ELECTROPHYSIOLOGICAL STUDIES ON THE ROLE OF PURINES IN THE SOMATOSENSORY SYSTEM IN THE DORSAL HORN OF THE SPINAL CORD OF THE CAT

by

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Short title: Role of purines in the somatosensory system of the spinal dorsal horn

to Janet
with love

ABSTRACT

This thesis focuses on the possible role of purines, in particular adenosine and adenine nucleotides, in the regulation of activity of neurones in the somatosensory system in the spinal cord. In electrophysiological studies done *in vivo* in the cat, single unit recordings were made from functionally identified neurones in the dorsal horn. Iontophoretic administration of ATP had a differential effect: most non-nociceptive neurones were excited whereas most nociceptive neurones showed a biphasic response (excitation followed by depression) or a depression. The differential nature of its effects raised the possibility that ATP may be a chemical mediator released by low threshold primary afferents which prompted investigation of the responses of dorsal horn neurones to low threshold inputs activated by cutaneously applied vibration. Non-nociceptive neurones were excited by vibration and nociceptive neurones had depressant or biphasic (excitatory/depressant) responses. Additional results indicated that depression and a subgroup of the biphasic responses are mediated by Pacinian corpuscle afferents and that the depression and depressant component of the biphasic response to vibration are mediated by adenosine. Interestingly, it was found that depression of nociceptive neurones by vibration and by application of ATP is enhanced by administration of the tachykinins physalaemin, substance P and neurokinin A.

Evidence presented in this thesis was brought together with that reported in the literature and a model was synthesized to account for the effects of vibration. The possibility is raised that ATP might be released from Pacinian afferents, act postsynaptically to cause excitation and then be converted extracellularly to adenosine to cause depression.

RÉSUMÉ

Cette thèse se concentre sur le rôle possible des purines, en particulier l'adénosine et les nucléotides de l'adénine, dans la régulation de l'activité des neurones du système somatosensoriel de la moëlle épinière. Lors d'études électrophysiologiques faites chez le chat *in vivo*, nous avons enregistré l'activité de neurones identifiés fonctionnellement au niveau de la corne dorsale. L'administration iontophorétique de l'ATP a eu différents effets: la plupart des neurones non-nociceptifs ont été excités alors que la plupart des neurones nociceptifs ont montré soit une réponse biphasique (excitation suivie d'une dépression) soit une dépression. La nature différente de ses effets soulève la possibilité que l'ATP puisse être un médiateur chimique libéré par les fibres afférentes primaires à seuil d'excitation peu élevé, ce qui nous a incités à étudier les réponses des neurones de la corne dorsale à des influx de fibres à faible seuil activées par l'application d'une vibration cutanée. Les neurones non-nociceptifs ont montré une excitation en réponse à la vibration alors que les neurones nociceptifs ont eu une réponse biphasique (excitation suivie d'une dépression) ou une dépression. Des résultats additionnels indiquent que cette dépression et un sous-groupe des réponses biphasiques sont médiés par les fibres afférentes des corpuscules de Pacinie et que la dépression et la composante de dépression de la réponse biphasique sont médiées par l'adénosine. Il est intéressant de noter que nous avons également trouvé que l'effet dépressif de la vibration de même que celui de l'ATP sur les neurones nociceptifs sont tous deux accrus par l'administration des tachikinines physalaemine, substance P et neurokinine A.

Les résultats présentés dans cette thèse et ceux rapportés dans la littérature ont été utilisés pour synthétiser un modèle expliquant les effets de la vibration: L'ATP serait libéré par les fibres afférentes des corpuscules de Pacinie et agirait de façon post-synaptique entraînant une excitation pour finalement être converti dans l'espace extracellulaire en adénosine et entraîner une dépression.

PREFACE

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Chapter 2:

Salter,M.W. and Henry,J.L. (1985) Effects of adenosine 5'-monophosphate and adenosine 5'-triphosphate on functionally identified units in the cat spinal dorsal horn. Evidence for a differential effect of adenosine 5'-triphosphate on nociceptive vs non-nociceptive units. *Neuroscience* 15, 815-825.

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Chapter 4:

Salter,M.W. and Henry, J.L. (1987) Evidence that adenosine mediates the depression of spinal dorsal horn neurones induced by peripheral vibration in the cat. *Neuroscience*, in press.

Chapter 5:

Salter,M.W. and Henry,J.L. Parametric studies on responses of spinal wide dynamic range neurones to cutaneously applied vibration in the cat: evidence implicating inputs from Pacinian corpuscles in a subset of responses. (To be submitted for publication).

Chapter 6:

Salter,M.W. and Henry,J.L. Purine-induced depression of dorsal horn neurones in the cat spinal cord: enhancement by tachykinins. *Neuroscience*, in press.

Chapter 7:

Salter,M.W. and Henry,J.L. Tachykinins enhance depression of spinal nociceptive neurones caused by cutaneously applied vibration in the cat. (To be submitted for publication).

This thesis is the work of the author done in the laboratory of Dr. J.L. Henry.

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CHAPTER 1

INTRODUCTION

"It is a sorry situation that despite considerable efforts we are totally in the dark as to the chemical transmitter at the primary sensory fibres, and both presynaptic and postsynaptic spinal cord inhibitory systems, not to mention other areas. Sooner or later one hopes that the blunderbus approach being pursued at present will lead to a breakthrough, but there is really little sign of this so far"

(Burgen, 1964)

I. PRELIMINARY REMARKS

i) Rationale for studying the physiology of neurones in the dorsal horn

As a medical student reflecting on the problems of patients with pain[1], I developed a fascination with understanding the mechanisms of pain and analgesia. This fascination has remained with me since then and has motivated my physiological studies of nociception and antinociception in the spinal cord.

I have chosen to do my studies in the dorsal horn because in nociceptive pathways this is the first level at which the activity of nociceptive neurones is regulated by integration of different excitatory and inhibitory inputs (for review see Dubner and Bennett, 1983). Nociceptive neurones are those excited by stimuli which damage or threaten to damage tissue, i.e. noxious stimuli (Sherrington, 1906). The inputs which regulate the activity of nociceptive neurones come from nociceptive and non-nociceptive primary afferents, and from supraspinal structures. The relevance of this integration of inputs to the human experience of pain derives from the observations that, while pain is usually associated with tissue-damaging stimuli, noxious stimulation does not always produce pain and pain can be experienced in the absence of noxious stimuli (for example Melzack, 1973; Merskey and Spear, 1967; Noordenbos, 1959; Wall, 1979). Therefore, the fact that the level of activity of nociceptive dorsal horn neurones is not only increased by noxious stimulation but is also controlled by other factors, such as innocuous stimulation (Wall and Cronly-Dillon, 1960) and even, apparently, level of attention (Dubner et al., 1981), suggests that these neurones might have an important role in the experience of pain.

On the basis of the apparent role for nociceptive dorsal horn neurones in the experience of pain, it seems reasonable to expect that the study of the physiology of nociceptive dorsal horn neurones might provide useful information about pain mechanisms. This expectation is reinforced by the previous successful clinical application of the results of other basic

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[1] The word "pain" is used according to the definition adopted by the International Association for the Study of Pain - "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" (Merskey et al., 1979). Thus, the physiological phenomena reported herein concern nociception and the possible correlation with pain is speculatory.

sciences studies on nociception. To cite two from many possible examples, the use of electrical stimulation of the dorsal columns for relief of pain (Wall and Sweet, 1966) was prompted by predictions made from physiological studies (Melzack and Wall, 1965) and the use of opioid peptides in treatment of pain (see Foley and Inturrisi, 1986) developed as a result of the discovery of the enkephalins (Hughes et al., 1975). My studies were thus undertaken with the hope that new information about the properties and behaviour of neurones in the dorsal horn may ultimately lead to improved treatment of patients suffering with pain.

ii) Rationale for studying adenine nucleotides

I chose to investigate adenine nucleotides because as I started my studies evidence was appearing which raised the possibility that these compounds might be chemical mediators in nociceptive pathways in the dorsal horn (see sections IV and V). I began my studies by examining the effects of adenine nucleotides on sensory neurones in the dorsal horn *in vivo* because prior to beginning my investigations the nature of these effects was unknown. It was anticipated that this avenue of research might yield useful new information because evidence from studies elsewhere in the central and peripheral nervous systems had indicated that adenosine and adenine nucleotides may be important regulators of neuronal function (for reviews see Burnstock, 1975; Phillis and Wu, 1981; Stone, 1981).

II. HISTORICAL PERSPECTIVE ON ADENOSINE AND ADENINE NUCLEOTIDES

i) Discovery of adenine and its nucleosides and nucleotides[2]

The purine base, adenine, was first isolated from extracts of yeast during biochemical studies on nucleic acids (Kossel, 1879). The adenine derivatives, adenosine and adenylic acid, were also first discovered in yeast extracts (Levene and Jacobs, 1909; Levene, 1919). Adenine, adenosine and adenylic acid were initially considered only as components of nucleic acids (Levene and Bass, 1931).

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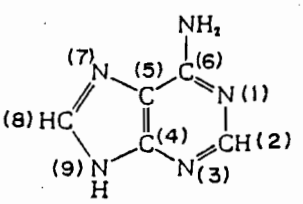
[2] A nucleoside is a compound consisting of a sugar (notably ribose or deoxyribose) with a purine or a pyrimidine base, connected by an N-glycosyl link. On the other hand, a nucleotide is the combination of a purine or a pyrimidine with a sugar and with one or more phosphate groups.

Following its discovery in yeast extracts, adenylic acid was also isolated from blood (Bass, 1914) and muscle (Embden and Zimmerman, 1927). However, it was soon recognized that muscle adenylic acid had different chemical properties and a different structure than yeast adenylic acid(Embden and Schmidt, 1929): adenylic acid derived from yeast is adenosine 3'-monophosphate and that derived from muscle is adenosine 5'-monophosphate (Fig. 1). Adenine nucleotides phosphorylated at the 5' position are the major forms present in animal cells whereas 3' derivatives occur naturally in plants (Green and Stoner, 1950) and are also the end products of hydrolysis of certain ribonucleotide linkages in animal cells (Lehninger, 1975). Hence, 3'-adenine nucleotides will not be considered further.

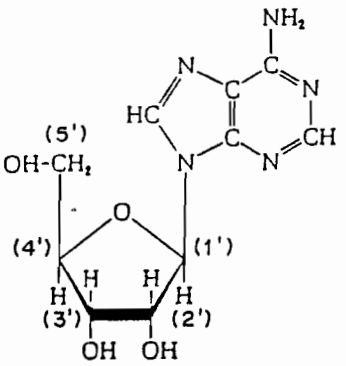
In the 1920's adenine nucleotides began to be recognized as potentially important metabolic intermediates and in this respect were thought to be concerned primarily with contraction of muscles. By 1927 Embden and Zimmerman had isolated the mono-phosphorylated nucleotide, adenylic acid, and two years later the first reports appeared which indicated that muscle also contains an adenine nucleotide with three phosphate groups (Fiske and Subbarow, 1929; Lohmann, 1929). At this time the actual structure of the compound was unknown but Lohmann postulated that two of the phosphates were linked; Fiske and Subbarow simply reported the stochiometric ratios of the component atoms. Three years following his initial report, Lohmann proposed that the triply phosphorylated compound was adenosine 5'-triphosphate (ATP). Final verification of Lohmann's structure for ATP (Fig. 1) did not come until this compound was totally synthesized by Todd's group 16 years later (Baddily et al., 1948).

The importance of ATP for bioenergetics became apparent in the early 1940's. In 1941 Lipmann proposed a general hypothesis in which the high energy phosphate bonds of ATP play a pivotal role in energy transfer in all living cells. Since this time ATP has been mainly considered primarily "a carrier of energy to those processes in cells which require energy input" (Lehninger, 1975). However, evidence which has accumulated in the last 25 years indicates that ATP may also function as a chemical mediator of intercellular communication. This evidence will be reviewed later in section III.

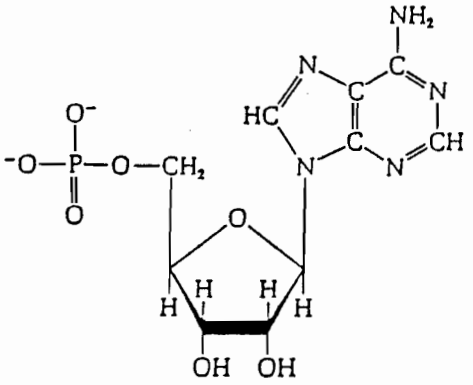
Adenine nucleotides containing greater than three phosphates also



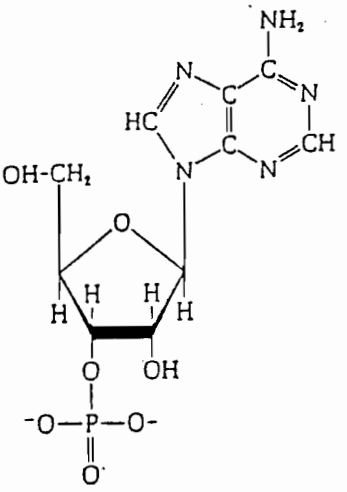
Adenine



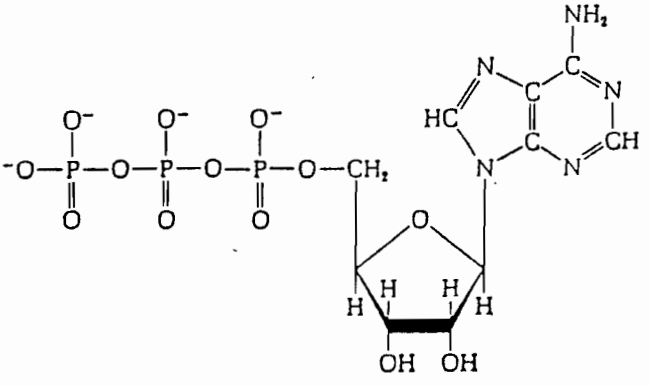
Adenosine



Muscle adenylic acid
(Adenosine 5'-monophosphate)



Yeast adenylic acid
(Adenosine 3'-monophosphate)



Adenosine 5'-triphosphate

Fig. 1 Structure of adenine, adenosine & some adenine nucleotides.

occur endogenously, for example, adenosine 5'-tetraphosphate has been extracted from muscle (Marrian, 1954). The function of adenosine 5'-tetraphosphate is largely unknown because this compound fails to serve as a substrate for many reactions utilizing ATP (Lieberman, 1955). Recently, Mowbray's group have isolated an oligomeric derivative of adenosine tetraphosphate from cardiac muscle (Hutchinson et al., 1986) and they have suggested that this derivative may function as a storage compound for nucleosides or for pyrophosphate groups (Lawson and Mowbray, 1986). There are at present no reports indicating that adenosine tetraphosphate or its oligomeric derivative is released from cardiac or other cells. Therefore, these compounds, unlike ATP and other adenine derivatives which do appear to be released from some types of cells (for review see Gordon, 1986), cannot be presently considered substances which mediate intercellular communication.

ii) *Early physiological studies on adenosine and adenine nucleotides*

Historically, the first investigation of the effects of adenosine and adenine nucleotides was reported by Drury and Szent-Györgyi in 1929. They extracted from heart muscle a bio-active compound which, based on its chemical properties, they deduced was muscle adenylic acid (AMP). Their classical paper laid the groundwork for many later physiological and pharmacological studies by describing a wide range of biological effects of AMP and adenosine in mammals *in vivo*.

Two parts of the study by Drury and Szent-Györgyi are of particular relevance to the present thesis. First, they showed that both AMP and adenosine have depressant effects on excitable tissues, cardiac and smooth muscle. Second, they provided evidence that these effects may be pharmacologically specific because, in contrast to AMP and adenosine, the purine bases, adenine and guanine, and the mononucleotide derivatives of inosine and guanine were found to be inactive. ATP was not tested as it had not yet been isolated.

In the 1930's ATP gradually became available in amounts sufficient to use in physiological experiments. In many experiments, intravenous injection of ATP was found to have cardiovascular effects similar to those of adenosine and AMP (for review see Green and Stoner, 1950). However, Gillespie (1934) found that ATP caused an increase in arterial blood

pressure before it induced hypotension, suggesting that the effects of ATP are not identical to those of AMP and adenosine.

When preparations of smooth muscle were examined it was found that the effects of administration of adenosine and were similar to the effects of administration of AMP regardless of which tissue was studied. On the other hand, in some tissues application of ATP had an effect similar to that of AMP or adenosine but in other tissues administration of ATP caused a different, sometimes opposite response. For example, all three compounds cause contraction when administered to the guinea-pig uterus (Bennet and Drury, 1931; Gillespie, 1934; von Euler and Gaddum, 1931; Zipf, 1931) while with the cat stomach and urinary bladder administration of ATP evoke contraction but adenosine and AMP are ineffective (Buchthal and Kahlson, 1944b). In addition, Gillespie (1934) found a biphasic effect of intravenous injection of ATP on motility of the small intestine in the cat while AMP and adenosine cause only inhibition (Drury and Szent-Györgyi, 1929).

An explanation for the similarities and the differences between the effects of adenosine (and AMP) and the effects of ATP can be found in Burnstock's (1978) proposal that there are two distinct purinergic receptors: at one receptor, (P_1), adenosine is much more potent than is ATP, and at the other receptor, (P_2), ATP is much more effective than is adenosine. In addition, it is important to note that ATP is rapidly converted to adenosine in the extracellular space (Manery and Dryden, 1979). Thus, the similarities between the effects of adenosine and of ATP may arise because administration of both compounds leads to activation of P_1-receptors and the differences may be due the fact that ATP rather than adenosine activates P_2-receptors. Further details concerning metabolism of purines and purine receptors are given in section III.

While many early studies examined the effects of adenosine and adenine nucleotides on peripheral tissue, few focussed on the effects of these compounds in the central nervous system. Buchthal and Kahlson were prompted by their demonstration that ATP evoked contraction of skeletal muscle (1944a) to investigate the effects of this nucleotide on "anterior horn cells" in the spinal cord. Injections of ATP into a vertebral artery at the level supplying the cervical cord evoked contractions of muscles in the upper forelimb while injections of AMP or of orthophosphate were

ineffective (Buchthal et al., 1947). These results indicated an excitatory effect of ATP on cervical motoneurones because the artery was occluded above the cervical level. Similar observations were made by Emmelin and Feldberg (1948). Subsequent studies have indicated that ATP fails to have a direct effect on spinal motoneurones (Curtis et al., 1960; Phillis and Kirkpatrick, 1978), hence it appears that the excitatory effects of intra-arterial injection of ATP were likely indirect.

Direct application of ATP onto neuronal tissue was subsequently investigated by Babskii and Malkiman (1950). Foreshadowing later studies using iontophoresis (for example Phillis et al., 1979), Babskii and Malkiman reported an excitatory effect of ATP when it was applied topically to the motor cortex.

Many anatomical, biochemical, physiological and pharmacological studies followed from the early work in the peripheral and central nervous systems. In 1972 Burnstock crystallized the available data into a coherent hypothesis and proposed that some neurones might use a purine - ATP - as a chemical mediator of synaptic transmission. The neurones specifically considered to use ATP were the non-cholinergic, non-adrenergic nerves in the autonomic system. Burnstock coined the term "purinergic" to describe these neurones much as Dale had introduced "cholinergic" and "adrenergic" to describe the other major components of the autonomic system nearly 40 years previously (Dale, 1933).

Since the original proposal of the purinergic hypothesis, evidence has continued to appear to support the concept that ATP may be a chemical mediator of synaptic transmission (for more recent reviews see Burnstock, 1981; Phillis and Wu, 1981; Stone, 1981). Other evidence has pointed to the additional possibility that ATP may function as a co-transmitter with "classical" transmitters such as acetylcholine (Silinsky, 1975) or noradrenaline (Sneddon et al., 1982; Sneddon and Westfall, 1984).

Finally, adenosine, which was recognized some time ago as an endogenous regulator of coronary blood flow (Berne, 1964), is now well known to have potent effects on the activity of neurones in the central nervous system (Phillis and Wu, 1981; Stone, 1981). Recently, the existence of "adenosinergic" neurones in the central nervous system was postulated on the basis some central neurones may contain particularly high levels of intracellular adenosine (Snyder, 1985).

III. BIOCHEMISTRY, PHYSIOLOGY AND PHARMACOLOGY OF ADENOSINE AND ADENINE NUCLEOTIDES

The purpose of this section is to refresh the reader on the principles of purine biochemistry, physiology and pharmacology particularly as these relate to adenine derivatives and to the nervous system. The relevant information has been summarized and the reader is referred to representative papers and to reviews if further details are required for specific topics. The literature pertaining specifically to the possibility that adenosine and ATP might be chemical mediators of synaptic transmission in the spinal dorsal horn is critically appraised in section IV.

i) Biosynthesis and intracellular metabolism

Adenine nucleotides and the enzymes which synthesize these compounds occur ubiquitously in living cells. The relevant biosynthetic reactions for adenine nucleotides and adenosine are summarized in Fig. 2. AMP can be seen as a central intermediate in the synthesis of adenine derivatives as it can be metabolized to form more highly phosphorylated derivatives and it can also be hydrolyzed to form adenosine.

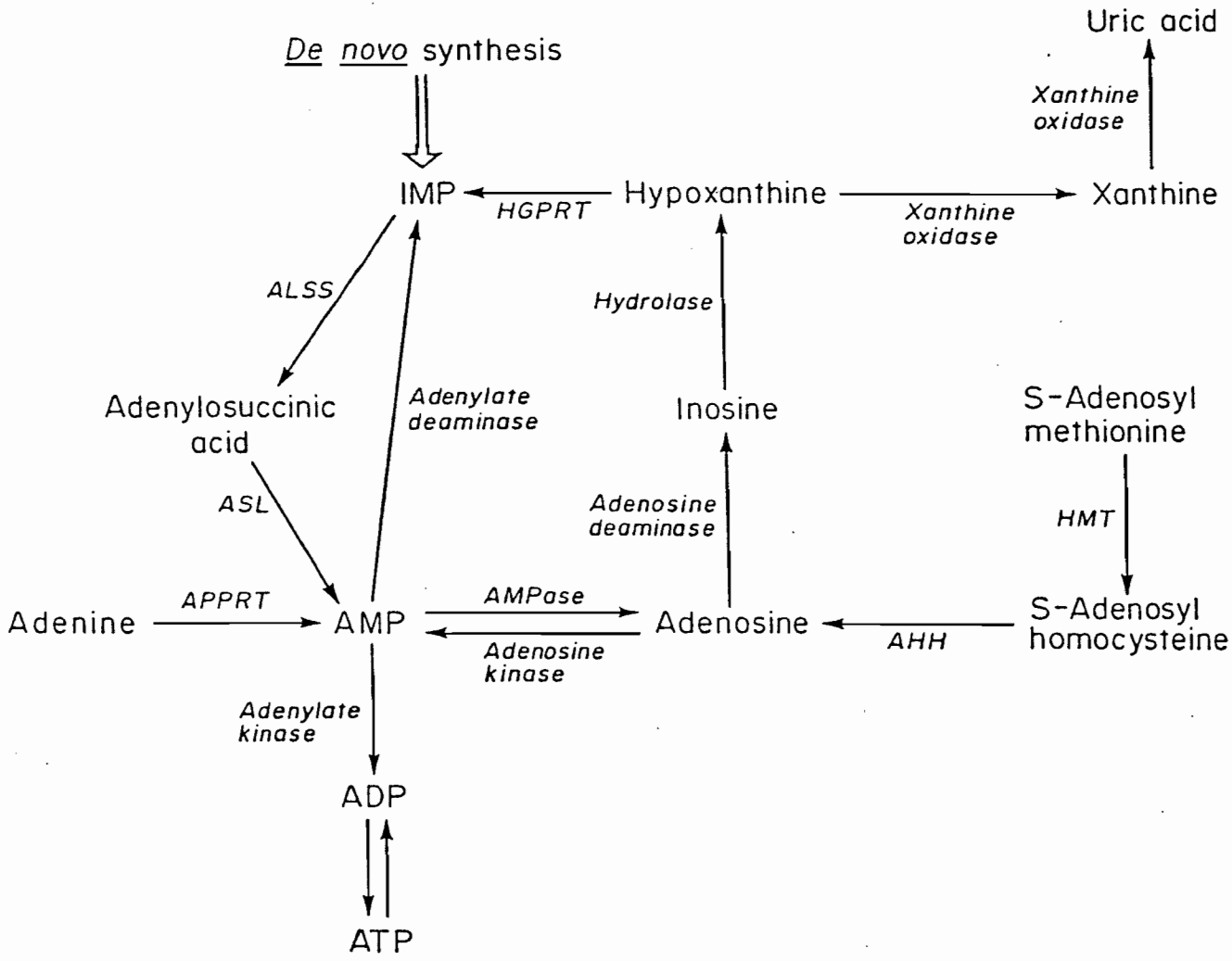
The three principal synthetic pathways leading to the formation of AMP are shown in Fig. 2. First, AMP is formed as a consequence of *de novo* purine synthesis which leads first to the formation of IMP and then by two reactions to generation of AMP. Second, AMP is formed from adenine which is taken up into cells and is then rapidly phosphorylated by adenine phosphoribosyltransferase (EC 2.4.2.7). Third, AMP is formed by the phosphorylation of adenosine by adenosine kinase (EC 2.7.1.20). Adenosine is taken up into cells as described in a later section and is formed from S-adenosylhomocysteine.

Once formed, AMP can be phosphorylated to generate ADP in a reaction catalyzed by the enzyme adenylate kinase (EC 2.7.4.3). ATP is then produced from ADP during oxidative and other phosphorylation reactions. Oxidative phosphorylation of ADP occurs within mitochondria and ATP is released into the cytoplasm by carrier-mediated exchange with ADP

ig. 2 Biosynthetic pathways for adenosine and adenine nucleotides.

bbreviations for enzymes:

AHH - adenosylhomocysteine hydrolase
ALSS - adenylosuccinate synthetase
AMPase- adenosine monophosphatase
ASL - adenylosuccinate lyase
APPRT - adenine phosphoribosyltransferase
HGPRT - hypoxanthine-guanine phosphoribosyltransferase
HMT - homocysteine methyltransferase


De novo synthesis
Uric acid
Xanthine oxidase
IMP
HGPRT
Hypoxanthine
Xanthine oxidase
Xanthine
ALSS
Hydrolase
Adenylosuccinic acid
Adenylate deaminase
Inosine
S-Adenosyl methionine
ASL
Adenosine deaminase
HMT
APPRT
AMPase
Adenine
AMP
Adenosine
S-Adenosyl homocysteine
AHH
Adenosine kinase
Adenylate kinase
ADP
ATP

(Lehninger, 1975).

Estimates of the level of ATP within cells are difficult to make due to the lability of this nucleotide. Nonetheless, by *in situ* freezing of rat brain tissue it has been possible to show that the concentration of ATP is about 3 mmol/kg whereas adenosine is present at a concentration of less than 1 μmol/kg (Nordstrom et al., 1977; Rehncrona et al., 1978). When the brain is allowed to become hypoxic before being frozen the tissue levels of adenosine rise rapidly and the ATP levels fall.

ii) Intracellular localization

In view of the fact that adenosine and adenine nucleotides occur ubiquitously in living cells the relevant question when these compounds are being considered as chemical synaptic mediators is whether they are stored in such a way that nerve stimulation can evoke their release from presynaptic elements. In Burnstock's original description of purinergic nerves it was suggested that ATP might be stored in and released from the large opaque vesicles which are found in the terminals of non-adrenergic, non-cholinergic nerves in the gut but which are absent from either adrenergic or cholinergic nerves (Burnstock, 1972). This suggestion was based on findings that the large opaque vesicles and the purinergic nerve-mediated response persist after destruction of adrenergic nerves using 6-hydroxydopamine or depletion of catecholamines using reserpine or metaraminol.

a) *Labelling of neurones with quinacrine.* Evidence consistent with the possibility that purinergic nerves contain releasable ATP came from studies using the fluorescent anti-malarial drug, quinacrine, which binds to ATP possibly via the adenine moiety (Ellison and Barr, 1972). Olson et al. (1976) used quinacrine to label putative purinergic nerve fibres in the gut. Further experiments revealed that quinacrine binding is decreased by depolarizing concentrations of potassium and of veratridine (Alund and Olson, 1979) and this decrease is calcium-dependent. The calcium-dependence of the release of the quinacrine is consistent with the possibility that the compound to which quinacrine is binding may be a neurotransmitter. Evidence that quinacrine actually binds to ATP under the conditions of the experiments is that analysis of fractions of microsomal preparations of gut tissue preloaded with [^{3}H]adenosine and

[^{14}C]quinacrine showed that the peak of ^{14}C and [^{3}H]ATP occur in the same fractions (Burnstock, 1981). While these experiments suggest that quinacrine is binding to ATP, quinacrine is also known to bind to nucleic acids (Ellison and Barr, 1972). Therefore, quinacrine cannot be considered, necessarily, a specific label for purinergic nerve fibres.

Despite the possible lack of specificity of quinacrine, nerve fibres labelled by this drug have been found in other peripheral tissues in addition to the gut where pharmacological evidence indicates that purinergic transmission occurs (for example Burnstock et al., 1979). On the other hand, quinacrine-positive fibres are absent in peripheral tissues, such as the iris, where there is no evidence of purinergic transmission (Burnstock, 1981).

In the brain, quinacrine-positive neuronal cell bodies are found in the cerebellum, cerebral cortex, hippocampus and basal ganglia; the latter also contains a dense plexus of quinacrine-positive fibres (Crowe and Burnstock, 1984). The labelling in cell bodies is consistent with binding of quinacrine to nucleic acids. However, further evidence is required before it will be known whether the labelled fibres are purinergic.

b) *Presence of adenine nucleotides in secretory granules and vesicles*. The possibility suggested by Burnstock (1972) was that ATP might be stored uniquely in secretory granules in purinergic nerves. Adenine nucleotides have been found as components of secretory granules or vesicles isolated from many sources including cholinergic neurones, chromaffin cells, peptidergic neurones and platelets (see Poisner and Trifaró, 1982). In cholinergic vesicles and chromaffin granules ATP constitutes up to 85% of the total adenine nucleotide content whereas in platelets up to 50% of the adenine nucleotides are ADP and AMP.

The question as to how ATP gains access to granules and vesicles is important when considering whether ATP may be able to enter vesicles in the absence of other catecholamines or acetylcholine. In this regard, ATP does not appear to be synthesized within chromaffin granules but rather it is taken up by a carrier-mediated mechanism (Aberer et al., 1978); a similar uptake mechanism is used to take up catecholamines (Bashford et al., 1975). That there is a mechanism for uptake of ATP into granules of chromaffin cells raises the possibility that a similar mechanism may also exist in other cell types.

Adenosine was not reported as a constituent of either chromaffin granules or cholinergic vesicles. This lack of presence of adenosine raises doubts as to whether it might be localized in vesicles in neurones in the central nervous system. Therefore, unless it can be demonstrated that adenosine does occur in vesicles in these or other neurones there will be serious doubts as to whether it can function like a "classical" transmitter as suggested by Snyder (1985).

iii) Release of purines

Evidence that nerve stimulation causes release of ATP came first from Holton and Holton (1954) who showed with the rabbit ear *in vivo* that electrical antidromic stimulation of sensory nerves causes an increase in ATP, ADP, AMP and adenosine in the venous outflow; ATP was assumed to have been released and ADP, AMP and adenosine were considered breakdown products.

Burnstock et al. (1970) demonstrated calcium-dependent release of purines following transmural electrical stimulation of the gut. Adenosine and inosine were the purines collected in the largest amounts but because exogenously administered ATP was rapidly degraded and because ATP, but not adenosine or inosine, mimicked the response to stimulation it was assumed that ATP was the compound actually released. Stimulation-evoked release of [^{3}H]ATP when the tissue was incubated with [^{3}H]adenosine was shown later by Su et al. (1971). The possibility that the released nucleotide was derived from muscle, rather than from nerves, was eliminated because the release persists when the muscle response is blocked with tetrodotoxin (Rutherford and Burnstock, 1978). These results are consistent with the possibility that ATP is released from purinergic nerves in the gut.

As far as the central nervous system is concerned, there is a large body of evidence showing stimulation-evoked release of purines *in vitro* and *in vivo* (for reviews see Fredholm and Hedqvist, 1980; Phillis and Wu, 1981; Stone, 1981; White, 1984). Nucleotides are rapidly catabolized when they are present extracellularly (Pull and McIlwain, 1972) and as such there has been considerable controversy as to whether purines are released primarily as ATP or as adenosine (Phillis and Wu, 1981; Stone, 1981). ATP, released from synaptosomes by depolarizing chemical stimuli,

was detected directly using the firefly enzyme luciferin-luciferase[3] (White, 1978). On the other hand, there is evidence which indicates that adenosine, *per se*, can also be released (MacDonald and White, 1985; Pons et al., 1980; Pull and McIlwain, 1973). A major problem which continues to plague all studies of release of purines in the central nervous system is that release by presynaptic terminals cannot be distinguished from release by postsynaptic or other elements of the preparations; hence, the exact source of the purines is unknown. Furthermore, the conditions used to evoke release are non-physiological and so the question remains as to whether the release measured during the experiments is physiologically relevant.

The co-localization of adenine nucleotides in secretory granules and vesicles with other chemical mediators mentioned in the previous section raises the possibility that these compounds might be released together. Studies on the co-release of adenine nucleotides and other substances have been carried out primarily using peripheral tissues. ATP appears to be released from chromaffin cells together with catecholamines (Douglas, 1968), from terminals of motoneurones along with acetylcholine (Silinsky, 1975) and from platelets with serotonin and histamine (DaPrada et al., 1982).

iv) Extracellular metabolism

As mentioned in the preceding section, interpretation of results from studies on the release of purines has been problematic due to the rapid extracellular breakdown of adenine nucleotides to adenosine. A scheme illustrating the extracellular metabolism is shown in Fig. 3. When ATP is present in the extracellular space it is rapidly metabolized to ADP by a Ca^{++}-Mg^{++} dependent ATPase (EC 3.6.1.3). This enzyme is found on the "ecto", that is to say the extracellular, surface of many cell types including skeletal muscle, cardiac cells and glia (Manery and Dryden, 1979). More recently, ecto-ATPase activity has been reported in synaptosomal preparations from brain (Nagy et al., 1983) and peripheral cholinergic nerves (Keller and Zimmerman, 1983), consistent with the possible presence of the enzyme at or near synapses.

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[3] Only ATP is detected using this assay because other adenine and purine nucleotides are not substrates for the reaction (McElroy and Green, 1956).

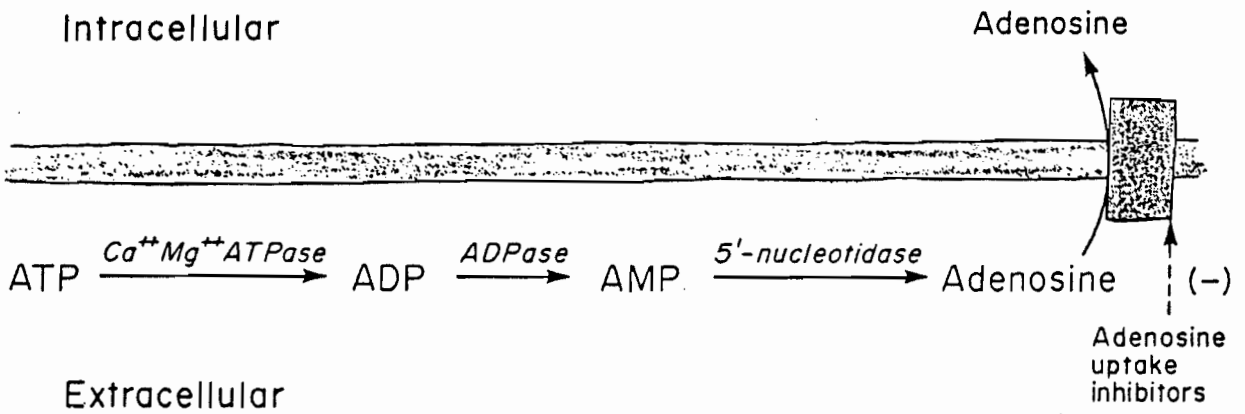

Fig. 3. Extracellular metabolism of ATP.

ADP is converted to AMP by an ecto-ADPase. The ecto-ADPase present on vascular endothelial cells has been well studied (Gordon, 1986). However, this enzyme has been poorly characterized in nervous tissue.

The most well characterized of the ecto-enzymes involved in purine metabolism is 5'-nucleotidase (EC 3.1.3.5). This enzyme catalyzes the conversion of mononucleotides to nucleosides (Manery and Dryden, 1979) and it is distinct from the AMPase which produces adenosine from AMP intracellularly (Pearson, 1985). 5'-Nucleotidase is widely distributed in the central nervous system (Scott, 1965, 1967). The concentration of this enzyme is increased in synaptosomal preparations (Pilcher and Jones, 1970; Marani, 1977) which raises the possibility that it may be present at synapses. Recently, the ultrastructural localization of the enzyme has been investigated using electron microscopic cytochemistry (Kreutzberg et al., 1986). The results of this study indicate that the enzyme is present in membranes of glia and neurones at or near synapses.

5'-Nucleotidase also occurs in myelin (Cammer et al., 1980; Heymann et al., 1984) but this observation is likely not significant for consideration of adenine nucleotides and adenosine as chemical mediators of synaptic transmission.

The function of extracellular metabolism of ATP may be seen as important in terminating its actions. However, another equally important function of this metabolism may be the generation of adenosine, which is clearly a biologically active compound.

iv) Uptake of adenosine

One important metabolic reaction which apparently does not occur extracellularly is the deamination of adenosine because the enzyme which catalyzes this reaction, adenosine deaminase (EC 3.5.4.4), is found only intracellularly (Wu and Phillis, 1984). The lack of extracellularly located adenosine deaminase is important because it indicates that adenosine must be taken up into cells before it can be metabolized. Thus, uptake of adenosine rather than extracellular chemical degradation appears to be the primary mechanism for terminating its actions.

Uptake of adenosine and of other nucleosides has been studied extensively using peripheral tissues (Plagemann and Wohlheuter, 1980) and more recently in the nervous system (for review see Wu and Phillis,

1984). The uptake process appears to occur by carrier-mediated, facilitated diffusion. Evidence that uptake of adenosine is important in terminating its effects comes from experiments using drugs such as dipyridamole and nitrobenzylthioinosine, which inhibit adenosine uptake. These drugs have been shown to potentiate effects of exogenously administered adenosine in the peripheral (Stafford, 1966) and in the central (Phillis et al., 1979) nervous systems.

Evidence has been presented that when adenosine is taken up by nerve terminals it can be subsequently recycled to form ATP which can then be released (Su et a., 1971; Burnstock, 1972). Re-utilization of released agents is well-described for catecholamines in adrenergic systems (Iversen, 1967) and for choline, which is actively taken up by cholinergic nerves to permit continued acetylcholine synthesis, in cholinergic systems (for example Collier and Katz, 1974; Collier and MacIntosh, 1969). Thus, the extracellular metabolism of ATP and the uptake of adenosine shows an homology between purinergic and other putative transmitter systems.

vi) Effects of adenosine and ATP

a_1) Presynaptic effects - adenosine. One of the most consistently reported effects of adenosine is to decrease evoked release of other neurochemical mediators (for reviews see Fredholm and Hedqvist, 1980; Phillis and Wu, 1981; Ribeiro and Sebastião, 1986). This effect has been observed both in the periphery and in the central nervous system. For example, using an *in vitro* slice preparation of hippocampus, exogenously applied adenosine causes a decrease in the release of aspartate (Corradetti et al., 1984), glutamate (Dolphin and Archer, 1983), noradrenaline (Jonzon and Fredholm, 1984), acetylcholine (Jackisch et al., 1984) and serotonin (Feuerstein et al., 1985).

The mechanism by which adenosine induces depression of release of putative transmitters is controversial. The evidence indicates that adenosine causes a decrease in effective excitation-secretion coupling and that calcium is involved but the precise mechanism of the effect is disputable. On one hand, evidence present by Henon and McAfee (1983) and by Ribiero et al. (1979) indicates that the effect is due to decreased influx of calcium through voltage-dependent calcium channels. On the other hand, evidence presented by Silinsky (1984) is consistent with the

possibility that adenosine may cause a reduction of the affinity for calcium of an intracellular component of the secretory system. In these studies it was impossible to rule out the mechanism suggested by the other results. Therefore, it is not possible to decide at the present time which mechanism is more likely. It should be noted that these two mechanisms are, however, by no means mutually exclusive.

To investigate whether the adenosine-induced decrease in release of putative transmitters may be relevant to synaptic transmission, electrophysiological studies have examined the effects of adenosine on neuronal responses evoked by electrical stimulation. As an example, evidence from experiments by Schubert and Mitzdorf (1979) in hippocampal slices is consistent with the possibility that adenosine depresses synaptic transmission by a presynaptic mechanism because the population responses to electrical stimulation of afferents were diminished when there was no obvious change in the excitability of the postsynaptic neurones. In a study of single units, Lekić (1977) provided other evidence indicative of a presynaptic effect: the synaptically evoked excitatory responses of Renshaw cells were decreased by iontophoretically applied AMP whereas the excitatory responses to application of acetylcholine, aspartate and glutamate were unaffected.

Recordings from single neurones in the cerebral cortex and elsewhere in the brain have shown that administration of adenosine and adenine nucleotides depresses the rate of discharge when these compounds are applied by iontophoresis (Phillis et al., 1974). The depressant effects are enhanced by adenosine uptake inhibitors and are blocked by P_1-purinergic antagonists (Phillis and Kostopoulos, 1975). Hence, it is likely that the depression caused by application of the nucleotides is due to their conversion to adenosine. The depression of firing rate was considered by Phillis et al. (1979) to be a presynaptic effect because recordings made intracellularly indicated that adenosine caused membrane hyperpolarization with little change in input resistance and also caused a decrease membrane potential fluctuations attributed to synaptic inputs. However, it is possible that a postsynaptic effect may explain most of their findings and thus, a presynaptic effect is by no means certain.

a_2) *Presynaptic effects - ATP.* Generally, there is little evidence that ATP *per se* decreases transmitter release. Rather, effects

of administration of this nucleotide appear to be due to its conversion to adenosine (for example see Silinsky, 1975). However, in the frog sympathetic ganglion ATP, but not adenosine, appears to inhibit release of acetylcholine (Silinsky and Ginsborg, 1983).

b_1) *Postsynaptic effects - adenosine.* A postsynaptic effect of adenosine on mammalian central neurones was suggested by Segal (1982) based on the findings that adenosine caused membrane hyperpolarization and decreased input resistance in hippocampal CA1 neurones *in vitro*. The hyperpolarization had an apparent reversal potential of -90 mV, consistent with the additional proposal that the ionic mechanism is an increase in potassium conductance. Adenosine-induced increases in potassium conductance have been subsequently confirmed in hippocampal neurones (Greene and Haas, 1985; Halliwell and Scholfield, 1984) and have been reported, as well, in postganglionic parasympathetic neurones (Akasu et al., 1984), atria heart muscle (Jochem and Nawrath, 1983) and *Xenopus* oocytes (Lotan et al., 1982). Additional postsynaptic effects have been reported: blockade of calcium-dependent action potentials (Proctor and Dunwiddie, 1983), which appears to be due to the increase in potassium conductance (Halliwell and Scholfield, 1984), and enhancement of afterhyperpolarization (Greene and Haas, 1984). The latter effect occurs independently of the increase in resting potassium conductance (Greene and Haas, 1985).

b_2) *Postsynaptic effects - ATP.* With peripheral tissue ATP, *per se*, has two distinct effects in different tissues. These effects are opposite in terms of the change in excitability of the postsynaptic cells. One effect, membrane hyperpolarization, has been observed in smooth muscle in the taenia coli of the guinea-pig (Burnstock et al., 1970). This effect is blocked by the bee venom, apamin (Vladimirova and Shuba, 1978), and hence appears to be due to activation of an apamin-sensitive, calcium-dependent potassium conductance (Banks et al., 1979). ATP also has inhibitory effects on smooth muscle in other tissues including the guinea-pig trachea (Christie and Satchell, 1980) and the rabbit portal vein (Hughes and Vane, 1967; Burnstock and Kennedy, 1985).

On the other hand, ATP-induced excitation has been reported in many tissues such as the vas deferens of the rat (Hogaboom et al., 1980; Sneddon and Westfall, 1984), the urinary bladder of the guinea-pig

(Kasakov and Burnstock, 1983) and of the rabbit (Hoyle and Burnstock, 1985), the anococcygeus muscle of the rat (Byrne and Large, 1984), and the atria (Flitney and Singh, 1980) and the oocyte of the frog (Lotan et al., 1982).

Excitatory effects of ATP have also been observed with neurones. In the central nervous system iontophoretic application of ATP increases the firing rate of neurones in the rat cerebral cortex (Phillis et al., 1979) and elsewhere in the brain (for review see Phillis and Wu, 1981). Additionally, ATP has been found to cause depolarization of neurones in the bullfrog sympathetic ganglia (Akasu et al., 1983a; Siggins et al., 1977) and in the snail subesophageal ganglia (Yatani et al., 1982).

The ionic mechanism of the depolarization caused by ATP appears to vary in different types of tissue: with neurones in the frog ganglion there is a decrease in potassium conductance (Akasu et al., 1983a) and in the snail neurones there is an increase in the conductance for calcium. For non-neuronal cells ATP-induced depolarization appears to be due to other ionic mechanisms, such as increased conductance for chloride in oocytes (Lotan et al., 1982) and for sodium and potassium in fibroblasts (Friedberg et al., 1985). In the rat vas deferens, the ATP-induced depolarization was not associated with a change in membrane conductance (Sneddon and Westfall, 1984). Thus, no single ionic mechanism can explain the excitatory effects of ATP in every tissue.

c) *Biphasic effects of administration of ATP*. Administration of ATP to the frog atria causes an initial excitation followed by an inhibition (Flitney et al., 1977; Goto et al., 1977). The inhibitory, but not the excitatory, effect of administration of ATP is blocked by methylxanthines and is potentiated by dipyridamole; these drugs have similar effects on the inhibition caused by adenosine (Burnstock and Meghji, 1981). These observations lead to the conclusion that the excitatory effect appears to be caused by ATP, *per se*, while the inhibitory effect seems to be due to adenosine likely formed by extracellular metabolism of ATP. A similar conclusion has been made concerning the biphasic effect seen when ATP is applied to the frog oocyte (Lotan et al., 1982).

Biphasic effects of administration of ATP have also been observed in the mammalian central nervous system: iontophoretic administration of ATP causes an increase and then a decrease in the rate of discharge (Phillis

et al., 1979). Application of adenosine caused only a decrease in the rate of discharge. This finding is consistent with the suggestion made by the authors that decrease in rate of discharge during the biphasic effect is caused by adenosine.

vii) Receptors for purines

In 1978 Burnstock introduced the concept that there are two classes of "purinergic" receptors[4]: P_1 and P_2. This classification was proposed on basis of a difference in the rank order of potency of agonists and of the existence of specific antagonists for the P_1-mediated effects. Thus, for P_1-mediated effects the rank order of potency of adenosine and adenine nucleotides is adenosine≥AMP>ADP≥ATP and for P_2-mediated effects the order of potency is reversed. In addition, P_1-mediated effects, but not P_2-mediated effects, are antagonized by methylxanthines.

It now appears that there are subclasses of both P_1- and P_2-receptors. These subclasses will be described briefly in the following sections; pharmacological properties of the subclasses of receptors are shown in Table 1.

a)*P_1-receptors*. While effects of adenosine and adenine nucleotides on electrical and mechanical responses of peripheral tissue were investigated by Burnstock and his colleagues, Sattin and Rall (1970) were the first to demonstrate that administration of adenosine elevates levels of cyclic AMP in brain slices. Subsequently, others have examined the effects of adenosine and synthetic analogues on the activity of the enzyme which synthesizes cyclic AMP, adenylate cyclase (EC 4.6.1.1), and it has been found that adenosine can either increase or decrease activity of this enzyme (for review see Wolff et al., 1981). The changes in adenylate cyclase activity appear to be due to an action on receptors located on the external cell surface because when adenosine is linked to macromolecules not taken up into cells the same effects are observed

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[4]"Purinergic" receptors appear to be activated specifically by adenosine, adenine nucleotides and synthetic analogues whereas derivatives of other purines such as inosine and guanine are inactive. Thus, the reader should keep in mind the fact that not all purines act on these receptors. It is beyond the scope of this thesis, however,to introduce new nomenclature such as "adeninergic" to refer to these receptors and thus the generally accepted term "puringeric receptors" will be used.

Receptor Type	Rank Order of Agonists	Proposed Antagonists
$P_1 = A_2 + A_2 + (A_3)$	ADO≥AMP>ADP≥ATP[3]	Methylxanthines[3] CAFF, THEO, 8-SPT[8]
A_1	L-PIA, CHA>NCPCA, NECA, D-PIA[4,11,13]	PD 116,948[1,9]
A_2	NECA, NCPCA>L-PIA, CHA, D-PIA[4,11,13]	PD 115,199[2]
(A_3)	(L-PIA, CHA, NECA>CADO, D-PIA)[12] (CADO>NCPCA>L-PIA, D-PIA)[7]	?
$P_2 = P_{2X} + P_{2Y}$	ATP≥ADP>AMP≥ADO[3]	Not Methylxanthines[3]
P_{2X}	APCPP, APPCP>ATP>MeS-ATP[5]	$ANAPP_3$[10] desens. APCPP[5]
P_{2Y}	MeS-ATP>>ATP>APCPP, APPCP[5]	Reactive blue 2[6]

Table 1. Pharmacological characteristics of purinergic receptors.

Abbreviations:
ADO - adenosine
$ANAPP_3$ - 3'-O-{3-[N-(4-azido-2-nitrophenyl)-amino]propionyl}adenosine 5'-triphosphate
APCPP - α,β-methyleneATP
APPCP - β,γ-methyleneATP
CADO - 2-chloroadenosine
CAFF - caffeine
CHA - N^6-cyclohexyladenosine
D-PIA - D-N^6-phenylisopropyladenosine
L-PIA - L-N^6-phenylisopropyladenosine
MeS-ATP - 2-methylthioATP
NCPCA - 5'-N-cyclopropylcarboxamide adenosine
NECA - 5'-N-ethylcarboxamide adenosine
THEO - theophylline
8-SPT - 8-sulphophenyltheophylline

References:
1. Bruns et al., 1987a
2. Bruns et al., 1987b
3. Burnstock, 1978
4. Burnstock & Buckley, 1985
5. Burnstock & Kennedy, 1985
6. Burnstock & Warland, 1987
7. Chin et al., 1985
8. Daly et al., 1985
9. Haleen et al., 1987
10. Hogaboom et al., 1980
11. Londos et al., 1980
12. Ribiero & Sebatião, 1986
13. van Calker et al., 1979

(Wolff et al., 1981).

It has been proposed that a different type of receptor mediates each of the effects on adenylate cyclase activity (Londos et al., 1980; van Calker et al., 1979) because two different rank orders of potency of synthetic adenosine analogues have been found and because there is a good correspondence between these two rank orders and the two different effects on adenylate cyclase activity. The nomenclature presently used for the two types of receptors, A_1 and A_2, follows the suggestion made by Londos et al. (1980). Using this nomenclature A_1-receptors are those which appear to mediate the decrease in adenylate cyclase activity and A_2-receptors appear to mediate the increase[5]. A point of difference between A_1 and A_2 receptors is that the affinity of A_1 receptors appears to be about a thousand times greater than the affinity of A_2 receptors (Bruns et al., 1980). Importantly, methylxanthines such as caffeine, theophylline and 8-sulphophenyltheophylline block both the increase and the decrease in adenylate cyclase activity caused by adenosine and synthetic analogues (Londos et al., 1980; van Calker et al., 1979; Bruns et al., 1980). Therefore, these methylxanthines appear to be antagonists at both A_1- and A_2-receptors. Furthermore, in view of the fact that P_1-mediated effects are blocked by methylxanthines, the antagonism by these compounds of A_1- and A_2-mediated effects supports the idea that A_1 and A_2 receptors are indeed subtypes of the P_1 class.

As is the case for most receptor-linked effects on adenylate cyclase activity, guanine-nucleotide regulatory proteins, G_s and G_i (Gilman, 1984), appear to transduce, respectively, the stimulatory effects of A_2-receptors and the inhibitory effect of A_1-receptors (for review see Patel and Marangos, 1984).

The A_1/A_2 dichotomy for P_1 receptors is now well established but recently, two groups have proposed the existence of a third type of adenosine receptor, A_3 (Chin et al., 1985; Ribeiro and Sebastião, 1986). Each group has suggested that A_1 and A_2 receptors cause their

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[5] Londos' group has also proposed a third type of adenosine receptor, the so-called "P" type (see Wolff et al., 1981). This receptor appears to be located intracellularly and methylxanthines are not antagonists at the P-receptor. Hence, this receptor is not subtype of Burnstock's P_1 class. Therefore, the P-receptor will not be considered further.

effects through changes in adenylate cyclase activity and that the A_3 receptor is not coupled to this enzyme. Rather, they suggest that A_3 receptors mediate the adenosine-induced effects which occur via decreasing calcium influx. Although it is presently controversial as to whether a third type of receptor exists, the important point as far as this thesis is concerned is that caffeine, theophylline and 8-sulphophenyltheophylline appear to be antagonists at A_1, A_2 and A_3 receptors (Chin et al., 1985; Ribeiro and Sebastião, 1986). Therefore, these compounds can not be used to distinguish between these different subtypes of P_1-receptors. Interestingly, within the last year, there is evidence for antagonists which may be specific for A_1 (Bruns et al., 1987a; Haleen et al., 1987) and A_2 (Bruns et al., 1987b) receptors. These new tools may help to decide the questions of whether A_3 receptors exist and if so, what are their functions.

With regard to the depression of neuronal firing rate, it has been suggested that this effect is mediated by A_2 receptors because NECA causes greater depression than L-PIA when these compounds are applied by iontophoresis using equal ejection currents (Phillis, 1982; Stone, 1982). However, in neither of these studies is there any evidence that the actual amounts of NECA and L-PIA applied were equal and so in light of evidence that equal ejection currents often release different amounts of different substances (Kelly, 1975; Purves, 1979), it seems that the conclusion that the depression is mediated by A_2-receptors is premature. Future experiments using specific antagonists may help to resolve the question of which subtype of P_1 receptors mediates depression of neuronal firing rate in the central nervous system.

Physiological and pharmacological studies have lead to the hypothesis that P_1-receptors are physical structures in membranes (for example see Phillis and Wu, 1981). Through the use of photoaffinity labelling with analogues of adenosine, several groups (Choca et al., 1985; Green et al., 1986; Klotz et al., 1986; Stiles et al., 1985) have isolated a single membrane glycoprotein of M_r approximately 38,000 which they have suggested may be the A_1 receptor. The finding of this glycoprotein may help to further elucidate the molecular mechanisms of the action of adenosine.

b) *P_1-mediated effects and adenylate cyclase.* Despite the

overwhelming evidence that adenosine and its analogues cause changes in the activity of adenylate cyclase, evidence that these changes in enzyme activity are responsible for the electrophysiological effects is inconclusive. Recent evidence has raised the alternative possibility that there is not a cause-and-effect relationship between effects on adenylate cyclase and effects on neuronal membrane properties and thus that these two types of effects may occur through parallel mechanisms. For example, with regard to the presynaptic effects of adenosine, Dolphin and Prestwich (1985) have found that the adenosine-induced decrease in release of glutamate from hippocampal slices is reversed by pertussis toxin. On the basis that pertussis toxin causes inactivation of G_i (Murayama and Ui, 1983; Katada and Ui, 1979), Dolphin and Prestwich suggested that the decreased transmitter release is caused by inhibition of adenylate cyclase. However, pertussis toxin also inactivates other guanine-nucleotide binding proteins not linked to the adenylate cyclase system, these include G_o (Sternweis and Robishaw, 1984). Therefore, the results of Dolphin and Prestwich fail to provide definitive proof of involvement of adenylate cyclase. In fact, Fredholm et al. (1986) have provided evidence that adenosine receptors mediating decreased glutamate release are coupled to a G protein but not to adenylate cyclase. They suggested, on the other hand, that the G protein indirectly blocks calcium influx. This suggestion is consistent with the findings of Hescheler et al. (1987) that G_o blocks a voltage-dependent calcium current in neuroblastoma cells. Interestingly, adenosine-induced inhibition of noradrenaline release also appears to be mediated through a G protein (Allgaier et al., 1987).

Whether the postsynaptic effects of adenosine are due to altered activity of adenylate cyclase is also uncertain in view of the evidence just mentioned. In addition, for other compounds there is evidence that receptor-mediated changes in adenylate cyclase activity do not mediate the changes in membrane properties which occur concomitantly. For example, like adenosine, serotonin and baclofen activate a potassium conductance in the hippocampal neurones (Andrade et al., 1986). Both of these latter compounds also decrease adenylate cyclase activity (DeVivo et al., 1986; Wojcik and Neff, 1984), but evidence from Nicoll's laboratory indicates the increase in potassium

conductance is not mediated via changes in adenylate cyclase activity (Andrade et al., 1986). A similar conclusion was reached concerning the muscarinic potassium current in the heart (Logothetis et al., 1987) which, like the receptor-mediated potassium conductance in the hippocampal neurones, is blocked by pertussis toxin (Pfaffinger et al., 1985; Sasaki and Sato, 1987).

Thus, it is possible that the effects of adenosine on ionic fluxes are separate from its effects on adenylate cyclase.

c) *P_2-receptors*. Burnstock and Kennedy (1985) have proposed a subdivision of P_2-receptors into two subtypes. This proposal was based largely on the rank order of potency of various agonists in different tissues. In support of a subdivision of the receptors they noted that the P_2-antagonist, $ANAPP_3$ (see Table 1), is not equally effective in all tissues and that in tissues where $ANAPP_3$ is most effective, responses to ATP can be diminished by desensitization using α,β-methyleneATP. The receptor type at which $ANAPP_3$ is an antagonist has been termed P_{2X} and P_{2Y} designates the other receptor. Originally, no specific antagonist of the P_{2Y}-subtype was proposed but recently Burnstock and Warland (1987) have provided evidence that reactive blue 2, a derivative of anthraquinone sulphoric acid, may be a specific P_{2Y}-antagonist.

In peripheral tissues, the P_{2X}-receptor appears to mediate the excitatory effects of ATP, whereas the inhibitory effects seem to occur via the P_{2Y}-receptor. Importantly, neither of the receptor subtypes appears to be associated with adenylate cyclase (Burnstock, 1978).

The question as to whether the peripheral subtypes of P_2-receptors exist in the central nervous system remains open at this time because pharmacological studies using specific agonists and antagonists are lacking at the present time. Interestingly, Fedan et al. (1985) have isolated from membranes of guinea-pig vas deferens two constituents which are labelled by $[^3H]ANAPP_3$. So it may soon be possible to raise antisera to these constituents and thus to investigate their occurrence in the central nervous system.

IV. ON ATP AND ADENOSINE AS CHEMICAL MEDIATORS OF SYNAPTIC TRANSMISSION IN THE DORSAL HORN

The first suggestion that an adenine nucleotide might be a chemical mediator of synaptic transmission was made by Holton and Holton (1954) based on their physiological and pharmacological studies of the vasodilatation caused by electrical stimulation of peripheral endings of sensory nerves. They stated "it is tentatively suggested that in certain sensory nerves, ATP is liberated by the arrival of the action potential at the nerve ending, and that it then either acts on a blood vessel (as in antidromic vasodilatation), or, in the central nervous system, as a synaptic transmitter". While this eloquent statement stands as a landmark in the history of the concept of purinergic transmission, it is important to examine the evidence which has accumulated since the time this statement was made to determine whether it remains possible to consider the possibility that ATP may be a neurotransmitter of primary afferent neurones.

The purpose of this section is first to provide a critical review of the evidence concerning this possible role for ATP and second, to present evidence which indicates that adenosine might also play a role in the regulation of neuronal activity in the dorsal horn.

i) *Presence.*

The fact that ATP and adenosine are ubiquitous within living cells precludes argument concerning their presence within neurones in the dorsal horn. Furthermore, as nerve terminals are known to be highly metabolically active these compounds are necessarily present there. However, as mentioned in section III, the mere presence of ATP and adenosine is insufficient to conclude that they are present in an intracellular location from which they can be released during synaptic transmission. As a first step in investigating the intracellular localization of ATP Alund and Olson (1979) have examined the binding of quinacrine within the spinal cord and elsewhere in the central nervous system. Their report was not extensive but they did find quinacrine-positive varicose fibres within the spinal cord noting them particularly within the substantia gelatinosa. Clearly, further studies are necessary

to document the extent of the labelled fibres, to look at the subcellular localization of the labelling and to determine the compound to which quinacrine binds within the labelled neurones.

Other histochemical evidence relating to purines in the spinal cord comes from Snyder's group (Braas et al., 1986). They incubated fixed sections of tissue with an antibody which binds adenosine but not adenine nucleotides or other purine nucleosides and nucleotides. They found that, in the dorsal horn and in discrete areas elsewhere in the central nervous system, the antibody labelled certain nerve cell bodies and fibres. Non-neuronal cells were not labelled and hence it seems that the labelling was not non-specific. However, while the antibody may specifically localize adenosine in the tissue sections, in the living animal this adenosine may be in the form of ATP or other nucleotides as the intracellular adenosine concentration is usually less than one-thousandth the concentration of ATP except in situations of hypoxia (Nordstrom et al., 1977; Rehncrona et al., 1978). Future studies on the subcellular localization of the adenosine-like immunoreactivity may indicate if the antibody is labelling adenosine (or adenine nucleotides) located at sites from which it could be synaptically released.

ii) *Release.*

The Holtons' original conclusion that ATP is released from peripheral endings of primary afferents was based on the fact that during electrical nerve stimulation which caused antidromic vasodilatation large amounts of adenine derivatives were collected in the venous effluent and that ATP mimicked the stimulation-evoked vasodilatation (Holton and Holton, 1954). The possibility that the released compounds came from sympathetic rather than from sensory nerves was eliminated because sympathectomy failed to abolish the release. Subsequent experiments using luciferin-luciferase, the firefly enzyme, confirmed that ATP was released and indicated further that the release of ATP did not occur after the primary afferents were cut and time was allowed for degeneration (Holton, 1959).

In view of the fact that ATP can be released from cells present in the vascular system such as platelets (for review see Gordon, 1986), it is important to determine whether the ATP measured by the Holtons was indeed released by primary afferent neurones or whether the primary afferents

caused release of ATP from another source. In this regard, White et al. (1985) have provided evidence consistent with the possibility that ATP is released from neurones in the dorsal horn. They found, using the luciferin-luciferase assay, that depolarizing concentrations of potassium caused a calcium-dependent release of ATP from synaptosomal preparations of the dorsal portion of the lumbar spinal cord. In addition, preparations from the dorsal cord released two to three times more ATP than did those from the ventral cord. In this study ATP was also released with depolarizing concentrations of veratridine (see also Yoshioka and Jessell, 1985). The veratridine-induced release was enhanced by lowering the calcium concentration; similar observations have been made concerning release of ATP from synaptosomal preparations elsewhere in the central nervous system (Potter and White, 1980). The explanation offered by the authors was that the veratridine-induced release might occur by a different mechanism than the potassium-induced release and they noted other studies in which release of GABA from brain slices did not require calcium (Benjamin and Quastel, 1972; Szerb 1979).

Unfortunately, in the study of White et al. synaptosomal preparations were not also taken from animals in which dorsal rhizotomies or spinal transections had been performed and hence it cannot be decided whether the source (assuming it is neuronal) of the released ATP is primary afferents, intrinsic or descending neurones. Thus, while the data of White et al. are consistent with the possibility that ATP may be released from central terminals of primary afferents, further studies are clearly necessary.

The possibility that adenosine, *per se*, is released in the dorsal horn is suggested from the recent report of Sweeney et al. (1986). In this study it was found that with synaptosomal preparations from the dorsal spinal cord there is a calcium-dependent release of adenosine upon stimulation with morphine; the effect was blocked by the opiate antagonist naloxone. It appears, in this case, that adenosine rather than ATP is released because the amount of adenosine collected is not decreased by blockers of ecto-5'-nucleotidase; a decrease in collection of adenosine would be expected if ATP is released. Release of adenosine from synaptosomes stimulated by potassium was not examined in this study. The morphine-induced release of adenosine was suggested as an explanation for the previously reported observation that administration of methylxanthines

at the spinal level antagonizes the antinociceptive effects of morphine (DeLander and Hopkins, 1986; Jurna, 1981; Sawynok et al., 1986).

Thus, in the dorsal horn as elsewhere in the central nervous system it appears that both adenosine and ATP can be released in a calcium-dependent manner. However, the source of the released purines and the functional significance of the release remains to be determined.

iii) *Electrophysiological effects*

a_1) *In vitro studies - adenosine.* Adenosine and its analogues have been found to cause depression of the monosynaptic reflex response in the ventral root evoked by electrical stimulation of the dorsal roots, in a preparation of isolated toad spinal cord (Phillis and Kirkpatrick, 1978). This effect occurs without a change in the DC potential recorded from the dorsal root suggesting that adenosine fails to cause a change in the membrane potential of the terminals of dorsal root fibres. In addition, there is no apparent change in the excitability of the postsynaptic, ventral horn neurones. These results are consistent with the suggestion that adenosine causes a presynaptic inhibition of the monosynaptic response and that this inhibition may be due to decreased excitation-secretion coupling in the terminals of the primary afferent fibres.

Subsequent studies using mammalian dorsal root ganglion (i.e. primary afferent) neurones in culture have shown that adenosine and structurally related analogues cause a decrease in voltage-dependent calcium currents recorded at the neuronal soma (Dolphin et al., 1986; MacDonald et al., 1986). This effect occurrs in the absence of any change in either resting membrane potential or resting membrane conductance. Thus, if the effects of adenosine observed at the soma of the dorsal root ganglion neurones also occur at the terminals, then the mechanism of the adenosine-induced presynaptic inhibition in the spinal cord may be a decrease in calcium conductance.

MacDonald et al. (1986) tested six different adenosine analogues and the rank order of potency was found to be different from that which would have been expected had the effect been mediated by either A_1 or A_2 receptors. In terms of the putative A_3 receptor, the order of potency resembled more closely that reported by Ribeiro and Sebastião (1986) than

that of Chin et al. (1985).

The possibility that adenosine may have a postsynaptic effect has been investigated using a slice preparation of rat spinal cord. It has been found that adenosine causes a membrane hyperpolarization of intrinsic neurones in the dorsal horn (Kangrga et al., 1987). This effect persists after blockade of synaptic transmission by lowering the extracellular calcium concentration and hence appears to be a postsynaptic effect. The hyperpolarization is accompanied by an increase in membrane conductance and the apparent reversal potential is less than -90 mV. Thus, the ionic mechanism of the hyperpolarization of dorsal horn neurones may be similar to the mechanism of the hyperpolarization caused by adenosine in the neurones in the hippocampus. This mechanism is an increased conductance to potassium.

It is interesting to note that with rat dorsal horn neurones in the spinal slices adenosine causes hyperpolarization whereas Jahr and Jessell (1983) have reported that rat dorsal horn neurones in culture are unaffected by adenosine. This difference may indicate that the cultured neurones lack functional P_1-receptors or that the uptake and degradation of adenosine is much greater in the cultures than in the slice preparation.

a_2) *In vitro studies - ATP.* Using an intracellularly perfused preparation Krishtal et al. (1983) provided evidence that ATP causes membrane depolarization in rat primary afferent fibres by increasing conductance to monovalent cations. ATP also causes depolarization and increases membrane conductance in cultured dorsal root ganglion neurones from the rat (Jahr and Jessell, 1983). In this case, however, the effects of ATP were blocked when sodium in the extracellular medium was 90% replaced with choline. Thus, the principal ionic mechanism may be increased conductance to sodium. The effect of ATP was not blocked by tetrodotoxin (TTX) and hence the sodium conductance activated appears to be different from the conductance responsible for the rising phase of the action potential. In the bullfrog, ATP causes depolarization of primary neurones but here the mechanism appears to be inactivation of a membrane potassium conductance (Morita et al., 1984). Thus, as mentioned in section III, there appears to be great variability in the ionic mechanism by which ATP causes depolarization. ATP also depolarizes terminals of

primary afferents in the isolated toad spinal cord (Phillis and Kirkpatrick, 1978).

The significance of these results in terms of the possibility that ATP might cause presynaptic inhibition is questionable because *in vivo* in cats ATP and slowly-degraded analogues fail to affect the excitability of primary afferent terminals (Stone, 1980), although Phillis and Kirkpatrick (1978) did find that ATP depolarizes terminals of primary afferents in the isolated toad spinal cord. Furthermore, whereas depression of synaptic transmission caused by administration of ATP in the central nervous system appears to be mediated by P_1-receptors (for review see Phillis and Wu, 1981), the ATP-induced depolarization of dorsal root ganglion neurones is not blocked by P_1-receptor antagonists (Jahr and Jessell, 1983).

Postsynaptic effects of ATP on rat dorsal horn neurones in culture were examined by Jahr and Jessell (1983). They found that administration of ATP caused depolarization of 27% of these neurones and the remainder were unaffected. As was the case with the dorsal root ganglion cells reported from the same study, the ionic mechanism of the effect on the dorsal horn neurones appears to be mainly a TTX-insensitive increase in conductance to sodium. It appears that the depolarization is caused by ATP, *per se*, because slowly-hydrolyzed analogues of ATP have the same depolarizing effect and because the effects of ATP and of the analogues are not blocked by P_1-receptor antagonists or by adenosine deaminase. The depolarization does not seem to be caused by chelation of calcium as disodium, calcium, magnesium and Tris salts of ATP are equally effective and calcium chelators such as EDTA had no effect. Chelation of calcium has been suggested as the mechanism of excitatory effects previously observed *in vivo* (Curtis et al., 1960; Galindo et al., 1967).

The fact that stable analogues of ATP are equally as effective as the nucleotide itself suggests not only that the excitation is not due to products of degradation of ATP but also suggests that the excitation is unlikely due to phosphorylation of membrane proteins or to metabolic effects of ATP. These findings are thus consistent with the possibility that the ATP-induced excitation is mediated by a membrane receptor such as the P_2-receptor proposed by Burnstock (1978). The receptor mediating the excitation of the cultured dorsal horn neurones is likely different from the P_{2X}-receptor proposed by Burnstock and Kennedy (1985) because

$ANAPP_3$ fails to antagonize this effect (Jahr, personal communication). As far as the P_{2Y}-receptor is concerned, this receptor consistently has been seen to increase a calcium-activated potassium conductance in peripheral tissue (Burnstock and Kennedy, 1985). Thus, if the depolarization of the cultured dorsal horn neurones is mediated through P_{2Y}-receptors then these must be linked to a different ionic mechanism than that to which they are linked in the peripheral tissue. On the other hand, it is possible that P_2-receptors on the dorsal horn neurones are different from either the P_{2X} or the P_{2Y} subtype found in the periphery.

b_1) *In vivo studies - adenosine.* The possibility that inhibitory effects of adenosine on synaptic transmission are due to changes in the excitability of primary afferent terminals was investigated by Stone (1980). He found that administration of adenosine hemisulphate or of AMP failed to affect the threshold for antidromic activation of primary afferent terminals in the dorsal column nuclei of the rat. This finding is therefore consistent with the lack of change in membrane potential and conductance reported with the *in vitro* studies of dorsal root ganglion neurones and of the toad spinal cord.

At the time of beginning this thesis project no *in vivo* studies had examined the effects of adenosine (or AMP) on single neurones at the sites of termination of somatic primary afferents, either in the spinal cord or in the brain stem. Subsequently, Salt and Hill (1983) have reported that neurones in the trigeminal nucleus caudalis (the so-called "medullary dorsal horn") of the rat are depressed by iontophoretic application of AMP. Neurones in the spinal dorsal horn of the cat have also been reported to be depressed by application of AMP (Salter and Henry, 1985a). In the latter paper, which is presented as Chapter 2 of this thesis, the effects observed in the spinal cord are discussed in relation to those seen in the brain stem.

b_2) *In vivo studies - ATP.* In view of the fact that somatic primary afferents terminate in the dorsal column nuclei as well as in the dorsal horn it is relevant to the present discussion that the effects of iontophoretic application of ATP on neurones in the cuneate nucleus were investigated by Krnjević's group (Galindo et al., 1967). Application of ATP caused either excitation or had no effect on these neurones.

Depressant or biphasic effects of application of ATP which have subsequently been seen elsewhere in the central nervous system (Phillis et al., 1979) were not observed in the cuneate nucleus. Interestingly, the proportion of neurones affected was related to the inputs the neurone received: approximately eighty percent of neurones with inputs from cutaneous afferents were excited by application of ATP whereas only twenty percent of units with proprioceptive inputs were affected. Thus, the results of Galindo et al., in the first physiological study of the effects of ATP in the somatosensory system, indicate that there may be functional specificity in the sensitivity of central neurones to adenine nucleotides.

Since beginning my thesis work on the effects of ATP on dorsal horn neurones two related studies have appeared. In the first, Salt and Hill (1983) investigated the effects of ATP on neurones in the trigeminal nucleus caudalis of the rat. The second study (Fyffe and Perl, 1984) appeared after my work on the effects of ATP had been submitted for publication (Salter and Henry, 1985a). In both of these studies experiments were done in cats but a major difference was that Fyffe and Perl examined the effects of ATP only in the superficial laminae of the dorsal horn whereas I have investigated neurones located throughout the dorsal horn. Several types of effects were observed in these studies and the significance of these effects will be discussed fully in Chapter 2. For the present discussion the most important observation, which was made in all three studies, was that application of ATP alters the rate of discharge of neurones in the dorsal horn, which is consistent with the possibility that ATP may act as a chemical mediator.

iv) *Binding sites for adenosine agonists and uptake inhibitors*

a) *Agonists.* In the hope of localizing and biochemically characterizing P_1-receptors in the central nervous system, the binding of analogues of adenosine which are resistant to enzymatic breakdown and to cellular uptake has been studied extensively (for example see Lee and Reddington, 1986a). Within the spinal cord, Goodman and Snyder (1982) used autoradiography and found high levels of binding of [^{3}H]N^6-cyclohexyladenosine ([^{3}H]CHA) in the dorsal horn particularly in the substantia gelatinosa. In view of the fact that [^{3}H]CHA appears to label binding sites corresponding to A_1 receptors (Bruns et al.,

1980), the results of Goodman and Snyder support the possibility that the dorsal horn contains high levels of this type of receptor.

In a more detailed investigation Geiger et al. (1984) quantitatively compared [^{3}H]CHA binding in the dorsal and ventral spinal cord; the maximum binding was forty percent greater in the dorsal cord. In another part of the study, injection of kainic acid into the dorsal part of the cord, which caused extensive loss of neurones but not of glia, greatly decreased the amount of [^{3}H]CHA binding. In addition, the amount of binding in the dorsal horn was not affected by dorsal rhizotomy or by spinal transection. While dorsal rhizotomy may not completely eliminate all primary afferents, these findings are consistent with the possibility that the [^{3}H]CHA binding sites, and thus possibly A_1 receptors, occur primarily on intrinsic neurones in the dorsal horn.

Recently, Choca et al. (1986) have provided evidence that there are agonist binding sites in the dorsal horn in addition to the putative A_1 sites. They proposed that these extra sites are A_2 receptors because they were labelled with [^{3}H]5'-N-ethylcarboxamide adenosine ([^{3}H]NECA), although this proposal may have been premature due to the lack of specificity of [^{3}H]NECA and the possibility that it may bind to a receptor different from those which have been previously characterized (Lee and Reddington, 1986a,b). Nonetheless, the results of Choca et al. are important because they raise the possibility that there may be more than one subtype of P_1-receptor in the dorsal horn. If in fact there is not a single type of receptor in the dorsal horn several points arise for further investigation, including determining which of the subtypes mediates the different effects of adenosine.

b) *Uptake sites.* Binding of [^{3}H]-labelled nitrobenzylthioinosine (NBI), an adenosine uptake inhibitor, has been used to localize and to characterize putative adenosine uptake sites in the central nervous system (Bisserbe et al., 1985; Marangos, 1984). In the spinal cord [^{3}H]NBI binding sites occur in both the dorsal and the ventral horns (Geiger and Nagy, 1985). It appears likely, therefore, that the dorsal horn contains uptake sites for adenosine. In view of the fact that uptake is the main means by which the action of adenosine is terminated (for review see Wu and Phillis, 1984) it may then be possible to use inhibitors of adenosine uptake in physiological experiments to prolong the effect of this

substance in the dorsal horn.

v) *Enzymes*

a) *FRAP*. The enzyme fluoride-resistant acid phosphatase (FRAP) (EC 3.1.3.1), has been found within the dorsal horn of all species examined and in rodents, the species most often studied, FRAP is most highly concentrated in the substantia gelatinosa (for review see Knyihar-Csillik and Csillik, 1981). FRAP is an intracellular enzyme which, unlike most acid phosphatases, is not found in lysosomes. Dodd et al. (1983) studied the specificity of FRAP and reported that it acted on 5'-mononucleotides of guanine, uridine and inosine but that AMP was not a substrate. In the periphery, this enzyme occurs in chromaffin and some other cells in which ATP is found in secretory granules (Knyihar-Csillik and Csillik, 1981; Dodd et al., 1983). Taking this evidence together Dodd et al. suggested that FRAP might function to segregate nucleotides within cells and that nucleotides may play some special role in cells containing FRAP. However, this enzyme is clearly different from the Mg^{2+}-ATPase which provides the electrochemical gradient responsible for uptake of ATP into granules; and cells, such as platelets, which do not contain FRAP also incorporate ATP into granules (Poisner and Trifaró, 1982). Therefore, FRAP is certainly not a marker for cells which might release ATP by exocytosis and the exact function of this enzyme remains to be determined.

In the dorsal root ganglia, activity for FRAP is confined to small diameter neurones and is found in about seventy percent of these (Dodd et al., 1983, 1984). In the dorsal horn, FRAP activity is found in nerve terminals particularly within the substantia gelatinosa (Coimbra et al., 1970). Nerve terminals containing FRAP activity appear to be primary afferents because dorsal rhizotomy eliminates enzyme activity in the dorsal horn (Coimbra et al., 1974; Dodd et al., 1983). The similarity in the distribution of FRAP activity and primary afferent nociceptors prompted the suggestion that FRAP activity occurs in this type of neurone and that FRAP may in some way be involved in nociception (Janscó and Knyihar, 1975; Knyihar and Csillik, 1977). However, until the function of FRAP is determined its specific role, if any, in spinal nociceptive mechanisms will remain unclear.

b) *Adenosine deaminase*. In recent years Nagy's group has used

immunocytochemistry to investigate the distribution of adenosine deaminase throughout the nervous system (Geiger and Nagy, 1986; Nagy et al., 1984a,b, 1986; Nagy and Daddona, 1985). They have found that adenosine deaminase-like immunoreactivity (ADA-LI) is distributed in neurones in various, discrete locations in the nervous system and on this basis have suggested that neurones containing ADA-LI might release adenosine as a neurotransmitter (Nagy et al., 1984b). In this regard it will be recalled that adenosine deaminase is the enzyme which degrades adenosine to inosine and hence, is functionally quite different from synthetic enzymes, such as glutamic acid decarboxylase (GAD), which have been used previously to localize neurones that might release other putative neurotransmitters, GABA in the case of GAD. Therefore, only if it can be clearly demonstrated that adenosine is synaptically released specifically from neurones which show ADA-LI will it be possible to use this as a marker for these neurones. Otherwise it is possible that ADA-LI-positive neurones have some other function, such as scavenging extracellular adenosine. Interestingly, Nagy et al. (1985) have found neurones showing ADA-LI are found in brain regions where the [^{3}H]NBI binding is highest. This correspondence is expected if a major function of neurones containing ADA-LI is to take up adenosine.

Despite the lack of certainty about whether neurones containing ADA-LI actually use adenosine as a neurotransmitter, it interesting to note that ADA-LI is found within a subpopulation of small diameter neurones in dorsal root ganglia (Nagy et al., 1984a). On the basis that the terminals of small diameter afferents are found mainly within the superficial laminae of the dorsal horn (Sugiura et al., 1986), the observation that ADA-LI is localized in small diameter primary afferents is consistent with another report that ADA-LI is found in nerve fibres primarily in lamina I and II of the dorsal horn (Nagy and Daddona, 1985). In view of the fact that ADA-LI is found in small diameter primary afferents and that some small diameter afferents are known to be nociceptors (Perl, 1984), it is possible that nociceptive primary afferents contain ADA-LI.

c) *5'-nucleotidase*. As described in section III, 5'-nucleotidase is an ecto-enzyme present in neuronal and glial membranes at or near synapses (Kreutzberg et al., 1986). Activity of 5'-nucleotidase in the spinal cord is present throughout the grey matter of dorsal and ventral horns (Suran,

1974b). This finding indicates that extracellular adenosine can be formed in an enzymatic reaction from AMP in the vicinity of spinal neurones.

Within the dorsal horn, the level of 5'-nucleotidase activity is highest in laminae I and II (Scott, 1967; Suran, 1974a) which is consistent with other reports that the highest levels of immunoreactivity for 5'-nucleotidase in the dorsal root ganglia are found in a subpopulation of small diameter neurones (Nagy and Daddona, 1985). The coincidence of these two observations raises the possibility that 5'-nucleotidase might be expressed in high levels on the terminals of nociceptive and other small diameter neurones. Further experiments will be required to decide between this possibility and the other main one that in laminae I and II the enzyme is present mainly on intrinsic neurones or on glia.

vi) *Summary*

Reviewing the literature one finds evidence consistent with the possibility that ATP and adenosine may be chemical mediators of synaptic transmission in the dorsal horn. The most important pieces of evidence which are presently known will be summarized briefly. First, ATP and adenosine can be released in a calcium-dependent manner from synaptosomal preparations derived from the dorsal horn. Second, ATP and adenosine have effects on both dorsal horn and primary afferent neurones. Third, biochemically identifiable structures related to the synthesis, binding and inactivation of these compounds are present in the dorsal horn. Undeniably, many relevant questions remain but it is hoped that answers to some of these might be found in this thesis.

V. RATIONALE FOR THE PRESENT STUDIES AND OVERVIEW OF THE THESIS

It is readily apparent from the preceding section that, although more than three decades have passed since the Holtons' original suggestion was made, the study of adenosine and ATP in the spinal dorsal horn is still in its infancy. At the time this thesis project was begun there was a paucity of definitive evidence that these compounds had any role in

synaptic transmission in the dorsal horn. However, five significant points motivated beginning this project. The first point was the evidence of the Holtons' which, although imperfect, provided the first clue that ATP might be a neurotransmitter in sensory pathways in the spinal cord. The second point was the study by Galindo, Krnjević and Schwartz on neurones in the cuneate nucleus which suggested that the sensitivity to ATP might depend on the function of the neurone. The third point was the work of Jahr and Jessell in tissue culture which also indicated that not all neurones are equally sensitive to ATP. The fourth point was the rapid growth of evidence, primarily from studies in the periphery by Burnstock and his colleagues, indicating that ATP has a physiological role in communication between excitable cells. Finally, studies in the cerebral cortex, primarily by the groups of Phillis and of Stone, suggested that the notion of purinergic transmission which had been developed for the periphery might be of relevance in the central nervous system as well.

For me, the Holtons' work was of particular significance because the phenomenon they studied, antidromic vasodilatation, appears to be mediated by small diameter primary afferent neurones (Hinsey and Gasser, 1930) and a large proportion of these neurones are nociceptive (for review see Perl, 1984). Therefore, the finding that ATP release occurred with antidromic vasodilatation plus the evidence that only a proportion of neurones are excited by ATP raised the possibility that ATP might be a excitatory chemical mediator in nociceptive pathways in the spinal cord.

Investigation of this possibility began with a study of the effects of iontophoretically applied ATP on dorsal horn neurones *in vivo*. This study is reported in Chapter 2; AMP was also tested for reasons described therein. On the basis that, in the studies of Galindo et al. and of Jahr and Jessell, not all neurones appeared to be equally responsive to ATP, it was considered possible that neurones in the dorsal horn might show differences in sensitivity to ATP related to the functions of these neurones. Of course there is no *a priori* reason why the chemical sensitivity should necessarily be related to function. In fact, however, in the case of ATP the experimental data revealed that the responses were well correlated with the functional properties of the neurones.

Using the experimental data, a hypothesis was made concerning which

physiologically elicited response of dorsal horn neurones might be mediated by ATP. As will become apparent, the simple idea which lead to doing these experiments (i.e. that ATP might be an excitatory mediator in spinal nociceptive pathways) was radically altered on the basis of the experimental results and it was suggested that ATP might be released from low threshold primary afferents. Included in this suggestion was the possibility that ATP, via extracellular conversion to adenosine, might mediate the depressant effects of low threshold inputs which had been described previously (Hillman and Wall, 1969).

With presently available pharmacological tools it is only possible to test *in vivo* the part of the hypothesis suggesting that the depressant effects of low threshold inputs might be mediated by adenosine. The evidence obtained was found to be consistent with this suggestion, as described in Chapter 4. In that chapter, the physiologically elicited response used to test the hypothesis was the response to cutaneously applied vibration. Before using this response it was necessary, first, to determine if this was the type of response which might be consistent with the hypothesis. Therefore, the physiological characteristics necessary to make this determination were examined as described in Chapter 3. Later, the parameters which control the response to vibration were investigated (Chapter 5). From the results of this investigation it was concluded that a subset of the vibration-induced responses are mediated by a specific class of primary afferents, the Pacinian corpuscle afferents.

During a few experiments in which the effects of ATP were being studied, neuronal responses to the tachykinin, physalaemin, (Erspamer et al., 1964) were also investigated. [Responses to tachykinins were being studied because another member of the tachykinin family, substance P, had been previously implicated as an excitatory mediator in spinal nociceptive pathways (Henry, 1976).] In the course of the experiments a novel observation was made: the magnitude of the depressant response to application of ATP was greater when the application was preceded by administration of physalaemin. Because the results had raised the possibility that purines might have a physiological role in the dorsal horn, a thorough investigation of the effects of tachykinins on purine-induced responses was made (Chapter 6). Then, when the studies on the vibration-induced responses indicated that the depression might be

mediated by purines, I felt compelled to examine the effects of tachykinins in responses to vibration (Chapter 7).

In an attempt to synthesize the results of this thesis and to incorporate relevant data from other studies in a coherent fashion, a theoretical model is tentatively suggested in Chapter 8. The main purpose of this model is, as Popper (1959) has suggested, to provide a falsifiable hypothesis which can be tested through future experiments.

CHAPTER 2

EFFECTS OF ADENOSINE 5'-MONOPHOSPHATE AND ADENOSINE 5'-TRIPHOSPHATE ON FUNCTIONALLY IDENTIFIED UNITS IN THE CAT SPINAL DORSAL HORN. EVIDENCE FOR A DIFFERENTIAL EFFECT OF ADENOSINE 5'-TRIPHOSPHATE ON NOCICEPTIVE VS NON-NOCICEPTIVE UNITS

"can we suppose that the discovery and identification of a chemical transmitter of axon-reflex vasodilatation would furnish a hint as to the nature of the transmission process at a central synapse? The possibility has at least some value as a stimulus to further experiment."

Dale, 1935

I. ABSTRACT

A study was done of the effects of iontophoretic application of adenosine 5'-monophosphate (AMP) and adenosine 5'-triphosphate (ATP) on functionally identified neurones in the spinal dorsal horn of the cat. AMP depressed nearly two-thirds of the 32 neurones tested regardless of functional type; the remainder were unaffected. ATP, on the other hand, had three types of effect: depression, excitation and a biphasic effect which consisted of excitation followed by depression. A significant difference was found when a comparison was made of the frequency of occurrence of each of these three types of effect in the samples of non-nociceptive (n=18) and of wide dynamic range neurones (n=42): of non-nociceptive neurones 61% were excited, 11% were depressed, 6% had a biphasic response, and 22% were unaffected; of wide dynamic range neurones 45% had a biphasic response, 19% were depressed, 14% were excited and 21% were unaffected (χ^2=16.2, P=0.001). The depressant effects of both AMP and ATP and the depressant phase of the biphasic effect of ATP seem to be mediated through activation of P_1-purinergic receptors because these effects were blocked by theophylline, a P_1-purinergic antagonist (Burnstock, 1978).

Thus the biphasic effect appears to consist of excitatory and depressant responses in the same neurone. The differential effects of ATP on non-nociceptive vs wide dynamic-range neurones are similar to the differential effects on these neurones observed during activation of low threshold primary afferents. This similarity, together with evidence that ATP might be released from primary afferent neurones (Holton & Holton, 1954; Holton, 1959), prompts us to suggest that ATP may be a chemical mediator of effects of low threshold primary afferent inputs in the spinal dorsal horn.

II. INTRODUCTION

Holton and Holton (1954) have shown that adenosine 5'-triphosphate (ATP) is released into the circulation following electrical stimulation of sensory nerves in the ear of the rabbit. Based on this evidence they concluded that ATP is released from the peripheral ends of primary sensory neurones and suggested that it might be released also from the central ends of these neurones, to function as a neurotransmitter. Since then abundant evidence has been presented that adenosine and its nucleotide derivatives may be important chemical mediators of synaptic transmission in the peripheral autonomic nervous system (Burnstock, 1972; 1981) and in the central nervous system (Phillis and Wu, 1981; Stone, 1981).

A possible role for ATP in chemical transmission at the first synapse in sensory pathways is further suggested by evidence that *in vivo* iontophoretic application of ATP excites neurones in the trigeminal nucleus caudalis (Salt and Hill, 1983) and in the cuneate nucleus (Galindo et al., 1967), and that *in vitro*, ATP depolarizes cultured dorsal horn neurones (Jahr and Jessell, 1983). Furthermore, with synaptosomal preparations from the dorsal spinal cord, there is a calcium-dependent release of ATP in the presence of depolarizing concentrations of potassium (White et al. 1985). Both of these studies showed that release of ATP from synaptosomes from the dorsal spinal cord is significantly greater than the corresponding release of ATP from synaptosomes derived from the ventral spinal cord.

Burnstock (1978) has distinguished between P_1- and P_2-purinergic receptors: adenosine and adenosine 5'-monophosphate (AMP) are much more potent activators of P_1-receptors than is ATP, whereas the order of potency is reversed for P_2-receptors. On the other hand, Bruns (1980) has demonstrated that the increase in intracellular cyclic AMP levels in neuroblastoma cells caused by ATP and AMP requires the conversion of these nucleotides to adenosine before receptors can be activated.

The terminals of primary afferent neurones in the spinal dorsal horn contain enzymes which may be involved in processing of nucleotides (Coimbra et al., 1970; Dodd et al., 1983; Dodd and Jessell, 1982; Knyihar and Csillik, 1977; Suran, 1974a,b), and hydrolysis of ATP has been

suggested as a necessary step in the ATP-induced depression of neurones in the cerebral cortex (Phillis et al., 1979). The levels of these enzymes are highest in the substantia gelatinosa (Coimbra et al., 1970; Knyihar and Csillik, 1977; Suran, 1974b) and identical enzymes are located in small neurones in dorsal root ganglia (Dodd et al., 1983; Nagy and Hunt, 1982). The substantia gelatinosa also contains high levels of binding sites for an analogue of adenosine, [^{3}H]cyclohexyladenosine, possibly indicating the presence of high levels of P_1-purinergic receptors (Goodman and Snyder, 1982). These observations, together with evidence that sensory fibres might release ATP, suggest that ATP and/or its related nucleotides or nucleosides may be involved in spinal mechanisms of sensation. To investigate this possibility a study was done of the effects of iontophoretic application of ATP and of AMP on functionally identified neurones in the dorsal horn of the spinal cord. The effects of citrate were also investigated because excitatory effects of ATP have been attributed to chelation of calcium (Galindo et al., 1967; Phillis et al., 1979) and the affinities for calcium of ATP and citrate are similar (Curtis et al., 1960). Preliminary results of this study have been previously reported (Salter and Henry, 1984a,b).

III. MATERIALS AND METHODS

i) *Animal preparation*

Experiments were done on 36 adult cats anaesthetized with alpha-chloralose (60 mg/kg i.v.) after induction with halothane (Somnothane, Hoechst). The carotid artery and the jugular vein were cannulated and a tracheal cannula was inserted via a tracheostomy.

The spinal cord was exposed in segments L1 and L5-L7 by laminectomy. The influence of supraspinal structures on the activity of dorsal horn cells was eliminated by transection of the spinal cord at the caudal end of the L1 segment; before transection, to minimize spinal shock, the L1 segment was cooled with topical application of ethyl chloride and/or was injected with 0.1-0.15 mL of 1.0% lidocaine hydrochloride.

Arterial pressure measured using a Statham pressure transducer

connected to the carotid catheter was displayed continuously on a Grass Model 5 polygraph. If the mean arterial pressure fell below 90 mm Hg, the animal was resuscitated with intravenous fluid (normal saline and/or 10% dextran) or by i.v. infusion of noradrenaline bitartrate (Levophed, Winthrop; 0.002% in normal saline).

The cats normally breathed spontaneously and end-tidal CO_2 concentration (measured using a Beckman LB2 Medical Gas Analyzer) was 3.5-5.0%. However, if the breathing pattern became irregular or if respiratory arrest occurred, the animal was paralysed with pancuronium bromide (Pavulon, Organon; 1 mg/kg i.v.) and mechanically ventilated.

Movement of the spinal cord at the recording site was minimized by clamping the L4 vertebra and the ischia tightly in a spinal frame, by firmly fixing the L5-vertebral body with lateral stabilizing bars and by securing the head in a head holder. Then the spinal cord was covered with a pool of warm mineral oil (38°C) to prevent drying and cooling. The fur on the hindlimb ipsilateral to the site of recording was cut unevenly to permit mechanical and heat stimulation of the skin surface while leaving some hairs which could be moved.

Rectal temperature was maintained around 38°C with a thermistor-controlled servomechanism. The spinal cord circulation was checked periodically throughout the experiment with a Zeiss dissecting microscope.

ii) *Recording*

Seven-barrelled micropipettes (overall tip diameter 4-8 μm) were used for recording extracellular single-unit spike activity and for iontophoresis. The central recording barrel was filled with 2.7 M NaCl (impedance 5-10 MΩ at 100 Hz in saline) and was connected via a high-impedance, unity-gain headstage to a Tektronix RM-122 amplifier and then to Tektronix 565 and 5111 oscilloscopes. Selected sweeps were recorded on Polaroid film from the storage oscilloscope (5111) with a Tektronix C-5B camera; the continuous discharge was recorded on Kodak Linagraph paper from the 565 oscilloscope using a Grass C4-K Kymograph camera.

The output from the vertical plate of the 565 oscilloscope was connected to a gating unit which served three functions: audio

monitoring, amplitude discrimination and frequency counting. Spikes were electronically counted by appropriate adjustment of the amplitude discriminator and the output of the frequency counter was connected to the Grass 5 polygraph to display continuous-time histograms of the spike activity (bin size 1 s).

Recording was done at the L5-L6 level. The electrode was inserted into the spinal cord 0.5-1.5 mm medial to the dorsolateral sulcus through a small hole made in the pia and arachnoid membranes. The electrode was advanced and retracted in 2 μm steps with a Burleigh Inchworm system. The Inchworm translator was held in an LPC micro-manipulator to permit movement of the electrode in three orthogonal directions.

iii) *Iontophoresis and solution*

Each of the outer barrels of the micropipette was filled with one of the following solutions: sodium L-glutamate (Sigma; 1.0 M, pH 7.4), disodium adenosine 5'-triphosphate (Sigma product no. A5394 "vanadium-free"[1]; 0.2 M, pH 7.0), sodium adenosine 5'-monophosphate (Sigma; 0.2 M, pH 7.0), theophylline (Sigma; 20 mM in 165 mM NaCl, pH 6.0), calcium chloride (0.1 M, pH 7.0), sodium citrate (0.2 M, pH 7.0) or control solution (165 mM NaCl, pH 7.0). All solutions were prepared using deionized water, stored frozen in aliquots and then thawed immediately prior to the experiment. L-Glutamate (glutamate), ATP, AMP and citrate were ejected using inward current; theophylline and calcium were passed with outward current. Retaining currents of 10 nA with the polarity opposite to that used to make ejections were maintained between applications to counteract leakage from the pipettes. The control solution was used to detect artifacts due to the local changes in current density around the electrode tip and for automatic current balancing in some experiments. The currents used during the ejection periods were produced by an IDS1 constant current iontophoresis unit manufactured by Cocyen Co. Ltd., Pointe Claire, Quebec.

The effects of ATP and AMP were tested on the on-going and on the

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[1] Vanadium compounds have been found to contaminate many commercial ATP samples and these compounds can affect ATPases and adenylate cyclase (Jandhyala & Horn, 1983). Therefore we used ATP that was essentially "vanadium-free".

glutamate-evoked activity, as well as on responses to natural and electrical stimulation within the cutaneous receptive field. The effects of the two compounds were compared between the different functionally defined groups.

iv) *Classification of units*

The following scheme (based upon those of Henry (1976) and Price and Dubner (1977)) was used to classify each unit's responses to different natural peripheral stimuli: a brisk response to a low-flow air stream or to a movement of single hairs was called a "hair" response; a brisk, rapidly adapting response to light mechanical stimulation of the skin in the absence of a hair response was called a "touch" response; a rapid onset, slowly adapting response to firm but innocuous mechanical stimulation without hair, touch or proprioceptive (see below) responses was called a "pressure" response; a response to passive movement of the hindlimb without responses to cutaneous stimulation was called a "proprioceptive" response; a brisk response to pinching the skin with a serrated forceps, followed by a prolonged afterdischarge was called a "nociceptive mechanical" response; a delayed onset, rapidly increasing response followed by a prolonged afterdischarge when the receptive field was heated with a 250 W infrared bulb was called a "nociceptive heat" response (in some experiments, a thermistor probe was placed in contact with the cutaneous receptive field; the maximal skin temperature during "nociceptive heat" responses always equalled or exceeded 43^{o} C). Responses to hair movement and/or to touch and pressure stimuli constituted the group of cutaneous non-nociceptive responses.

Responses to electrical stimulation of the receptive field were examined for each unit with a cutaneous receptive field. Bipolar stimulating electrodes, separated by 5 mm, were inserted into the receptive field. The electrodes were connected to Grass SD9 stimulator which was set to deliver single pulses 150 μs in duration. The magnitude of the pulses was gradually increased from 0.1 V to determine the threshold for the earliest spike response. Threshold was considered to be the stimulus intensity at which a response was observed in 5 of 10 trials.

Responses were examined to greater stimulus voltages to a maximum of 20 times threshold or 100 V, whichever was less.

Each unit was classified into one of the following functional groups according to its profile of responses to peripheral stimuli: wide dynamic range (WDR) units had both nociceptive and non-nociceptive responses; nociceptive specific units had only nociceptive responses; non-nociceptive units had only non-nociceptive responses; proprioceptive units had only proprioceptive responses.

IV. RESULTS

Recordings were made from 91 dorsal horn units of which 73 are included because all of the following criteria were satisfied: testing of responses to peripheral stimulation was complete; mean arterial pressure was 90 mm Hg or greater; to eliminate recordings from fibres, the unit was excited by application of glutamate. Forty-six of these units were wide dynamic range, 21 were non-nociceptive, 2 were nociceptive specific, 2 were proprioceptive and 2 had no detectable peripheral input.

The characteristics of the responses to natural stimulation of the receptive fields were similar to those reported in previous studies (Henry, 1976; Price and Browe, 1973; Price and Dubner, 1977; Willis et al., 1974). The relative number of each functional type of neurone was not different in the samples tested either with ATP (n=66) or with AMP (n=32). For each unit ATP and AMP were applied initially with low currents (5-30 nA) for short time periods (15-20 s); higher currents (up to 150 nA) and longer application periods (up to 2 min) were used with unresponsive units before there was considered to be no effect. Effects were considered genuine responses if they were reproducible, reversible and not mimicked by control current.

i) *Effect of AMP*

Application of AMP caused depression of 20 of the 32 neurones tested; the remainder were unaffected. This depression was manifested by a

decrease in the on-going rate of discharge, in glutamate-evoked activity and/or in responses to peripheral stimulation. The depressant responses to AMP were seen with iontophoretic currents of 15-150 nA. Excitatory responses were not observed even at the highest ejecting currents. The magnitude of depression varied directly with the ejecting current and with the duration of ejection. The onset was delayed (30-90 s), maximal depression occurred 30-90 s after the end of ejection and full recovery occurred gradually over the 2-5 min after the end of application. With some neurones there was a reversible increase in spike amplitude of 10-20% when the depression was maximal, but in most cases spike amplitude remained constant during the effect of AMP.

An example of the depressant effect of AMP on the response to noxious cutaneous heat applied to the receptive field is shown in Fig. 1. Depression began near the end of the 90 s application of AMP and was deepest 30-60 s after the end of ejection; 3 min later the responses to the noxious heat stimuli had clearly returned to control levels. While there was a minor decrease in background activity, the effect on the heat-evoked activity was greater. In particular, the maximal firing rate and duration of the after-discharge were decreased.

A similar time course of depression was seen when AMP was tested on the activity induced by glutamate. Seventy second applications of AMP were tested on the response to glutamate, as shown in Fig. 2. AMP failed to cause depression when ejecting currents of 60 nA or less were used. However, within 5 s of the end of ejection of 80 or 120 nA of AMP the responses to glutamate were diminished and the smallest responses were observed 30-60 s later. Full recovery from the 80 nA dose occurred 30-60 s after the maximal depression. After the 120 nA dose, responses to glutamate failed to return completely to control levels.

(Note: During the applications of AMP shown in Fig 2 a commonly occurring artefact of iontophoresis was encountered which is fully described in the figure legend. This artefact should not be confused with real effects on neuronal excitability. Thus neither this neurone, nor any other, showed excitation caused by AMP.)

ii) *Effects of ATP*

Three effects of ATP applications were observed: 11 neurones were depressed, 20 were excited and 20 had a biphasic response which consisted of excitation followed by depression. Fifteen neurones were unaffected by ATP ejection. The total population of neurones studied included 42 wide dynamic range, 18 non-nociceptive, 2 nociceptive specific and 2 proprioceptive neurones, as well as 2 neurones for which a peripheral input was not detected.

The depressant effect of ATP application was observed with ejecting currents of 25-100 nA. This effect began 30-60 s after the start of ejection and was maximum 15-120 s after the end of application and there was gradual recovery over 1-3 min after the maximum effect. ATP depressed on-going discharge, glutamate-evoked activity and responses to natural and electrical stimulation of the receptive field. An example of the depressant effect of ATP is illustrated in Fig. 3. Responses of this WDR neurone to periodic stimulation of the receptive field by an air stream began to decrease 30-60 s after the beginning of ATP application, the lowest levels of the air-induced response occurred about 90-120 s later (i.e. 2 min after ejection was stopped) and there was a gradual recovery over the subsequent 2-3 min.

In terms of excitation, application of ATP potentiated the response of the non-nociceptive neurone illustrated in Fig. 4 to electrical stimulation of its receptive field. In general, the rate of development of excitation and its duration upon termination of the current varied directly with the magnitude of current (for the neurones studied the time delay before excitation was observed and the duration of the effect after the end of ejection ranged from 5 to 30 s). Usually spike amplitude was unchanged during excitatory responses but in some cases, especially when high ejecting currents were used, a progressive, reversible decrease in spike amplitude occurred during excitation; this effect was not mimicked by current control.

The biphasic effect of ATP application consisted of an initial excitatory phase which began 5-30 s after the start of application and lasted 10-45 s. Excitation was followed by depression which was maximum 15-90 s following the end of ejection. Full recovery occurred gradually

over 1-5 min after the end of ejection. An example of the biphasic effect of ATP is shown in Fig. 5. The firing rate of this spontaneously active WDR neurone began to increase within 5 s of the beginning of ejection; increased firing persisted for 40 s after the end of application and was followed by depression of firing which gradually returned to the control level over the subsequent 2 min (note that the rate of discharge failed to return completely to the control level after the end of the first application). In some neurones such as the one illustrated in Fig. 5, there was variability in the magnitude of either the excitatory or the depressant phases. Despite this variability, the qualitative character of the biphasic response was preserved for up to several hours of repeated application. The relative magnitudes and time courses of the excitatory and depressant phases varied from one neurone to another. No correlation was found between the magnitude of the excitatory phase and the duration of the depressant phase; such a correlation would have been expected if the depressant phase was a post-excitatory depression.

Importantly, biphasic responses did not develop in neurones which initially showed only excitatory or depressant effects and therefore the three classes of effects were distinct.

iii) *Comparison of effects of AMP and ATP*

Twenty-five neurones were tested both with AMP and with ATP. A statistically significant relationship (χ^2=7.94 with 3 degrees of freedom, $p<0.05$) was found between the effects of AMP and of ATP. In particular, all 7 neurones depressed by ATP were depressed by AMP. In 6 cases the depression caused by AMP was more pronounced that that caused by ATP ejected with the same current (for example see Fig. 6), while the other neurone was depressed more deeply by ATP. The time courses of the depressant responses to both compounds were similar: the onset of each was delayed, maximal depression occurred after the end of ejection and there was prolonged, gradual recovery.

iv) *Correlation of responses to AMP and ATP with functional type*

Numbers of WDR and non-nociceptive neurones responding to AMP are compared in Table 1. This comparison shows that the proportions of

neurones depressed by AMP was not significantly different in the two groups.

In contrast to AMP, ATP had a differential effect on WDR vs non-nociceptive neurones. Table 2 shows that the frequency of occurrence of the different effects of ATP is significantly different ($p=0.001$) between the two groups: the predominant effect on WDR neurones was the biphasic response whereas that of non-nociceptive neurones was excitation. Table 2 also shows that the distribution of the different types of response in the group of WDR neurones was much less skewed towards the biphasic response than was the distribution of responses of non-nociceptive neurones toward excitation.

v) *Effect of theophylline on responses to AMP and ATP*

Iontophoretically applied theophylline (20-150 nA) wholly or partially blocked the inhibitory response to AMP in each of four trials. In one case the response to AMP gradually diminished over a 40 min period of application of theophylline and there was no recovery of the response to AMP during the subsequent hour (spike amplitude and heat-evoked responses were carefully monitored and remained relatively constant throughout the recording period). In the other 3 cases theophylline was applied for only 5-20 min; in these instances there was rapid recovery of the inhibitory response to AMP after the end of theophylline application.

In the first case above, ATP was also tested and it caused depression when tested prior to application of theophylline. This depressant response to ATP was absent, however, 30 min after the start of theophylline ejection and 30 min after the end of the theophylline application ATP caused excitation. This post-theophylline excitation was similar in its time course to the excitatory responses seen without theophylline pre-treatment.

The effect of theophylline on the biphasic response to ATP was tested in 2 cases: with one neurone there was no effect of theophylline, with the other the inhibitory phase was absent 16 min after the start of theophylline application and began to return 5 min after the termination of theophylline.

vi) *Effect of citrate*

Citrate (25-150 nA) excited 15 of the 18 neurones tested; the remainder were unaffected. The excitatory effect of citrate on responses to electrical stimulation of the cutaneous receptive field is illustrated in Fig. 4. At ejecting currents of 90 and 150 nA excitation began within 1-3 s of the start of citrate application and ceased within 2-6 s of its termination. Excitation developed more slowly with 50 nA of ejecting current but again the responses to electrical stimulation returned rapidly to control levels at the end of citrate application. Figure 4 also shows that depression following excitation was a variable finding. This type of depression is also illustrated in Fig. 3: depression of responses to periodic stimulation of the receptive field by an air stream began almost immediately following the end of citrate ejection and full recovery occurred within 45 s. The effect of citrate was more rapid than the effect of ATP but was slightly slower than that of glutamate.

Eight of 9 WDR and 6 of 8 non-nociceptive neurones were excited by citrate (χ^2 (corrected)=0.01, with one degree of freedom P>0.50). Thus citrate did not have the differential effect on WDR vs non-nociceptive neurones found with ATP. Citrate excited the one neurone tested which had no identifiable peripheral receptive field.

V. DISCUSSION

This study has demonstrated that the iontophoretic application of AMP and ATP can change the activity of neurones in the spinal dorsal horn. AMP caused depression of nearly two-thirds of the neurones tested. Effects of ATP were more diverse, consisting of depression, excitation or a biphasic effect.

i) *Depressant responses to AMP*

Application of AMP caused depression of on-going and glutamate-evoked activity as well as responses to peripheral stimulation. Excitatory and biphasic responses which have been reported in the cerebral cortex

(Phillis et al., 1979) and in the olfactory bulb and the caudate nucleus (Kostopoulos and Phillis, 1977) were not observed. Inhibitory responses occurred in proportions of WDR and of non-nociceptive neurones which failed to be statistically different, and therefore AMP did not seem to have a differential effect on either type of neurone. Blockade of AMP-induced depression by theophylline, a P_1-purinergic antagonist, suggests that the effect is mediated through P_1-purinergic receptors. Depression of glutamate-evoked activity may indicate that AMP acts directly on dorsal horn neurones because glutamate-induced excitation is primarily a direct postsynaptic effect (Puil, 1983; Shapovalov et al., 1978); however, this finding is not an entirely reliable means of defining the locus of action of AMP.

Some adenosine may have been formed from AMP after it is ejected from the pipette (Stefanovic et al., 1976) and this adenosine may be required to cause depression (Bruns, 1980); alternatively, both AMP and adenosine might be active (Burnstock, 1978; Moody et al., 1984). In either case conversion of AMP to adenosine is probably an important, possibly necessary, step in the development of the depressant response of dorsal horn neurones to application of AMP. Iontophoretic application of adenosine was attempted in 4 experiments. However, in each of many different micropipettes the resistance of the barrel containing adenosine was too great to permit useful current ejection even though the concentration of adenosine was well below the limit of solubility. Thus further investigation is required to determine if conversion of AMP to adenosine is essential for the effect.

ii) *Responses to ATP*

ATP depressed 17% of the neurones tested. This effect was similar to that of AMP in that the time courses of both were essentially identical. Interestingly, both responses could also be blocked by theophylline. Thus the depressant effect of ATP may be mediated through activation of P_1-purinergic receptors. As extracellular conversion of ATP to AMP and then to adenosine can occur (Bruns, 1980), this conversion may have occurred in cases where ATP produced depression. If this is the case, then only adenosine might be active in producing depression (Phillis et

al., 1979). Alternatively ATP may have produced depression directly, but because both adenosine and AMP are more potent activators of P_1-receptors than is ATP (Burnstock, 1978; Moody et al., 1984) the direct depression by ATP may have been a relatively minor component of the response in comparison to the depression induced by the AMP and/or adenosine which were derived from the exogenously applied ATP. Either possibility is supported by our observation that the depressant effect of ATP was generally less profound than that of AMP whenever the two were tested on the same neurone with identical ejecting current and duration.

A small amount of non-enzymatic hydrolysis of ATP would be expected to occur within the micropipette at body temperature (Seki and Hayashi, 1982). This spontaneous hydrolysis would lead to the release of small amounts of AMP from the pipette along with the ATP. Thus released AMP may have accounted for the very small numbers of depressant or biphasic responses of non-nociceptive neurones.

Thirty percent of the neurones were excited by ATP. Similar excitatory effects of ATP have been observed by others in the trigeminal nucleus caudalis (Salt and Hill, 1983) and in the cuneate nucleus (Galindo et al., 1967). Salt and Hill (1983) did not report a preferential excitation of non-nociceptive neurones in the trigeminal system similar to that which we have found in the spinal cord (*vide infra*). Galindo et al. (1967) reported that prolonged (but not short-term) excitation in the cuneate nucleus was associated with low pH (2-3) of the solution in the pipette. However, pH effects fail to explain the prolonged excitatory effect we observed because the pH of our ATP solution was always 7.0 and the excitatory effect was never mimicked by current and pH controls.

Excitatory effects of ATP on central neurones have been attributed to chelation of extracellular calcium (Galindo et al., 1967; Phillis et al., 1979) because compounds which chelate calcium (of which ATP is one) excite neurones when applied iontophoretically (Curtis et al., 1960; Galindo et al., 1967; Phillis et al., 1979; Salt and Hill, 1983). In our experiments the excitatory response to citrate, which is also a calcium-chelator, often began more rapidly and ended sooner than did the excitatory response to ATP. In addition, citrate-induced excitation was often followed by depression which began immediately after the end of ejection and which

recovered rapidly. Citrate failed to show a differential effect on WDR vs non-nociceptive neurones and in preliminary experiments we have found that the effect of citrate seems to be blocked more easily by concomitant application of calcium than is the excitatory effect of ATP. Thus it appears that the excitatory response to citrate may have been elicited via a different mechanism than that mediating the excitatory or biphasic effects of ATP. These findings indicate that calcium chelation may account for only part of the mechanisms underlying the excitatory effect of ATP and they support results which have shown that excitatory effects of ATP may be independent of calcium chelation (Jahr and Jessell, 1983; Phillis and Kirkpatrick, 1978; Siggins et al., 1977). Also, the excitatory effect of ATP is unlikely to be mediated by P_1-purinergic receptors because a neurone in which the depressant responses to AMP and ATP had been abolished by theophylline then showed an excitatory response to ATP. Others have reported that the excitatory effect of ATP is unaffected by theophylline and by other P_1-antagonists (Jahr and Jessell, 1983; Phillis et al., 1979).

A biphasic effect of application of ATP was observed with 30% of the neurones tested. Biphasic effects of ATP have been observed in other laboratories with iontophoretic application *in vivo* onto neurones in the cerebral cortex (Phillis et al., 1979) and the trigeminal nucleus caudalis (Salt and Hill, 1983), and with application of ATP onto isolated heart cells (Goto et al., 1977; Flitney and Singh, 1980) and oocytes (Lotan et al., 1982). In each of these systems the biphasic effect invariably was short-latency excitation followed by depression and hence these biphasic effects are similar to the one we have observed in the dorsal horn. In one of our two trials, as in the heart cells (Burnstock and Meghji, 1981) and in the oocyte (Lotan et al., 1982), the inhibitory phase was blocked by theophylline, indicating that this phase was a P_1-effect. These findings suggest that the biphasic effect consists of excitatory and depressant responses from the same neurone.

Fyffe and Perl (1984) have recently reported effects of ATP on neurones in the superficial dorsal horn. While their results are generally consistent with our hypothesis that ATP might be a chemical mediator of synaptic transmission released specifically from low threshold

primary afferent neurones they found that excitatory effects of ATP were associated with neurones receiving inputs from low threshold axons conducting in the range of C-fibres; we observed no such association, nor has this been reported in other studies (Galindo et al., 1967; Salt and Hill, 1983). Furthermore, Fyffe and Perl did not report depressant or biphasic effects of ATP which are commonly observed with central neurones (Phillis et al., 1979; Phillis and Kirkpatrick, 1978; Salt and Hill, 1983). These differences between the results of Fyffe and Perl on the one hand and our own results, as well as those of others, on the other hand, may be related at least in part to the particular method of recording and iontophoresis used by Fyffe and Perl, which was a single-barrelled electrode with a tip diameter of <0.1 μm, used for both recording and iontophoresis.

iii) *Significance of the differential effects of ATP on wide dynamic range vs non-nociceptive neurones*

To us, the most important finding of our study was the differential effects of ATP on WDR vs non-nociceptive neurones; i.e. that non-nociceptive neurones were mostly excited by ATP while WDR neurones showed depressant and/or excitatory effects This differential responsiveness suggests that the activity of non-nociceptive and WDR neurones might be differentially regulated by ATP. Interestingly, differential regulation of activity in non-nociceptive and nociceptive neurones is a cornerstone of the "gate theory of pain" (Melzack and Wall, 1965). Melzack and Wall proposed that the interaction of input from large and small diameter primary afferents is critical in regulating the activity of nociceptive neurones in the dorsal horn. Indeed the differential effects on dorsal horn neurones of iontophoretically applied ATP are similar to those reported with activation of low threshold primary afferent inputs: non-nociceptive neurones are excited while WDR neurones show excitation, depression or a biphasic response (Hillman and Wall, 1969). The similarity of the effects of ATP and of activation of low threshold primary afferents prompts us to suggest that ATP might be a chemical mediator of synaptic transmission released specifically by low threshold primary afferent neurones.

This suggestion appears contrary to that of Jahr and Jessell (1983) who proposed that ATP is released from unmyelinated (nociceptive) primary afferent neurones. A possible prediction from their hypothesis is that non-nociceptive dorsal horn neurones should be unaffected, or at least should not be excited, by iontophoretic application of ATP and therefore their suggestion seems inconsistent with our results. They based this conclusion on their evidence that ATP excites a subpopulation of dorsal horn neurones in culture (Jahr and Jessell, 1983) together with other evidence that ATP is released from the peripheral ends of primary afferents by electrically stimulated antidromic activation which also produces vasodilation (Holton and Holton, 1954; Holton, 1959), that unmyelinated fibres must be activated to produce antidromic vasodilatation (Celander and Folkow, 1953; Hinsey and Gasser, 1930) and that injection of ATP causes vasodilatation (Holton and Holton, 1954). However, dorsal horn neurones in culture cannot be classified functionally. In addition, the studies of release failed to control for the effects of stimulus intensity and thus because myelinated fibres must be activated when unmyelinated ones are, Holton (1959) and Holton and Holton (1954) were neither able to separate release from the two types of neurones nor able to investigate possible interactions between myelinated and unmyelinated fibres. Finally ATP which was injected was largely converted to adenosine (Holton, 1959) and adenosine is now well-known as a potent vasodilator (Berne et al., 1983).

As the depressant effects of application of ATP may require, or are at least facilitated by conversion of ATP to AMP and then possibly to adenosine, the differential effects of ATP we have observed could be accounted for by the possibility that WDR neurones are associated with higher levels of purinergic receptors or with higher levels of enzymes capable of hydrolysing ATP than are non-nociceptive neurones. However, the finding that non-nociceptive neurones may be inhibited by AMP suggests that they do have at least some purinergic receptors. Therefore our evidence seems to support the latter possibility, that greater levels of enzymes capable of hydrolysing ATP are associated with WDR neurones. This possibility is consistent with anatomical data which have shown that Ca^{++}-Mg^{++}ATPase and acid phosphatase activity in the dorsal horn are

greatest in small-diameter primary afferents (Dodd et al., 1983, 1984; Knyihar and Csillik, 1977; Nagy and Hunt, 1982) and small-diameter primary afferents are more likely associated with WDR than with non-nociceptive neurones. Furthermore, the only effect of ATP on neurones in the cuneate nucleus was excitation (Galindo et al., 1967) and these neurones receive no small-diameter primary afferent input. The results of Salt and Hill (1983) may indicate that the function of ATP is different in the trigeminal and spinal systems as they found no differential effects of ATP in the trigeminal nucleus caudalis, but it seems alternatively possible that they may not have investigated carefully the biphasic and depressant effects which might have differentiated between non-nociceptive and WDR neurones.

Our results suggest that ATP might play a physiological role in the regulation of nociception in the dorsal horn of the spinal cord, specifically by mediating the actions of low threshold primary afferent input. These results might be useful in the development of future analgesics.

Table 1. Effects of AMP on wide dynamic range vs non-nociceptive neurones (numbers represent numbers of neurones).

N-N, non-nociceptive

The one nociceptive specific neurone tested was depressed.

	Depression	No effect
WDR	13	10
N-N	6	2

χ^2 (corrected) = 0.25, with 1 degree of freedom p=0.62

Table 2. Effects of ATP on wide dynamic range vs non-nociceptive neurones.

N-N, non-nociceptive

In addition to the 60 neurones included in this table there were 2 nociceptive specific neurones (1 depressed, 1 no effect), 2 proprioceptive neurones (both excited) and 2 neurones with no detectable peripheral input (1 excited, 1 no effect).

	Excitation	Depression	Biphasic effect	No effect
WDR	6	8	19	9
N-N	11	2	1	4

χ^2 = 16.2, with 3 degrees of freedom p=0.001.

Fig. 1. Effect of AMP on the response of a wide dynamic range neurone to periodic application of noxious radiant heat to the cutaneous receptive field.

A. Pen-recorder trace showing continuous-time histogram of the rate of discharge of this neurone. A reproducible response was elicited from this neurone by automatically controlled, periodic application of the heat stimulus (H) to the receptive field; periods of application are indicated by the short bars below the record. AMP was applied during the period indicated by the long horizontal bar below the record (ejecting current is in nA). Ordinate shows the firing rate in impulses per second.

B. Diagrammatic representation of the receptive field of the neurone whose activity is shown. The neurone responded to touch in the blackened area only, and to pinching the skin with serrated forceps in both the blackened and hatched areas.

C. Oscilloscope records of selected heat-evoked responses. Records a), (b) and (c) correspond to the respective responses indicated in A. The trace below record (c) shows the output of a calibrated thermistor probe which was in contact with the receptive field. Skin temperature was identical for each application of heat. The period of heat application is indicated by the bar below the temperature trace. Skin temperature is in C.

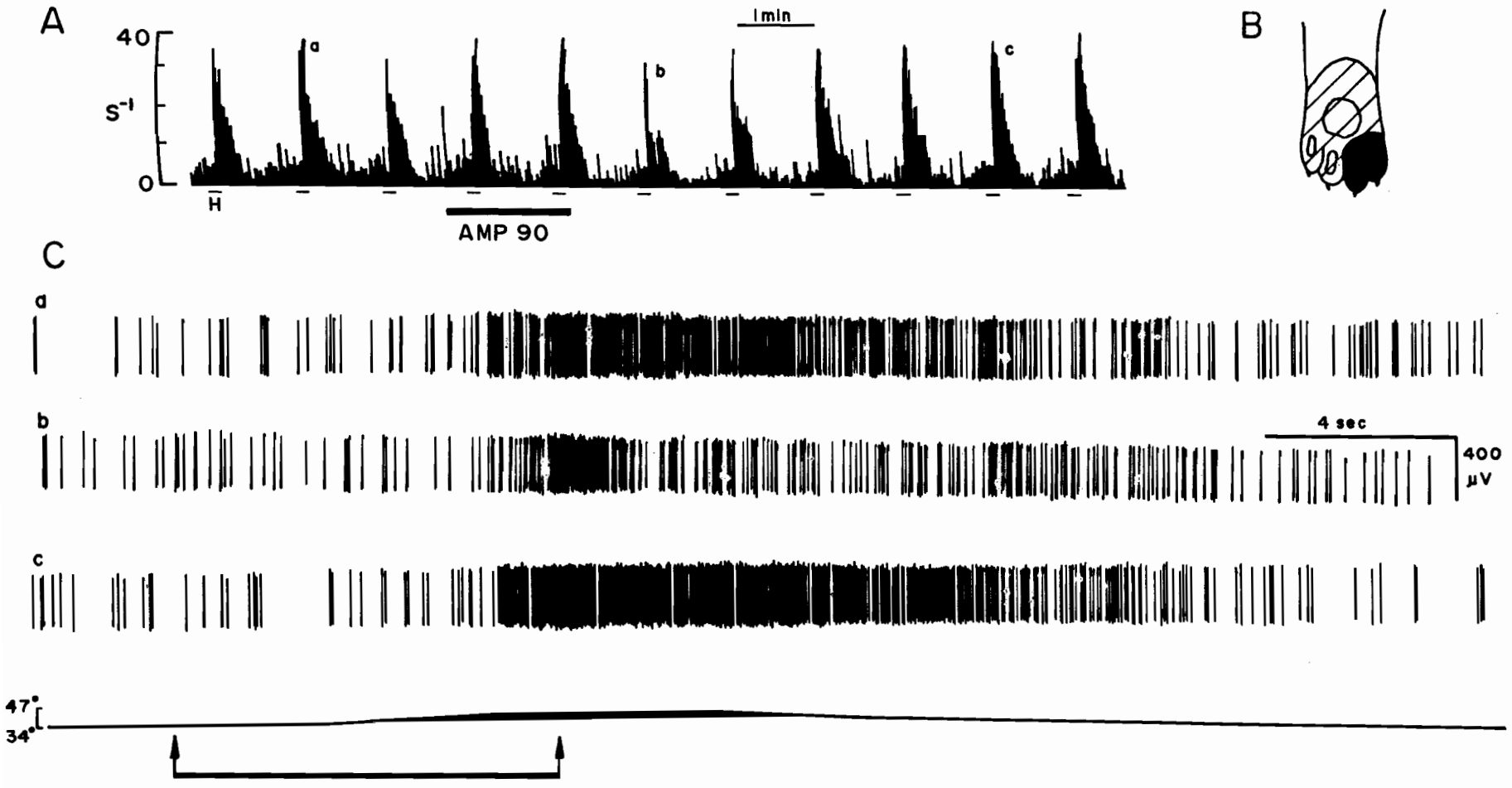
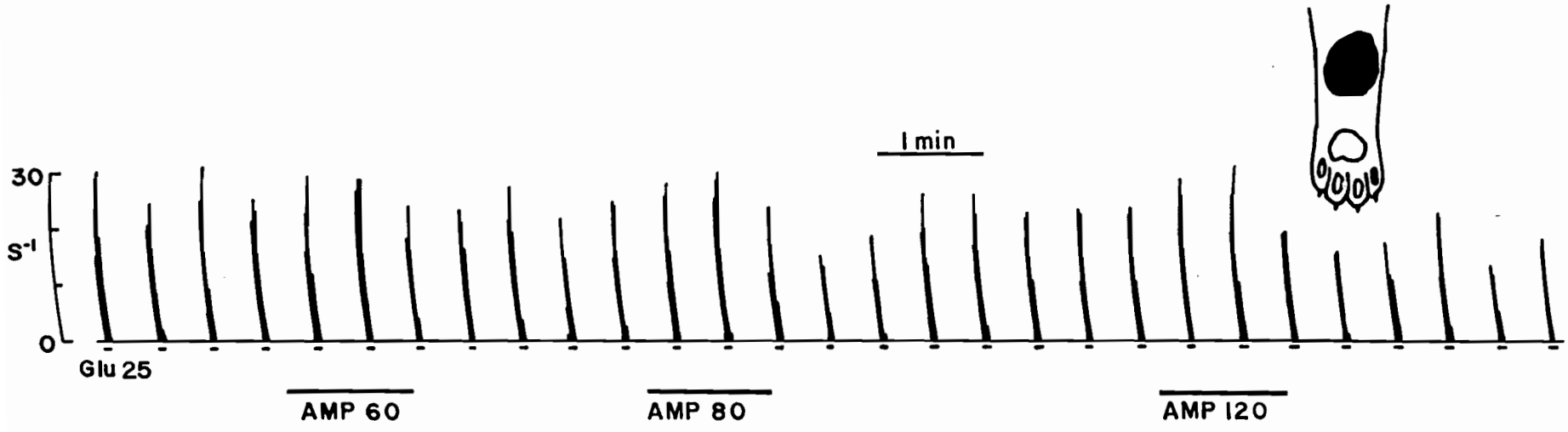

A
40
S⁻¹
0
a
b
c
1 min
H
AMP 90
B
C
a
b
c
4 sec
400
μV
47°
34°

Fig. 2. Pen-recorder trace showing effect of AMP on responses of a non-nociceptive neurone to iontophoretic application of glutamate.

This neurone, which was excited by hair movement but by no other natural, peripheral stimulus, was silent; but reproducible responses were induced by automatically controlled, periodic applications of glutamate (Glu, short bars below the record). AMP was applied during the periods indicated by the long bars below the record. After the final application of AMP the responses to glutamate diminished and failed to return to control levels. The slight increase in the glutamate-evoked responses during the AMP applications at ejecting currents of 80 and 120 nA was considered an artefact due to passing inward current because within 1 s after the end of AMP ejection the glutamate-evoked responses were not facilitated and also because passing of outward current through a barrel containing NaCl depressed glutamate-evoked responses and this depression recovered within 1 s after the current was turned off (not shown). Inset: the receptive field is illustrated by the blackened area.

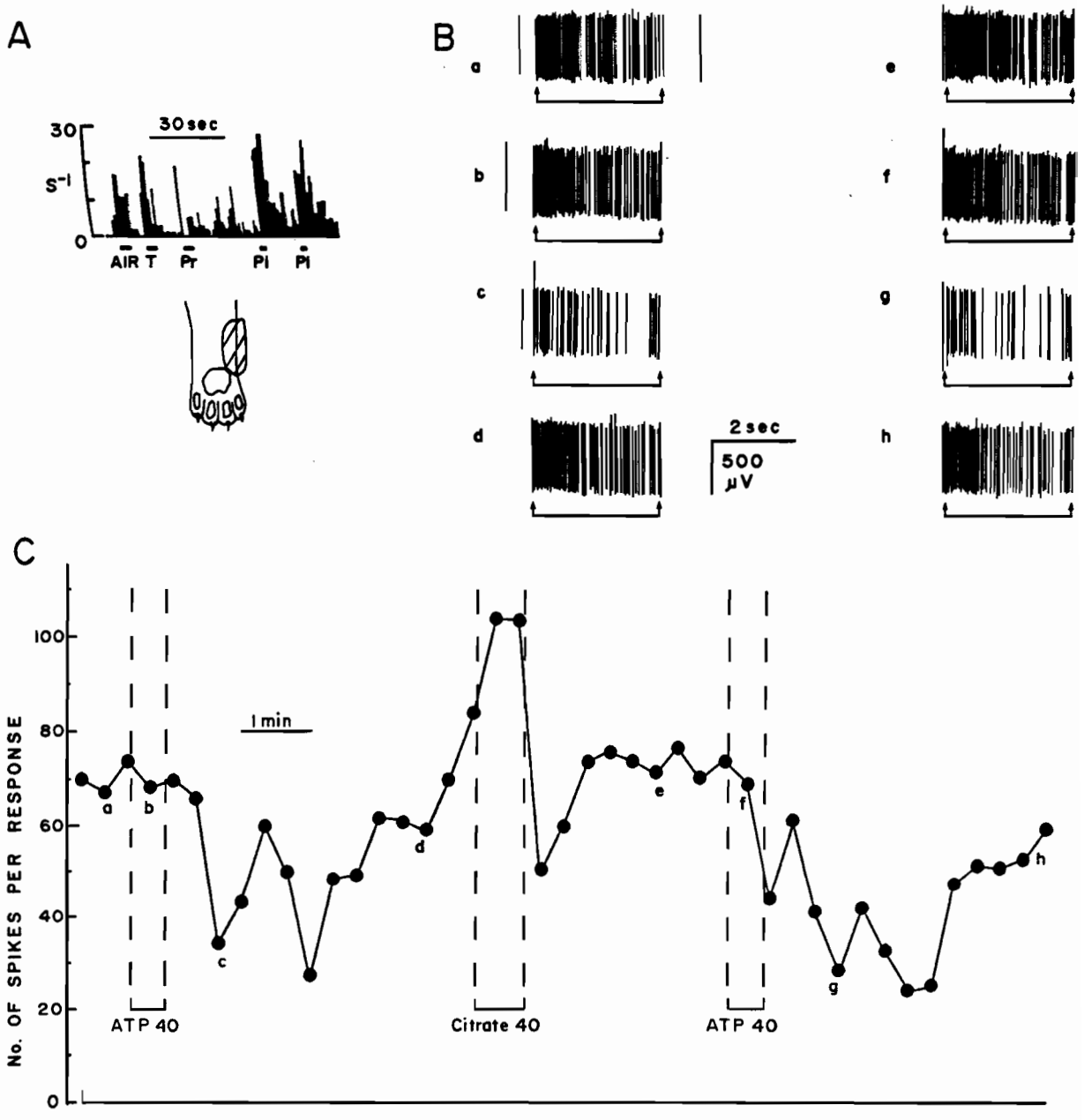

1 min
30
S-1
0
Glu 25
AMP 60
AMP 80
AMP 120

'ig. 3. Depressant effect of ATP.

A. Pen-recorder trace showing the firing rate of a WDR neurone. The eceptive field was stimulated by an air-stream (Air), by light touch (T), y firm but innocuous pressure (Pr) and by noxious pinch (Pi) for the eriods indicated by the bars below the record. Note the afterdischarge o noxious pinch which is absent from the responses to innocuous stimuli. he receptive field is shown by the hatched area in the diagram below the en-recorder trace.

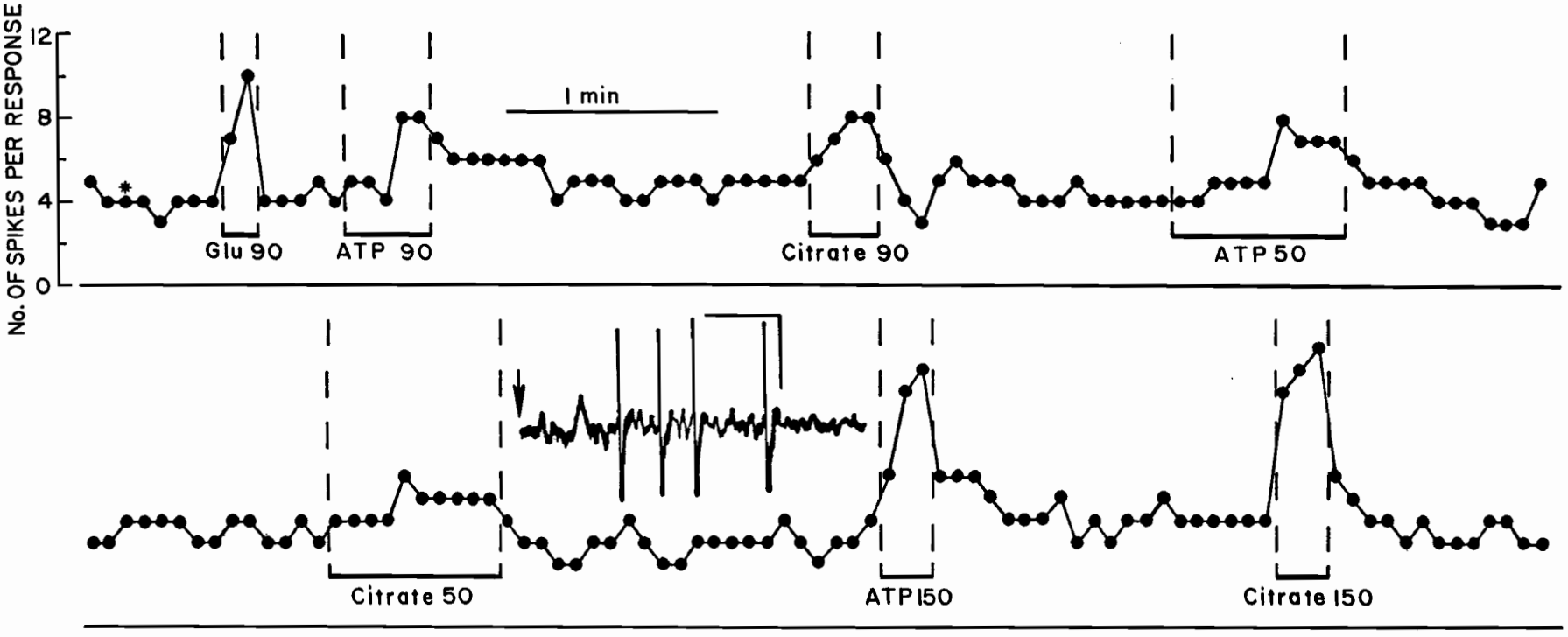
B. Oscilloscope records of responses to an automatically controlled ir stream applied periodically to the receptive field. The air stream as applied for the period indicated by the bar below each record. Each ecord corresponds to the respective response indicated by the letters elow the graph in C. The large deflections at the beginning of the air pplications in records (c), (f), and (g) were artefacts caused by ctivation of the mechanism which controlled the air stream.

C. Graph illustrating the number of spikes in each response to pplication to the air stimulus. ATP and citrate were applied during the eriods indicated by the bars below the graph. Ejection currents are in A.


A
30
S⁻¹
0
30sec
AIR T Pr Pi Pi
B
a
b
c
d
e
f
g
h
2sec
500
μV
C
No. OF SPIKES PER RESPONSE
100
80
60
40
20
0
1 min
a
b
c
d
e
f
g
h
ATP 40
Citrate 40
ATP 40

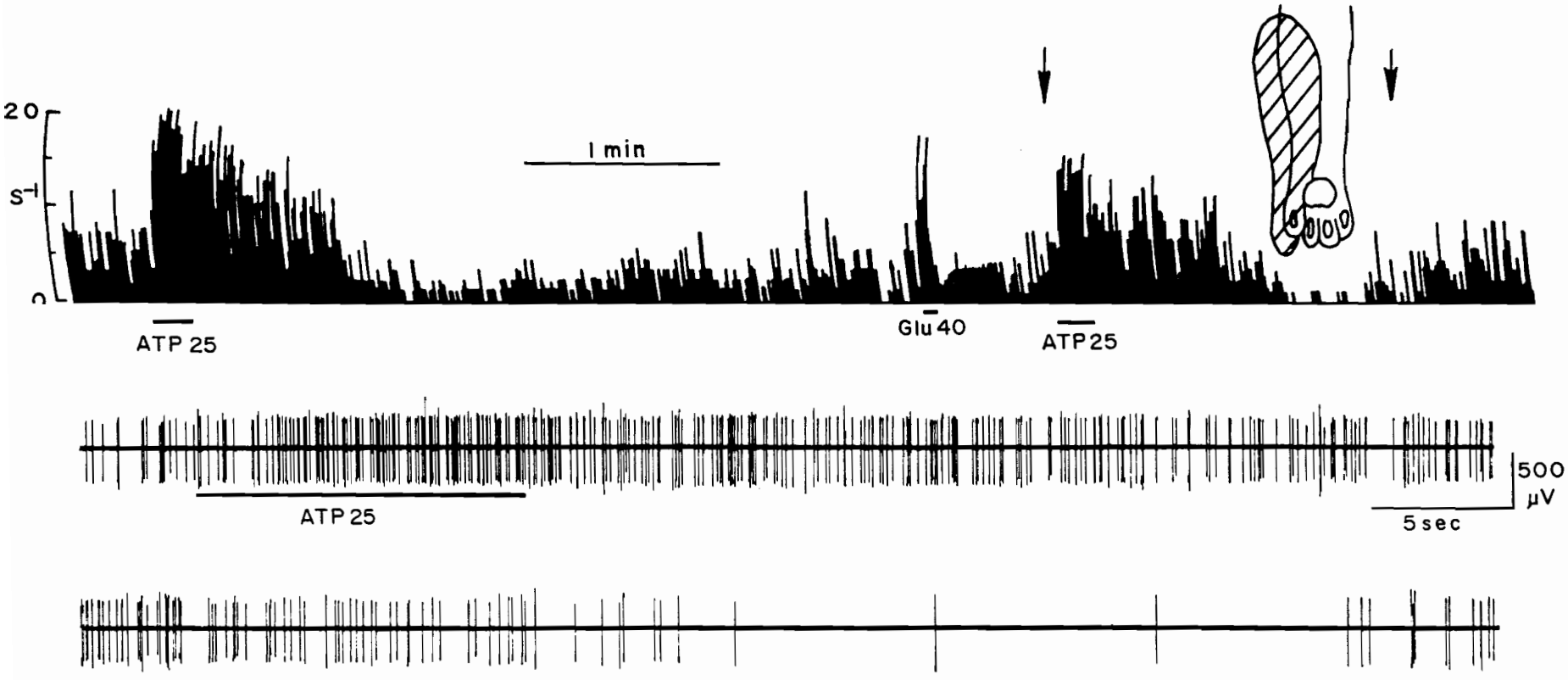
g. 4. Excitatory effect of ATP.

Graph illustrating the number of spikes per response to automatically ntrolled, repetitive electrical stimulation of the receptive field. The per and lower panels are continuous. Applications of glutamate (Glu), P and citrate are indicated by the bars below the graph. Electrical imuli were 60 V (1.5-times threshold) and were delivered at a rate of $2 \cdot s^{-1}$. This neurone showed only touch and pressure responses to tural peripheral stimulation. Inset: photograph of a response to ectrical stimulation. This record corresponds to the response indicated the asterisk in the upper panel. The arrow indicates when the stimulus s delivered. Calibration bars: vertical, 300 μV; horizontal, 10 ms. ection currents are in nA.


No. OF SPIKES PER RESPONSE
12
8
4
0
1 min
Glu 90
ATP 90
Citrate 90
ATP 50
Citrate 50
ATP150
Citrate 150

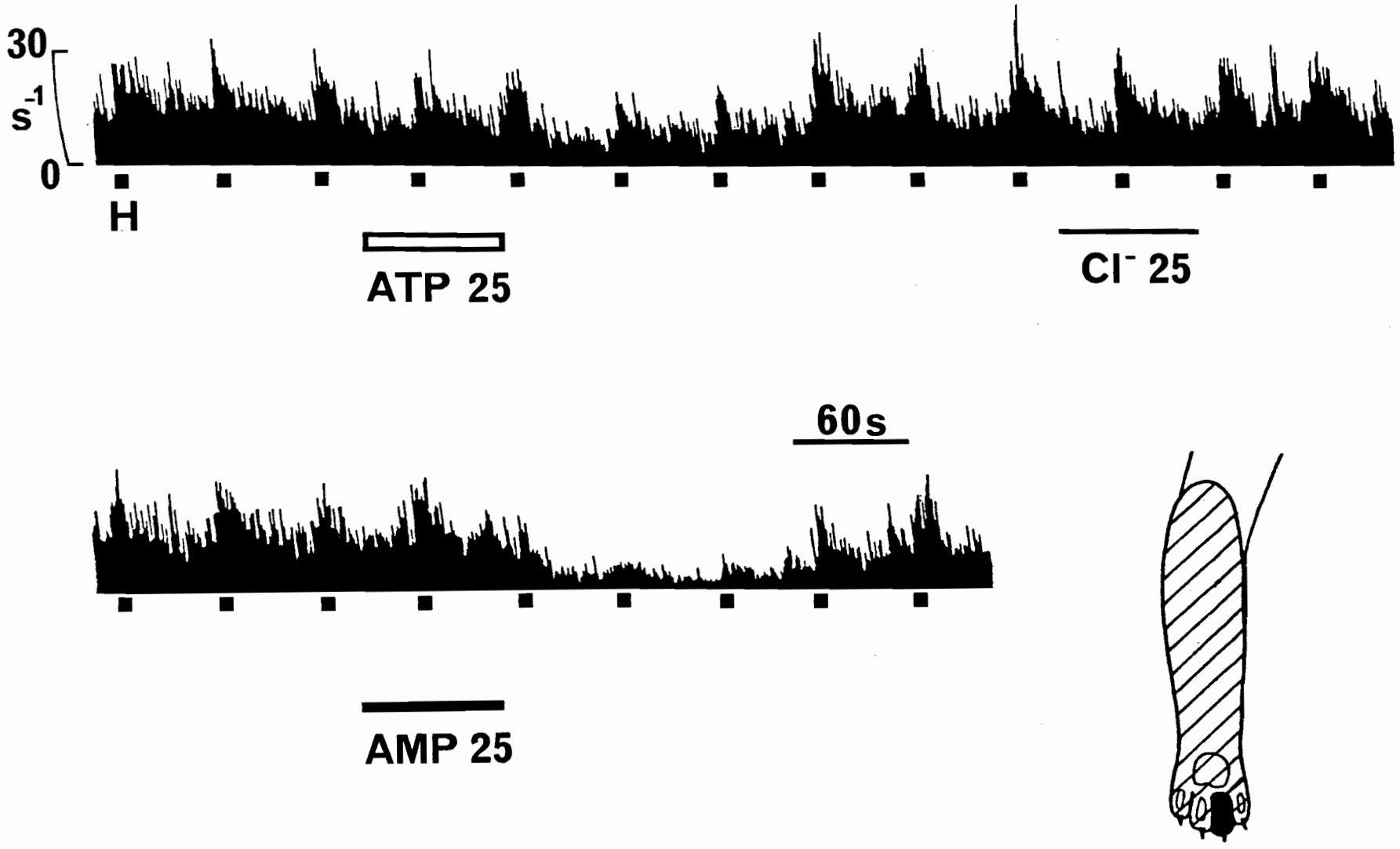
Lg. 5. Biphasic effect of ATP.

Upper: the firing rate of a WDR neurone is illustrated by this en-recorder trace. Periods of application of glutamate (Glu) and ATP are ndicated by the bars below the record. Inset: the receptive field of ne neurone is shown by the hatched area in the diagram. This neurone was xcited by hair movement and noxious mechanical and thermal stimuli. ower: oscilloscope record made during the second ATP application (the uration of the record is shown by the arrows above the pen-recorder race. Application of ATP is indicated by the bar below the upper film cord). The upper and lower film records are continuous. Ejection urrents are in nA.


20
S⁻¹
0
1 min
ATP 25
Glu 40
ATP 25
ATP 25
500
μV
5 sec

ig. 6. Comparison of effects of ATP and AMP on a wide dynamic range neurone.

This WDR neurone exhibited a reproducible excitatory response to eriodic, automatically controlled applications of the heat stimulus (H) hich were made during the periods shown by the short bars below the en-recorder trace. The upper and lower records are continuous. pplication of ATP (open bar below upper record) caused a decrease in the eat-evoked responses and in the on-going activity. On the other hand, jection of inward current of the same magnitude through the barrel ontaining the control solution (Cl^-, closed bar below upper record) was ithout effect. Applications of AMP (bar below lower record) caused epression that was deeper and more prolonged than that caused by pplication of ATP. The excitatory receptive fields for touch and noxious inch are represented in the diagram using the same scheme as in Fig. 1.


30
s-1
0
H
ATP 25
Cl- 25
60s
AMP 25

CHAPTER 3

DIFFERENTIAL EFFECTS OF CUTANEOUSLY APPLIED VIBRATION ON NOCICEPTIVE AND NON-NOCICEPTIVE NEURONES IN THE SPINAL DORSAL HORN OF THE CAT

I. ABSTRACT

Extracellular single-unit recordings were made from dorsal horn neurones in the lumbar spinal cord of cats which were anaesthetized with sodium pentobarbital (n=55) or were anaemically decerebrated (n=2). On the basis of responses to natural noxious and innocuous cutaneous stimuli each neurone was classified functionally as wide dynamic range (WDR; n=193), non-nociceptive (n=30), nociceptive specific (n=3) or proprioceptive (n=12). Vibration was then applied to the hind limb using a feedback-controlled mechanical stimulator; the probe of the stimulator was placed at sites inside and outside the excitatory receptive fields.

With WDR neurones vibration (80 Hz; 0.3-1.0 mm) had 3 distinct types of effect: excitation, depression and a biphasic effect which consisted of excitation followed by depression. Thirty-five WDR neurones were tested with the stimulator probe placed inside that part of the receptive field from which low intensity cutaneous stimuli elicited excitation; 31 neurones were excited by the vibratory stimulation, none was depressed and 4 showed the biphasic effect. On the other hand, when the probe was positioned outside the receptive field for low intensity stimuli, 7 WDR neurones were excited, 76 were depressed, 88 showed the biphasic effect and 7 were unaffected. On-going activity and activity evoked by iontophoretic application of glutamate were decreased during the depressant response and the depressant phase of the biphasic effect. This finding raises the possibility that the depression of activity was due to a postsynaptic inhibition of the neurones being studied.

All non-nociceptive neurones were excited by vibration; depressant or biphasic effects failed to occur. Excitation could be elicited by placing the probe either inside or outside the receptive field for non-vibrational stimuli.

Comparison of the frequency of occurrence of the 3 different types of effect showed that when the probe was positioned outside the receptive field there was a differential effect of vibration on the groups of WDR vs non-nociceptive neurones (χ^2=143, 3 df, $p<0.0001$).

All nociceptive specific neurones were depressed by vibration.

The differential effect of vibration on nociceptive vs non-nociceptive neurones prompts us to suggest that the increase in pain threshold and the clinical analgesic effects elicited by vibration may be mediated at the

spinal level by a decrease in the rate of firing of nociceptive neurones and/or by excitation of non-nociceptive neurones.

II. INTRODUCTION

The aim of the present study was to systematically investigate the general population of neurones in the dorsal horn and to examine their responses to standard vibrational stimulation. In the only other study to focus specifically on the responses of dorsal horn neurones to vibration, Wall and Cronly-Dillon (1960) studied neurones responding to all types of cutaneous stimuli. In view of the fact that the dorsal horn contains neurones which respond specifically to restricted types of stimuli (Perl, 1984), the population of neurones in the present study was more diverse than that of Wall and Cronly-Dillon. An important part of the current studies was that each neurone was functionally classified on the basis of its responses to natural, non-vibrational stimuli. In addition, the range over which stimulation parameters were varied was greater here than in the study by Wall and Cronly-Dillon and we have also investigated the effects of the level of excitability on the vibration-induced responses. Indeed, the conclusions drawn from our study are different from those drawn from the study of Wall and Cronly-Dillon.

The present study developed from a systematic survey of the responses of dorsal horn neurones to innocuous mechanical and electrical stimulation of the skin. Responses to vibration proved to be particularly interesting because of the different effects which were obtained from nociceptive and non-nociceptive neurones. The current investigation has concentrated on characterizing the responses of these neurones. Evidence will be reported in the following Chapter as to the neurochemical basis of one of the vibration-induced responses of wide dynamic range (WDR) neurones and a study of the parameters which affect the vibration-induced responses of these neurones is given in Chapter 5.

Preliminary results of the present study have been previously reported (Salter and Henry, 1986b).

III. MATERIALS AND METHODS

i) *Animal preparation*

Experiments were done on 57 adult cats: 55 were anaesthetized with sodium pentobarbital (40 mg/kg, i.p.; supplemental doses 5 mg/kg i.v.

every 3 hours) and 2 were anaemically decerebrated under anaesthesia induced by halothane (Somnothane, Hoechst) which was subsequently discontinued. Throughout the experiments the carotid arterial blood pressure was continuously displayed on a Grass Model 5 polygraph. The mean arterial pressure was maintained above 80 mm Hg with intravenous infusion of 10% dextran (Macrodex, Pharmacia) or noradrenaline bitartrate (Levophed, Winthrop; 0.002% in normal saline), if necessary.

Spinal segments L5 to L7 were surgically exposed for recording and were covered with warm mineral oil to prevent drying and cooling. In all experiments, the spinal cord was transected at the first lumbar level to remove descending influences and to eliminate the possibility that the vibration-induced effects on lumbar dorsal horn neurones might be mediated via supraspinal structures. Prior to the transection 0.1 mL of 1.0% lidocaine hydrochloride were injected into the L1 segment to minimize spinal shock.

Bilateral pneumothoraces were made and the animals were paralyzed with pancuronium bromide (Pavulon, Organon; 1 mg/kg i.v., repeated when required) and ventilated artificially. End-tidal CO_2 concentration was maintained between 3.5 and 5.0%.

Rectal temperature was maintained at 38°C with a servo-controlled infrared bulb. Spinal cord circulation was visually monitored periodically using a dissecting stereomicroscope.

ii) *Recording and Data Acquisition*

Single unit spikes were recorded extracellularly with multibarrelled glass micropipettes (overall tip diameter 4-8 μm). A solution of 2.7 M NaCl was placed in the central recording barrel (impedance 4-10 MΩ measured at 100 Hz). The raw data were amplified, displayed on oscilloscopes and recorded on magnetic cassette tape as detailed in Salter and Henry (1987a). Film records were taken from the oscilloscopes by displaying the data from the tapes.

Spikes were observed visually and were detected electronically by appropriate adjustment of an amplitude discriminator in a gating unit. The gating unit also functioned as a frequency counter (bin width 1 s) and the output was continuously displayed on a Grass Model 5 polygraph.

The amplitude discriminator produced a Schmitt trigger output which

was led to a digital-to-analogue multiplexer. The multiplexer had a total of 3 input channels. One was connected to the output of the amplitude discriminator. Each of the 2 other channels received trigger pulses, one from the iontophoresis unit and the other from a Grass stimulator. The occurrence of a trigger in any of the 3 channels caused the multiplexer to put out a rectangular voltage pulse. Occurrence of a trigger in a given input channel was encoded uniquely for that channel in the amplitude of the output pulses: the amplitudes for the different channels were 1, 2 or 4 V. The pulses were 0.95 ms in duration and when the pulses overlapped temporally the output was the algebraic sum of the voltages. Thus, the output voltage had only discrete levels between 0 and 7 V, and any given level represented a unique combination of triggers. The output of the multiplexer was connected to a Data Translation DT2801-A analogue-to-digital board in an IBM personal computer.

Data sampling was continuous at a sampling frequency of 2 kHz. The digitized input from the multiplexer was decoded using software to determine in which of the multiplexer input channels a trigger had occurred. The intervals between spikes and between trigger pulses were computed to $\pm$0.5 ms and stored on fixed disk.

In a few cases attempts were made to determine the central delay for the response to vibration. In these cases, a silver ball electrode was placed on the dorsal root entry zone and the cord dorsum potential (Gasser and Graham, 1933) was monitored. The central delay was calculated as the time between the afferent volley in the cord dorsum potential and the start of the response to vibration.

iii) *Iontophoresis*

One of the outer barrels of the micropipette contained a solution of sodium L-glutamate (1M, pH 7.4, Sigma). Glutamate was used to increase systematically the excitability of selected neurones and thus to investigate the effect of the level of excitability on the response to vibration. An outward current (10 nA) was passed between applications of glutamate to minimize leakage.

Other barrels of the micropipette contained NaCl (165 mM) or Pontamine Sky Blue 6BX (0.5% in 0.5 M sodium acetate, Gurr). Inward current was passed through the barrel containing NaCl to detect artefacts due to local

changes in current density at the tip of the electrode. In some experiments other neuroactive compounds such as ATP, substance P or physalaemin were contained in other barrels; these compounds are the topics of other papers.

Currents used for iontophoretic ejections were produced by a constant current iontophoresis unit (Cocyen Co. Ltd.).

iv) *Histological identification of recording sites*

Sites of recording were marked with deposits of Pontamine Sky Blue by passing inward current (5-10 μA) for 5-10 min through the barrel containing this dye. At the end of the experiment the section of spinal cord containing the deposits was removed, fixed and embedded according to the method of Salter and Henry (1987a). The location of the deposits was determined by microscopic examination of serial sections (15-30 μm) which had been counterstained with thionin.

v) *Functional classification of units*

The following natural peripheral stimuli were used to classify each unit on a functional basis: movement of single hairs, light touch of the skin with a tissue paper, firm manual pressure (judged to be non-noxious when applied to the experimenter), noxious pinch of the skin with a serrated forceps, noxious heating of the skin (temperature >47°C), and passive movement of the limb. According to its responses to these stimuli a unit was classified as non-nociceptive, wide dynamic range (WDR), nociceptive specific or proprioceptive. Further details of the classification scheme are given elsewhere (see Chapter 2). Importantly, the response to vibration was not a criterion used in the classification. For each unit the excitatory receptive fields for hair movement, light touch and noxious pinch were represented on a schematic diagram of the hindlimb.

In some cases bipolar needle electrodes were inserted into the receptive field and the response to electrical stimulation of the receptive field was determined. In other cases the superficial peroneal or tibial nerves were exposed at the ankle and were stimulated electrically using hook electrodes.

vi) *Vibrational stimulation*

Vibration was generated by a feedback-controlled mechanical stimulator (Chubbuck, 1966) to which was attached the stimulator probe. The probe consisted of a plexiglass rod 5 cm in length and 10 mm in diameter, squared off at the ends. To test the response of a given unit to vibration the probe was positioned to contact with the skin so that a constant force of up to 0.5 N was applied during the periods between vibration. This force did not change throughout the testing and only occasionally were slight adjustments of the position of the stimulator necessary to keep the force constant. Care was taken to avoid positioning the probe directly over major peripheral nerves.

Trains of rectangular voltage pulses produced by a Grass S88 stimulator were used to drive the mechanical stimulator. The frequency of the pulses within the trains was 80 Hz, the maximum frequency at which the probe returned completely to the rest position after each pulse. The duration of each voltage pulse was 6 ms. Using these parameters the peak displacement varied linearly with the input voltage (0.1 mm/V to a maximum of 1.0 mm). The transducer of the mechanical stimulator acted as a low pass filter (-3 dB at 85 Hz). Thus, the displacement of the the probe was curvilinear (see for example Fig. 2) and when the peak displacement was 0.6 mm or more the trajectory approximated a sinusoid. The initial motion of the probe was always towards the limb.

The trains of pulses were 2.5-3.5 s in duration and were given periodically at regular intervals every 20-25 s; train duration and repetition rate were constant for a given unit. To ensure that the probe displacement was stable it was monitored during the testing via an output from the control unit of the mechanical stimulator.

The position of the stimulator probe was a variable investigated during the study. As WDR, non-nociceptive and nociceptive specific units are well known to have cutaneous receptive fields for non-vibrational stimuli (Price and Dubner, 1977; Willis and Coggeshall, 1978), the effect of vibration when the stimulator probe was placed inside the receptive field was compared with the effect when the probe was placed outside the receptive field.

It is important to note that for the remainder of the thesis the term "receptive field" will be used only with respect to non-vibrational

stimuli. This specific use of "receptive field" is made because of the well established use of this term with respect to non-vibrational stimuli (Price and Dubner, 1977; Willis and Coggeshall, 1978) and because it was the non-vibrational receptive fields that were used as references when assessing the effects of varying the position of the stimulator probe.

Most units had receptive fields located on the distal foot and, therefore, attempts were made to minimize stimulation of this part of the foot when the stimulator probe was elsewhere on the hindlimb. To do this the limb was positioned as follows: the ankle was draped over a horizontal bar such that the foot was free of contact with surrounding objects. The hindlimb was able to flex and to extend slightly at the ankle but was otherwise fixed and unable to abduct or to adduct.

The effects of the vibratory stimulation on on-going activity were investigated. Units which failed to show spontaneous activity were excited with iontophoretic application of glutamate to provide a stable rate of firing. In some cases, glutamate was also used to increase the firing rate of slowly firing units. Effects of vibration on responses evoked by other stimuli applied to the receptive field were not investigated to avoid the possibility that the vibration might alter the peripheral transduction of the other stimuli.

vii) *Data analysis*

Quantitative analysis of data was done off-line. To determine the effect of vibration the number of spikes during the period of each stimulation was measured and compared with the number of spikes during a control period of the same duration immediately preceding each stimulation. The percentage change in the number of spikes was calculated for each application. The average percentage change was determined using at least 5 consecutive applications and statistical significance was calculated using the paired *t*-test or the sign test, as appropriate. The correlation coefficient for the number of spikes during the stimulation vs the number of spikes during the control period was calculated by the method of least squares (Snedecor and Cochran, 1967) and p was determined using Fisher's table (Fisher, 1970).

Effects were considered statistically significant for $p<0.05$. Numerical values are reported as the mean ± 1 standard error of the mean.

Peristimulus time histograms (PSTHs) displaying the rate of firing in relation to the application of vibration were constructed to show time dependence of the effects.

IV. RESULTS

Recordings from 238 units are included in these results. No difference was apparent in the effects of vibration on units recorded in anaesthetized (n=220 units) compared with unanaesthetized (n=18 units) animals and hence data from these 2 preparations are considered together. Thirty units were classified as non-nociceptive, 193 as WDR, 3 as nociceptive specific and 12 as proprioceptive. In this sample, WDR units were specifically selected for detailed investigation and are therefore over-represented compared with the general population of units in the dorsal horn we have previously reported (see Chapter 2). All units reported here were excited by iontophoretic application of glutamate. Therefore, the results are considered to exclude recordings from fibres and it was concluded that the recordings were made from an excitable area, likely the soma-dendritic region, of neurones intrinsic to the dorsal horn.

For a given neurone the effects of vibration were reversible and reproducible and hence were considered to be genuine responses. Responses to vibration were stable, varying in average magnitude less than 10%, over periods of greater than 4 hours.

i) *Effects of vibration on WDR neurones*

Vibration at 80 Hz (amplitude 0.3 to 1.0 mm) had 3 distinct effects on the rate of firing of WDR neurones: excitation, depression and a biphasic effect (excitation followed by depression).

a) *Response depends on site of application of vibrational stimulation.* The most important factor in determining if excitation or one of the other 2 responses occurred appeared to be whether the probe was inside or outside the excitatory receptive field for low threshold stimuli. With the probe at any given location only one of the 3 effects was observed. For example, with the neurone shown in Fig. 1A, when the probe was positioned inside the receptive field for light touch (Fig. 1Aa),

there was a marked increase in the firing rate during the period of vibration. On the other hand, when the probe was applied outside the receptive fields for light touch and for noxious pinch, stimulation using the same parameters caused a burst followed by depression, i.e. the biphasic effect (Fig. 1Ab). Depression in the absence of preceding excitation was observed in other units when the probe was positioned outside the low threshold receptive field (Fig. 1Bb).

Vibration applied inside the receptive field for low threshold stimuli was tested on 35 neurones: 31 were excited and 4 showed the biphasic effect. On the other hand, of the 178 WDR neurones for which vibration was applied outside the low threshold receptive field 7 were excited, 76 were depressed, 88 showed the biphasic effect and 7 neurones were unaffected. In all cases in which the biphasic effect was observed, the total number of spikes during the period of vibration was less than the total number of spikes during the control period (for example Fig. 1Ab). Whether depression or the biphasic effect was observed did not appear to depend on whether the probe was inside the receptive field for high threshold stimuli but outside the low threshold receptive field, or outside both receptive fields.

In addition to being an important factor in determining whether excitation or one of the other 2 responses occurred, the position of the probe relative to the low threshold receptive field was important in controlling the magnitude of the depressant and biphasic effects. The neurone illustrated in Fig. 1B, for example, was excited by vibration when the probe was placed inside the receptive field for touch. However, when the probe was located inside the receptor field for noxious pinch but far from the touch receptive field, vibration caused an abrupt decrease in the rate of discharge without any preceding excitation (Fig. 1Bb). After the probe had been moved closer to the receptive field for touch, the magnitude of the depression was greatly reduced.

Fig. 1 illustrates an additional consistent observation, that vibration had no effect on either spike amplitude or spike shape. Therefore, the effect of vibration cannot be accounted for by movement of the electrode at the site of recording.

b) *Latency of responses of WDR neurones.* The vibration-induced excitation began 8-15 ms (mean: 9.6$\pm$0.8 ms, n=10) after the onset of the

stimulation. In the cases measured the central delay was 1.2-1.5 ms (n=3). In addition, it was possible on occasion to observe a small field which preceded the first spike by 1 ms or less. Interestingly, the initial burst of the excitatory response occurred at a longer latency than the latency of the excitation due to electrical stimulation of the receptive field (for example compare Fig. $2A_1$ and $2A_2$).

The latency for the excitatory component of the biphasic effect was similar to the latency of the excitation (for example compare Fig. $2C_1$ and $2C_2$). In addition, the latency of the excitatory component of the biphasic response to vibration was longer than the latency of the excitation caused by electrical stimulation of peripheral nerves (for example compare Fig. $2B_1$ and $2B_2$).

The earliest depression observed was 10 ms after the onset of vibration (Fig. 3); the shortest central delay which could be determined with accuracy was 2-3 ms. Thus, the minimum values for latency and for central delay were both greater with depression than with either excitation or with the excitatory component of the biphasic effect. After the beginning of the depression the rate of discharge decreased abruptly to its lowest level: sometimes the decline from the control to the lowest level of discharge occurred in less than 1 ms (Fig. 3).

c) *Time course of responses of WDR neurones.* Examples which illustrate the 3 different types of response are shown as peristimulus time histograms (PSTHs) in Fig. 4.

c_1) *Excitation.* The vibration-induced excitation appeared to consist of 3 parts: there was an initial burst which was followed by a period lasting up to 100 ms during which the rate of discharge was less than at the peak which immediately followed this period (in one-third of cases the lowest discharge rate during this period was less than the control level before vibration); during the remaining period the rate of discharge peaked and then decreased slowly or stayed at the peak level throughout the remainder of the vibration. Although the pattern of the excitation was consistent, the relative magnitude and duration of the different parts varied from neurone to neurone and also for the same neurone depending upon stimulus location, stimulus intensity and level of excitability. However, with a given neurone the excitatory response to vibration was reproducible for periods of up to 4 hours when the on-going

rate of discharge of the neurone, and the location and amplitude of stimulation remained constant.

Upon termination of vibration an afterdischarge was never observed. In fact with 6 neurones, immediately after the period of vibrational stimulation the rate of discharge decreased to below the control level (Fig. 4A). This post-vibrational depression recovered gradually and for most neurones full recovery occurred within less than 1 s.

c_2) *Depression.* During the depressant response, the discharge rate either remained maximally depressed throughout the period of vibration (Fig. 4B) or, after reaching its lowest level, increased slightly during the remainder of vibration (for example Fig. 5A). In no case did the rate of discharge return completely to the control level during the period of vibration. With two-thirds of the neurones which showed depression, the rate of discharge remained depressed after the period of stimulation and gradually returned to the control level (Fig. 4B); the depression typically lasted less than 2 s after the vibration ended, although in one case complete recovery took a full 9 s. In the remaining one-third of the cases, vibration-induced depression was followed by an excitation which decayed to the control level within 500 ms.

c_3) *Biphasic effect.* In all cases the biphasic effect consisted of a short-latency excitation followed by depression throughout the remainder of the period of vibrational stimulation (Fig. 4C). The duration of the excitatory component varied with different neurones and with the parameters of stimulation (see Chapter 5). The excitatory component ranged from a single spike to a burst (for example Fig. 2C2) which lasted up to 20 ms. During the ensuing depressant component of the biphasic effect, as was the case for the depression, the discharge rate either remained at the lowest level (Fig. 4C) or gradually increased slightly (for example Fig. 10Ac). In addition, the depressant component outlasted the period of vibration (Fig. 4C) or there was post-vibration excitation. The time course of the biphasic effect caused by vibration was the same when the probe was applied inside the low threshold receptive field (4 neurones) or when the probe was placed outside this receptive field (88 neurones).

d) *Control experiments.* To differentiate between effects of

vibration and effects of displacement of the skin, a single long mechanical pulse was applied as illustrated in Fig. 5B. This long pulse was of the same duration and the same amplitude as the trains of pulses used to produce vibration. For the long pulse, there was depression which lasted less than 200 ms at the onset of the pulse and there was a shorter period of depression following the end of the pulse. On the other hand, for the vibration-induced depression (Fig. 5A) the effect persisted throughout the period of stimulation. Similar testing was done in other cases for the excitatory response: excitation occurred with the transient and not with the constant part of the stimulation by the long pulses.

Fig. 5 provides evidence, in addition to that shown in Fig. 1, against the possibility that the effects of vibration were due to movement of the electrode at the site of recording: the depression caused by vibration when the probe was positioned on the hindlimb ipsilateral to the recording site (Fig. 5A) was not mimicked when the probe was placed on a homologous region of the contralateral hindlimb (Fig. 5C).

Two types of experiment were done to control for the possibility that the depressant component of the biphasic effect represented a post-excitation depression. First, the duration of the vibration was systematically varied and it was found that the duration of the depression varied directly with the duration of vibration. In addition, it was found that the duration of depression was independent of the duration of the excitatory component. Second, the firing rate was briefly increased by application of glutamate; this increase was not followed by depression with the same time course as seen during the biphasic effect. These findings suggest that the depressant component was not a post-excitation depression.

e) *Amplitude-response relationships for WDR neurones.* The relationship between the amplitude of stimulation and the magnitude of the response has been investigated as described in detail in Chapter 5.

f) *Vibration causes depression of glutamate-evoked activity of WDR neurones.* Glutamate was used to increase the excitability of the neurones under study. One such case is illustrated in Fig. 6. This neurone was silent in the absence of overt stimulation. Iontophoretic application of glutamate evoked an on-going discharge and also increased the number of spikes during the excitatory responses to the cutaneous

stimuli (Fig. 6A). Importantly, the glutamate-evoked activity was abolished by vibration (Fig. 6B and 6C). In all cases tested (n=54), cutaneously applied vibration blocked discharge either evoked or increased by glutamate. This blockade of activity was observed both with neurones which showed the depressant effect and with those which showed the biphasic effect. A quantitative analysis of the relationship between the level of excitability and the magnitude of the vibration-induced depression is given elsewhere (see Chapter 7).

ii) *Effects of vibration on non-nociceptive neurones*

a) *Vibration-induced excitation.* Non-nociceptive neurones were tested using vibration in a similar way as were WDR neurones. Unlike WDR neurones, vibration only excited non-nociceptive neurones (Fig. 7). Vibration caused excitation whether the stimulator probe was placed inside (12 neurones tested) or outside (21 neurones tested) the receptive field. However, the magnitude of the response was greater when the probe was inside the receptive field: with the neurone illustrated in Fig. 7, for example, the average number of spikes during the period of vibration was 12.4 ± 3 (10 trials) when the probe was applied outside the receptive field and was 118 ± 7 (10 trials) when the probe was inside the receptive field ($t=10^{-5}$, $p=10^{-7}$).

b) *Latency and time course of excitation.* The excitatory response of non-nociceptive neurones began with a short-latency burst (mean latency: 10 ± 0.7 ms, 10 trials). When the amplitude of stimulation was sufficiently great there was excitation during the remainder of the stimulation (for examples see lower 2 insets in Fig. 8A). In these cases, the burst was followed by a period of decreased rate of discharge lasting up to 200 ms. Subsequently, the discharge rate peaked and then either remained at the peak level or, as can be seen in the lower 2 insets in Fig. 8A, the discharge rate gradually declined and reached a plateau level which was maintained during the last half of the vibration. Of the neurones which fired spontaneously or for which a baseline rate of firing was evoked by glutamate, half showed a decrease in the rate of discharge following vibration. These findings indicate that the excitatory response of non-nociceptive neurones was similar in latency and time course to the excitatory response of WDR neurones.

c) *Amplitude-response relationships for vibration-induced excitation of non-nociceptive neurones.* When the stimulator probe was located inside the receptive field, it was noticed that the number of spikes during the vibration appeared to be directly related to the stimulation amplitude. This apparent relationship prompted a systematic investigation of the relationship between the amplitude of the mechanical stimulation and the magnitude of the vibration-induced response with 5 non-nociceptive neurones when the stimulator probe was positioned outside the receptive field.

The neurone shown in Fig. 8A responded to the vibratory stimulation when the amplitude was 0.4 mm or greater. Above threshold, the magnitude of the response varied directly with the amplitude of stimulation. The relationship between the number of spikes during the vibration vs the stimulation amplitude was approximately linear ($r=0.996$, $p<0.01$). The lowest amplitude caused only a single spike which occurred at the onset of stimulation (upper trace in the inset). At greater amplitudes spikes also appeared later during the period of stimulation. In Fig. 8B amplitude-response curves are represented for the 5 neurones tested. In every case the number of spikes during the response increased progressively with increasing stimulus amplitude over the range tested.

d) *Effect of glutamate on excitation of non-nociceptive neurones.* In cases where vibration evoked only a burst at the onset of stimulation (for example the upper trace in the inset in Fig. 8B) it was considered possible that vibration may have been causing a biphasic effect like that observed with WDR neurones except that this possible biphasic effect was unseen because the on-going rate of discharge was zero. To investigate this possibility, iontophoretic application of glutamate was used to increase the excitability of non-nociceptive neurones.

As would be expected on the basis of an increase in excitability, the total number of spikes during the vibration-induced excitation was increased by application of glutamate (Fig. 9). As illustrated in Fig. 9A when the excitability was increased, vibration was found to cause excitation in the period following the initial burst. In no case did increasing the level of excitability reveal a biphasic effect of vibration on non-nociceptive neurones.

iii) *Comparison of effects of vibration on WDR vs non-nociceptive neurones*

The numbers of WDR and non-nociceptive neurones responding to vibration applied to the receptive field are compared in Table 1. This comparison indicates that the rate of discharge of WDR and non-nociceptive neurones was similarly affected by vibration applied at this site.

On the other hand, when the probe was located outside the receptive field vibration had a differential effect on neurones in the 2 functional groups; non-nociceptive neurones were excited whereas most WDR neurones were depressed or showed the biphasic effect (Table 2).

Although care was taken in attempting to use similar stimulation when testing the 2 groups of neurones, it is possible that there was a bias in the responses due to subtle differences in the location of the stimulator probe or in the parameters of stimulation. If such a bias had occurred the conclusion may have been erroneous that vibration applied outside the receptive field had a differential effect.

Fig. 10 illustrates an attempt to control for possible variability in location of the probe or in stimulation parameters. During a single penetration of the electrode, the WDR neurone (Fig. 10A) was recorded first and its responses to vibration were determined (Fig. 10Ab and 10Ac). When recording from this neurone had been completed the electrode was advanced to search for another single unit. Importantly, the stimulator probe was left undisturbed at the position indicated by the arrows in the insets. The non-nociceptive neurone shown in Fig. 10B was then isolated, 400 μm deeper than the WDR neurone. The response of this neurone was then determined (Fig. 10Bc) using parameters identical to those used when testing the WDR neurone. Comparing Figs. 10Ac and 10Bc illustrates the differential effect of vibration with the probe applied outside the low threshold receptive field. Importantly, the receptive field of the non-nociceptive neurone is contained completely within the receptive field for innocuous mechanical stimuli of the WDR neurone. Therefore, it is unlikely that the spread of vibration from the site of application into the receptive field of the non-nociceptive neurone was greater than the spread into the receptive field of the WDR neurone; in fact the opposite seems more likely as the receptive field of the WDR neurone was larger. For the non-nociceptive neurone, the stimulator probe

was then moved to the position indicated by the 'x' in the inset, just on the edge of the receptive field. Again, vibration was tested using the same parameters of stimulation as used when testing the WDR neurone. With the probe in this position the excitatory responses of both neurones were similar: there was a short latency response (8 ± 0.01 ms for the non-nociceptive neurone and 9 ± 0.1 ms for the WDR neurone, $t=6.7$, $p=10^{-5}$; 10 trials were used in each case to determine the latency), in both cases the burst at the onset of vibration was followed by a period of decreased excitation and during the remainder of vibration the discharge occurred in phase with the stimulation.

In one other case when it was possible to record in succession from 2 neurones of the 2 different functional types, a differential effect of vibration was found when the stimulator probe was located outside the low threshold receptive field. In this case the WDR neurone was depressed by the vibration and the non-nociceptive neurone was excited.

Therefore, the differential effect of vibration applied outside the receptive field appears to represent a real difference in the responses of WDR and non-nociceptive neurones.

iv) *Entrainment of discharge of non-nociceptive and WDR neurones.*

The WDR and the non-nociceptive neurone illustrated in Fig. 10 both had their rate of firing entrained to the mechanical stimulation during the third part of the excitatory response (Fig. 10Ab and 10Bb). In total, 6 of the 12 non-nociceptive neurones and 2 of the 31 WDR neurones excited by vibration exhibited a phasic discharge entrained to the stimulation; in the remaining cases the discharge was not entrained to the stimulation. Discharge entrained to vibrational stimulation occurred only when the stimulator probe was positioned inside the low threshold receptive field.

v) *Effects of vibration on nociceptive specific neurones*

Vibration was tested on 3 nociceptive specific neurones. An important characteristic used in classifying this type of neurone was the afterdischarge in response to briefly pinching the skin using a serrated forceps (Fig. 11A). The response of WDR neurones to pinch was also characterized by an afterdischarge but this was not observed with non-nociceptive neurones (for example compare Fig. 10Aa and 10Ba). An

example of the vibration-induced effect on nociceptive specific neurones is shown in Fig. 11B. With the stimulator probe positioned outside the receptive field, vibration depressed the firing rate of this neurone; the time course of this depression was similar to the time course of the vibration-induced depression of WDR neurones. All 3 nociceptive specific neurones were depressed by vibration regardless of the location of the stimulator probe relative to the receptive field; excitation or the biphasic effect was never seen.

vi) *Effects of vibration on proprioceptive neurones*

Twelve proprioceptive neurones were tested with vibration. These neurones lacked any identifiable cutaneous receptive field but they were excited or depressed by passive flexion/extension of the ankle or of joints of the foot. All proprioceptive neurones were excited by vibration and the magnitude of the excitation was graded with respect to stimulation amplitude. The latency of this excitation was similar to that of the vibration-induced excitation of neurones with cutaneous receptive fields. While there was often a gradual decline in the discharge rate during the period of vibration (Fig. 12A), the response of proprioceptive neurones was different from the excitatory response of WDR and non-nociceptive neurones because the second part of the response was not observed with proprioceptive neurones and they lacked the post-vibration depression.

An example of the response of proprioceptive neurones is illustrated in Fig. 12. The discharge rate of this neurone increased during the period of vibration. The scatter graph in Fig. 12C illustrates that the number of spikes in the response to vibration varied directly ($r=0.98$, $p<0.01$) with the baseline rate of discharge as it was systematically raised using glutamate.

vii) *Correlation of site of recording with response to vibration*

Forty-five sites of recording (for examples see Fig. 5 and Fig. 10) were marked by deposits of Pontamine Sky Blue in a total of 30 experiments. The locations of these deposits, identified in stained histological sections, are represented in the composite drawing in Fig. 13. The recording sites were found in Rexed's laminae I and III-VI (Rexed,

1954) for the WDR neurones (n=40), in laminae III-V for the non-nociceptive neurones (n=4) and in lamina I for the nociceptive specific neurone (n=1).

With respect to the vibration-induced responses of the WDR neurones for which the recording sites were marked, the proportion of neurones which showed either the depressant or biphasic response to vibration failed to differ significantly from the proportions of these responses in the total population studied (corrected χ^2=0.18, p=0.67). Therefore, the sample of WDR neurones represented in Fig. 13 is considered representative of the total population. Interestingly, the proportion of neurones which exhibited the biphasic as opposed to the depressant response was greater when the recording site was located in laminae V or VI (11/13) than when the site of recording was in laminae I, III or IV (12/26; corrected χ^2=3.8, p=0.05).

V. DISCUSSION

i) *Vibration-induced responses are synaptically evoked by activation of rapidly conducting primary afferents*

In this study we have described 3 distinct responses of functionally-identified neurones in the spinal dorsal horn to vibration applied to the hindlimb. The control experiments provide evidence against the possibility that the vibration-induced responses were caused by movement of the electrode at the recording site. In addition, the finding that the responses to vibration failed to occur when the stimulator probe was positioned on a homologous region of the contralateral hindlimb indicates that the effects of vibration were likely due to an activation of sensory inputs in the periphery, ipsilateral to the site of recording.

The minimum latency to the start of the excitation, of the depression and of the excitatory component of the biphasic effect correspond to

conduction velocities[1] of 50, 37.5 and 50 $m \cdot s^{-1}$ respectively which indicates that at least the earliest parts of these responses were mediated by primary afferents whose axons conduct in the A$\alpha\beta$ range (Erlanger and Gasser, 1937). As innocuous mechanical stimuli, such as vibration, activate rather than inhibit primary afferent neurones in the A$\alpha\beta$ class (Darian-Smith, 1984) we conclude that the vibration-induced effects are mediated by activation rather than by inhibition of neurones in this class. This activation likely occurred at the level of the peripheral receptor because we minimized the likelihood of direct activation of axons by avoiding positioning the probe over major peripheral nerves. Consistent with activation of peripheral receptors was the finding that responses to electrical stimuli, which would presumably bypass any time delay introduced by transduction of the movement of the probe into action potentials, occurred at shorter latencies than responses to vibration. As the spinal cord was transected at L1, the vibration-induced effects could not have been mediated through supraspinal structures.

Thus, the vibration-induced discharge of primary afferents appears to have led to the responses of the dorsal horn neurones. Synaptic connections of rapidly conducting primary afferents to dorsal horn neurones have been well described in anatomical studies at both the light (Brown, 1981) and electron (Ralston et al., 1984) microscopic level and hence it seems reasonable to suggest that the vibration-induced responses were likely synaptically mediated.

ii) *Vibration-induced excitation*

The majority of WDR neurones and all non-nociceptive neurones were excited by vibration when the stimulator probe was positioned inside the receptive field for innocuous stimuli; only nociceptive specific neurones failed to be excited by vibration. The excitatory responses of WDR and

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[1] The distance used was about 300 mm (the average distance from the tip of the toe to the L7 segment measured by laying a thread along the path of the peripheral nerves in several animals) and the time was the minimum latency - 2 ms. Two ms were subtracted to account for the delay caused by peripheral activation of the primary afferents which at the least was 1 ms (cf. Fig 2A1 and 2A2), and to allow for synaptic delay (1 ms). Thus, the values for conduction velocity represent the minimum for the earliest component of each response. These values would be greater for polysynaptic responses.

non-nociceptive neurones were similar in latency and time course and hence will be considered together during this portion of the Discussion.

The first part of the excitation was a burst which occurred at a minimum latency of 8 ms. The central delay measured for the excitation caused by vibration is similar to the central delay for the excitatory postsynaptic potential (EPSP) following electrical stimulation of peripheral nerves (Hongo et al., 1966,1968). The length of the central delay may indicate that some of the dorsal horn neurones studied here were connected monosynaptically with the primary afferents meditating the effects of vibration.

The second part of the excitatory response is a period of depressed activity relative to the first and third parts. The decrease in excitation does not appear to represent a post-excitatory depression of activity because the number of spikes during this period were not inversely related to the number during the first part (Salter and Henry, unpublished observations). Instead, this decrease may be caused by an inhibitory input during the second part which is consistent with the finding that in a third of cases the mean rate of discharge falls below the control level. Interestingly, proprioceptive neurones failed to exhibit this depressant part of the response which may indicate that the presumed inhibitory input causing the depression is absent from this type of neurone.

The third part of the excitation is particularly interesting because with 50% of non-nociceptive neurones the excitation occurred phasically with a period equal to that of the stimulation. This phasic excitation may have resulted from the entrainment of the discharge of primary afferents which is known to occur during vibration (Hunt, 1961; Lindblom and Lund, 1966; Talbot et al., 1968). Although previous reports have indicated that dorsal horn neurones which would be termed WDR neurones using our classification may be entrained by vibrational stimulation of the skin (Honda et al., 1986; Wall and Cronly-Dillon, 1960), we believe that this is the first report that the discharge of non-nociceptive neurones will entrain to this type of stimulation. The possible functional implications of this entrainment will be discussed subsequently.

The decrease in rate of firing which followed the vibration-induced

excitation may have been due to a post-excitatory depression or may have been caused by activation of an inhibitory input with a prolonged time course.

iii) *Vibration-induced depression*

Forty-three percent of WDR neurones and all nociceptive specific neurones exhibited the depressant response; non-nociceptive neurones were never depressed by vibration. Importantly, with the stimulation frequency used in the present study (80 Hz) depression could be elicited only when the stimulator probe was applied outside the excitatory receptive field for innocuous mechanical stimuli. The present results are similar to the finding of Wall and Cronly-Dillon (1960) that vibration, using a frequency of 60 Hz applied outside the receptive field, sometimes produced depression of dorsal horn neurones which were excited by noxious and innocuous cutaneous stimuli and hence would appear to be WDR neurones using our classification; effects of vibration on neurones activated specifically by noxious stimuli were not investigated by Wall and Cronly-Dillon.

The finding that the discharge evoked by glutamate is blocked by vibration is consistent with our suggestion that the effects of vibration are elicited synaptically in the spinal cord. In addition, because the excitatory effect of glutamate is primarily postsynaptic (Puil, 1983), the depression of glutamate-evoked activity raises the possibility that the mechanism of the depression involves a postsynaptic inhibition. This effect of vibration on glutamate-evoked activity is consistent with findings from a recent study of spinocervical tract neurones recorded intracellularly (Brown et al., 1987). It was noted that vibration applied to the foot pads by a 500 Hz tuning fork caused hyperpolarization in some of the 10 cases examined; this hyperpolarization was interpreted as an inhibitory postsynaptic potential (IPSP). Intracellular studies by others have shown that dorsal horn neurones are hyperpolarized by activation of primary afferents with electrical stimulation of peripheral nerves (Kolmodin and Skoglund, 1958; Hongo et al., 1966,1968; Hunt and Kuno, 1959a; Steedman et al., 1985). Blockade of glutamate-evoked activity does not, however, rule out the possibility that the vibration-induced depression may be mediated by a presynaptic mechanism.

The central delay and the minimum latencies for the depression were found to be longer than the central delay and the minimum latency for the excitation, respectively. These results are consistent with findings from the intracellular studies noted previously which revealed that the central delay for EPSPs is shorter than the central delay for IPSPs. An explanation for the longer central delay with depression is that the depression may be a polysynaptic response whereas excitation may be monosynaptic; this explanation has been advanced by previous authors to account for the longer delay of the IPSPs (Hongo et al., 1968). However, other possibilities may account for the longer latency of the depression and 2 of these possibilities will be mentioned. First, it seems possible that different types of A$\alpha\beta$ primary afferents might mediate depression and excitation. As not all primary afferents in the A$\alpha\beta$ class have the same the axonal conduction velocity (Burgess and Perl, 1973) the afferents mediating the depression might conduct more slowly than those causing the excitation. On the basis of the cord dorsum potential it may not be possible to determine the time of arrival of the volley in the more slowly conducting group. A second possibility also bears mentioning. In view of increasing evidence for slowly acting chemical mediators of synaptic transmission (for example Cuello, 1983; Libet, 1986) there seems to be no valid reason to assume, as is apparently done at present (for example Mountcastle, 1984), that the synaptic delay is identical at excitatory and inhibitory synapses in the dorsal horn. Thus, the possibility must also be considered that the synaptic delay might be longer at the inhibitory synapses and thus that depressant as well as excitatory effects of vibration may be mediated monosynaptically. At present no evidence is available to distinguish between this possibility and the possibility of polysynaptic depression.

The increase in firing rate which occurred during the remainder of the vibration-induced depression may represent adaptation much as the excitation appeared to adapt during the third part of the response.

After the end of the period of vibration the rate of firing either remained depressed or was increased relative to the control rate. The persistent decrease in the rate of discharge may reflect a prolonged time course of depression because the depression caused by single short mechanical pulses has a duration of up to 300 ms (see Chapter 5). In

contrast, the increase in the firing rate after vibration may have been caused by a post-depression excitation.

iv) *The biphasic effect of vibration*

Forty-nine percent of WDR neurones responded to vibration with excitation followed by depression when the stimulator probe was located outside the receptive field. The biphasic effect shared properties of the excitatory and depressant effects: the latency of the excitatory component was similar to the latency of the excitatory response and the time course of the depressant component was similar to the time course of the depressant response. These similarities raise the possibility that the biphasic effect is the net effect of excitatory and depressant responses of the same neurone. The finding that the initial component of the biphasic effect was always excitation can be thus explained on the basis that the latency of the excitatory response was shorter than the latency of the depression. The decrease in rate of discharge which occurred following the burst at the onset of stimulation may have been caused by depression being of greater magnitude than excitation. A corollary to this possible explanation for the biphasic effect is that neurones which are depressed by vibration may show this response because they fail to receive or do not respond to excitatory inputs activated when the stimulator probe is positioned outside the receptive field.

When the stimulator probe was positioned outside the low threshold receptive field, the biphasic response to vibration was observed with neurones in laminae I and III-VI whereas the depressant response was seen mainly in laminae I, III and IV. The basis for the difference in the locations of neurones exhibiting these 2 responses may be related to the findings that each type of A$\alpha\beta$ primary afferent appears to have a specific pattern of projections to the dorsal horn and not all types of afferent project to every lamina (Brown, 1981). Therefore, neurones lacking excitatory inputs activated by vibration with the probe applied outside the low threshold receptive field may be more common in the more superficial laminae because that may be the location of the projection for the afferents causing the depression. On the other hand, it is possible that in the these laminae, neurones more commonly occur which receive but which do not respond to the afferent input which is excitatory for

neurones with the biphasic effect. The lack of responsiveness might be caused, for example, if neurones which were depressed received synaptic input from the primary afferents causing the biphasic effect (in other neurones) but lacked receptors for the as yet unidentified excitatory chemical mediator responsible for the excitatory component of the biphasic response. The possibility that dorsal horn neurones may not have receptors for a chemical mediator released by primary afferents is consistent with previous studies reporting that not all dorsal horn neurones are excited by glutamate (Schneider and Perl, 1985) and ATP (Fyffe and Perl, 1984; Jahr and Jessell, 1983; Chapter 2), substances which have been suggested as possible chemical mediators of synaptic transmission of primary afferent neurones.

In comparing the results of the present study with those of Wall and Cronly-Dillon (1960) it is noted that all of the neurones recorded by Wall and Cronly-Dillon showed a response to vibration which was termed biphasic. Their response appears to be different from the biphasic effect we report here because in their case the response was evoked by stimulation inside the receptive field. This stimulation evoked excitation which occurred repetitively, in phase with the stimulation. Each period of excitation was followed by a period of depression during which responses to cutaneous stimuli were decreased. The interpretation of Wall and Cronly-Dillon that the period of decreased responsiveness was necessarily due to an inhibition seems unwarranted as they were unable to control for the effects of the preceding excitation.

v) *Significance of the vibration-induced effects on dorsal horn neurones*

We suggest that the results of the present study may be relevant to the neural mechanisms underlying perception of cutaneously applied vibratory stimulation and to the analgesic effects of vibration in humans.

a) *Relevance to perception of vibration.* The present results indicate the magnitude of the excitatory response of non-nociceptive and proprioceptive neurones to vibration increases monotonically with increasing amplitude of stimulation and a similar observation has been made concerning the excitation of WDR neurones (see Chapter 5). In addition, for non-nociceptive and WDR neurones the magnitude of the

excitation was greater when the probe was applied inside the low threshold receptive field than when the probe was applied outside the receptive field, which indicates that these neurones respond differentially to stimulus location. Thus, dorsal horn neurones exhibited a graded response and were sensitive to the site of application of vibration, both of which are considered important properties of neurones, located elsewhere in the central nervous system, which are thought to underlie perception of vibration (Mountcastle, 1984).

An interesting feature of the responses of non-nociceptive neurones was entrainment of the discharge to the vibrational stimulation when the probe was applied inside the receptive field. We also observed that a small proportion of WDR neurones will entrain to vibration at 80 Hz. Using lower frequencies of stimulation, entrainment of firing of WDR neurones has been observed previously in the cat by Wall and Cronly-Dillon (1960) and also in the monkey by Honda et al. (1986). Entrainment of spike discharge to vibration is considered an important characteristic of the neuronal mechanisms thought to underlie discrimination of the frequency of oscillating mechanical stimuli (Merzenich and Harrington, 1969; Talbot et al., 1968). Thus, it seems possible that if there are non-nociceptive and WDR neurones in the dorsal horn in humans these neurones may constitute part of the neuronal population which underlies a conscious appreciation of vibration frequency, particularly for frequencies of 80 Hz and below which are in the range corresponding to the perception of flutter (for review see Mountcastle, 1984).

b) *Relevance to vibration-induced analgesia.* A novel finding of the present study is the differential effect of vibration on WDR vs non-nociceptive neurones when the stimulator probe was applied outside the low threshold receptive field: all non-nociceptive neurones were excited whereas only 4% of WDR neurones were excited and of the remainder, slightly more WDR neurones exhibited the biphasic as opposed to the depressant response. This difference in responsiveness to stimulation applied outside the low threshold receptive field occurred despite the fact that the proportions of WDR and non-nociceptive neurones excited by vibration were not different when the stimulator probe was positioned inside the receptive field. Interestingly, nociceptive specific neurones showed only the depressant response regardless of the

position of the probe. Thus, vibration-induced depression was restricted to nociceptive neurones.

Previous studies have indicated that cutaneously applied vibration decreases pain intensity in both experimental (Bini et al., 1984; Ekblom and Hansson, 1982; Pertrovaara, 1979; Sherer et al., 1986; Sullivan, 1968; Wall and Cronly-Dillon, 1960) and clinical situations (Lundeberg, 1983, 1984; Lundeberg et al., 1984; Ottoson et al., 1981). On the basis of the results of the present study we are prompted to suggest that the analgesic effects of vibration may be mediated at the spinal level by depressant and biphasic effects on nociceptive neurones and/or by excitatory effects on non-nociceptive neurones. The stimulation amplitudes and frequencies used in the human studies are in the range expected to cause all of the neuronal effects and therefore it seems inappropriate to exclude any of these as possibly contributing to vibration-induced analgesia.

Table 1. Effects of vibration applied inside the low threshold receptive field on WDR vs non-nociceptive (N-N) neurones (numbers represent numbers of neurones).

	Excitation	Depression	Biphasic	No effect
WDR	31	0	4	0
N-N	12	0	0	0

χ^2(corrected)=0.39, p=0.53

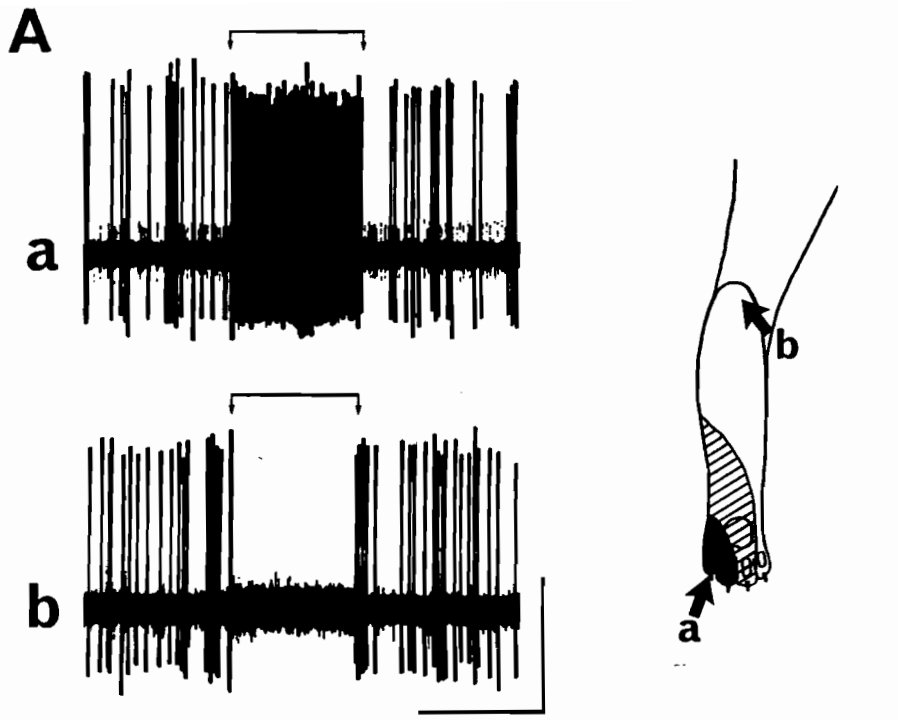
Table 2. Effects of vibration applied outside the low threshold receptive field on WDR vs non-nociceptive neurones (N-N).

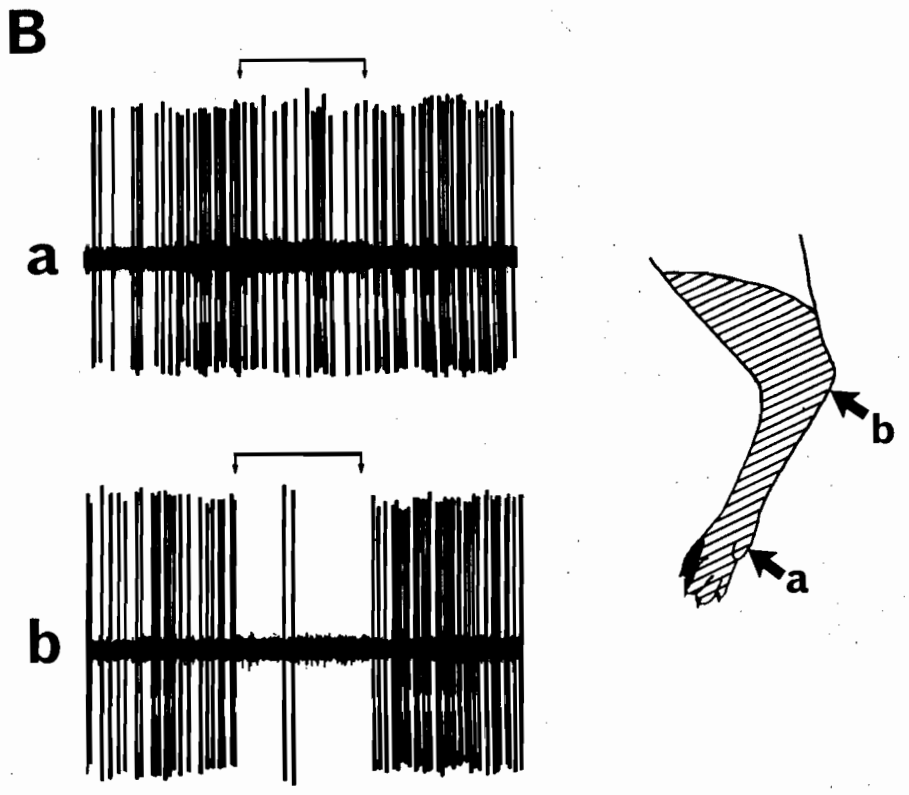
	Excitation	Depression	Biphasic	No effect
WDR	7	76	88	7
N-N	21	0	0	0

χ^2=143, with 3 degrees of freedom, $p<0.0001$

ig. 1 Effects of vibration on two WDR neurones.

Vibration was applied during the periods indicated by the bar above ach film record. Each record shows a single sweep of the oscilloscope; ie pair of records in A were from a different neurone than those in . The receptive fields for mechanical stimuli for each neurone are llustrated in the diagram to the right of the respective records. The eurones were excited by low intensity mechanical stimuli (hair movement : light touch) applied to the blackened area and by noxious pinching in oth the blackened and hatched areas. For each neurone, the probe of the timulator was applied in 2 different positions; the arrow in each of the agrams and in all subsequent figures indicates the position of the probe id, in addition, the orientation of its long axis. The upper record of ich pair was taken with the probe in position "a" and the lower record th the probe in position "b". The stimulation amplitude was 0.5 mm for g. 1A and 0.3 mm for Fig. 1B. The calibration bars apply to all the lm records: vertical 250 μV, horizontal 3 s.


A
a
b
b
a


B
a
b
b
a

ig. 2 Onset of excitatory and biphasic effects.

Film records as shown from 3 different WDR neurones; each record is a ingle oscilloscope sweep. The vibrational stimulation continued beyond he end of the traces.

A1 and A2. This neurone was excited by electrical and by ibrational stimulation in the receptive field for low intensity echanical stimuli. The arrow above the upper trace indicates the time of single electrical stimulus (0.5 mA; 1.1×threshold). The line below ne lower trace shows the displacement output from the mechanical timulator (stimulation amplitude: 0.4 mm; 1.1×threshold). In all races showing the displacement output of the mechanical stimulator an pward deflection indicates movement of the probe towards the foot and the races begin at the start of the vibrational stimulation.

B1 and B2. A single electrical stimulus (3.0 μA; 1.5× nreshold) was delivered to the common peroneal nerve at the time ndicated by the arrow above the upper trace. For the lower trace, the robe of the mechanical stimulator was applied 2 cm distal to the ankle, utside the excitatory receptive fields. The amplitude of the stimulation as 0.8 mm. The burst in B2 was followed by depression and hence this as considered to be a biphasic effect of vibration.

C1 and C2. For the upper trace, the probe of the stimulator was pplied inside the receptive field and for the lower trace it was placed utside the receptive field. The stimulation amplitude was 0.5 mm. fferent responses from the same neurone are shown in Fig. 1A.

Calibration bar: horizontal 4 ms; vertical 150 μV for microelectrode cordings only.

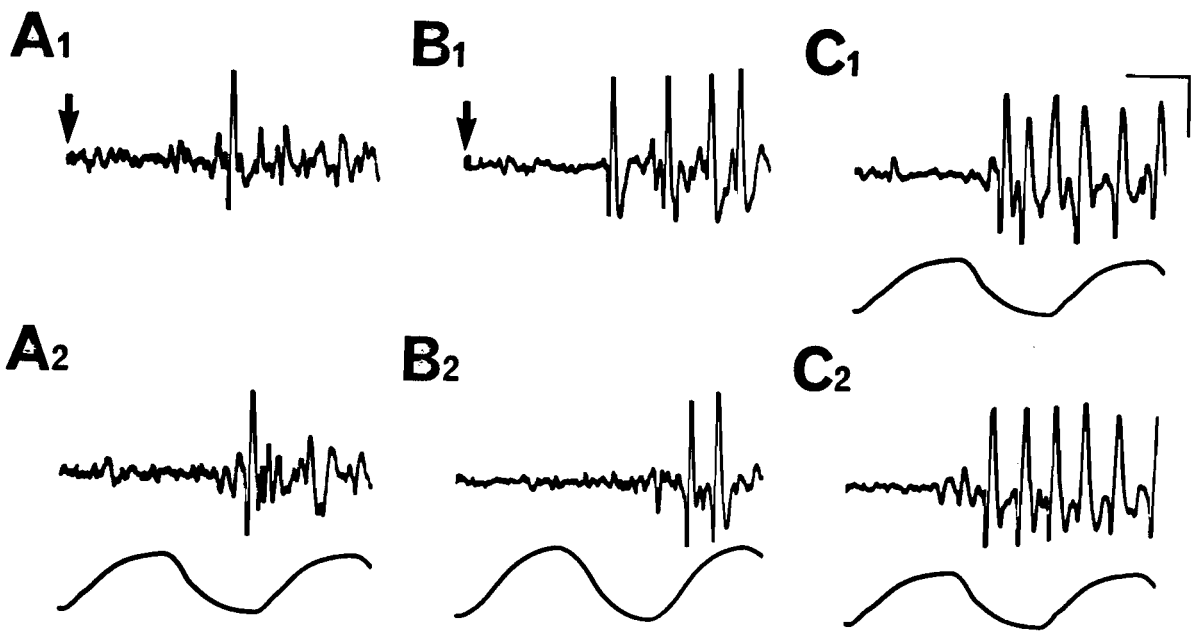

A1
B1
C1
A2
B2
C2

Fig. 3 Onset of vibration-induced depression.

The upper record is a PSTH compiled using 200 sequential applications of vibration. The rate of discharge is shown as the number of spikes per bin and the bin width is 1 ms. The stimulator probe was placed at the position indicated by the arrow in the diagram to the right. The lower record shows the displacement output from the mechanical stimulator; the duration of vibration was 3 s and hence vibration continued beyond the period of the records. The amplitude of stimulation was 0.4 mm. The receptive fields for noxious pinch and for innocuous mechanical stimuli are represented in the diagram using the same scheme as in Fig. 1.

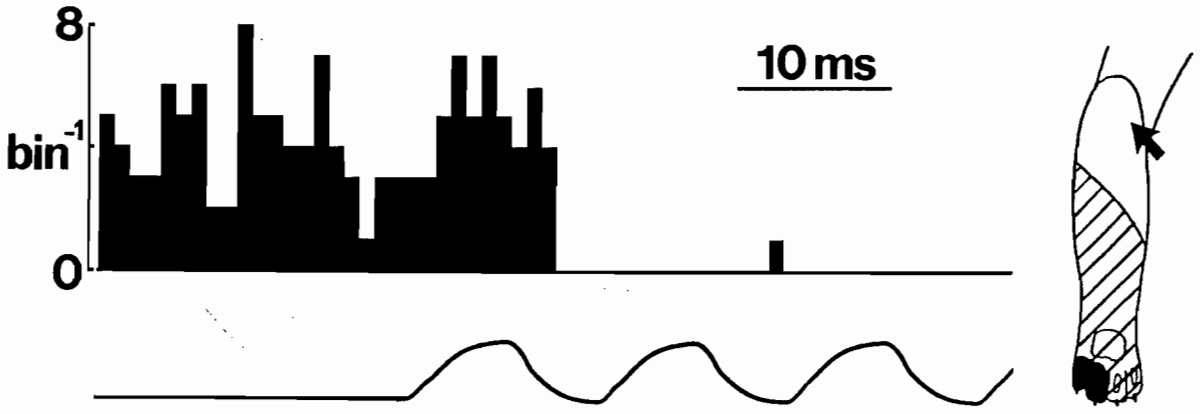
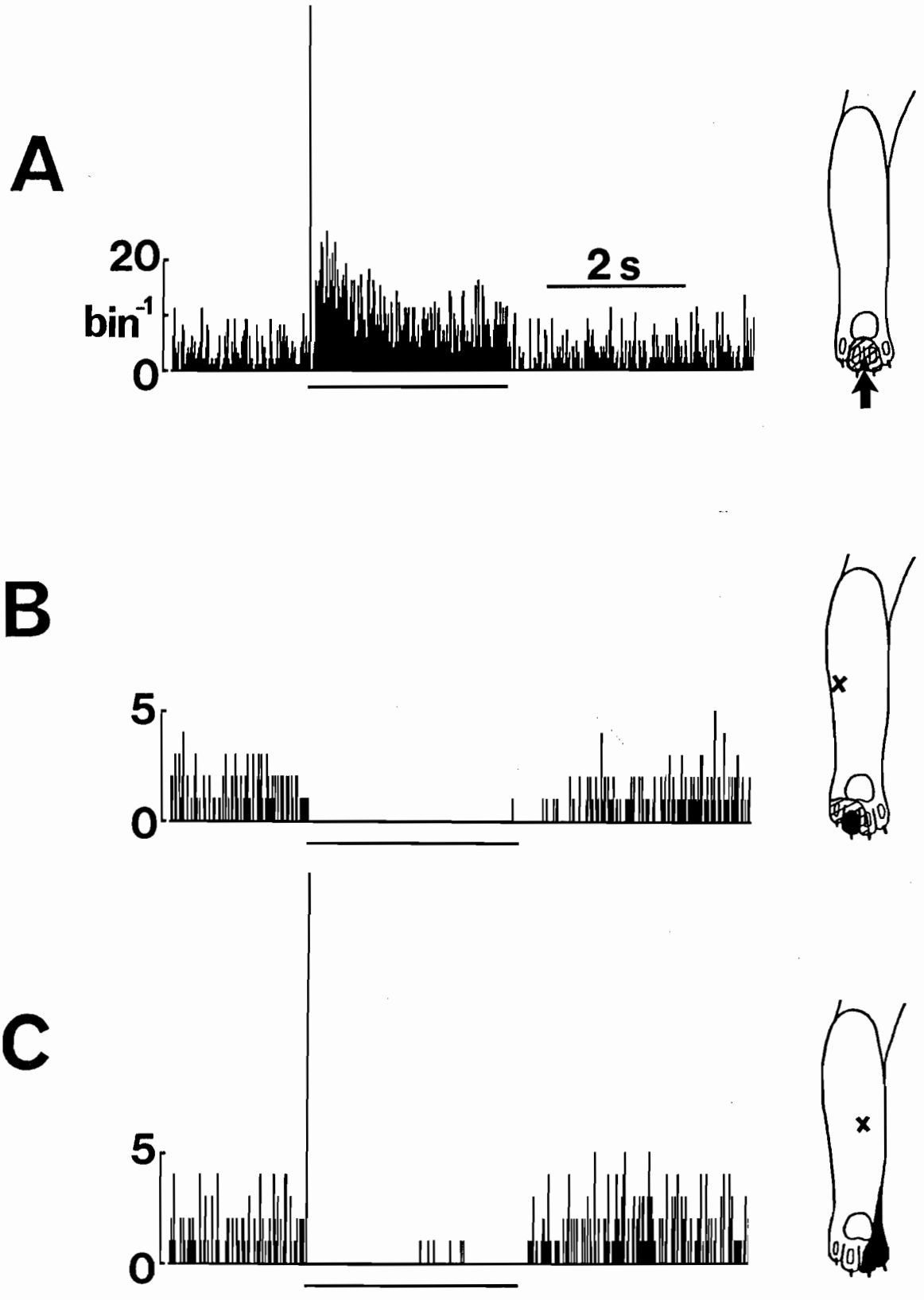

8
bin-1
0
10 ms

Fig. 4 Excitatory, depressant and biphasic effects of vibration with 3 different WDR neurones.

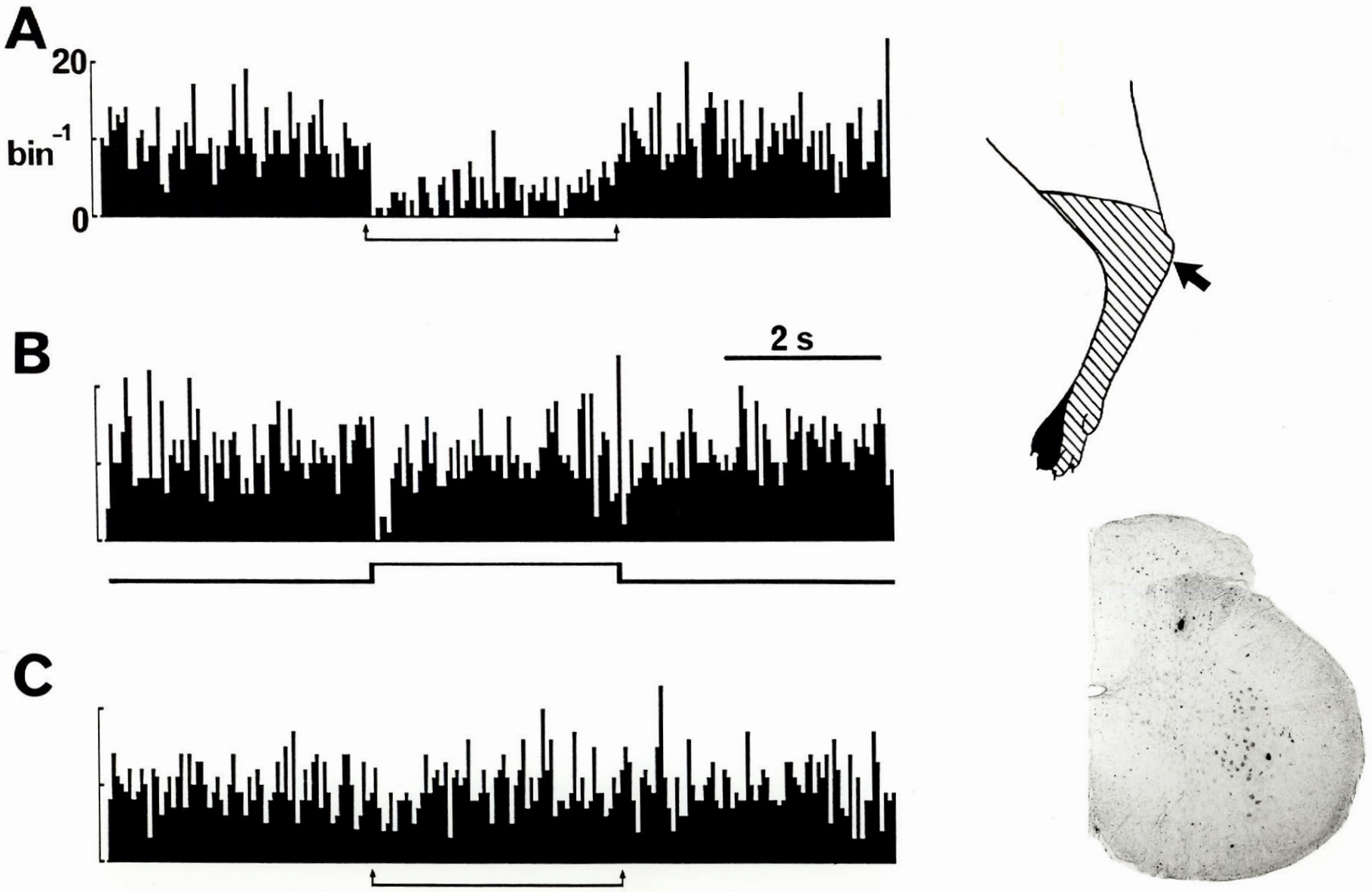
Each record shows a PSTH compiled from 10 consecutive applications of vibration; the period of vibration is indicated by the line below each record. For each PSTH the bin width is 20 ms but notice that the vertical scale for A is different from that in B and C. The receptive fields for noxious and innocuous mechanical stimuli for each of the neurones are represented in the diagrams to the right of each PSTH. For A and B the hatched and blackened areas have the same significance as in Fig. 1. For the neurone illustrated in C the receptive fields for touch and pinch were coincident and these are shown by as blackened area.

The PSTH in A illustrates the excitatory effect and the stimulator probe was positioned inside the low threshold receptive field as indicated by the arrow in the diagram to the right. In the cases of B, the depressant effect, and for C, the biphasic effect, the stimulator probe was placed outside the receptive fields at the position indicated by the 'x'. The 'x' in this and subsequent figures also signifies that the long axis of the probe was perpendicular to the plantar aspect of the foot. For B and C the amplitude of stimulation was 0.5 mm and it was 0.3 mm in the case of A.


A
20
bin-1
0
2 s
B
5
0
C
5
0

.g. 5 Effects of vibration on a WDR neurone in lamina IV are not mimicked by single long pulses or by vibration applied to the contralateral hindlimb.

Three PSTHs are shown (bin width: 50 ms). Each histogram was nstructed using 15 trials. The bar below the histogram in A indicates e period of vibration with the stimulator probe in the position shown by e arrow in the diagram to the right. In B, the line below the stogram indicates the duration a single long mechanical pulse applied at e same position as in A. For C, the probe was moved to a similar sition on the contralateral hind limb and vibration was applied for the riod indicated by the bar below the record. In all cases the imulation amplitude was 0.5 mm. The blackened and hatched areas in the agram on the upper right have the same significance as in Fig. 1. The cation of the dye deposit can be seen in lamina IV in the otomicrograph. Magnification factor: 12×


A
20
bin^{-1}
0
B
2 s
C

ig. 6 Activity evoked by glutamate is blocked by vibration.

A. Continuous time histogram showing the rate of discharge of this eurone. The receptive field was stimulated by the movement of single airs (H), by touching the skin (T) and by firm manual pressure (Pr). At he times indicated by the arrows, noxious pinch (Pi) was applied briefly o the receptive field; the duration of the pinch was less than 1 s. lutamate (Glu) was applied by iontophoresis during the period indicated y the long line below the record. The ejection current indicated is in A.

B. Film record showing a single response to vibration during pplication of glutamate. The stimulator probe was applied at the osition shown in **D**. The amplitude of stimulation was 0.5 mm. alibration bars: vertical 150 μV; horizontal 3 s.

C. The continuous time histogram begins 8 min after the one shown in ; the vertical and time scales in **C** are the same as in **A**. Ejection f glutamate was stopped after the end of the record in **A** and in **C**, he period of glutamate application is shown by the line below the ecord. At the time indicated by the arrow the ejection current was ecreased from 35 to 30 nA. Vibration applications (V) are indicated by he short bars below the histogram.

D. Diagram illustrating the receptive fields. The blackened and atched areas have the same significance as in Fig. 1. The 'x' shows the osition of the stimulator probe.

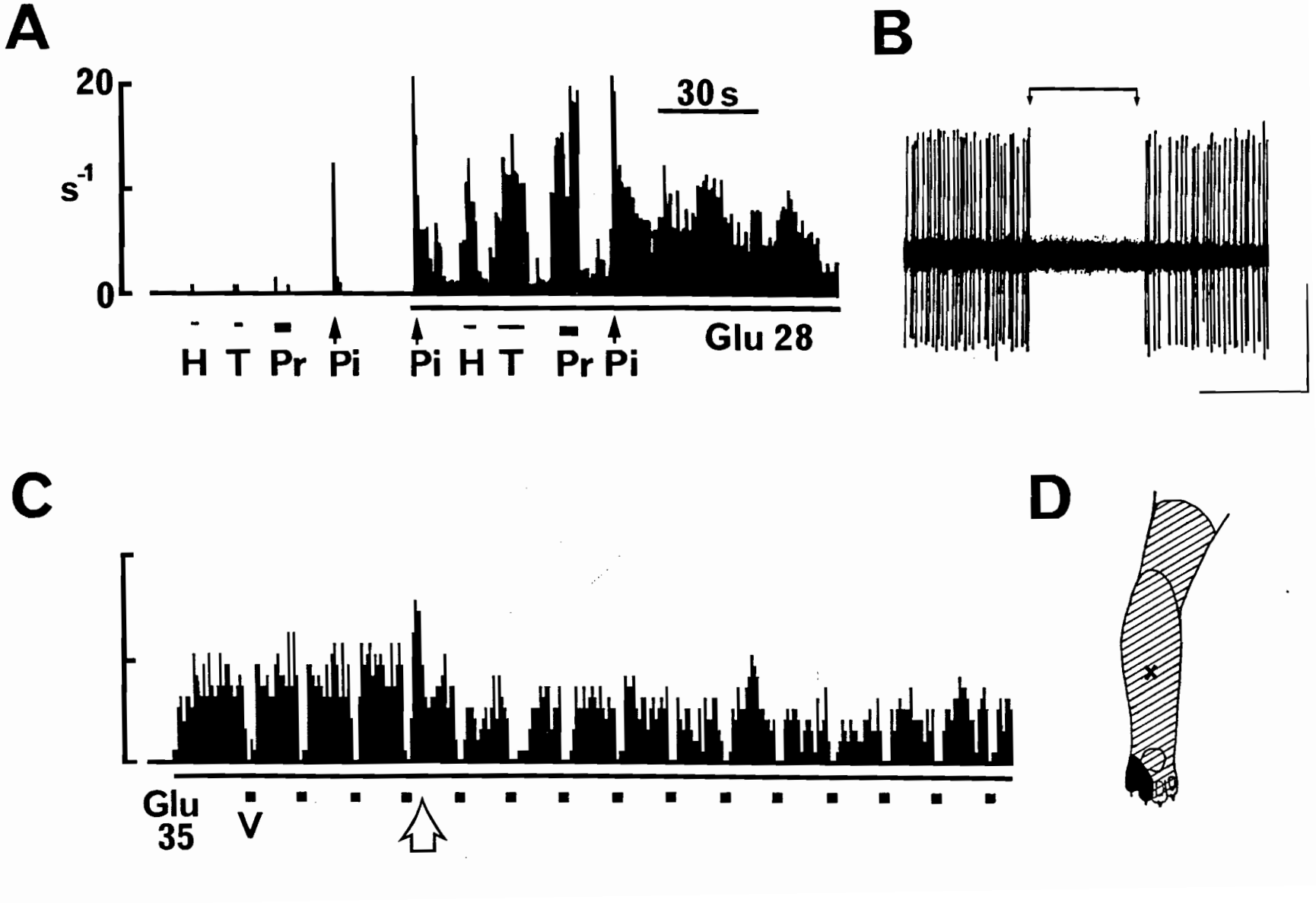
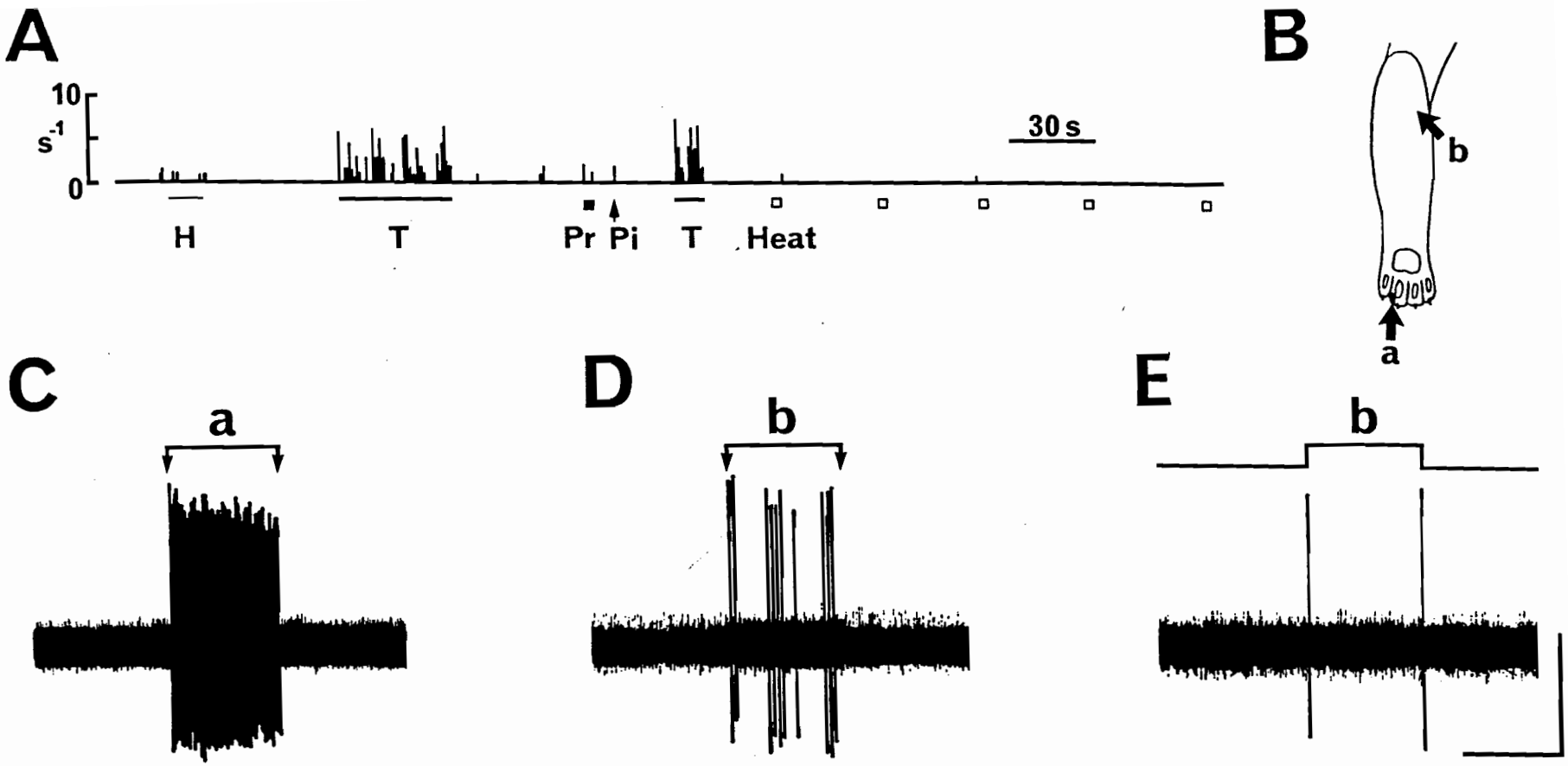

A
20
s-1
0
30 s
H T Pr Pi Pi H T Pr Pi
Glu 28
B
C
Glu
35
V
D

Fig. 7 Excitation of a non-nociceptive neurone by vibration.

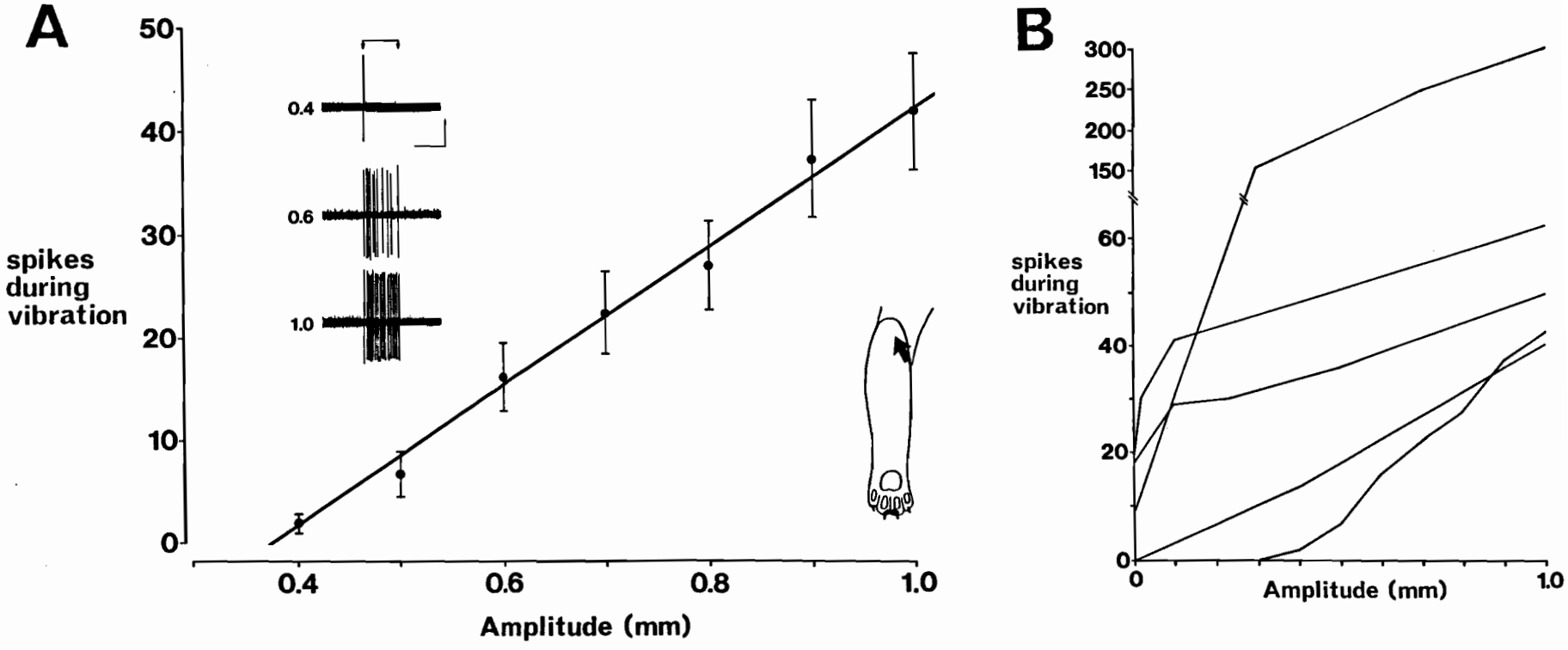
The rate of discharge is shown in the continuous time histogram in A. The receptive field was stimulated by hair movement (H), touch with a tissue paper (T) and firm pressure (Pr). At the time indicated by the arrow, the receptive field was pinched (Pi) briefly. The open squares below the record illustrate the period of application of noxious radiant heat to the receptive field. The receptive field is shown by the blackened area on the second toe from the left in the diagram in **B**. The arrows indicate the 2 positions where the stimulator probe was located. The film records in **C** and **D** show single vibration-induced responses with the probe in positions "a" and "b", respectively. With the probe in position "b" a single long pulse was applied as indicated by the line above the trace in **E**. In **C,D** and **E** the stimulation amplitude was 1.0 mm; the calibration bars are 100 μV and 3 s.


A
10
s-1
0
30 s
H
T
Pr
Pi
T
Heat
B
b
a
C
a
D
b
E
b

ig. 8 Amplitude-response relationships for non-nociceptive neurones.

The amplitude-response curve for a single non-nociceptive neurone is
iown in the graph in A: the dots indicate the average number of spikes
or 10 trials using stimulation amplitudes of 0.4 to 1.0 mm. The line is
ie least-squares regression line for the data points and the equation is
=1.96x-26.5. The error bars indicate ± one standard error of the mean.
ie film records show examples of responses for stimulation amplitudes of
.4, 0.6 and 1.0 mm. Calibration bars: vertical 200 μV; horizontal 3 s.
ie receptive field is illustrated by the blackened area in the diagram
id the position of the stimulator probe is shown by the arrow.

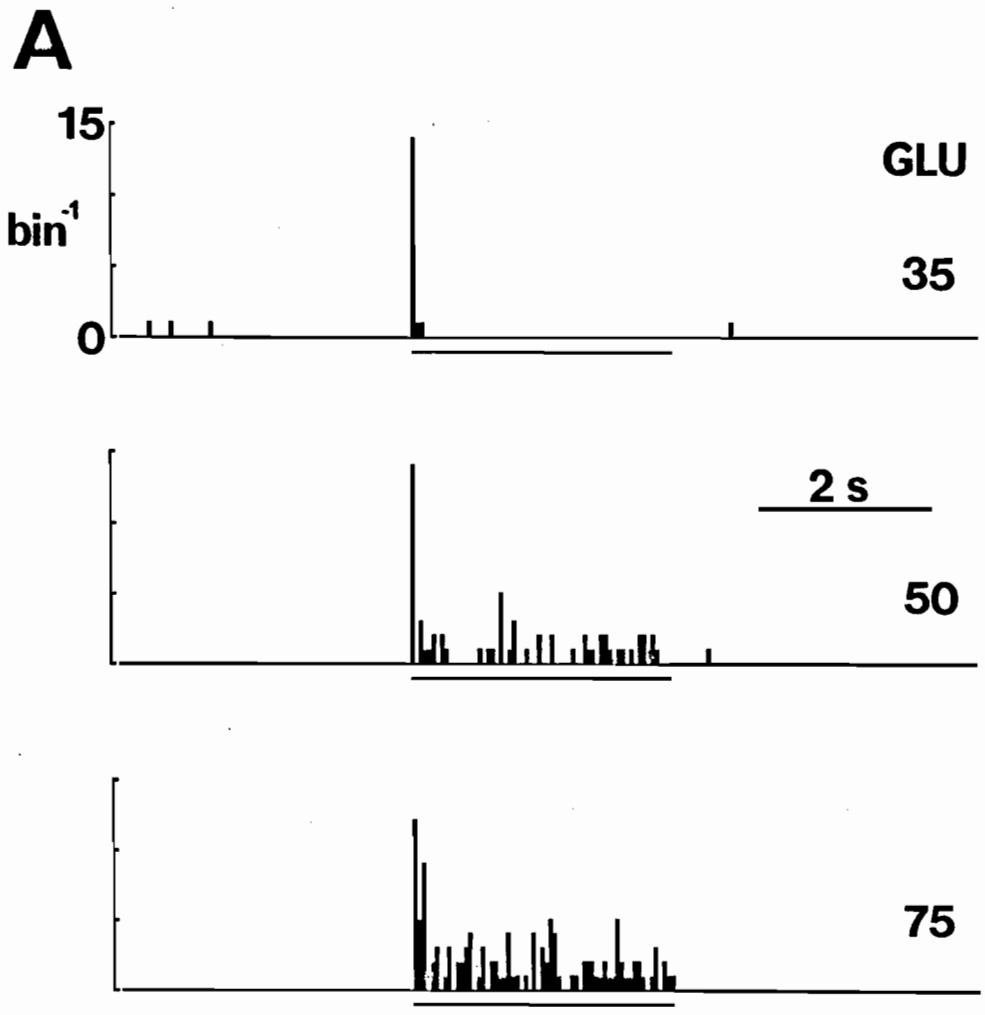
Amplitude-response curves for 5 non-nociceptive neurones are shown in
ie graph in B. In contrast to A, the data points are joined by line
:gments. The points on the y-axis indicate the average number of spikes
ıring the control period.


A
spikes during vibration
50
40
30
20
10
0
0.4
0.6
1.0
0.4
0.6
0.8
1.0
Amplitude (mm)
B
spikes during vibration
300
250
200
150
60
40
20
0
0
1.0
Amplitude (mm)

.g. 9 Vibration-induced excitation of a non-nociceptive neurone during ejection of glutamate.

Each of the 3 PSTHs in A was constructed using a series of 10 ›nsecutive applications of vibration (amplitude of stimulation: 1.0 mm). ıe period of vibration is indicated by the bar below each record and the .n width for the PSTHs is 50 ms. The ejection current, in nA, for .utamate (Glu) is indicated on the right in each PSTH. To allow for :abilization, in each case the ejection began at least 20 s before the .rst application of vibration in the series. Using ejection currents :eater than 75 nA on-going rate of discharge increased (not shown). The :ceptive field and location of the stimulator probe are illustrated in

The film record in C shows an example of a vibration-induced :sponse during ejection of glutamate using a current of 75 nA. ılibration bars: vertical 150 μV; horizontal 3 s.


A
15
bin-1
0
GLU
35
2 s
50
75

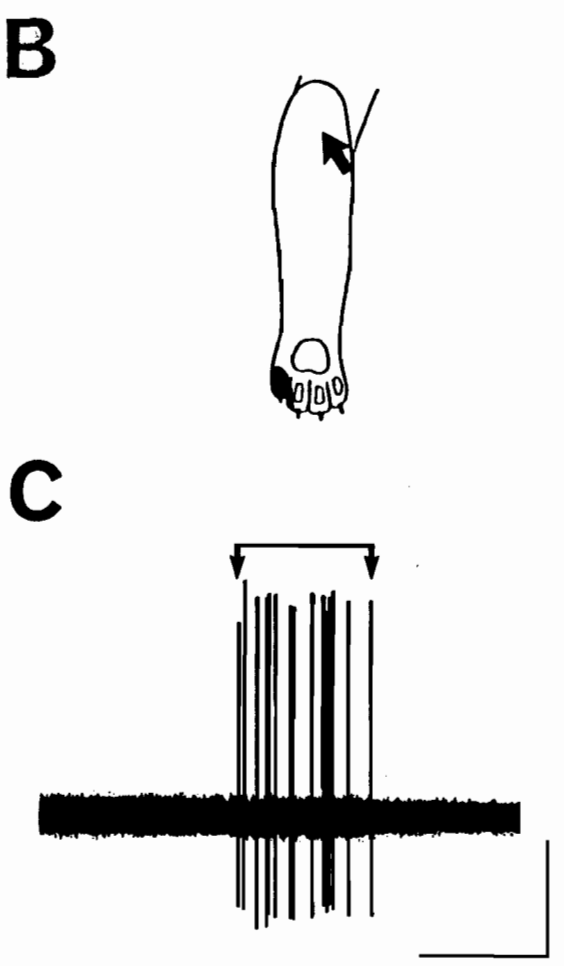

B
C

Fig. 10 Comparison of effects of vibration on a WDR and on a non-nociceptive neurone.

Data for a WDR neurone are shown in A and for a non-nociceptive neurone are in B; these neurones were studied sequentially during a single penetration of the electrode (see text for details). The records in a,b and c were constructed in the same way for each neurone. In each case, the continuous time histogram in a shows the rate of discharge and illustrates the responses to hair movement (H), light touch (T), firm pressure (Pr) and noxious pinch (Pi). The PSTHs in b and c show responses to vibration (amplitude 1.0 mm); each histogram was each made using 10 trials and the bin width is 50 ms. For b the stimulator probe was located in the position indicated by the x in the diagram illustrating the receptive field and for c the probe was in the position shown by the arrow. The line below the histogram in b is a representation of the displacement output of the mechanical stimulator.

The receptive field for the WDR neurone is shown in the right-hand diagram between A and B; this neurone was excited by mechanical stimuli applied throughout the hatched area. The receptive field for the non-nociceptive neurone is illustrated by the blackened in the diagram to the left.

Below the diagrams is a photomicrograph showing a histological section which contains a dye deposit at the site of recording of the WDR neurone (A). The recording site for the non-nociceptive neurone (B) was determined in another section. Magnification factor: 20 ×.

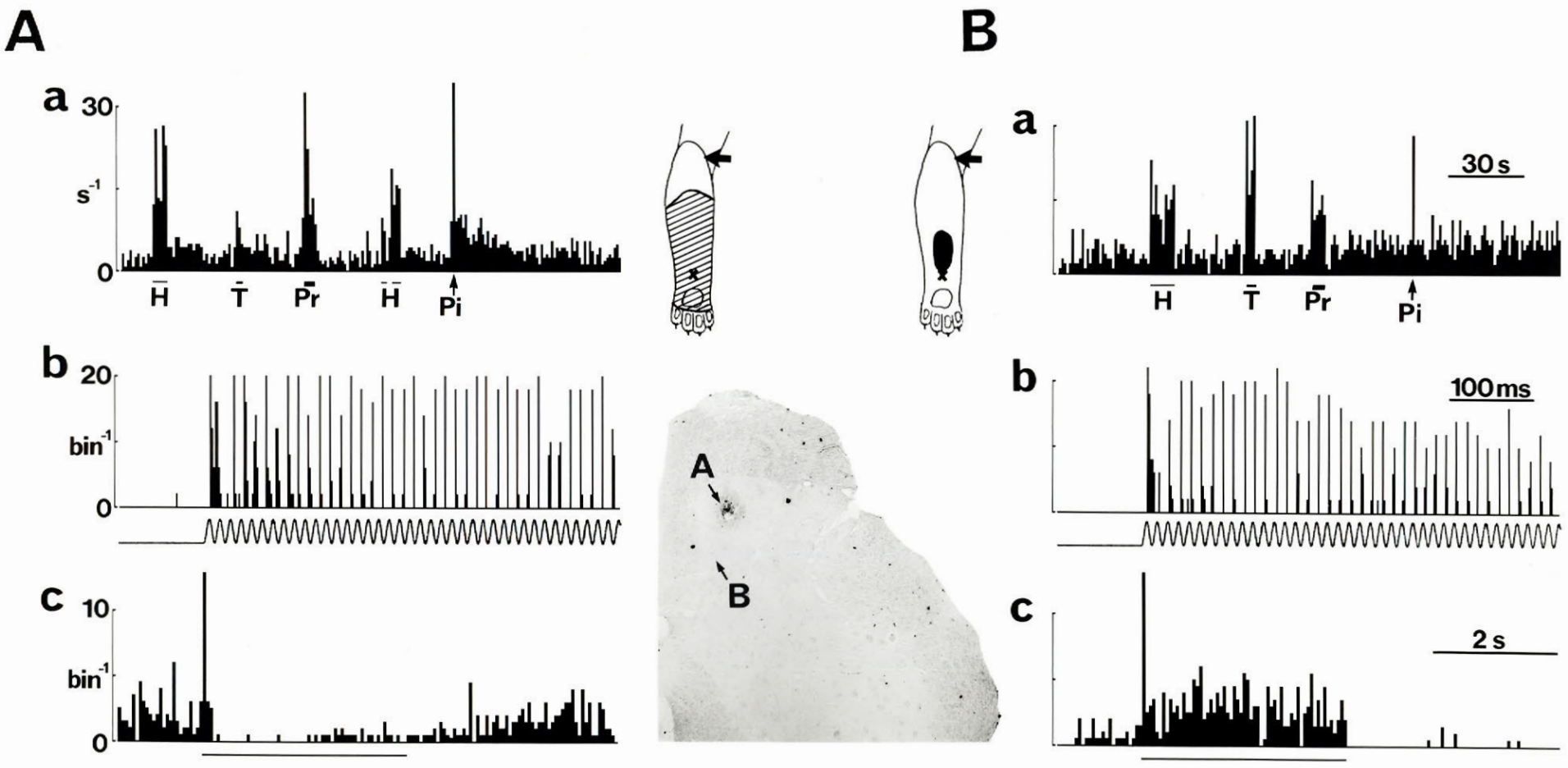

A
a
30
s-1
0
H
T
Pr
H
Pi
b
20
bin-1
0
c
10
bin-1
0
A
B
B
a
30 s
H
T
Pr
Pi
b
100 ms
c
2 s

Fig. 11 Vibration-induced depression of a nociceptive specific neurone.

A. The rate of discharge is shown in the continuous time histogram. The following stimuli were applied to the receptive field: movement of single hairs (H); light touch (T); firm, innocuous pressure (Pr); noxious manual squeeze (S) and noxious pinch (Pi). The receptive field is represented as the blackened area in the diagram to the right. The arrow indicates the location of the stimulator probe.

B. The PSTH was constructed using 15 consecutive stimulations. The period of vibration is shown by the bar below the record. The bin width is 50 ms.

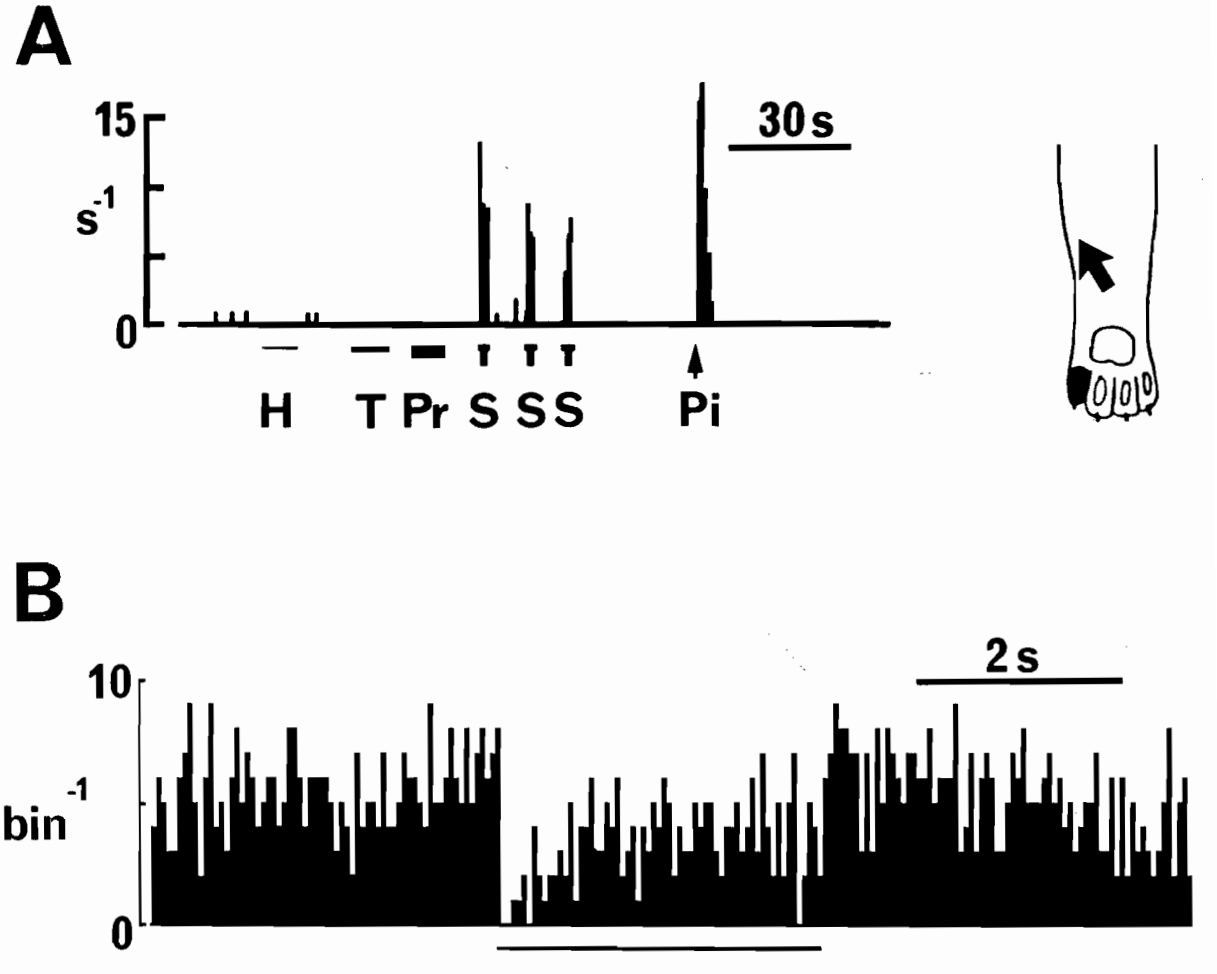
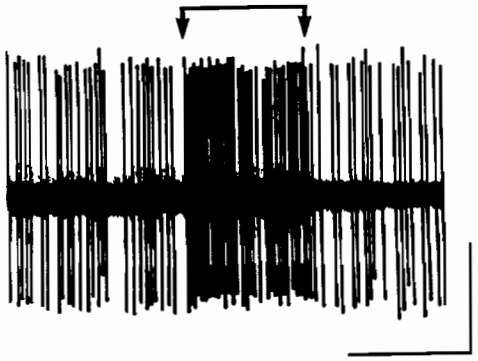

A
15
s-1
0
30 s
H
T
Pr
S
S
S
Pi
B
10
bin-1
0
2 s

Fig. 12 Vibration excites a proprioceptive neurone.

An example of a response to vibration is shown in the film record in A; the calibration bars are 150 μV and 3 s. The activity was evoked by application of glutamate. The location of the stimulator probe is shown in the diagram in B. The stimulation amplitude was 0.7 mm. In the graph in C the number of spikes during the control period before each application of vibration is plotted against the number of spikes during the corresponding application. The increase in the number of spikes before the vibration was caused by systematically increasing the ejection current for glutamate. The line is the regression line for the points ($r=0.98$, $p<0.01$); the equation of the line is $y=1.1x+7.7$.

A



B

C

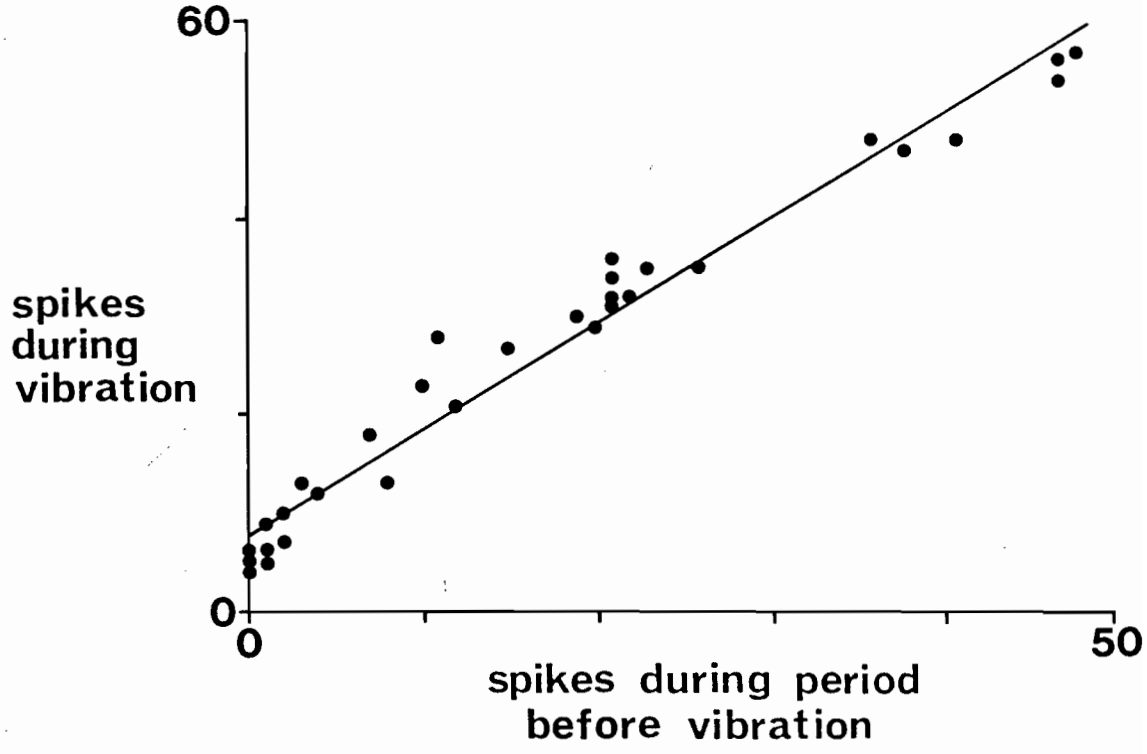
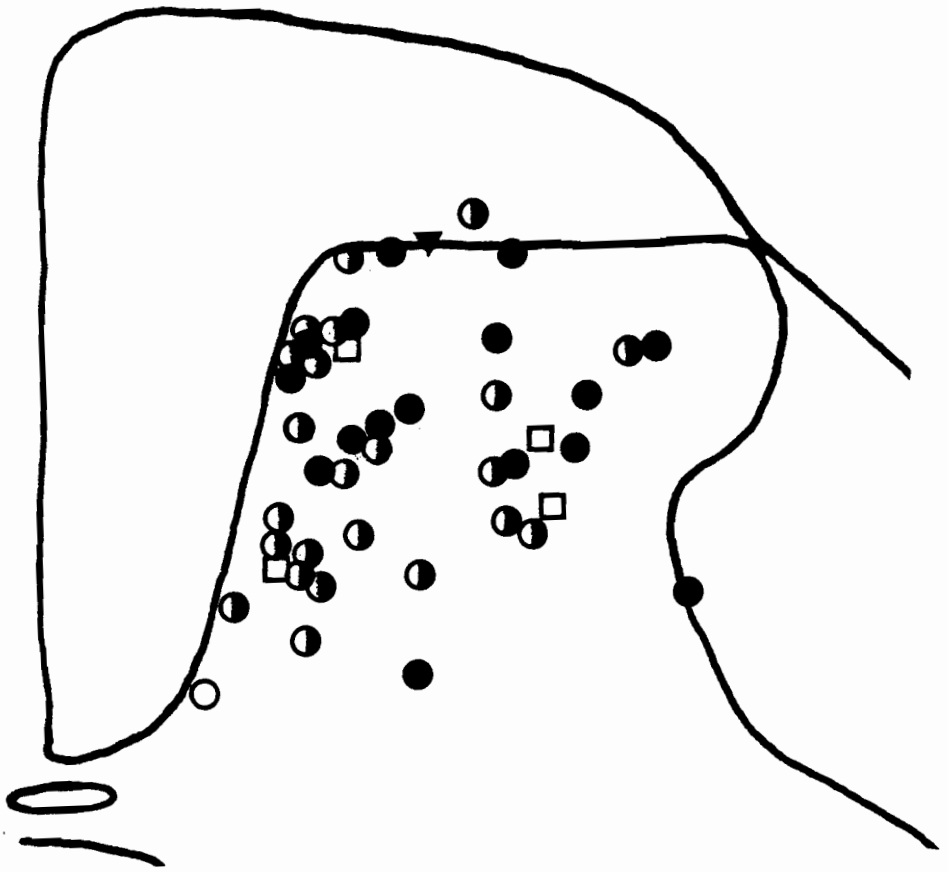

60
0
0
50
spikes during vibration
spikes during period before vibration

Fig. 13 Histological location of sites of recording.

Sites of recording for 45 neurones are represented on a schematic diagram of the dorsal aspect of one-half of the seventh lumbar segment. Recording sites are shown as dots for WDR neurones (n=40), as squares for non-nociceptive neurones (n=4) and as triangles for nociceptive specific neurones (n=1). The effect of vibration when the stimulator probe was positioned outside the receptive field is indicated by the shading of the symbols: open symbols - excitation (n=5), closed symbols - depression (n=17) and half-filled symbols - biphasic effect (n=23).



CHAPTER 4

EVIDENCE THAT ADENOSINE MEDIATES THE DEPRESSION OF SPINAL DORSAL HORN NEURONES INDUCED BY PERIPHERAL VIBRATION IN THE CAT

I. ABSTRACT

Nociceptive neurones in the dorsal horn of the cat spinal cord are depressed by vibration applied to the ipsilateral hind limb. The present study investigated the pharmacological properties of this depression because of the possibility that it represents the neural basis at the spinal level for the analgesic effects of vibration in humans. Experiments were done in cats anaesthetized with sodium pentobarbital and acutely spinalized at the first lumbar level. Extracellular recordings were made from nociceptive neurones in the lower lumbar segments. The depression and the depressant component of the biphasic response induced by vibration to the hindlimb was attenuated by administration of the P_1-purinergic (adenosine) receptor antagonist, caffeine (20-60 $mg \cdot kg^{-1}$ i.v.); the maximum attenuation was 100%. Effects of caffeine began within 2 min after the start of injection (1-3 min injection period), were greatest in the 10 min period after the end of injection and lasted for up to 2 hours. Importantly, another P_1-purinergic receptor antagonist, which does not cross the blood-brain barrier, 8-sulphophenyltheophylline (8-16 $mg \cdot kg^{-1}$), had no effect on the depression when given intravenously (n=5); however, when administered by iontophoresis 8-sulphophenyltheophylline blocked the depression in 2 of 6 units. Dipyridamole (1.0-2.0 $mg \cdot kg^{-1}$ i.v.), an inhibitor of adenosine uptake, potentiated the depression in 2 of 5 cases. These results prompt us to suggest that depression induced by vibration may be mediated by adenosine via the activation of P_1-purinergic receptors.

On the other hand, the $GABA_A$ antagonist, bicuculline, failed to attenuate vibration-induced depression when administered either intravenously (0.2-0.4 $mg \cdot kg^{-1}$; n=5) or by iontophoresis (n=10) and the glycine antagonist, strychnine (0.2-0.6 $mg \cdot kg^{-1}$; n=3) and the opiate antagonist, naloxone (0.1-0.4 $mg \cdot kg^{-1}$; n=4) were similarly ineffective. These findings suggest that vibration-induced depression of these units occurs without involvement of bicuculline-sensitive GABA receptors, strychnine-sensitive glycine receptors and naloxone-sensitive opiate receptors.

In view of the fact that vibration-induced depression is evoked synaptically, this study is the first evidence in the central nervous system of a synaptic response which is mediated by adenosine.

In addition, we suggest that the analgesic effects of vibration in humans may be mediated at the spinal level by activation of P_1-purinergic receptors.

II. INTRODUCTION

Nociceptive neurones in the spinal dorsal horn are depressed by peripheral vibration (Chapter 3; Wall and Cronly-Dillon, 1960). This depression is likely mediated by rapidly conducting, non-nociceptive primary afferent neurones (Melzack and Wall, 1965; Wall, 1978) because vibration activates this type of primary afferent fibre (Hunt, 1961; Hunt and McIntyre, 1960b) and because activation of large diameter, presumably non-nociceptive, primary afferents by electrical stimulation also depresses nociceptive dorsal horn neurones (Foreman et al., 1976; Handwerker et al., 1975; Hillman and Wall, 1969; Hongo et al., 1968; Lindblom et al., 1977; Steedman et al., 1985; Wagman and Price, 1969). Thus, a depression of these nociceptive neurones may represent, at the spinal level, the neural basis of the analgesic effect of vibration and of electrical stimulation of peripheral nerves in humans (Melzack, 1973; Melzack and Wall, 1965; Melzack and Wall, 1982).

We have reported recently that iontophoretic application of adenosine 5'-triphosphate (ATP) has a differential effect on nociceptive versus non-nociceptive dorsal horn neurones (see Chapter 2). The main effects on nociceptive neurones were a biphasic excitatory/depressant effect or depression, whereas on non-nociceptive neurones the effects were predominantly excitatory. ATP-induced excitation of units receiving non-nociceptive primary afferent input has also been reported by Fyffe & Perl (1984). The principal suggestion from both laboratories was that ATP may be a chemical mediator of synaptic transmission released by low threshold primary afferents.

The depressant effects of iontophoretic application of ATP may be mediated by adenosine, formed by hydrolysis of ATP, with the subsequent activation of P_1-purinergic (Burnstock, 1978) (adenosine) receptors (see Discussion in Chapter 2). Thus, the possibility was considered that P_1-purinergic receptors may mediate the depressant effect of low threshold primary afferent inputs on dorsal horn nociceptive neurones. In the present study, vibration was used to activate these primary afferents and the pharmacological properties of the resultant depression of dorsal horn neurones were investigated by intravenous administration of agents which affect purinergic mechanisms. These agents were caffeine and

8-sulphophenyltheophylline (8-SPT), P_1-purinergic receptor antagonists (Burnstock, 1978; Daly et al., 1985), as well as dipyridamole, which blocks uptake of adenosine and hence potentiates its effects (Phillis et al., 1979; Wu and Phillis, 1984). Caffeine and 8-SPT were also administered by iontophoresis into the local vicinity of the neurones studied.

Results of this study have been previously reported in part (Salter and Henry, 1986a).

III. MATERIALS AND METHODS

i) *Animal preparation*

Experiments were done on adult cats anaesthetized with sodium pentobarbital (40 $mg \cdot kg^{-1}$, i.p.); anaesthesia was maintained with supplemental doses (5-10 $mg \cdot kg^{-1}$, i.v.) every 3 hours throughout the experiment. The carotid artery and the external jugular vein were cannulated. Arterial blood pressure was displayed continuously on a Grass polygraph. Mean arterial pressure was above 80 mm Hg and was maintained, if necessary, with intravenous infusion of noradrenaline bitartrate (Levophed, Winthrop; 0.002% in isotonic saline). Adequate perfusion of the spinal cord was ensured as the spinal cord circulation was monitored visually throughout the experiment with a dissecting stereomicroscope.

The spinal cord was exposed for recording in segments L5 to L7 and covered with warm mineral oil to prevent drying. The cord was transected at L1 to eliminate the influence of supraspinal structures on the activity of lumbar dorsal horn neurones; transection also ensured that changes observed following intravenous administration of drugs were not due to actions at supraspinal sites. Before transection the L1 segment was injected with 0.1 mL of 1.0% lidocaine hydrochloride to minimize spinal shock.

After bilateral pneumothorax, animals were paralyzed with pancuronium bromide (Pavulon, Organon; 1 $mg \cdot kg^{-1}$) and were artificially ventilated according to the parameters of Kleinman & Radford (1964). End-tidal CO_2 concentration was maintained at 3.5-5.0%.

Rectal temperature was maintained at 38° C using a DC-driven

infra-red bulb and a servo-controller.

ii) *Recording and data acquisition*

Extracellular single unit spikes were recorded with seven-barrelled micropipettes (overall tip diameter 4-10 μm). The central recording barrel was filled with 2.7 M NaCl (impedance 4-10 MΩ) and was connected via a high-impedance, unity-gain headstage to a Tektronix RM-122 amplifier and then to Tektronix 565 and 5111 oscilloscopes. The continuous discharge was recorded from the display of the 565 oscilloscope onto Kodak linagraph paper using a Grass Kymograph camera. The amplified raw data were recorded on magnetic cassette tape and film records were made, as well, by re-displaying the data from the tape.

The output of the 565 oscilloscope was led to a gating unit. Spikes were detected, electronically, by appropriate adjustment of an amplitude discriminator in a gating unit. The gating unit also served as a frequency counter (bin width 1 sec) and the output was continuously displayed on a Grass Model 5 polygraph.

The amplitude discriminator produced a Schmitt trigger output which was led to a digital-to-analogue multiplexer. This multiplexer also received trigger pulses from the iontophoresis unit and the Grass stimulator. These three different inputs were encoded as output voltage pulses of distinct amplitudes. The output of the multiplexer was connected to a Data Translation DT2801-A analogue-to-digital board in an IBM personal computer.

Data sampling was continuous at a sampling frequency of 2 kHz. The input from the multiplexer was decoded by the software and the intervals between spikes or between trigger pulses were computed to $\pm$0.5 ms and stored on fixed disk. Software for sampling and analysis were developed in our laboratory.

iii) *Iontophoresis and solutions*

Outer barrels of the micropipettes were used for iontophoresis and were each filled with one of the following solutions: sodium L-glutamate (1 M, pH 7.4, Sigma); 8-SPT (20 mM, pH 7.0, Research Biochemicals Inc.); (-)-bicuculline methiodide (10 mM in 165 mM NaCl, pH 5.5, Sigma);

Pontamine Sky Blue 6BX (1% in 0.5 M sodium acetate, Gurr) and control solution (165 mM NaCl, pH 5.5). All solutions were prepared with double distilled water. L-Glutamate (glutamate) was kept as a stock solution; for all other compounds individual aliquots were made sufficient for one experiment. Solutions were stored frozen and then thawed on the day of the experiment.

Glutamate was ejected with inward current; other compounds were ejected using outward current. Currents were produced by a constant current iontophoresis unit (Cocyen Co. Ltd). Retaining currents of 10 nA with the polarity opposite to that used to make ejections were maintained between applications to counteract leakage from the pipettes by diffusion. The control solution was used to detect artifacts due to local changes in current density, pH or ionic composition at the electrode tip.

iv) *Histological identification of recording sites*

Selected recording sites were marked with a deposit of Pontamine Sky Blue by passing inward current (5-10 μA) for 8-10 min through a barrel containing this compound. The method of fixation and staining were modified from those of Henry (1976). In the present experiments the fixative was a phosphate buffered solution of 1% paraformaldehyde and 1.25% glutaraldehyde; toluene rather than xylene was used to clear the tissue. Serial transverse sections (15 or 25 μm) were stained with thionin to show the lamination of the dorsal horn. Dye deposits were retained in the tissue sections better with this histological method than with that used previously.

v) *Intravenous administration of drugs*

Drugs for intravenous administration were delivered via a catheter in the external jugular vein. The dosages were as follows: caffeine, 20-60 $mg \cdot kg^{-1}$ (Fisher); 8-SPT, 8-16 $mg \cdot kg^{-1}$; dipyridamole, 0.1-5 $mg \cdot kg^{-1}$ (Sigma); bicuculline hydrochloride, 0.2-0.4 $mg \cdot kg^{-1}$ (Research Biochemicals Inc.); strychnine sulphate, 0.2-0.6 $mg \cdot kg^{-1}$ (British Drug Houses); naloxone hydrochloride, 0.1-0.4 $mg \cdot kg^{-1}$ (Narcan, Endo). 8-SPT, dipyridamole, bicuculline and strychnine were dissolved immediately prior to use; caffeine and naloxone were made as

stock solutions and stored at 4°C. Caffeine and 8-SPT were dissolved close to the limit of their solubility; however, to administer the dosages required, it was necessary to inject volumes of up to 10 mL and hence injections were made over a period of 1-3 min. Compounds other than caffeine and 8-SPT were dissolved in less than 2 mL of vehicle and were administered over a period of less than 1 min. The vehicle for all compounds except dipyridamole, was sterile isotonic saline; for dipyridamole dimethylsulfoxide was used. Intravenous injections of vehicle alone were made to control for possible effects of injection.

vi) *Classification of neurones*

Each unit was carefully classified according to its responses to natural peripheral stimulation; details of the classification scheme are described elsewhere (see Chapter 2). Briefly, the stimuli were low-flow air stream, movement of single hairs, gentle to firm manual pressure, pinching with a serrated forceps, heating to noxious temperature (>47°C) with an infrared bulb and passive movement of the limb. In some cases bipolar needle electrodes were inserted into the receptive field and the response was determined to electrical stimulation of the skin.

vii) *Vibration stimuli*

A feedback-controlled mechanical stimulator (Chubbuck, 1966) was used to generate vibration. The stimulator probe was placed firmly on the medial or plantar surface of the ankle. Each unit studied had an excitatory cutaneous receptive field to low intensity mechanical stimuli on the distal hindlimb. Accordingly, to minimize excitatory inputs the stimulator probe was always placed outside of this receptive field (see insets in Figs. 1-8). The ankle was draped over a horizontal bar and was able to flex and to extend slightly but the hindlimb was otherwise fixed and unable to abduct or to adduct.

The mechanical stimulator was driven by trains of rectangular voltage pulses from a Grass S88 stimulator. The frequency of pulses within the trains was 80 Hz and the pulse duration was 6 ms. The amplitude of the pulses was adjusted individually for each unit until a reproducible depression was achieved. The displacement of the stimulator probe was monitored throughout the experiment to ensure that pulse amplitude

remained stable. Constant duration trains, 2.5-3.5 s in length, were applied periodically at regular intervals every 20-25 s. Train duration and repetition rate were constant for a given unit. The 20-25 s interval between applications of vibration was sufficient for the firing rate to return completely to the control level for several s prior to initiation of the next train.

Pharmacological agents given to test their effects on vibration-induced depression were administered only after responses to the regular, periodic application of vibration had been of uniform amplitude and time course over a period of at least 10 min. The vibration amplitude was adjusted so that the magnitude of depression was submaximum during the period prior to administration of pharmacological agents to ensure that a dynamic part of the stimulus-response relationship was used. It has been found previously that vibration-induced depression remains stable for periods of up to 4 hours in the absence of pharmacological intervention.

viii) *Data analysis*

Data analysis by computer was done off-line. The average magnitude of the vibration-induced depression (D) was calculated using 10 or more consecutive applications of vibration. The statistical significance of D was determined using a paired t-test. D was calculated before and after administration of pharmacological agents.

As described below D often depended upon the baseline rate of firing. Thus, if drug administration caused a change in baseline firing rate it was possible to calculate a change in D which did not represent a real change in the effect of vibration (for an example see Fig. 7). It was therefore critical to control for possible variation in D due to changes in baseline rate of discharge alone. Thus, the discharge rate was controlled systematically with iontophoretic application of glutamate. Glutamate was used for this purpose because it to considered to have primarily a direct, postsynaptic excitatory effect (Puil, 1983). In terms of data analysis, the relationship of the numbers of spikes during the periods of vibration (test periods) versus the numbers of spikes during periods of equal duration just before each corresponding application (control periods) was evaluated using a linear regression analysis by the method of least squares. Then, an analysis of covariance (Snedecor and

Cochran, 1967) was done to determine if the pharmacological agents affected D independently of changes in the baseline rate of firing. Effects were considered statistically significant for $p<0.05$. Percentage change in D was then calculated at a baseline firing rate which was the average of that before and after drug administration.

Numerical values are reported as the mean $\pm$ 1 standard error of the mean.

IV. RESULTS

Data from 34 neurones are included in these results. All units studied were wide dynamic range; that is, they were excited by both noxious and innocuous cutaneous stimuli to the respective receptive field. An afterdischarge characterized responses to noxious pinch (see for example Fig. 3B); this afterdischarge was absent from responses to non-noxious mechanical stimuli. Responses to noxious and innocuous mechanical stimuli illustrated in Fig. 3B are typical of those of wide dynamic range neurones in the spinal dorsal horn. All units were tested with glutamate and, to eliminate recordings from fibres, only those units which showed clear excitation were included in the results.

On the basis of stereotaxic depth measurements from the dorsal surface of the spinal cord and of physiological responses, all units studied had features similar to those previously reported for neurones in laminae I and III-VI (Price and Dubner, 1977). In the cases attempted the recording sites were identified in histological sections; one example is shown in Fig. 2 and another in Fig. 8.

Intravenous administration of pharmacological agents was tested on only one unit in each experiment. To avoid possible persisting effects after intravenous injection this unit was the final one studied during any recording session; in 6 experiments this was also the only unit studied. Effects of pharmacological agents were considered genuine only if they were reversible, reproducible and not mimicked either by intravenous injection of vehicle in the case of intravenous drug administration or by passing similar current through the barrel containing control solution in the case of iontophoretic drug application.

i) *Response to vibration*

Characteristics of the response to vibration are described elsewhere (see Chapter 3). In brief, depression began 15-50 ms after the start of vibration, the nadir of the depression generally occurred within the first 100 ms of the stimulation. The firing rate either remained depressed or showed partial recovery gradually throughout the remainder of stimulation, and following the end of the vibration there was a return to control levels within the next 500 ms.

An increase in rate of firing during the first 8-20 ms of stimulation was observed in approximately 50% of units. These units showed the biphasic response described in detail in Chapter 3. Some units also showed an excitatory off-response to application of vibration.

A statistically significant correlation coefficient for the linear regression comparing the numbers of spikes in control versus test periods suggested a linear relationship between these numbers. When the intercept of the regression line was different from zero the magnitude of depression varied depending upon the number of spikes in the control period (i.e. upon the baseline rate of firing): an intercept less than zero indicated that the magnitude of depression decreased as the baseline firing rate increased (for example see Fig. 7A). The opposite relationship between the magnitude of depression and baseline rate of discharge occurred when the intercept was greater than zero. Only rarely was the intercept of the regression line zero; in these cases the magnitude of depression was independent of the baseline firing rate (see for example Fig. 1C, lower line).

ii) *Effects of i.v. administration of caffeine on response to vibration*

Effects of intravenous injection of caffeine were studied in 11 cats. The doses used in the present study were selected because they have been previously shown, by others, to reduce the depressant effects of iontophoretic application of adenosine on neurones in the cerebral cortex (Phillis et al., 1979; Stone and Taylor, 1980).

a) *Nature of the effects of caffeine.* The principal effect of administration of caffeine was attenuation of the depression induced by vibration. An example of this effect is illustrated by the oscilloscope

traces in Fig. 1A. The response to vibration was unaffected by intravenous injection of 10 mL of isotonic saline (Fig. 1Aa vs Fig. 1Ab). However, by comparing Fig. 1Ac with Fig. 1Ab it is apparent that the vibration-induced depression was attenuated by 80% 45 s after the end of administration of caffeine (50 $mg \cdot kg^{-1}$ in 10 mL isotonic saline). Thirty-four min after caffeine injection, the depression had recovered to 75% of the level before caffeine administration (Fig. 1Ad). Thus, this effect of caffeine satisfied the criteria stated above for a genuine response. It is important to note that this effect occurred without any change in the amplitude or in the configuration of the spike.

Administration of caffeine similarly caused a statistically significant attenuation of the vibration-induced depression in 10 of the 11 units tested. Greater than 50% attenuation was observed with 8 of these units.

b) *Time course of the effects of caffeine*. With the unit illustrated in Fig. 1 the response to vibration began to change about 1 min after the start of the injection of caffeine and the magnitude of the vibration-induced depression gradually decreased over the next 2-3 min (Fig 1B, middle record). The response to vibration was then stable for a period of 8-9 min. Over the following 20 min period the magnitude of the vibration-induced depression recovered gradually to stabilize at the level shown in the lower record in Fig. 1B.

The time course of the effect of caffeine administration on the depressant response to vibration was consistent from unit to unit: vibration-induced depression was noticeably affected within 2 min of the start of injection (for another example see Fig. 2C), the effect of caffeine was greatest in the 10 min period after the end of administration and recovery occurred gradually. A stable level of the vibration-induced response was reached over the next 20-30 min; this level was maintained throughout the subsequent one and a half hour period.

The degree of recovery was inversely related to the magnitude of the attenuation caused by caffeine which was dependent on the dose (*vide infra*). In 2 cases, with low doses of caffeine the response to vibration recovered totally. In the other cases, at higher doses, the maximum recovery was 89%.

c) *Effects of caffeine on the response to vibration are not due to*

changes in the on-going rate of discharge. The rate of firing of the unit shown in Fig. 1 increased slightly beginning about 1 min after the start of the injection of caffeine (Fig. 1B, middle record). Therefore, an investigation was made of the relationship between the number of spikes during the vibration versus the number of spikes in the control period before vibration. In this case, the examination was done on 2 parts of the response because vibration caused an increase in the rate of firing at the onset of stimulation (Fig. 1Aa & 1Ab). As this increase lasted approximately 500 ms after administration of caffeine (Fig. 1Ac) the first part to be analyzed was the initial 500 ms period of the response and the second part was the remainder of the period of vibration.

For the first part, the net effect of vibration changed from 64.0±12% depression to 219±40% excitation. Analysis of covariance indicated that this change was not due to the increase in the baseline firing rate during the period after caffeine injection

In Fig. 1C, the number of spikes in the second part of each response to vibration application is plotted versus the number of spikes in the corresponding period before the respective application; each point in the scatter graph corresponds to a single response. The least squares regression lines which are shown were calculated from the points taken in the periods immediately before and immediately after administration of caffeine ($r=0.42$, $0.10>p>0.05$ and $r=0.77$, $p<0.01$, respectively). The slope of the line from the former period was significantly less than that of the line from the latter period ($F(1,36)=18.0$, $p=3\cdot10^{-4}$). Thus, the caffeine-induced change in the second part of the response was statistically significant, and furthermore, was independent of the increase in the baseline rate of firing.

The effect of caffeine injection on baseline rate of discharge varied between the different units. With some units the magnitude of this effect also varied upon repeated administrations to that unit: with 2 units the baseline was unchanged by all injections, with 3 it was increased by at least one injection, and in 4 one or more injections decreased the baseline firing rate. In addition, with 2 units the rate of firing was maintained at a constant elevated level by iontophoretic application of glutamate. These 2 units are illustrated in Figs. 3 and 4. With all units except one, analysis of covariance showed that administration of

caffeine caused a statistically significant attenuation of the vibration-induced depression (see Fig. 3D for another example). Thus, caffeine attenuated vibration-induced depression independent of any effect on baseline firing rate.

d) *Relationship of the magnitude of the effects of caffeine to the dose*. The magnitude of the effect of caffeine on vibration-induced depression was directly related to the amount administered. This relationship is illustrated in Fig. 2. As this unit did not show the period of increased rate of firing at the onset of vibration seen in Fig. 1, the test period was the entire duration of the application of vibration. Three initial injections of caffeine (20 $mg \cdot kg^{-1}$) failed to alter significantly the response to vibration. After a fourth injection of caffeine the mean number of spikes in the control period was not different from the pre-injection level (106.1 ± 1.2 vs 106.4 ± 1.7, respectively; $t=0.05$, $p>0.40$) but the mean number during the test period was greater than in the corresponding period prior to caffeine administration (49.9 ± 1.0 vs 32.1 ± 0.8, respectively; $t=4.45$, $p=5 \cdot 10^{-4}$).

Forty-eight min after this caffeine injection, and immediately prior to the next, the vibration-induced depression had recovered completely. Subsequent administration of a dose of caffeine (40 $mg \cdot kg^{-1}$) greater than the previous doses caused a greater attenuation of the depression: the number of spikes in the test period after this dose was greater than the corresponding number after the previous dose (59.1 ± 1.1 vs. 49.9 ± 1.0, respectively; $t=2.0$ $p=0.05$) while the numbers of spikes in the control periods failed to differ (107.8 ± 1.0 vs 106.1 ± 1.2, respectively; $t=0.3$, $p>0.30$).

The degree of attenuation after the final dose of caffeine (40 $mg \cdot kg^{-1}$) was greater than that after the dose of 20 $mg \cdot kg^{-1}$ but was not significantly different from that induced by the earlier dose of 40 $mg \cdot kg^{-1}$. Therefore, the difference between the effects of these doses was not likely due to accumulation of caffeine but rather due to a greater efficacy of the larger dose.

The relationship between the dose of caffeine and the magnitude of the effect on vibration-induced depression was investigated in the population by comparing the effects of the first dose administered to each unit.

Effects of first doses were compared to avoid the possibility of cumulative effects of repeated doses. First doses of 40-60 $mg \cdot kg^{-1}$ (4 units) caused significantly more attenuation of vibration-induced depression than did first doses of 20-30 $mg \cdot kg^{-1}$ (7 units; 75.8±10.1% vs 26.4±4.0%, respectively, p=0.04). With 4 of the latter units greater doses of caffeine were administered and these caused greater attenuation of the depression (mean: 71.3±8.8%).

With 2 units first doses of 20 $mg \cdot kg^{-1}$ attenuated vibration-induced depression by more than 50%. These large effects of low doses of caffeine are important because they aid in distinguishing effects of caffeine mediated by blockade at P_1-purinergic receptors from other possible effects of caffeine which occur with higher concentrations (*vide infra*).

For the unit in which vibration-induced depression was unaffected by administration of caffeine only a single dose of 20 $mg \cdot kg^{-1}$ was given. Considering the dose-response relationship described above the possibility exists that the depression in this unaffected unit would have been attenuated by a greater dose of caffeine.

e)*Relationship of the increase in rate of firing at the onset of vibration to net excitatory effects after administration of caffeine.* In all cases, before caffeine administration the net effect of vibration was depression. With 5 units, after caffeine injection the net effect of vibration changed from depression before caffeine injection to excitation afterwards (see Figs. 3 & 5). Interestingly, net excitation after caffeine was seen only with units which exhibited a biphasic response to vibration during the period before caffeine administration. Units which lacked the initial excitation all failed to show net excitation after caffeine (p=0.015, Fisher's exact test). Thus, in the former cases caffeine may have completely blocked the depression caused by vibration and may have unmasked an excitatory effect of vibration.

f) *Relationship of the effects of caffeine to changes in arterial blood pressure.* Administration of caffeine generally had a biphasic effect on arterial blood pressure and therefore it was considered important to determine if effects on vibration-induced depression were mediated by effects on arterial pressure. Upon injection, the arterial pressure decreased transiently; the minimum pressure occurred about 1 min

after the end of administration. Subsequently, the arterial pressure gradually increased and usually reached a final pressure 5-10 mm Hg greater than that before injection (note that the record in Fig. 2 ends before the pressure was greater than the pre-injection level).

Three lines of evidence indicate that the attenuation of vibration-induced depression by administration of caffeine was not caused by the decrease in arterial blood pressure. First, in 2 cases the attenuation of the depression began before the decrease in arterial pressure. Second, intravenous injections of pancuronium bromide or dipyridamole which caused transient decreases in arterial pressure similar to those caused by injection of caffeine failed to mimic its effect on the vibration-induced depression. Finally, in one experiment the pressure was maintained with noradrenaline and the infusion rate was decreased to mimic the change in arterial pressure which had been caused previously by injection of caffeine. Vibration-induced depression was unaffected by decreasing the rate of infusion, whereas administration of caffeine had attenuated the depression.

iii) *Effects of i.v. administration of 8-SPT on the response to vibration.*

The P_1-purinergic antagonist, 8-SPT, was administered in 5 cats because unlike caffeine, this compound reportedly fails to cross the blood-brain barrier (Snyder et al., 1981). Therefore, 8-SPT was used to determine whether the attenuation of vibration-induced depression by caffeine was an effect in the spinal cord or in the periphery. 8-SPT is approximately 10 times more potent than caffeine both in inhibiting binding of [^{3}H]-cyclohexyladenosine to membrane preparations from rat brain (Daly et al., 1985) and in blocking adenosine-induced increase in cyclic AMP generation in human fibroblasts (Bruns et al., 1980). Therefore, doses of 8-SPT used (8-16 $mg \cdot kg^{-1}$; 21-42 $\mu mole \cdot kg^{-1}$) were considered equivalent to caffeine doses of 40-80 $mg \cdot kg^{-1}$ (210-420 $\mu mole \cdot kg^{-1}$), doses which blocked vibration-induced depression (*vide supra*).

The lack of effect of 8-SPT on vibration-induced depression is illustrated in Fig. 3. A reproducible depressant response to periodic application of vibration was first established (Fig. 3Aa). Comparison of

the oscilloscope records in Fig. 3Aa and 3Ab shows that this response was unaffected by administration of 8-SPT (8 $mg \cdot kg^{-1}$). In contrast to this lack of effect of 8-SPT, subsequent administration of caffeine (40 $mg \cdot kg^{-1}$) attenuated the vibration-induced depression (Fig. 3Ad).

8-SPT also failed to alter the relationship between the numbers of spikes in the control vs test periods (Fig. 3C). On the other hand, caffeine caused a statistically significant change in this relationship and there was partial recovery one hour after the caffeine injection (Fig. 3D).

With all 4 remaining units vibration-induced depression was unaffected by administration of 8-SPT. Thus, intravenous administration of 8-SPT failed to mimic the effect of equivalent intravenous doses of caffeine. It was concluded therefore, that the effect of caffeine on vibration-induced depression was an action mediated in the spinal cord.

iv) *Effects of iontophoretic application of 8-SPT on the response to vibration*

Administration of 8-SPT into the local vicinity of the units being studied was examined to provide additional evidence that the blockade caused by intravenous injection of caffeine was a central effect. In initial experiments attempts were made to apply caffeine by iontophoresis. These experiments proved fruitless: little caffeine was apparently released from the pipettes because the barrel containing caffeine became blocked as evident from the fact that the impedance increased markedly when passing current.

Even experiments with 8-SPT proved unusually difficult, not because the pipettes became blocked, but rather because the vibration-induced depression gradually faded away. The depression often disappeared completely and irreversibly within minutes of beginning recording whenever micropipettes were used which contained 8-SPT. Interestingly, when pipettes containing 8-SPT were used the proportion of wide dynamic range units in which the net effect of vibration was excitation was increased: 3 of 61 units had net excitatory responses to vibration when pipettes did not contain 8-SPT (Salter and Henry, 1986b) versus 6 of 20 when 8-SPT was present (χ^2(corrected)= 5.05, $p<0.05$). These problems of fading of depression and of excitation persisted despite attempts to decrease

leakage by increasing the current used to retain 8-SPT in the pipette.

In spite of these problems, reliable results were obtained from 6 units on which 8-SPT was tested. With these units vibration-induced depression reached a stable level after having decreased slightly in magnitude during the first minutes of recording. Effects of 8-SPT on vibration-induced depression in one of these units are illustrated in Fig. 4. The baseline firing rate decreased gradually during the first trial with 8-SPT (30 nA, 170 sec), remained below the pre-ejection level after the end of application and gradually recovered completely 8 min after the end of ejection. Therefore, after the second trial with 8-SPT (60 nA, 120 s followed by 90 nA, 80 sec) glutamate was applied to maintain the baseline firing rate at the level in the period prior to 8-SPT application. For purposes of analysing the relationship between the numbers of spikes in control and test periods, points taken in the 5-min period immediately before each 8-SPT application were combined as their distributions failed to differ. Similarly, points taken in the 3-min period after each 8-SPT application were combined. Correlation coefficients for regression lines computed in periods before and after 8-SPT applications were statistically significant ($r=0.74$, $p<0.01$ and $r=0.95$, $p<0.01$, respectively). The slope of the line made after the ejection of 8-SPT was greater than that of the line made beforehand ($F(1,51)=7.12$, $p=0.01$). Interestingly, the slope of the line made of points from periods after the 8-SPT ejections was not different from unity and the intercept was not different from zero. These findings indicate that vibration-induced depression was 100% attenuated during this period. Therefore, application of 8-SPT reversibly blocked the vibration-induced depression.

In one other case, application of 8-SPT (50 nA, 5 min) also caused a statistically significant blockade (43%) of the depression induced by vibration; this effect of 8-SPT was reversible. However, with the remaining 4 units ejections of 8-SPT (30-120 nA, 5-10 min) failed to affect vibration-induced depression. Longer periods of application may have caused attenuation of depression in these units. However, to avoid causing irreversible changes in depression such longer applications were not made.

The results of these experiments not only confirm that the effect of

caffeine on vibration-induced depression occurred centrally but they also indicate that the lack of effect of intravenous administration of 8-SPT was truly due to a failure of this compound to gain access to the central nervous system rather than to a lack of efficacy.

v) *Effects of i.v. administration of dipyridamole on the response to vibration*

The adenosine uptake inhibitor, dipyridamole, was administered in 5 experiments. These experiments were done to determine if dipyridamole could potentiate vibration-induced depression as would be expected if this depression was mediated by adenosine. The doses used in the present study have been previously reported, by others, to potentiate depressant effects of iontophoretic application of adenosine on neurones in the cerebral cortex (Phillis et al., 1979; Phillis and Kostopoulos, 1975).

Following dipyridamole administration the baseline discharge rate of 2 units was increased, in one case it was decreased and in another it was unchanged; in the remaining case the firing rate was elevated with glutamate prior to injection of dipyridamole and was held constant by slight adjustments of the glutamate ejecting current.

In the experiment illustrated in Fig. 5 administration of 0.1 $mg \cdot kg^{-1}$ (not shown) and 0.5 $mg \cdot kg^{-1}$ of dipyridamole failed to affect the response to vibration (Fig. 5A). Eighteen min after the latter injection, a third dose, of 1.0 $mg \cdot kg^{-1}$, was given; in this case the mean magnitude of depression was increased by 37% (Fig. 5B). It is important to note that the excitatory off-response to vibration was markedly reduced after dipyridamole administration. This reduction of the off-response appears to indicate that the depressant effect of vibration was prolonged following administration of dipyridamole. Twenty min after injecting dipyridamole the magnitude of depression during the period of vibration was unchanged but the off-response had reappeared (Fig. 5C, left record). At this time administration of caffeine (40 $mg \cdot kg^{-1}$) completely attenuated the depression and the net effect of vibration was excitation (Fig. 5C, right record). Dipyridamole (2.0 $mg \cdot kg^{-1}$) was subsequently given 6 min after the caffeine injection (not shown). This administration was made during the period when vibration-induced depression was completely blocked. Four min after injection of

dipyridamole the magnitude of the depression had returned nearly to the level prior to any drug injection. This change in the response was not likely due to a rapid recovery from the effects of caffeine because this time course was never observed without administration of dipyridamole (*vide supra*). Therefore, it was concluded that administration of dipyridamole caused a genuine potentiation of the vibration-induced depression.

For another unit dipyridamole (1 $mg \cdot kg^{-1}$) increased the magnitude of the depression by 47% and as with the unit just described, a subsequent dose (2 $mg \cdot kg^{-1}$) also potentiated the depression after it had been blocked by caffeine. In each of three other experiments administration of dipyridamole failed to have a statistically significant effect on vibration-induced depression; in one of these a dose of 0.5 $mg \cdot kg^{-1}$ was given; in another, 2 doses were each 1.0 $mg \cdot kg^{-1}$; in the third a single dose of 2.0 $mg \cdot kg^{-1}$ was used.

vi) *Effects of i.v. administration of bicuculline on the response to vibration*

GABA has been implicated as a possible mediator of the inhibition of dorsal horn neurones caused by activation of low threshold primary afferents (Duggan and Foong, 1985; Game and Lodge, 1975). Therefore, the possibility was considered that GABA might mediate the depressant effects of vibration and so bicuculline hydrochloride, a $GABA_A$ receptor antagonist, was administered in 5 cats. The doses used have been reported to block the depression of C-fibre-evoked responses in the anterolateral fasciculus caused by electrical stimulation of the dorsal columns (Duggan and Foong, 1985).

In one case, the baseline rate of discharge was unchanged after administration of bicuculline, in another there was a transient increase in firing rate and in a third the firing rate showed a sustained increase. In case the effects of bicuculline were related to an increase in firing rate, with the 2 remaining units the firing rate was maintained at a constant elevated level with ejection of glutamate.

The unit in which a transient increase in rate of discharge occurred after bicuculline administration is illustrated in Fig. 6. In this unit the magnitude of depression was 88.5 ± 1.6% in the 3-min period before and

88.8±1.5% during the 5-min period just after bicuculline injection; the difference between these values was not statistically significant. Thus, bicuculline administration failed to affect vibration-induced depression.

For another unit, vibration-induced depression was also unaffected by bicuculline (0.2 $mg \cdot kg^{-1}$) and for one unit 2 doses of 0.2 $mg \cdot kg^{-1}$ failed to affect the depression. With 2 remaining units the magnitude of vibration-induced depression was greater after injection of bicuculline (0.2 $mg \cdot kg^{-1}$). In each of these cases second doses (0.2 $mg \cdot kg^{-1}$ in one case and 0.4 $mg \cdot kg^{-1}$ in the other) failed to have any further effect. Thus, intravenous injection of bicuculline failed to block the depression induced by vibration.

vii) *Effects of iontophoretic application of bicuculline on the response to vibration*

It was considered important to apply bicuculline into the local vicinity of the neurones studied to validate the results of the experiments in which bicuculline was administered intravenously because of the evidence stated above implicating GABA in mediating depression of dorsal horn neurones caused by electrical stimulation of low threshold primary afferents. Bicuculline methiodide, a water-soluble derivative of bicuculline more potent than the alkaloid itself (Pong and Graham, 1972; 1973) was selected for use in iontophoretic experiments because it has been reported to reduce the inhibition of dorsal horn neurones caused by electrical stimulation of the dorsal columns (Duggan and Foong, 1985).

Application of bicuculline as bicuculline methiodide (30-90 nA; 5-11 min) increased the baseline firing rate of all wide dynamic range units tested. The firing rate began to increase 5-30 s after the start of ejection and was maximum at the end of application. Following ejection, recovery occurred gradually over a period up to twice the duration of the application. A striking feature of the response to bicuculline methiodide, which has been reported previously (Game and Lodge, 1975), was the appearance of bursts of spikes with internal frequencies often greater than 500 Hz. Bursting was manifest as skewing of the interspike interval distribution towards shorter intervals in the period of bicuculline-induced excitation versus the period of excitation to glutamate, as shown in Fig. 7B. For all units, bicuculline was applied

for a longer period than that necessary to induce bursting.

In the experiment illustrated in Fig. 7 the magnitude of vibration-induced depression was 62.9±4.0% during the period before application of bicuculline when the unit was spontaneously active. During the period when the firing rate was maximally increased by bicuculline (56% increase in mean rate) vibration-induced depression was 42.0±1.9%, indicating an apparent 33% attenuation of the depression. However, the effect on the depression of increasing the baseline firing rate had been examined by systematically varying the glutamate ejection current in a 4-min period prior to application of bicuculline. For purposes of analysis, points taken during this period were combined with those during the period of spontaneous activity. The correlation coefficient for the combined group was 0.91 ($p<0.01$) and that for the group of points taken during the period when bicuculline-induced excitation was maximum was 0.62 ($p<0.05$). Neither the slopes nor the elevations were different for the regression lines of these 2 groups. Therefore, the apparent attenuation of depression by bicuculline was due solely to the increase in firing rate. Similar testing was done in all units in which bicuculline was studied (n=10); in no case was vibration-induced depression affected by bicuculline independent of its effect of increasing the baseline firing rate.

viii) *Effects of i.v. administration of strychnine on the response to vibration*

Glycine is another neuroactive agent which has been implicated as possible mediator of the depressant effects on dorsal horn neurones caused by electrical stimulation of low threshold primary afferents (Game and Lodge, 1975). Therefore, the glycine antagonist, strychnine, was tested in 3 cats. Doses of strychnine used in the present study were greater than those which have been shown to reduce direct inhibition of spinal motoneurones (Curtis, 1959; Curtis et al., 1971) and the depression of dorsal horn neurones caused by exogenously applied glycine (Curtis et al., 1968).

In one case, illustrated in Fig. 8, the vibration-induced depression had been blocked after caffeine administration (Fig. 8A). Seventy-two min after caffeine injection the depression had stabilized at 67% of the

initial level. Subsequent injection of strychnine (0.6 $mg \cdot kg^{-1}$, Fig. 8B) failed to alter either the baseline firing rate or the response to vibration. However, another injection of caffeine again attenuated the depression. Interestingly, with this unit the magnitude of the depression during the 150-ms period at the onset of vibration was 100% and remained at this level after injections of caffeine or of strychnine.

Vibration-induced depression of another unit was unaffected by strychnine (0.2 $mg \cdot kg^{-1}$). With the remaining unit, strychnine increased the magnitude of the depression (45.9±5.4% before, 72.9±6.9% after, t=3.1, p=0.007). Thus, strychnine failed to block the depressant response to vibration.

ix) *Effects of i.v. administration of naloxone on the response to vibration*

The possibility that the depressant response to vibration might be mediated by an endogenous opioid was considered because enkephalin may be a major inhibitory transmitter in the dorsal horn (Henry, 1978; Randić and Miletić, 1978; Zieglgansberger and Tulloch, 1979b) and because the antinociceptive effect of electrical stimulation of peripheral nerves is reportedly mediated by enkephalin (Woolf, 1985; Woolf et al., 1980). Hence, the opiate antagonist, naloxone, was studied in 4 units. The dose used was in the range found in earlier studies to antagonize the depressant effects on dorsal horn neurones of the iontophoretic (Calvillo et al., 1974) and of the intravenous (Calvillo et al., 1976) administration of morphine.

The baseline rate of discharge was increased after naloxone administration in 2 cases in which naloxone was tested on spontaneously active units. One of these cases is illustrated in Fig. 2. The other 2 units were initially silent and were induced to fire by application of glutamate; the ejecting current required to maintain the baseline firing rate at a constant level was unchanged after naloxone injection in both of these units. With all units, the magnitude of vibration-induced depression was unaffected by naloxone.

V. DISCUSSION

We have previously reported that peripheral vibration depresses nociceptive dorsal horn neurones in the cat. The present study investigated the pharmacological basis of this depression to provide evidence on which neurochemical agent or agents might mediate this phenomenon.

i) *Adenosine appears to mediate vibration-induced depression in the spinal dorsal horn*

The principal finding of the present study was that intravenous administration of the P_1-purinergic receptor antagonist, caffeine, attenuated the depression caused by vibration. As caffeine is distributed throughout all body compartments after intravenous injection (Axelrod and Reichenthal, 1953; Yesair et al., 1984), another P_1-purinergic antagonist, 8-SPT, was used to localize the site of action of caffeine. Importantly, 8-SPT failed to affect vibration-induced depression when this compound was administered systemically; however, the depression was blocked when 8-SPT was given locally by iontophoresis. As 8-SPT reportedly fails to cross the blood-brain barrier (*vide supra*), its lack of effect when given peripherally is consistent with a site of action within the central nervous system when caffeine was administered systemically.

Blockade of the vibration-induced depression by iontophoretically applied 8-SPT supports a central site of action for caffeine and indicates furthermore that the precise site of action is within the spinal dorsal horn at the level of recording. From these results we suggest that vibration-induced depression is mediated at the spinal level by activation of P_1-purinergic receptors. The decrease in discharge rate caused by vibration was blocked regardless of whether vibration caused the depressant or the biphasic response reported in Chapter 3. Therefore, it appears that the chemical basis of the depression and of the depressant component of the biphasic response are the same.

This suggestion is consistent with anatomical data showing that the dorsal horn contains high levels of binding sites for slowly-metabolized analogs of adenosine (Geiger et al., 1984; Goodman and Snyder, 1982). These binding sites may reveal P_1-purinergic receptors.

The results of the present study together with those of our previous

investigation of the effects of iontophoretically applied purines (see Chapter 2) provide 3 direct lines of evidence to support the suggestion that activation of P_1-purinergic receptors is the principal mechanism of vibration-induced depression.

1) *Nociceptive dorsal horn neurones are depressed both by vibration and by exogenously applied purines*. As peripheral vibration causes a depression of nociceptive dorsal horn neurones, evidence which is necessary to support our suggestion concerning the mechanism of this depression is that iontophoretic application of purines which activate P_1-purinergic receptors also causes depression of these neurones (see Chapter 2).

2) *P_1-purinergic receptor antagonists block the depressant effects both of vibration and of exogenously applied purines*. In view of the fact that the depressant effects of iontophoretic administration of ATP and of AMP on neurones in the spinal dorsal horn are attenuated by iontophoretic application of the P_1-purinergic antagonist, theophylline (see Chapter 2) and that purine-induced depression elsewhere in the central nervous system is attenuated by intravenous administration of caffeine and theophylline (Phillis et al., 1979; Stone and Taylor, 1980; Taylor and Stone, 1978) and by iontophoretic application of 8-SPT (Phillis et al., 1979), it seems reasonable to conclude that caffeine and 8-SPT will block P_1-purinergic receptors in the dorsal horn. As such, the present study provides a second line of evidence necessary to support our suggestion; namely, caffeine and 8-SPT attenuate vibration-induced depression.

The apparent direct relationship between the dose of caffeine and the magnitude of its effect in attenuating the depression caused by vibration indicates that this effect of caffeine is not a non-specific effect. It is important to determine that the actions of caffeine were selective at P_1-purinergic receptors and therefore, we provide the following 4 points.

2a) At the doses used in the present study, caffeine failed to affect the depression of neurones in the cerebral cortex caused by iontophoretic application of GABA, of cyclic AMP or of noradrenaline (Taylor and Stone, 1978).

2b) The time course of the effects of caffeine on vibration-induced depression parallels that of the concentration of caffeine in

cerebrospinal fluid (CSF) following intravenous injection in dogs (Teschemacher et al., 1968). Importantly, in this other study, a rapid decline occurred in the concentration of caffeine in the CSF during the period 15-40 min following injection, a time at which the depression recovered rapidly in the present study. The decrease in concentration was likely due to redistribution of caffeine from the extracellular to the intracellular space because the elimination half-life of caffeine is approximately 3.5 hr (Rall, 1980). Thus, the time course of the effects of caffeine on vibration-induced depression matches that of its concentration in the extracellular space. This point is important because blockade at P_1-purinergic receptors would be expected to occur at an extracellular site whereas other reported effects would be expected to occur intracellularly.

2c) The predicted concentration of caffeine after i.v. administration is sufficient to cause blockade at P_1-purinergic receptors but is less than that necessary to cause effects by other mechanisms. At a dose of 20 $mg \cdot kg^{-1}$ caffeine attenuated vibration-induced depression by up to more than 50%. Based on the pharmacokinetic study described above, a dose of 20 $mg \cdot kg^{-1}$ would be expected to give a peak concentration in the CSF of 168 μm. This concentration just exceeds that necessary to achieve a 50% blockade of P_1-purinergic receptors but is less than that required for other reported effects of caffeine such as inhibition of cyclic nucleotide phosphodiesterase (Fredholm, 1985).

2d) Iontophoretic application of 8-SPT, an antagonist more selective than caffeine (Daly et al., 1985; Wu and Phillis, 1984), attenuated vibration-induced depression. This finding is important because it establishes that P_1-purinergic receptor antagonists other than caffeine can block this depression.

3) *Dipyridamole potentiates the depressant effects both of vibration and of exogenously applied purines.* The results of the present study indicate that intravenous administration of dipyridamole increases both the magnitude of vibration-induced depression and its duration. Because dipyridamole blocks uptake of adenosine and potentiates the depressant effects of exogenously applied adenosine (*vide supra*), the present finding further supports the suggestion that vibration-induced depression is due to activation of P_1-purinergic receptors. Moreover, this finding

suggests that adenosine is the agent causing activation of these receptors in this case. Therefore, we suggest that vibration-induced depression is mediated by adenosine.

The finding that administration of dipyridamole produced a statistically significant depression in only 40% of units suggests that this compound may have had only weak effects. Dipyridamole may have caused only a weak potentiation because, in addition to inhibiting uptake of adenosine, dipyridamole reportedly blocks release of adenosine (Fredholm et al., 1980). Thus, the lack of effect of dipyridamole in a proportion of units may have been due to these 2 conflicting actions on extracellular adenosine concentration.

Vibration-induced depression: a synaptic response mediated by adenosine. The depression of nociceptive dorsal horn neurones induced by peripheral vibration is a synaptic response elicited by natural stimulation of primary afferent neurones (see Chapter 3). Thus, the present study provides the first evidence, in the central nervous system, of a synaptic response mediated by adenosine. In view of the fact that intravenous administration of caffeine or iontophoretic application of 8-SPT may have completely blocked the depression, we suggest that adenosine may be the principal neurochemical agent mediating vibration-induced depression.

Evidence that the dorsal horn contains the cellular components necessary to use adenosine as a chemical mediator of synaptic transmission comes from anatomical studies which have indicated that the dorsal horn contains the enzymes necessary to produce (Dodd et al., 1983; Suran, 1974a,b) and to degrade adenosine (Nagy and Daddona, 1985; Nagy et al., 1984b). Interestingly, high levels of adenosine-like immunoreactivity have been recently reported in the dorsal horn (Braas et al., 1986). In addition, binding of inhibitors of uptake of adenosine is present in high levels in the dorsal horn (Geiger and Nagy, 1985); this binding may show sites of adenosine uptake.

Adenosine-mediated synaptic responses have been previously reported in *in vitro* studies of parasympathetic ganglia (Akasu et al., 1984) and of a sympathetic neurone-heart cell co-culture (Furshpan et al., 1986). These studies lend support to the notion that adenosine may be a chemical

mediator of synaptic transmission.

ii) *Possible endogenous sources of adenosine*

There is abundant evidence that activity in neurones may be accompanied by release of purines (for review see Fredholm & Heldqvist (1980), Phillis & Wu (1981), and Stone (1981)). As adenosine can be rapidly formed by extracellular hydrolysis of ATP (Manery and Dryden, 1979), it is of particular relevance that Holton (1959) concluded from studies of the effects of electrical stimulation of peripheral nerves in the ear of the rabbit that ATP is released by peripheral terminals of primary sensory neurones. Furthermore, it was suggested that ATP may also be released from the central terminals of primary afferent neurones (Holton and Holton, 1954). Recently, synaptosomal preparations from the dorsal spinal cord have been shown to release ATP by depolarizing concentrations of potassium (White et al., 1985). Importantly, the release of ATP depends on the presence of calcium and is significantly greater than the corresponding release from synaptosomes derived from the ventral spinal cord. Therefore, one potential source of adenosine is ATP.

On the other hand, adenosine, *per se*, appears to be released by depolarizing stimuli from brain slices (Kuroda and McIlwain, 1974; Pons et al., 1980; Pull and McIlwain, 1973) and from synaptosomes (MacDonald and White, 1985). Depolarizing stimuli also cause release of adenine nucleotides in addition to ATP (Pull and McIlwain, 1977); these nucleotides may be subsequently converted to adenosine.

The possibility is suggested that vibration-induced depression may be mediated by adenosine formed from ATP. This possibility is specifically emphasized as we have previously suggested that ATP may be a chemical mediator of synaptic transmission released by low threshold primary afferents (see Chapter 2). As cutaneous vibration activates low threshold primary afferents (Darian-Smith, 1984) the results of the present study are consistent with the possibility that ATP is released from central terminals of low threshold primary afferents activated by vibration. ATP may then be converted to adenosine to activate P_1-purinergic receptors.

Other potential sources of adenosine cannot be ruled out by the present study and therefore, the possibility remains that adenosine, ATP or other adenine nucleotides may be released from sources other than

primary afferent neurones.

iii) *Site of action: pre- or postsynaptic?*

We have reported that activity evoked by glutamate is depressed by vibration (see Chapter 3), raising the possibility that this depression is mediated, at least in part, by a direct effect on the neurones under study. Furthermore, many dorsal horn neurones are hyperpolarized following electrical activation of low threshold primary afferents (Hongo et al., 1968; Steedman et al., 1985). Adenosine has been reported, in *in vitro* studies, to have a direct hyperpolarizing effect on hippocampal CA1 neurones (Segal, 1982) and vesical parasympathetic neurones (Akasu et al., 1984). Thus, the suggestion that vibration-induced depression is mediated by adenosine is consistent with a postsynaptic site of action.

On the other hand, adenosine appears to inhibit release of putative neurotransmitters both at peripheral and central synapses (for review see Fredholm & Heldqvist, 1980). A presynaptic site of action cannot be ruled out by the present study. This site of action is consistent with the proposed presynaptic inhibitory effects of activation of large diameter primary afferents (Wall, 1958).

iv) *Vibration-induced depression is resistant to blockade by bicuculline*

Administration of bicuculline, either intravenously as bicuculline hydrochloride or by iontophoresis as bicuculline methiodide, failed to block the depression induced by vibration. These findings indicate that activation of the bicuculline-sensitive receptor for GABA is not involved in mediating this depression.

Previous reports have indicated that bicuculline reduces inhibition of dorsal horn neurones caused by electrical stimulation of large diameter primary afferents in peripheral nerve (Game and Lodge, 1975) or by stimulation of the dorsal columns (Duggan and Foong, 1985). Three points must be considered which distinguish the present study from these other two. First, in the other studies electrical stimulation was used whereas a natural stimulus was utilized in the present study. This difference may be important because vibration would be expected to activate a functionally specific subpopulation of low threshold primary afferents whereas such functional specificity, which might be important in

determining the type of depressant mechanisms activated, would be lacking with electrical stimulation.

The second point relates to the interpretation of data in the study of dorsal column stimulation. In the present study it has been clearly shown that increasing baseline discharge rate, alone, can cause a reduction in the magnitude of depression when calculated simply as a percentage. Game & Lodge (1975) reported that the duration of inhibition following electrical stimulation of peripheral nerves was shorter when the firing rate was increased; therefore, they attempted to control the firing rate by application of D,L-homocysteic acid. However, Duggan & Foong (1985) failed to systematically control for effects of increased activity caused by bicuculline. Therefore, it is difficult to adequately assess the real effect of bicuculline in their study.

The third point is that in each of the other studies electrical stimulus caused excitation. This excitation was followed by inhibition and it was this inhibition which was studied and found to be sensitive to bicuculline. On the other hand, in the present study excitation occurred in only 50% of the units and only at the beginning of the vibration. Thus, depressant mechanisms different from those which occur after excitation may have been activated in the present study. Interestingly, in another study of inhibition of dorsal horn neurones caused by electrical stimulation of primary afferents, peripheral nerves were stimulated with parameters that minimized excitation. The inhibition produced in this case was resistant to bicuculline and to another GABA blocker, picrotoxin (Chung et al., 1984).

v) *Strychnine fails to attenuate depression caused by vibration*

The possibility was investigated that vibration may have expressed its depressant effects via a mechanism involving glycine. The glycine antagonist, strychnine, failed to attenuate vibration-induced depression indicating that this depression is not likely mediated by a glycinergic mechanism. In contrast to the present study, in the study described above by Game & Lodge (1975) the early component of the depression following electrical stimulation of peripheral nerves was sensitive to strychnine. Thus, strychnine-sensitive and strychnine-resistant depressions may occur in the dorsal horn.

vi) *Vibration-induced depression is unaffected by naloxone*

Experiments were done to determine whether vibration-induced depression may be mediated via an endogenous opioid. Administration of naloxone failed to reduce the depressant effect of vibration indicating that this effect is independent of a naloxone-sensitive receptor. This finding is consistent with clinical reports that analgesia due to cutaneous vibration and to transcutaneous electrical nerve stimulation are resistant to naloxone (Freeman et al., 1983; Hansson et al., 1986; Lundeberg, 1983; Pertovaara et al., 1982; Sjolund and Eriksson, 1979; Woolf et al., 1978). Naloxone-sensitive analgesia caused by electrical stimulation of peripheral nerves has been reported (Chapman and Benedetti, 1977) but the interpretation of the data in this study has been questioned due to lack of adequate controls for possible hyperalgesic affects of naloxone (Freeman et al., 1983).

vii) *Possible relevance to clinical analgesia*

Vibration applied to the skin decreases both clinical (Lundeberg, 1983, 1984; Lundeberg et al., 1984) and experimentally-induced (Bini et al., 1984; Ekblom and Hansson, 1982; Pertovaara, 1979; Sherer et al., 1986; Sullivan, 1968; Wall and Cronly-Dillon, 1960) pain. In view of the fact that vibration has a preferential depressant effect on nociceptive rather than on non-nociceptive neurones in the dorsal horn (see Chapter 3), the results of the present study may provide some insight into the chemical mediation of vibration-induced analgesia. Our results prompt us to suggest that the analgesic effects of vibration may be mediated at the spinal level by activation of P_1-purinergic receptors.

Consistent with this suggestion are behavioural studies which have indicated that administration of slowly-degraded analogs of adenosine inhibit nociceptive responses in experimental animals (Ahlijanian and Takemori, 1985; Holmgren et al., 1983, 1987; Post, 1984; Yarbrough and McGuffin-Clineschmidt, 1981).

Vibration-induced depression of dorsal horn neurones together with vibration-induced elevation of pain threshold led to the suggestion that inputs from large and small diameter primary afferents interact in the spinal cord to regulate the activity of nociceptive neurones and that

activity in large diameter fibres is inhibitory (Melzack and Wall, 1965; Noordenbos, 1959). A consequence of this suggestion was the discovery that electrical stimulation of peripheral nerves or of the dorsal columns, sufficient to activate large diameter primary afferents, causes analgesia in humans (Wall and Sweet, 1966). As vibration likely activates primary afferents with large diameter fibres (see Chapter 3) the possibility is raised by the present results that analgesic effects of transcutaneous electrical nerve stimulation and of dorsal column stimulation may involve purines at the spinal level.

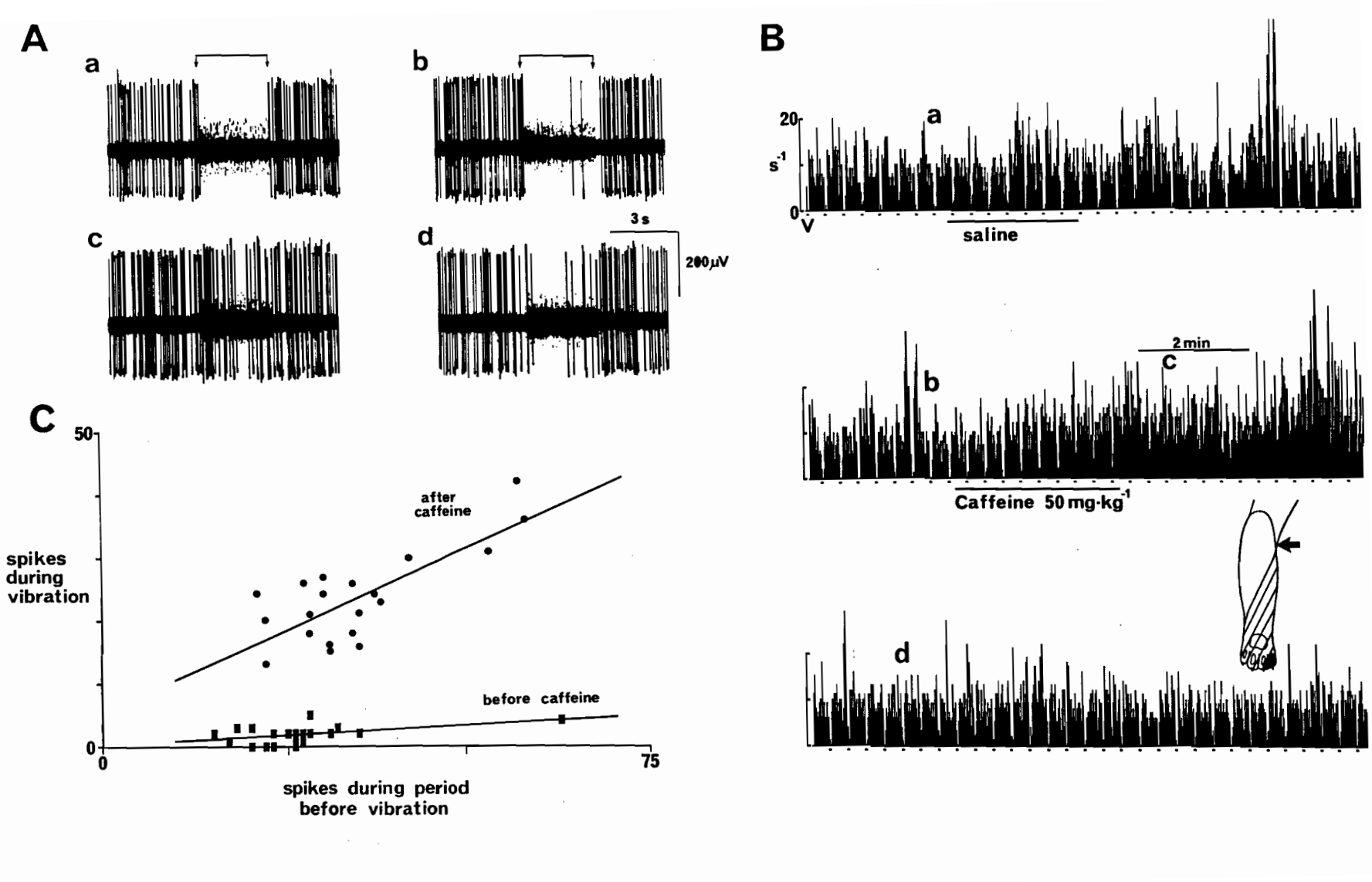
The results of the present study indicate that P_1-purinergic receptors may have a physiological role in the regulation of nociception at the spinal level. This physiological role may be exploited to advantage by some currently-used analgesic drugs. For example, nortryptiline which is useful in the treatment of chronic pain (Feinmann, 1985; Gomez-Perez et al., 1985) is a potent inhibitor of adenosine uptake (Phillis and Wu, 1982). Thus, its analgesic effects may be mediated by inhibition of adenosine uptake in the spinal cord. The present results indicate that the spinal purine system may be important in the development of future analgesics and in increasing the efficacy of treatments currently used to alleviate pain.

Fig. 1 Effects of caffeine on response to vibration applied periodically to the periphery.

A. Oscilloscope records of responses to application of vibration. The period of vibration (3.1 s) is indicated by the bar above the upper 2 records. Each record is a single oscilloscope sweep and corresponds to the respective response indicated by the letters above the records in B.

B. The rate of discharge of this unit is illustrated by the continuous-time histograms generated by the computer. The ordinate shows the firing rate in spikes·s^{-1}. Before any pharmacological agents were injected a reproducible response was obtained to vibration (V) applied during the periods indicated by the short bars below the records. The period of injection of physiological saline (10 mL) is indicated by the bar below the top record. Caffeine (50 mg·kg^{-1}) was administered in the period shown by the bar below the middle record; this record begins 4 min after the end of the one above. The bottom record begins 32 min following caffeine administration. Inset: diagrammatic representation of the receptive field to mechanical stimulation. This unit was excited by air movement, light touch and innocuous pressure to the blackened area only, and by pinch with a serrated forceps to both the blackened and patched areas. In this and all subsequent figures, the arrow indicates the position of the mechanical stimulator probe and its orientation. The amplitude of the mechanical pulses was 0.5 mm.

C. The number of spikes during the period beginning 500 ms after the onset of vibration (test period) versus the number in the corresponding period prior to vibration (control period) are shown in the scatter graph. Each point was taken from a single application of vibration during the period immediately before (squares) or after (circles) administration of caffeine. Lines are the least squares regression lines calculated before and after caffeine injection.

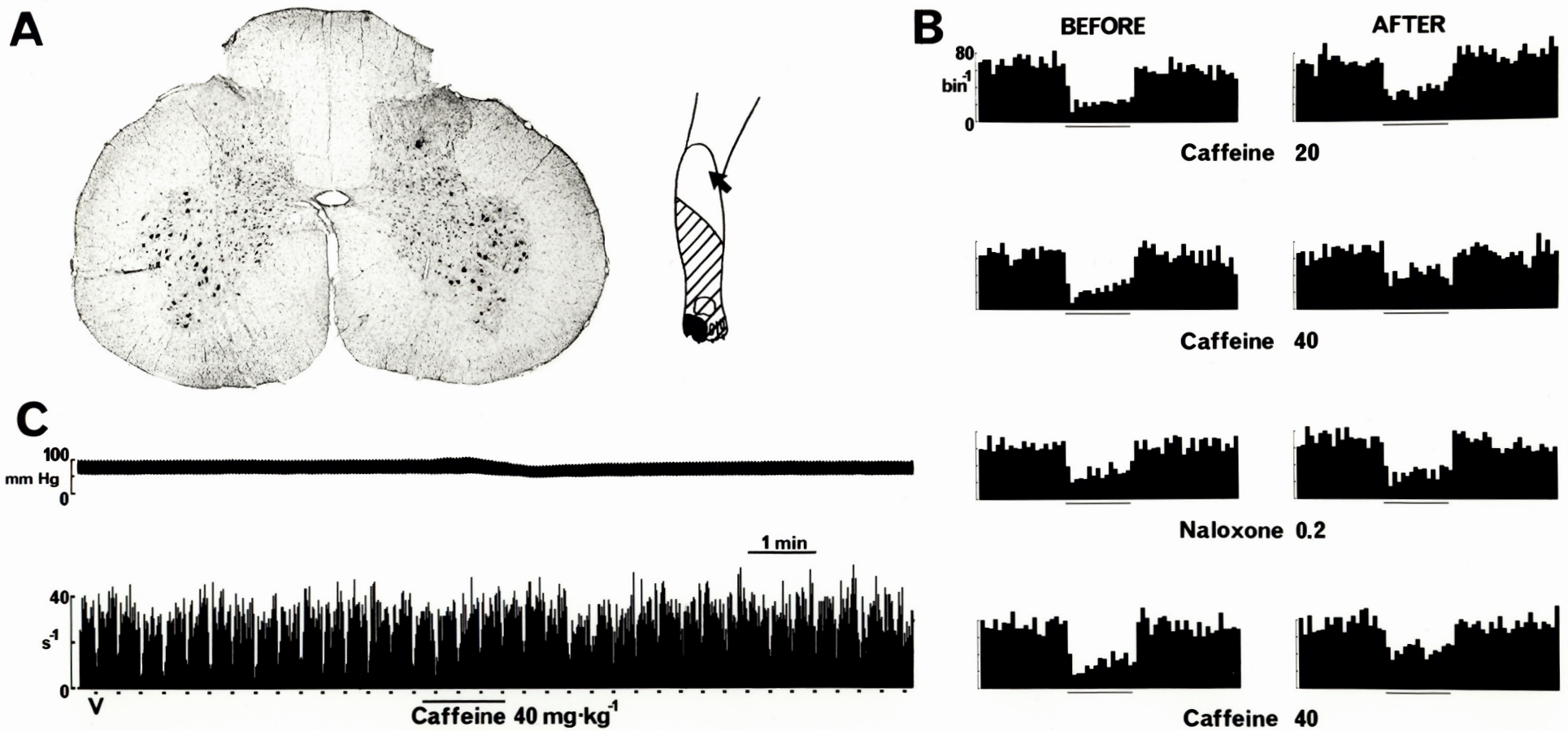

A
a
b
c
d
3 s
200 µV
B
20
s-1
0
V
a
saline
2 min
b
c
Caffeine 50 mg·kg-1
d
C
50
0
75
spikes during vibration
after caffeine
before caffeine
spikes during period before vibration

ig. 2 Effects of caffeine and naloxone on vibration-induced depression.

A. Photomicrograph of a transverse histological section of the pinal cord containing deposit of Pontamine Sky Blue left at the recording ite; the deposit is located on the border of Rexed's laminae IV and V. agnification: 10×. The receptive field of this unit is illustrated n the diagram to the right. The unit was excited by hair movement, light ouch and innocuous pressure (blackened area only) and by noxious pinch blackened and hatched areas). Mechanical pulses, 0.3 mm in amplitude, ere applied to the location shown by the arrow.

B. PSTHs were constructed immediately before and after intravenous njections. Ten consecutive applications of vibration were used to ompile each PSTH; the bars below each record indicate the period of ibration. The pharmacological agent administered in the interval between ijacent PSTHs is indicated below each pair; doses are in $mg \cdot kg^{-1}$. ıe interval between the 2 upper caffeine injections was 48 min. Naloxone ıs given 72 min after the second and was followed 8 min later by the inal injection of caffeine. The baseline firing rate increased slightly :ter naloxone administration but the regression line made during the ıriod before naloxone injection was not significantly different from the ine made after this injection. For all caffeine injections illustrated :fects were statistically significant.

C.The upper record is a pen recorder trace showing arterial blood :essure. The lower record is a continuous time histogram illustrating ıe firing rate in $spikes \cdot s^{-1}$. The 2 records are temporally nchronous. The period of intravenous injection of caffeine (40 $\cdot kg^{-1}$) is indicated by the long bar below the record; this injection rresponds to the first injection of this dose shown in B. Before ffeine injection the response to periodic applications of vibration (V; ort bars below the record) had returned completely to the level eceding the previous injection.


A
B
BEFORE
AFTER
80
bin-1
0
Caffeine 20
Caffeine 40
Naloxone 0.2
Caffeine 40
C
100
mm Hg
0
1 min
40
s-1
0
V
Caffeine 40 mg·kg-1

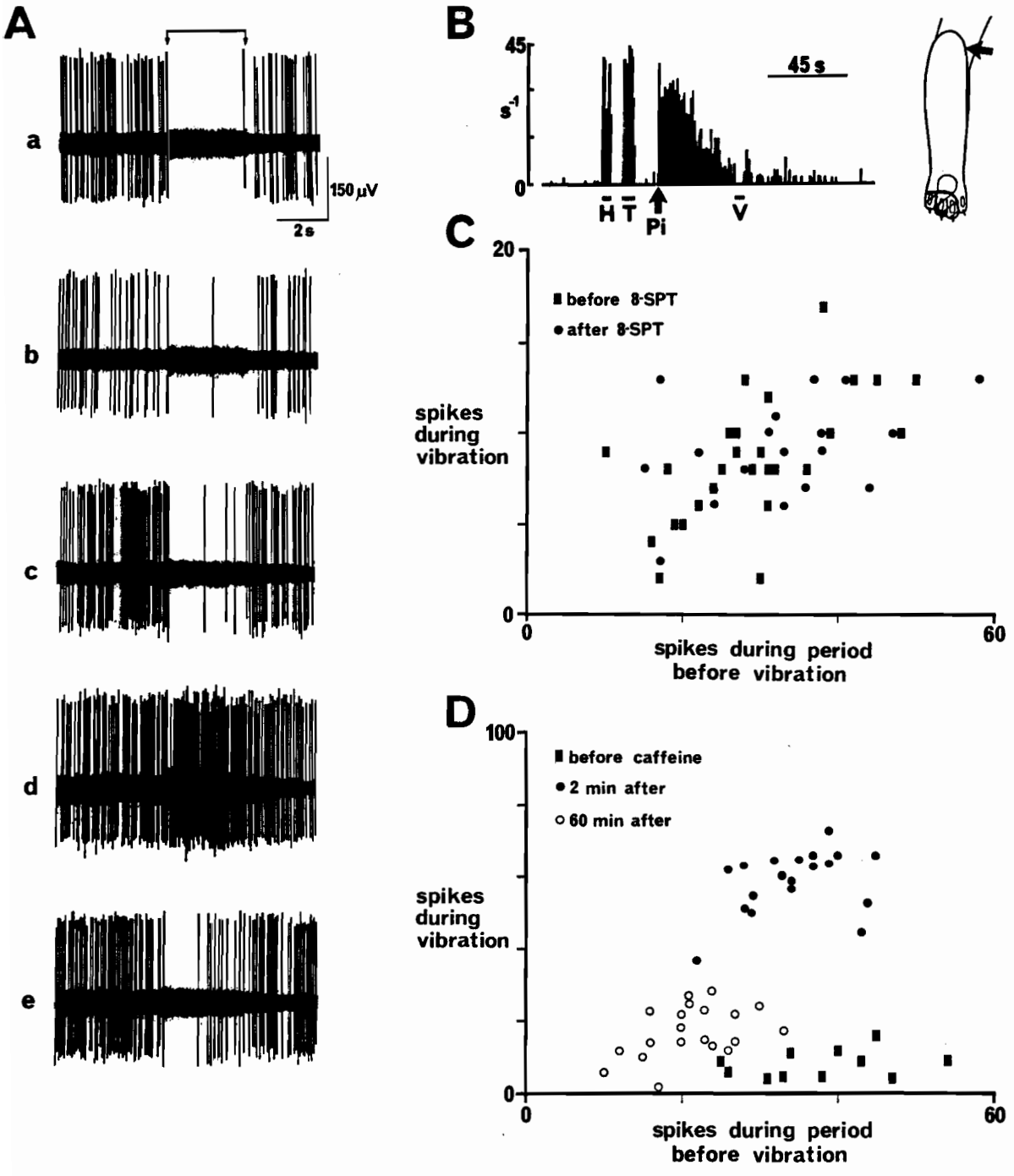
'ig. 3 Comparison of effects of 8-SPT and caffeine on vibration-induced depression.

A. Each of the oscilloscope records shows the response to a single .pplication of vibration (bar above records). Response "a" occurred 5 s efore intravenous administration of 8-SPT (8 $mg \cdot kg^{-1}$; 3 min injection eriod) and "b" was taken 150 s after the end of the injection. Records c" and "d" occurred at corresponding times before and after intravenous dministration of caffeine (40 $mg \cdot kg^{-1}$; 3 min injection period). weep "e" was taken one hour after caffeine injection. Note that before dministration of caffeine 1-3 spikes occurred at the onset of each ibration application. The baseline firing rate was maintained at a onstant level by adjustment of the ejecting current for glutamate.

B. Continuous time histogram showing the rate of discharge of this nit. The receptive field was stimulated by movement of single hairs (H) nd by touching the glabrous skin with tissue paper (T) during the periods ndicated by the bars below the record. At the time indicated by the rrow noxious pinch (Pi) was applied briefly to the receptive field; the ıration of pinching was less than one s. V indicates the period of pplication of vibration (amplitude 1.0 mm) to the location shown by the rrow in the diagram. The receptive fields to innocuous (blackened area) ıd to noxious (blackened and hatched areas) mechanical stimuli are also llustrated.

C and D show scatter graphs in which each point corresponds to the ımber of spikes in a period of vibration (test period) versus the number ı the corresponding period prior to that application (note that the :dinate scales are different). With this unit the test period was the ıtire period of vibration. In the upper graph, points corresponding to ɔnsecutive vibration applications were taken in periods immediately :fore (squares) and after (circles) injection of 8-SPT ($r=0.76$, $p<0.01$ ıd $r=0.43$, $p<0.05$, respectively; see Fig. 3C). The slopes and the .evations of the regression lines failed to differ ($F(1,28)=1.1$, $p=0.31$ ıd $F(1,29)=0.16$, $p=0.66$, respectively). In the lower graph points are ıown for consecutive applications in the periods immediately prior to .ffeine injection (squares), beginning 2 min after the start of injection :illed circles) and one hour after administration (open circles).

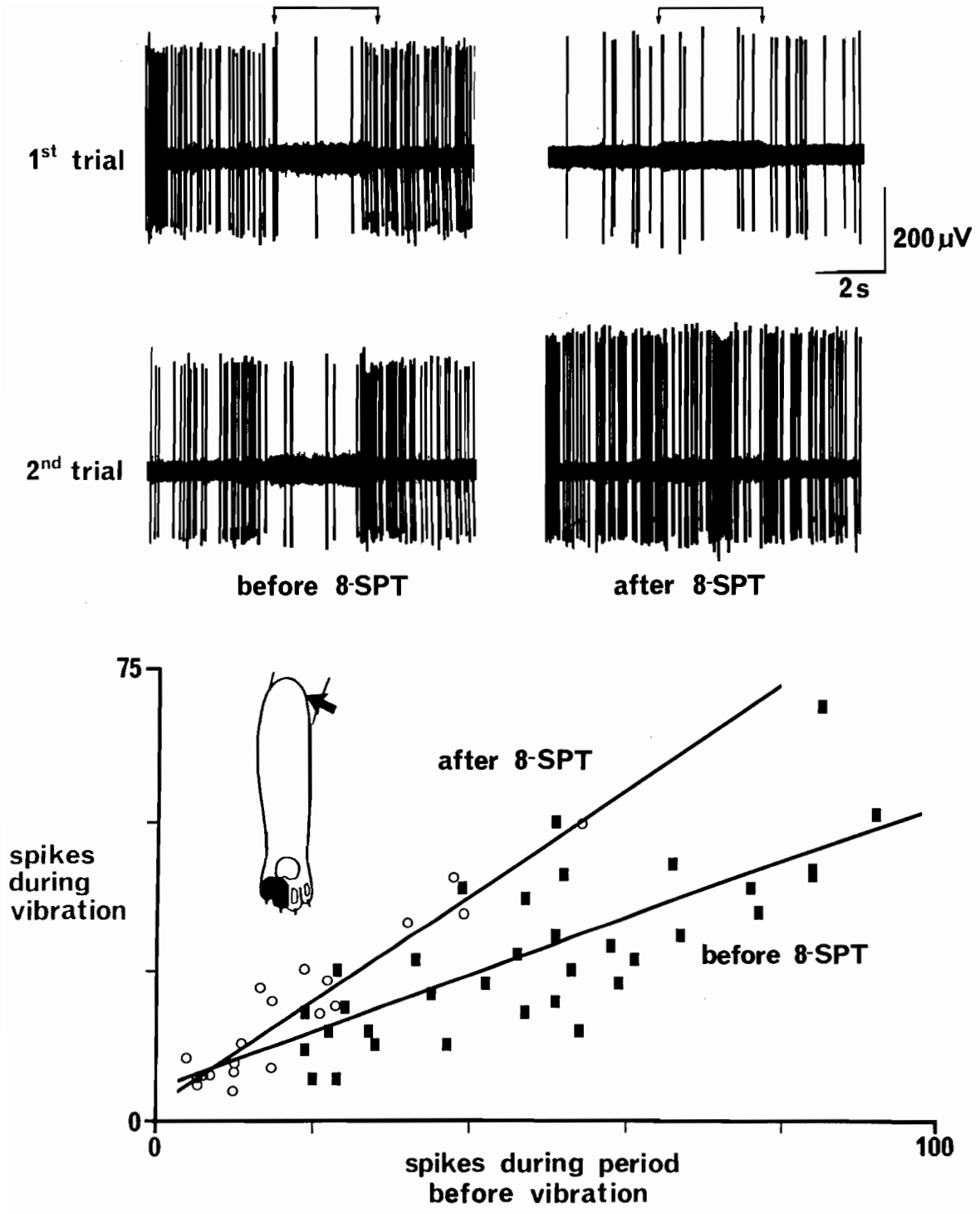
orrelation coefficients for points in these periods were 0.73 ($p<0.01$), .38 ($0.05<p<0.10$) and 0.44 ($0.05<p<0.10$), respectively. For clarity, the egression lines are not shown. The slopes of the regression lines alculated in the periods before and after caffeine injection failed to iffer, but the elevation of the line from the latter period was greater $F(1,29)=382.1$, $p=10^{-6}$). Note that the mean baseline firing rate in the eriod one hour after the injection was less than that during the other 2 eriods. The elevation of the regression line one hour after caffeine dministration was greater than that of the line before caffeine, hence ecovery was incomplete.


A
a
b
c
d
e
150 µV
2 s
B
45
s⁻¹
0
45 s
H̄ T̄
Pi
V̄
C
20
before 8-SPT
after 8-SPT
spikes during vibration
0
0
60
spikes during period before vibration
D
100
before caffeine
2 min after
60 min after
spikes during vibration
0
0
60
spikes during period before vibration

ig. 4 Iontophoretic application of 8-SPT blocks vibration-induced depression.

Oscilloscope records each show single, representative responses to ibration approximately 2 min before (left) and 2 min after (right) pplication of 8-SPT. The period of vibration is indicated by the bars bove the upper 2 records; amplitude of stimulation was 1.0 mm. The pper 2 traces were taken relative to the first 8-SPT application and the ower pair to the second. Seventeen min were left between the pplications of 8-SPT. Note that the spike amplitude was greater in the eriod just after versus just before each application of 8-SPT. Prior to he second application the spike amplitude had returned to the level bserved in the period before the first application. The changes in pike amplitude occurred without apparent movement of the electrode.

Each point in the scatter graph corresponds to the number of spikes uring the period of a single application of vibration versus the number n the corresponding period prior to that application. Points were taken rom consecutive periods of vibration immediately before (squares) and mmediately after (circles) applications of 8-SPT; lines are least quares regression lines. This unit was excited by innocuous and by oxious mechanical stimuli to the blackened area illustrated in the iagram.


1st trial
200 µV
2 s
2nd trial
before 8-SPT
after 8-SPT
75
spikes during vibration
after 8-SPT
before 8-SPT
0
0
100
spikes during period before vibration

ig. 5 Dipyridamole potentiates vibration-induced depression.

Each record shows a PSTH made from 10 consecutive applications of ibration either in the period immediately before (left) or immediately fter (right) intravenous administration of the pharmacological agent ndicated below each pair of records. The period of vibration is ndicated by the bar below each record; the pulse amplitude was 0.6 mm. uring the period of testing intravenous drugs the baseline firing rate as held constant by adjustment of the glutamate ejection current (70 nA efore any injections). The slopes of regression lines corresponding to he periods before versus after dipyridamole injection (1.0 $mg \cdot kg^{-1}$) ailed to differ but the elevation of the latter line was less $F(1,17)=5.39$, $p=0.03$). Similar analysis indicated that the attenuation f depression by caffeine was statistically significant. The receptive ields to mechanical stimuli are shown in the diagram between the middle nd lower pairs of records. The unit was excited by hair movement, light ouch and innocuous pressure to the area near the tip of the second toe rom the right and by noxious pinch to the blackened and hatched areas.

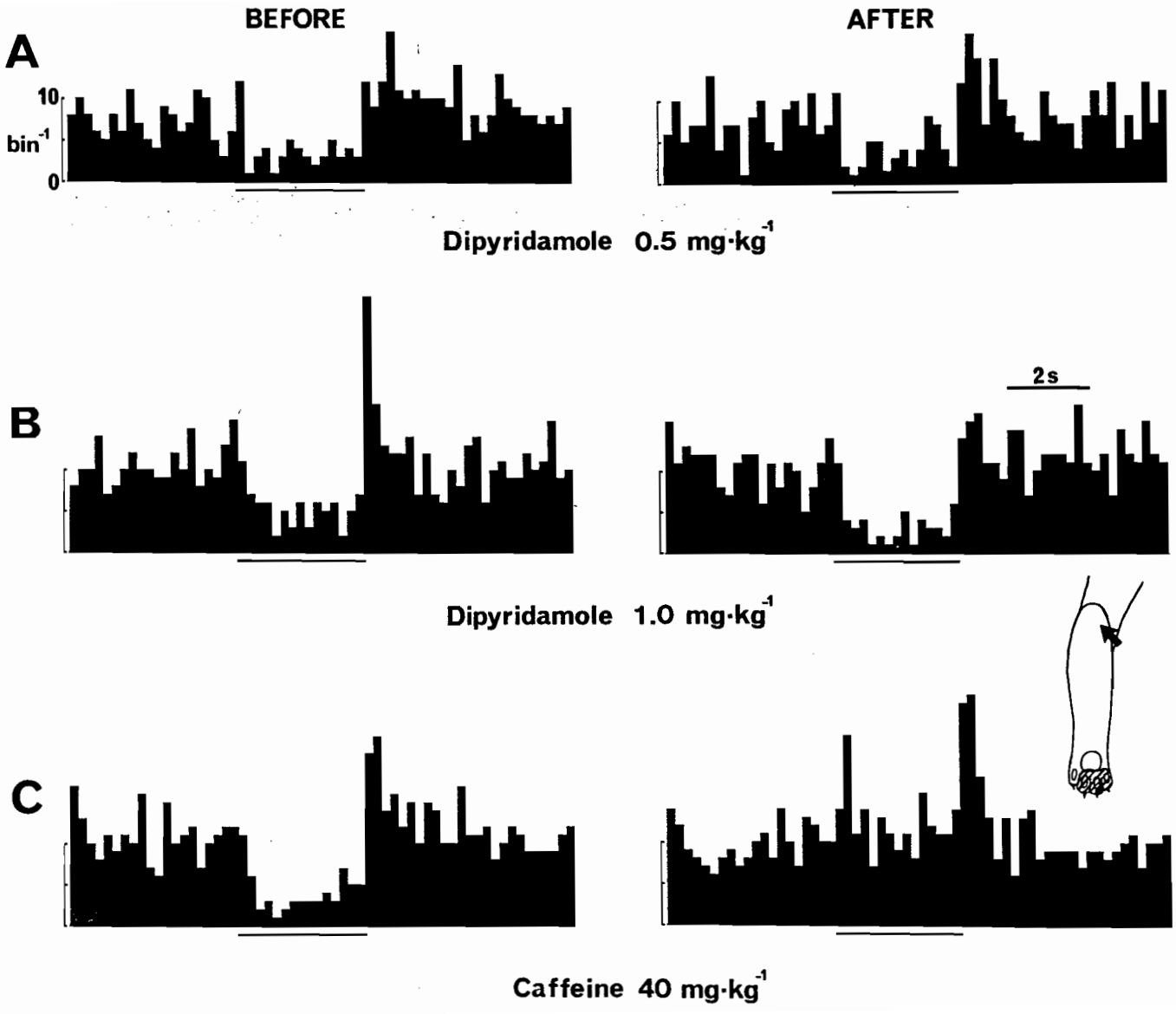

BEFORE
AFTER
A
10
bin-1
0
Dipyridamole 0.5 mg·kg-1
B
2s
Dipyridamole 1.0 mg·kg-1
C
Caffeine 40 mg·kg-1

Fig. 6 Intravenous administration of bicuculline fails to affect the response to vibration.

The firing rate of this unit is illustrated in the continuous-time histogram in A. A reproducible response was elicited by vibration (V, short bars below record) during the period before administration of bicuculline (BIC, long bar below record). The PSTH in B was compiled from 7 consecutive applications of vibration in this period. The same number of applications after injection of bicuculline was used to construct the PSTH in C. Vibration was applied during the period indicated by the bar below each PSTH. The vibration application immediately after the end of bicuculline injection was excluded from the histogram in C because the baseline firing rate just prior to this application was markedly greater than that during any period before bicuculline administration. Regardless of whether the data point corresponding to this application was included or excluded the effect of bicuculline failed to be statistically significant. The blackened and hatched areas in the diagram have the same significance as in the preceding figure. The cross shows the location of the probe of the mechanical stimulator and also signifies that its long axis was perpendicular to the plantar surface of the foot. The amplitude of the mechanical pulses was 0.6 mm.

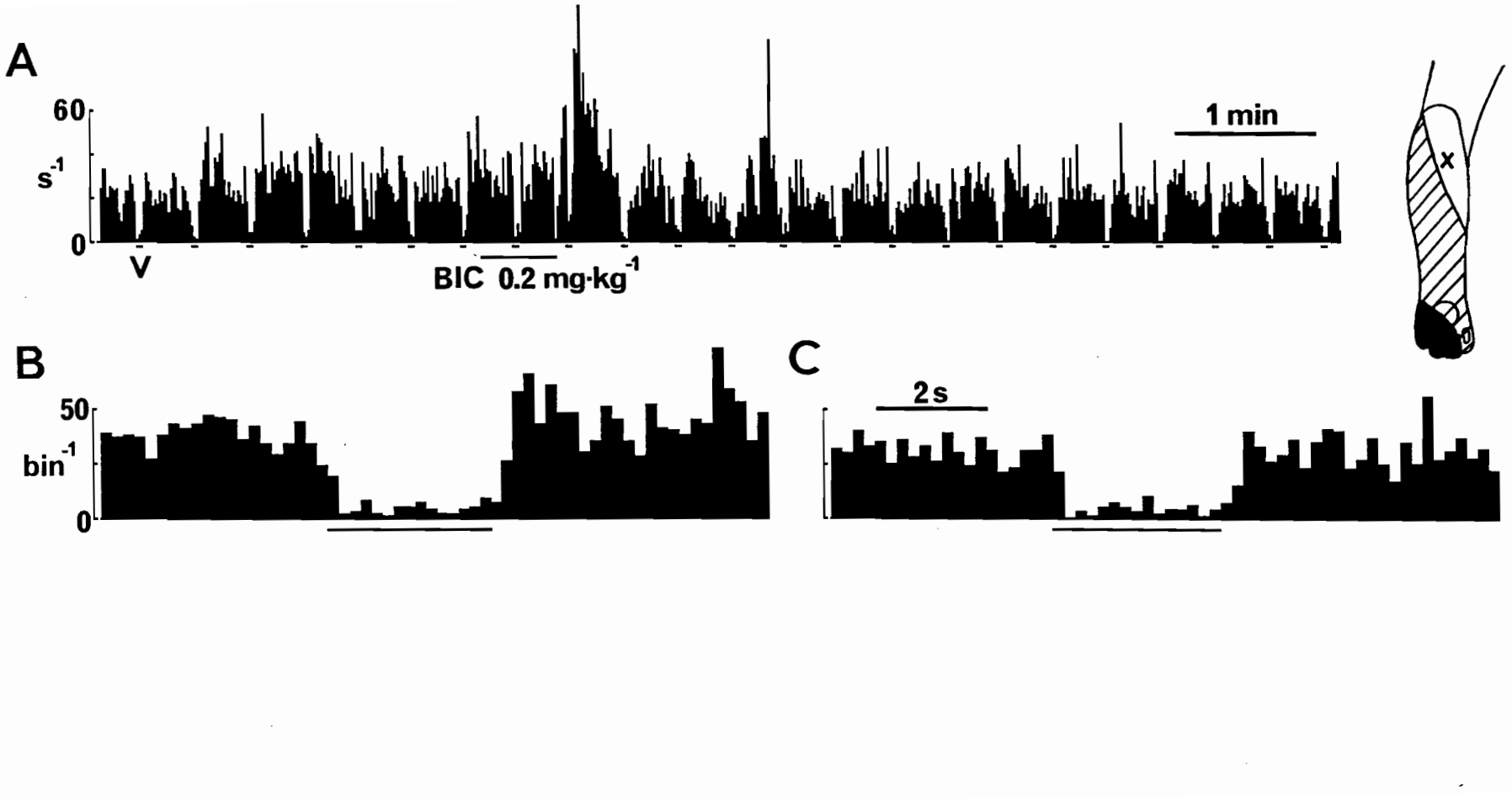

A
60
s-1
0
V
BIC 0.2 mg·kg-1
1 min
x
B
50
bin-1
0
C
2 s

Fig. 7 Vibration-induced depression is unaffected by iontophoretic application of bicuculline.

A. Scatter graph: each point was taken from a single application of vibration during the period of spontaneous activity and of excitation by glutamate before bicuculline (closed circles) or during the period of maximum excitation caused by application of bicuculline (30 nA, 6.5 min, open circles). Regression lines are not shown for clarity. Comparing these 2 groups of points, neither the slopes nor the elevations of the regression lines were different ($F(1,32)=2.97$, $p=0.09$ and $F(1,33)=0.39$, $p>0.40$, respectively).

Inset: Innocuous and noxious mechanical stimuli to the blackened area excited this unit. The amplitude of the mechanical pulses applied to the location indicated by the arrow was 1.0 mm.

B. Interspike interval distributions are shown in histograms (bin width 0.5 ms) compiled during periods of excitation by glutamate (GLU, upper) or by bicuculline (BIC, lower); means were 24.5 ms and 23.2 ms ($t=0.84$, $p>0.40$), respectively, during these periods. Comparison of the interspike intervals in these periods using the Mann-Whitney test indicated that the distributions were different ($z=6.8$, $p<10^{-7}$).

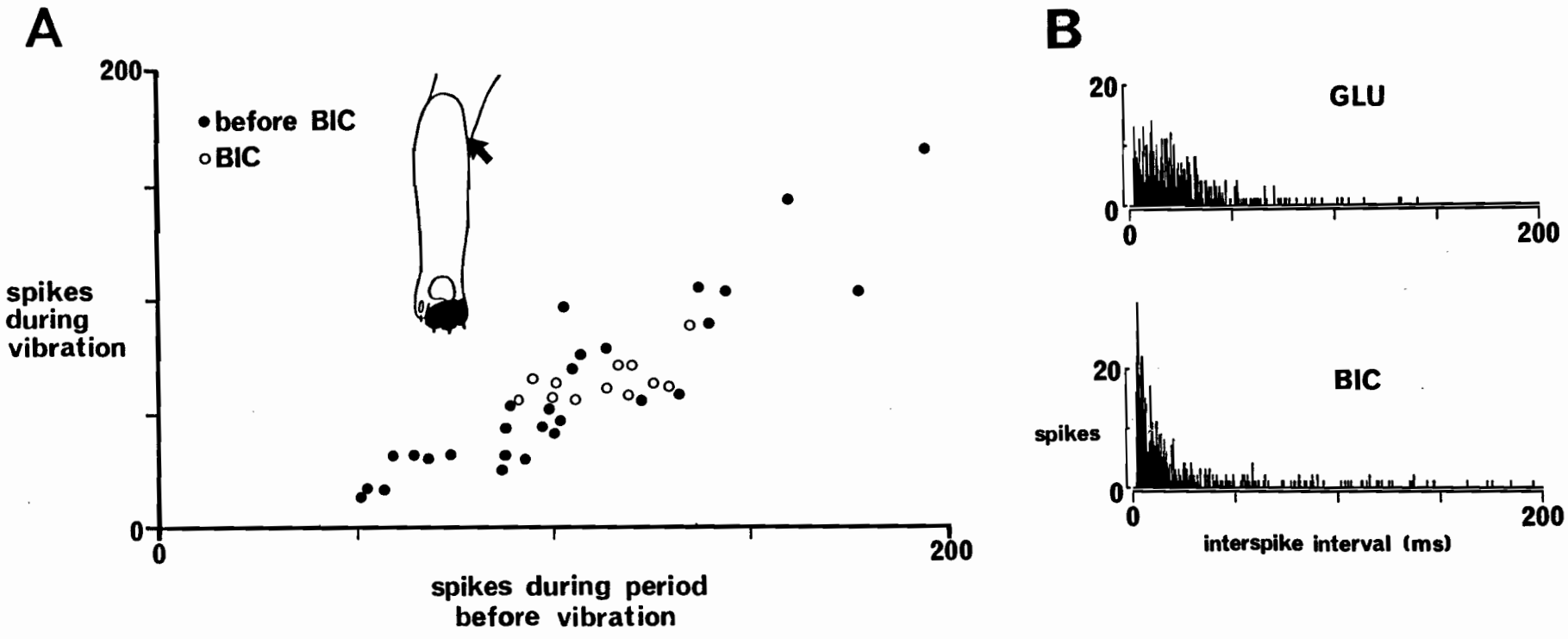
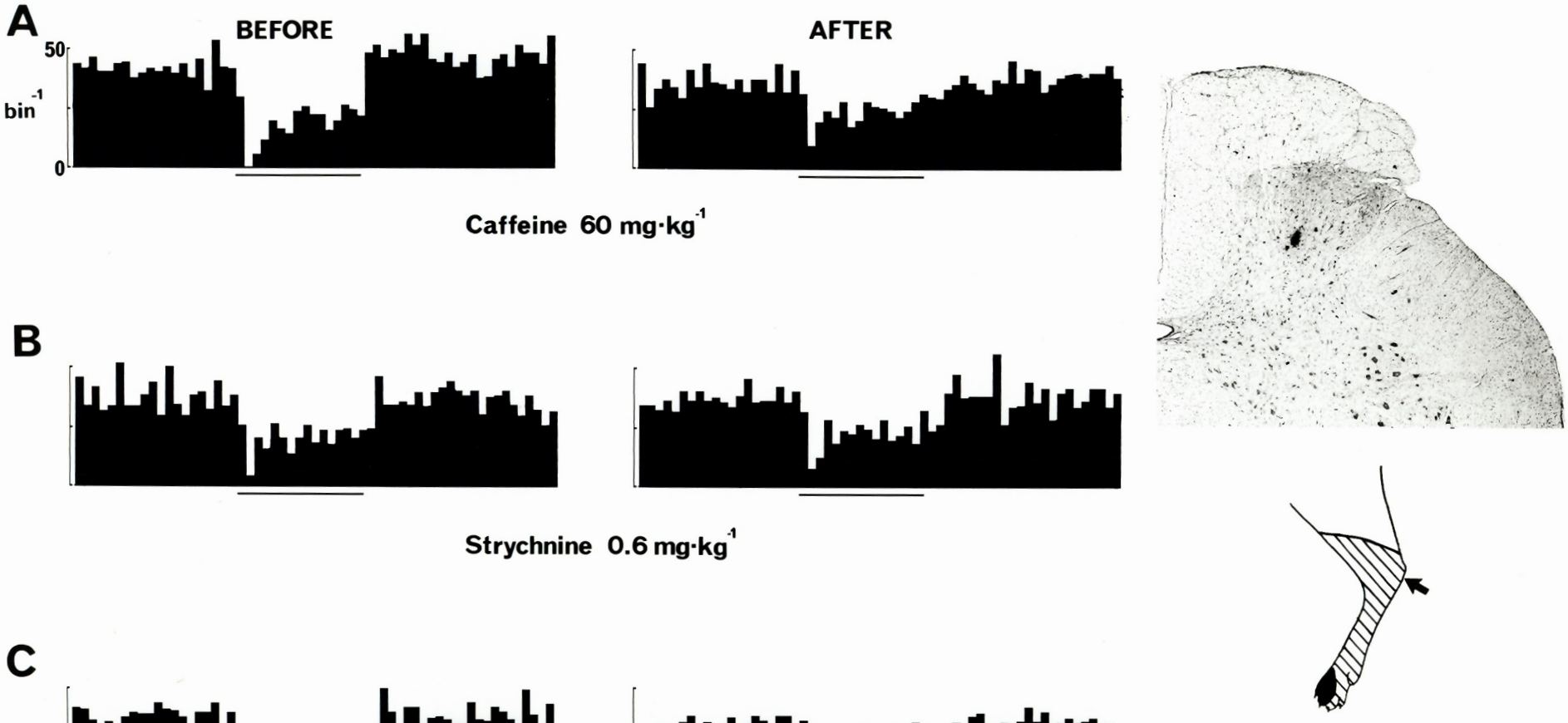

A
200
0
0
200
• before BIC
○ BIC
spikes during vibration
spikes during period before vibration
B
20
0
GLU
0
200
20
0
BIC
spikes
0
200
interspike interval (ms)

Fig. 8 Vibration-induced depression of a lamina IV unit is unaffected by strychnine.

Ten consecutive applications of vibration (bars below records) were used to compile each PSTH. These applications occurred during the periods immediately before (left) and just after (right) the period of injection of the pharmacological agent indicated below each pair of records. The interval between upper injections of caffeine and strychnine (i.e. between A and B) was 72 min; 32 min before administration of strychnine dipyridamole (5 $mg \cdot kg^{-1}$) was injected without effect on vibration-induced depression. The final dose of caffeine shown was given 7.5 min after strychnine injection. A histological section containing the dye deposit is shown in the photomicrograph; the deposit is located in Rexed's lamina IV. Magnification for the photomicrogragh: 20×. Below the photomicrograph is a diagram in which are illustrated the receptive fields to innocuous (blackened area) and noxious (blackened and hatched areas) mechanical stimuli. Mechanical pulses, 0.9 mm in amplitude, were applied at the location indicated by the arrow.


A
BEFORE
AFTER
50
bin-1
0
Caffeine 60 mg·kg-1
B
Strychnine 0.6 mg·kg-1
C
Caffeine 60 mg·kg-1

CHAPTER 5

PARAMETRIC STUDIES ON RESPONSES OF WIDE DYNAMIC RANGE SPINAL NEURONES TO CUTANEOUSLY APPLIED VIBRATION IN THE CAT: EVIDENCE IMPLICATING INPUTS FROM PACINIAN AFFERENTS IN A SUBSET OF RESPONSES

I. ABSTRACT

In the present study the effects of varying the duration, amplitude and frequency of vibrational stimulation on the responses of wide dynamic range (WDR) neurones to this stimulation were systematically investigated. Extracellular recordings are included from 60 WDR neurones considered a representative subgroup of the population reported in Chapter 3. The amplitude of stimulation (i.e. the peak-to-peak amplitude of the excursion of the stimulator probe) was varied from 0.001 to 1 mm. Frequencies of 10, 20, 40, 80, 120 and 240 Hz were examined.

Durations of stimulation of 0.125 to 3.75 s (i.e. 10-300 cycles at 80 Hz) were found to be acceptable for use in the study of the amplitude- and frequency-response relationships. For the remainder of the study the durations used were between 2.5 and 3.5 s.

When the probe of the mechanical stimulator was located outside the receptive field for low threshold (i.e. innocuous) stimuli, vibration at a frequency of 80 Hz caused a decrease in the rate of discharge during the period of stimulation. For the sample studied in detail (24 neurones), the magnitude of the decrease varied directly with the stimulation amplitude. The frequency-response relationship was examined in 22 cases using a stimulation amplitude of 0.15 mm. On average, a statistically significant net decrease occurred only at frequencies of 120 and 240 Hz. Excitation occurred at a frequency of 40 Hz and there was no statistically significant effect at other frequencies.

It was noted previously that most neurones showed either depressant or biphasic responses to vibration when the probe of the mechanical stimulator was positioned outside the low threshold receptive field: in either case the total number of spikes during the period of stimulation was decreased. The group of neurones which exhibited depression (group DPR) appeared to be homogeneous in terms of amplitude-response relationships. On the other hand, the amplitude-response data indicated that the group with the biphasic response appeared to consist of 2 subgroups (BPH_1 and BPH_2). For groups DPR and BPH_1 the average decrease during vibration was monotonically related to the amplitude of stimulation and the amplitude-response curves for the 2 groups failed to differ at any point. In group BPH_2 vibration caused

an increase in the number of spikes at stimulation amplitudes less than 0.3 mm and a decrease at greater amplitudes.

With regard to the frequency-response relationships using 0.15 mm stimulation, vibration caused a statistically significant average decrease in the rate of discharge in group DPR at frequencies of 120 and 240 Hz, 240 Hz stimulation being more effective. For group BPH_1 the effect was statistically significant only at 240 Hz. For both of these groups there was no effect at other frequencies. In contrast, an increase in the number of spikes during the stimulation occurred, on average, with group BPH_2 at frequencies of 40 and 80 Hz and a decrease was observed with stimulation at 240 Hz.

The similarity in the amplitude- and frequency-response curves for groups DPR and BPH_1 is consistent with the possibility that the vibration-induced effects in these groups may be mediated by a single class of primary afferent. In view of the fact the most effective stimulation frequency was 240 Hz it is possible that the vibration-induced effects in groups DPR and BPH_1 are mediated by Pacinian afferents. This suggestion is supported by the additional finding that depressant and biphasic effects were observed using stimulation amplitudes as low as 2 μm at a frequency of 240 Hz, which would be expected to selectively activate Pacinian corpuscle afferents.

The amplitude- and frequency-response data for group BPH_2 raises the additional possibility that neurones in this group have an excitatory input from rapidly adapting primary afferents in addition to depressant and biphasic inputs from Pacinian corpuscle afferents.

II. INTRODUCTION

In Chapter 3 the responses of dorsal horn neurones to vibration were described and the responses compared for neurones in different functionally defined groups. In contrast to the qualitative description given previously, the present chapter is concerned with a quantitative study of the parameters which control the responses of neurones in one specific group, the wide dynamic range (WDR) neurones. These neurones were studied specifically because they have been implicated as mediating responses to noxious cutaneous stimuli in experimental animals (Dubner and Bennett, 1983; Price and Dubner, 1977; Price and Mayer, 1975; Yaksh and Hammond, 1982) and also possibly in humans (Mayer et al., 1975).

Three distinct types of response of WDR neurones were described in Chapter 3 - excitation, depression and a biphasic effect. The aim of the present study was twofold: first, to investigate the effects of systematically varying the duration, amplitude and frequency of the vibrational stimulation on each of these 3 types of response and second, to describe any differences and similarities in the amplitude- and frequency-response relationships for the different responses. In view of the fact that primary afferents in the different classes respond preferentially, but not exclusively, to mechanical stimuli of particular amplitudes and frequencies (for reviews see Burgess and Perl, 1973; Darian-Smith, 1984), it was anticipated that by studying a wide range of amplitudes and frequencies evidence might be obtained regarding which class or classes of primary afferents mediate the 3 types of response.

III. MATERIALS AND METHODS

i) *Experimental preparation and recording*

The methods of investigation used in this study are essentially the same as those described in detail in Chapters 3 and 4. In brief, the experiments were done on cats anaesthetized with sodium pentobarbital (40 mg/kg, i.p.) or anaemically decerebrated under halothane which was then discontinued. All animals were acutely spinalized at the first lumbar level and the spinal cord was exposed for recording in segments L5-L7. Extracellular single-unit spikes were recorded using one barrel of

multibarrelled micropipettes. One of the other barrels was filled with a solution of L-glutamate (1M, pH 7.4). Each unit was classified functionally on the basis of responses to natural, non-vibrational stimuli. Raw data were recorded on magnetic cassette tape and interspike and interstimulation intervals were stored on fixed disk with an IBM personal computer.

ii) *Vibrational stimuli*

Vibration was produced using a Chubbuck mechanical stimulator (Chubbuck, 1966) and was tested on the on-going activity and on the activity evoked by iontophoretic application of glutamate.

a) *Stimulation parameters when investigating amplitude-response relationships.* When the amplitude-response relationships were examined the same frequency of stimulation was used as in Chapter 3, 80 Hz. To generate vibration the mechanical stimulator was driven using trains of rectangular voltage pulses produced by a Grass S88 stimulator. The frequency within the trains was 80 Hz and the duration of each voltage pulse was 6 ms. As described in Chapter 3, using this pulse duration the peak displacement of the stimulator probe varied linearly with the input voltage and the displacement of the probe had a curvilinear trajectory. Amplitudes of stimulation of 0.05 to 1.0 mm were routinely utilized when investigating amplitude-response relationships, although in some cases amplitudes of stimulation as small as 0.001 mm were used.

b) *Stimulation parameters when investigating frequency-response relationships.* When the frequency-response relationships were investigated sinusoidal stimulation was used. The sinusoids were generated using the computer: a DT 2801A board (Data Translation, Marlboro, MA) in the computer was programmed to produce sinusoidal voltage oscillations and the analogue output of this board was led to the input of the mechanical stimulator. Effects of stimulation at frequencies of 10, 20, 40, 80, 120 and 240 Hz were studied routinely and in some cases 1 and 5 Hz were also used.

In view of the fact that high frequency inputs were attenuated by the transducer of the mechanical stimulator (-3 dB at 85 Hz), it was necessary to increase the amplitude of the voltage sinusoids when frequencies of greater than 80 Hz were examined in order to maintain constant

peak-to-peak amplitude of the displacement. In addition, at frequencies greater than 80 Hz the stimulator probe failed to return to the control position between the cycles, causing an offset in the baseline position during the stimulation; the greatest offset (0.13 mm) occurred with stimulation at 240 Hz. Thus, when the frequency-response relationships were investigated a step displacement was made concurrently with the vibration so that a constant baseline offset of 0.13 mm occurred at all frequencies. To ensure that the input to the simulator produced the required amplitude and frequency of the excursion of the probe, its displacement was monitored via an output from the controller of the mechanical stimulator.. Data could not be sampled with the computer when it was being used to generate sinusoids; therefore, the interspike and interstimulus intervals were collected by replaying the magnetic tapes containing the raw data.

c) *Stimulation parameters when comparing effects of pulses and sinusoids.* For reasons fully described in section IV, in some cases trains of pulses were used in which the frequency was less than 80 Hz. In these cases, as was the case when the amplitude-response relationships were investigated, the voltage pulses used to drive the mechanical stimulator were 6 ms in duration. This duration of the voltage pulse caused the displacement of the mechanical stimulator to follow a trajectory that approximated one cycle of an 80 Hz sinusoid (for example see Fig. 2 in Chapter 3). Thus, the maximum frequency for which effects of pulses and sinusoids could be compared was 80 Hz.

iii) *Transection and electrical stimulation of peripheral nerves*

To test the suggestion that vibration-induced effects are mediated by activation of primary afferent neurones (see Chapter 3) and to ensure that the effects were not produced artefactually, by movement at the site of recording, 4 experiments were done in which the superficial and deep peroneal nerves and the tibial nerves were exposed at the level of the ankle joint. To prevent drying, the nerves were covered with gauze which had been dampened with saline. At the appropriate time during the experiment the nerves were transected using scissors. The central cut end was suspended on bipolar, hook electrodes for electrical stimulation. In 2 cases the nerves were suspended on the electrodes prior to transection

so that electrical stimuli could be delivered to the intact nerves and in the other 2 cases the nerves were placed on the electrodes only after transection. The electrical stimuli were rectangular pulses delivered using a constant-current, stimulus-isolation unit (Grass PSIU6) which was driven by a Grass S88 stimulator. The pulse duration was 0.10 ms.

iv) *Data analysis*

The method of data analysis will be described in detail because this paper is primarily concerned with quantification of the vibration-induced responses.

The net effect of vibration was determined by measuring the number of spikes during the period of each stimulation and comparing this number to the number of spikes during the control period. The control period, which had the same duration as the period of the stimulation, was the period immediately preceding each stimulation, except in cases where the effects of the duration of the stimulation were investigated. In these cases only, the control period was the 1 s period immediately preceding each stimulation. The rationale for choosing the control period preceding each stimulation was to control for possible variation in the magnitude of the effect depending on the rate of firing.

The percentage change in the number of spikes during the period of vibration compared with the control period was calculated for each stimulation. The average percentage change was determined using at least 5 consecutive stimulations. In cases where duration of the control period was 1 s the number of spikes in this period was multiplied by the duration of stimulation for calculation of the percentage change. A positive value for percentage change indicates that the net effect was an increase in the number of spikes during the vibration compared with the control level and conversely, a negative value indicates a decrease in the number of spikes. Statistical significance was determined using the paired *t*-test or the sign test, as appropriate. Means of percentage change for groups of units were compared using a two-tailed *t*-test or the Mann-Whitney U-test, as appropriate. Effects were considered statistically significant for $p<0.05$.

Numerical values are reported in the text as the mean $\pm$ one standard error of the mean.

IV. RESULTS

Parametric data from 60 WDR neurones are included in these results. These neurones were part of the group of 193 WDR neurones reported in Chapter 3. Thus, all of the neurones included here were shown to be excited by iontophoretic application of glutamate and the sites of recording were in Rexed's laminae I and III-VI (Rexed, 1954).

The parametric data will be presented in 4 sections. In the first section the responses will be considered when the stimulator probe was positioned outside the receptive field for low threshold mechanical stimuli. This section is presented first because, as reported in Chapter 3, vibration evoked primarily depressant or biphasic responses using this stimulus location and because we have previously presented evidence as to the possible neurochemical basis of the depression and the depressant component of the biphasic response (see Chapter 4). The second section of the results will be used to compare the parametric data for the depressant response with that for the biphasic response. In the third section parametric data will be presented for the situation in which the stimulator probe was located inside the low threshold receptive field. The final section will report on experiments designed specifically to investigate the possibility that the depressant and biphasic responses might be mediated by activation of Pacinian corpuscle afferents.

i) *Parametric data for responses evoked when the stimulator probe was positioned outside the receptive field for low threshold mechanical stimuli*

a) *Comparison of effects of various durations of vibrational stimulation.* When investigating the parameters which determine the responses to vibration it was considered important first to establish the most appropriate duration for the stimulation. On the basis of data such as illustrated for one neurone in Fig. 1, durations of 10 to 300 cycles at 80 Hz (i.e. 0.125-3.75 s) were considered optimal. Stimulation of less than 10 cycles had the possible advantage that many trials could be made in a short period because the stimulation could be repeated every 2-3 s. However, this advantage was outweighed by the large variability in the

magnitude of the responses. This variability may have arisen because of the variability in the on-going rate of discharge which is a feature of many dorsal horn neurones (for example Steedman et al., 1985). Stimulation longer than 300 cycles was not considered appropriate because compared with shorter durations, the magnitude of the response was lower and the variability in the magnitude was greater.

For the remainder of the study the duration of stimulation used was 200 to 250 cycles (i.e. 2.5-3.1 s) because this was in the range considered optimal and because the magnitude of the response could be easily monitored during the experiment (for example see Fig. 5 in Chapter 3). The intervals between the stimulations were 20 to 25 s. This duration of interval was sufficient to allow complete recovery between each stimulation. Using these temporal parameters responses were reproducible for greater than 4 hours. The duration of the stimulation and the interstimulation interval were constant throughout the period of testing for any given neurone.

b) *Effects of transection and electrical stimulation of peripheral nerves*. Results from one of the 4 experiments are illustrated in Fig. 2. The control response to vibration is shown in Fig. 2A. The deep and superficial peroneal nerves were transected during the intervals between Fig. 2A and 2B, and Fig. 2B and 2C, respectively. Each of these transections failed to affect the magnitude of the vibration-induced depression. In contrast, the tibial nerve was cut in the interval between Fig. 2C and 2D, and there was no effect of vibration after this transection.

When the nerves were transected the on-going rate of discharge decreased presumably because of alterations of the peripheral input. Therefore, glutamate was ejected to maintain a relatively constant rate of firing. As glutamate increases the firing rate primarily through a postsynaptic effect (Puil, 1983), the possibility existed that the change from Fig. 2C to 2D may have occurred if the effect of vibration was indirect. However, electrical stimulation of the central end of the transected tibial nerve mimicked the depressant effect of vibration (Fig. 2E) indicating that depression could still be evoked by activation of peripheral inputs. Therefore, it seems likely that the lack of response in Fig. 2D was not due to the use of glutamate to increase the rate of

discharge.

In one of the remaining 3 cases vibration also caused depression and in the 2 other cases it elicited the biphasic response. The depressant response and the depressant component of the biphasic response were abolished after transection of the peripheral nerves. In one case of the biphasic response the excitatory component was not present after transection and in the other case vibration caused excitation afterwards. The nerves were transected in a different order in each case and no consistent pattern emerged to implicate any of the 3 nerves as being the primary one mediating the effects of vibration.

c) *Comparison of effects of various amplitudes of vibrational stimulation.* The effect of varying the amplitude of stimulation was investigated systematically with 24 neurones; 7 of these exhibited vibration-induced depression and 17 showed the biphasic response. When examining the amplitude-response relationship the frequency of stimulation was 80 Hz. This frequency was chosen because 80 Hz stimulation had been used previously when the differential effect of vibration on nociceptive vs non-nociceptive neurones was observed (see Chapter 3). The upper limit of the range of stimulation amplitudes was 1.0 mm in all cases; the lower limit was 0.05-0.1 mm for 13 neurones and for the remaining 11 the lower end of the range was 0.001 mm.

Results for one neurone are illustrated in Fig. 3. The film records in Fig. 3A illustrate that the number of spikes during the period of stimulation decreased progressively when the amplitude of the stimulation was increased from 0.1 to 0.5 mm. The amplitude-response curve in the graph in Fig. 3B shows that the average decrease in the number of spikes during the period of stimulation was greatest with an amplitude of 0.5 mm and that the magnitude of the decrease was maintained when the stimulation amplitude was increased up to 1.0 mm. As illustrated by the inset in the upper right, this neurone showed the biphasic effect of vibration described in Chapter 3.

Individual amplitude-response curves from the 24 neurones are illustrated in Fig. 4; the data are shown in terms of the percentage change (Fig. 4A) and the total number of spikes (Fig. 4B) during the vibration. The points on the y-axis in the graph in Fig. 4B indicate the number of spikes in the control period. Thus, it can be calculated that

the baseline rate of firing of the different neurones varied from 2.5 to 105 spikes$\cdot s^{-1}$. Importantly, differences in the amplitude-response curves failed to correlate with differences in the baseline rates of discharge.

Sixteen of the neurones represented in Fig. 4 were unselected, that is they were studied during experiments in which amplitude-response curves were constructed for all WDR neurones which could be investigated for a sufficient length of time to acquire a full range of data points. The remaining 8 neurones had been selected for intensive study; assessment of the amplitude-response relationship was part of investigations made during the studies reported in Chapters 3, 4 and 7; the full range of amplitudes was not examined in every case. No systematic differences were apparent between the unselected vs selected groups. Hence, the data are reported together and the neurones sampled are considered representative of the population of WDR neurones normally encountered during our experiments.

Averages of the individual amplitude-response curves for percentage change and for total number of spikes are plotted in Fig. 4C. These average curves indicate that vibration caused a decrease in number of spikes which was graded with respect to stimulation amplitudes greater than 0.05 mm. The increase in the average number of spikes observed using 0.05 mm stimulation failed to be statistically significant ($t=1.5$, $p>0.10$). This increase was due to excitation of a subpopulation of neurones which exhibited the biphasic effect as will be described in section ii.

By interpolating between points it was calculated that, on average, the number of spikes during the vibration was decreased 50% when the stimulation amplitude was 0.45 mm; the modal value was 0.40 mm.

d) *Comparison of effects of various frequencies of vibrational stimulation.* Frequency-response curves were constructed for 22 unselected neurones, as described in the preceding section.

For the neurone shown in Fig. 5, frequency-response curves were made using 2 amplitudes of stimulation, 0.15 and 0.60 mm. The smaller stimulation amplitude was used because this was the maximum amplitude which could be generated at a frequency of 240 Hz. The greater amplitude was chosen because this was in the range where the percentage change using

a frequency of 80 Hz was expected to be most negative, on the basis of the population response. At both amplitudes, vibration failed to have a statistically significant effect at frequencies of 1, 5, 10, 20 and 40 Hz. With the greater amplitude, a statistically significant decrease in the number of spikes during the stimulation was observed at 80 Hz and at the additional frequency examined in this case, 60 Hz. With the smaller amplitude, the percentage change at 80 Hz was less negative than for the greater amplitude and maximum depression with 0.15 mm stimulation occurred only with frequencies of 120 and 240 Hz.

Individual frequency-response curves for the 22 neurones are shown in Fig. 6A; in each case the amplitude of stimulation used was 0.15 mm. The curves illustrate that each neurone had a unique frequency-response relationship. In all cases, however, the percentage change was most negative for frequencies of 120 or 240 Hz. For the whole sample, on the average, (Fig. 6B) only the points for these 2 frequencies were significantly less than zero, with the greater effect at 240 Hz. At 40 Hz the average percentage change was significantly higher than zero.

e) *Comparison of effects of pulses vs sinusoidal stimulation at frequencies of 80 Hz and less.* In an attempt to determine whether the lack of significant effect of sinusoidal stimulation at frequencies below 80 Hz was due to the slower repetition rate or to the longer rise time to the peak of the displacement with each sinusoid, experiments were done in which the 80 Hz sinusoidal waveform was held constant but the interval between each sinusoid was increased, producing lower overall frequencies. In these experiments, trains of pulses (amplitude: 1.0 mm) were used because with each pulse the trajectory of the probe was approximately sinusoidal (Chapter 3).

Data from a representative neurone are illustrated in Fig. 7. From the graph in Fig. 7A it is clear that pulses were more effective than sinusoids at frequencies of 20 Hz and less, whereas the effects of pulses and sinusoids were indistinguishable at 40 and 80 Hz. The PSTH in Fig. 7B illustrates that single pulses caused depression lasting approximately 300 ms. Thus, the magnitude of the depression produced by trains of pulses below 40 Hz may be accounted for by summation of the responses to individual pulses.

As similar results were obtained with all of the 13 neurones tested

using pulses at low frequencies, it appears that the reason for the limited effectiveness of sinusoidal stimulation at frequencies below 40 Hz may be the longer rise time of the displacement rather than the lower repetition rate.

ii) *Comparison of depressant vs biphasic responses*

a) *Comparison of amplitude-response relationships.* As depression and the biphasic effect were the predominant responses of WDR neurones to vibration when the probe was applied outside the receptive field (see Chapter 3), it was considered important to compare the amplitude-response relationships of neurones showing depression with those of neurones showing the biphasic response. This comparison was made using the data collected for the entire sample (Fig. 4); the stimulation frequency was 80 Hz. In Fig. 4A it can be seen that with some neurones the percentage change was positive for stimulation amplitudes of 0.2 mm or less; this effect was statistically significant in 6 cases and the increase was more than 25% in each of these cases. Each of the 6 neurones which exhibited excitation at stimulation amplitudes of 0.2 or less showed the biphasic response with amplitudes of stimulation greater than those causing excitation. The remaining 11 neurones with biphasic responses to vibration failed to show excitation (or depression) at any stimulation amplitude. Similarly, all 6 neurones which showed the depressant response at any given stimulation amplitude never showed biphasic or excitatory responses at any other amplitude. Therefore, it seemed reasonable to divide the neurones into 3 groups for purposes of analysis: the group which exhibited only depression (group DPR), the group which showed only the biphasic response (group BPH_1) and the group which showed excitation at low stimulation amplitudes and the biphasic response at greater amplitudes (group BPH_2).

The average amplitude-response curves for the 3 groups are illustrated in Fig. 8A. The curve for group BPH_2 is clearly different from the curve for group BPH_1 and for group DPR. Interestingly, none of the points on the curve for group BPH_1 was different from the corresponding point on the curve for group DPR.

b) *Comparison of frequency-response relationships.* When analyzing the frequency-response relationships for neurones with biphasic and

depressant responses, the group exhibiting the biphasic response was subdivided using the same criterion as in the preceding section: noting that the amplitude of stimulation used for the frequency-response studies was 0.15 mm, neurones which showed a statistically significant excitation at 80 Hz (group BPH_2, n=4) were considered separately from those which failed to show excitation (group BPH_1, n=9). Neurones which exhibited depression with 80 Hz stimulation (group DPR) showed only depression.

Average frequency-response curves for the 3 groups are shown in Fig. 8B. For groups DPR and BPH_1 there was no significant effect at frequencies of 80 Hz and less. At 120 Hz, the effect of stimulation was statistically significant for group DPR but not for group BPH_1; in addition, the difference between the groups was significant ($t=2.1$, $p=0.05$). At 240 Hz, the effect was statistically significant in both groups but the difference between the groups was greater than at 120 Hz.

Stimulation at 240 Hz caused a statistically significant negative percentage change in group BPH_2. On the other hand, 40 Hz stimulation caused excitation of neurones in group BPH_2. Thus, the net excitatory effect at 40 Hz in the general population was likely due to effects in group BPH_2. The increase was also statistically significant at 80 Hz as would be expected on the basis of the criterion used to identify members of group BPH_2.

c) *Excitation of neurones in group BPH_2 using low amplitude stimulation.* Experiments were done to investigate the possibility that the excitation observed with low amplitude stimulation in group BPH_2 might be due to spread of vibration from the site of application of the probe to the receptive field. Amplitude-response curves were constructed for 2 different probe locations as shown in Fig. 9. In each position the probe was applied with a force[1] of 440 mN.

When the probe was positioned inside the low threshold receptive field, the percentage change increased in a nearly linear fashion with the log of stimulation amplitude ($r=0.997$, $p<0.01$). On the other hand, when the probe was located outside this receptive field, the amplitude-response

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[1] It was noticed with some neurones that the magnitude of the vibration-induced response could by increased by increasing the constant force which was applied between stimulations. Therefore, when studying the amplitude- and frequency-response relationships this force was 400-500 mN.

curve was biphasic. At the peak of the curve (stimulation amplitude: 0.01 mm) the increase was 62±13%. This value was not significantly different from the value calculated by interpolation from the curve with the probe located inside the receptive field (63%; t=0.1, p>0.40). If the excitatory input causing both of these responses had originated in the low threshold receptive field it would be predicted, on the basis that vibration amplitude declines with the square of distance (von Gierke et al., 1952), that for any given amplitude of stimulation the excitation when the probe was applied inside the receptive field would be greater than when the probe was positioned outside the receptive field. As the magnitudes of the excitations were not different for small stimulation amplitudes (0.01 mm and less), the excitatory inputs activated when the probe was placed outside the receptive field for low threshold stimuli likely did not arise from this receptive field.

iii) *Parametric data for responses evoked when the stimulator probe was positioned inside the receptive field for low threshold mechanical stimuli*

a) *Amplitude-response relationships.* Vibration at 80 Hz caused excitation of nearly all WDR neurones when the stimulator probe was applied inside the receptive field (Chapter 3). This effect was graded and the excitation increased with increasing stimulation amplitude (for example see Fig. 9).

b) *Frequency-response relationships.* Using a stimulation amplitude of 0.15 mm, frequency-response curves (Fig. 10A) were constructed for 13 unselected neurones, as defined above. These curves illustrate that between the different neurones there was considerable variability in the frequency-response relationship.

The average frequency-response curve (Fig. 10B) indicates that within the population all frequencies of stimulation caused excitation. However, the average effect was statistically significant only at 40 and 80 Hz.

iv) *Effects of low-amplitude, high-frequency stimulation*

We have previously suggested that depressant and biphasic effects of vibration are mediated by primary afferents which adapt rapidly (Chapter 3). To differentiate between 2 of the classes of A$\alpha\beta$ cutaneous afferents

which show rapid adaptation - rapidly adapting and Pacinian corpuscle afferents (see Burgess and Perl, 1973; Darian-Smith, 1984) - vibration was tested using a frequency of 240 Hz and an amplitude of 0.02 mm or less (this type of stimulation will be termed low-amplitude, high-frequency stimulation). These parameters were selected because a previous report by Hämäläinen and Pertovaara (1984) indicated that in the hindlimb of the cat amplitudes of 0.02 mm at 240 Hz are below the tuning threshold of all rapidly adapting primary afferents but are greater than the tuning threshold for approximately 75% of Pacinian corpuscle afferents.

a) *Effects when the stimulator probe was positioned outside the low threshold receptive field.* Eleven neurones which showed either the depressant or biphasic response to vibration using stimulation amplitudes greater than 0.15 mm were tested with low-amplitude, high-frequency stimulation. All of these neurones were unaffected by this stimulation.

b) *Effects when the stimulator probe was positioned inside the low threshold receptive field.* Results from one of the 12 neurones tested are shown in Fig. 11. With this neurone, vibration caused depression when the probe was located outside the receptive field for low threshold stimuli but when the probe was placed inside this receptive field the response depended upon the amplitude and frequency of stimulation. Using an amplitude of 0.15 mm vibration had a biphasic effect with 240 Hz stimulation (Fig. 11A). Depression occurred alone with 240 Hz stimulation for amplitudes as small as 0.002 mm (Fig. 11B) and there was no effect at 0.001 mm (Fig. 11C). On the other hand, with a stimulation frequency of 40 Hz vibration caused excitation when the amplitude was 0.15 mm (Fig. 11D) and had no effect when the amplitude was 0.002 mm (Fig. 11E).

With 5 of the 12 neurones, it was possible to evoke either depression or the biphasic response using low-amplitude, high-frequency stimulation when the stimulator probe was located inside the low threshold receptive field; with the remaining neurones, when the probe was placed inside this receptive field low-amplitude, high-frequency stimulation had no effect. All of the neurones affected by the low-amplitude, high frequency stimulation exhibited a biphasic effect using greater amplitudes of stimulation at the same frequency. For 2 of the neurones affected, vibration caused depression using amplitudes smaller than those which elicited the biphasic effect (for example the neurone illustrated in Fig.

11). With the 3 other affected neurones, both components of the biphasic response were observed even at the smallest effective amplitude of stimulation.

V. DISCUSSION

This report presents data from a quantitative investigation of parameters which affect the responses of WDR neurones to cutaneously applied vibration. The findings that the depressant and biphasic effects of vibration were abolished after transection of the peripheral nerves and that these effects were mimicked by electrical stimulation of these nerves provide support for the suggestion made in Chapter 3 that these types of vibration-induced responses of dorsal horn neurones are mediated by activation of primary afferents in the periphery. Furthermore, the afferents which produce these responses appear to have peripheral endings in the foot because the nerve transection was made at the level of the ankle.

i) *Pacinian corpuscle afferents appear to mediate the depressant and biphasic responses to cutaneously applied vibration*

In the following sections arguments will be presented that the depressant and biphasic responses to vibration may be mediated by Pacinian corpuscle primary afferents.

a) *Depressant response*. All neurones showed similar amplitude- and frequency-response relationships for the vibration-induced depression produced when the stimulator probe was located outside the low threshold receptive field. In addition, the time course of depression was similar in each case (see Chapter 3). It therefore seems reasonable to suggest that with each neurone depression is mediated by the same type or types of primary afferents.

Evidence that a single type rather than multiple types of primary afferent mediate the depression comes from examination of the amplitude- and frequency-response curves for the depression. The curves decreased monotonically with increasing stimulation amplitude and frequency, respectively. In view of the fact that different classes of primary

afferents have different mechanical thresholds (Burgess et al., 1968) and different amplitude- and frequency-response relationships (Hämäläinen and Pertovaara, 1984; Merzenich and Harrington, 1969; Talbot et al., 1968), the monotonic gradation of the curves for the depression is consistent with the possibility that this response is mediated primarily by a single type of primary afferents.[2] The gradation of the depression can thus be accounted for on the basis that as the amplitude and frequency of stimulation increase more primary afferents of one type will be recruited and those already activated will fire at increasingly faster rates.

The present study and that reported in Chapter 3 have provided evidence that the primary afferents which mediate the depression appear to be activated by mechanical transients. Therefore, on the basis of reports by others concerning responses of primary afferents to mechanical stimuli (for reviews see Burgess and Perl, 1973; Darian-Smith, 1984), 4 types of afferents must be considered as possible candidates: Pacinian corpuscle afferents, rapidly adapting afferents, D hair afferents and muscle spindle afferents.

D hair afferents are sensitive to vibratory stimulation and they are activated with amplitudes in the range of those which will activate Pacinian corpuscle afferents (Brown and Iggo, 1967; Merzenich and Harrington, 1969). However, the conduction velocity of D hair afferents is in the Aδ range and is thus too slow to account for the latency of the depression which requires fibres which conduct in the A$\alpha\beta$ range (see Chapter 3). The other types of afferents which may possibly mediate the depression have axonal conduction velocities in the A$\alpha\beta$ range and hence, none can be eliminated on the basis of conduction velocity.

From the frequency-response curve it is apparent that the magnitude of the depression was greatest when the stimulation frequency was 240 Hz. This finding raises the possibility that the vibration-induced depression is mediated by Pacinian corpuscle afferents because these are activated preferentially at this frequency (Hunt, 1961; Sato, 1961; Skoglund, 1960, Talbot et al., 1968). However, this finding is not conclusive because it

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[2] For the present report the simplest case will be discussed, that is, vibration-induced effects will be considered to be mediated by a single class of primary afferents unless there is direct evidence to the contrary (i.e. the case of the biphasic response in group BPH_2). The possibility has not been eliminated, however, that in some cases effects of vibration may be mediated by more than one class of primary afferent.

is possible that 240 Hz stimulation at the amplitude used when investigating the frequency-response relationship would also activate rapidly adapting afferents (Johansson et al., 1982; Hämäläinen and Pertovaara, 1984) and muscle spindle afferents (Brown et al., 1967; Burke et al., 1976; Matthews and Stein, 1969).

Evidence from the present study which specifically indicates that activation of Pacinian afferents causes depression, is that depression was elicited by vibration using a frequency of 240 Hz with amplitudes as small as 0.002 mm. Studies in cats (Hämäläinen and Pertovaara, 1983,, 1984), in monkeys (Merzenich and Harrington, 1969; Talbot et al., 1968) and in humans (Johansson et al., 1982) have shown that this amplitude is below the threshold of all cutaneous afferents except Pacinian corpuscles.

As far as muscle spindle afferents are concerned, the type most sensitive to vibration, the primary endings, can be activated by vibration with amplitudes as small as 0.005 mm (Brown et al., 1967). However, activation by this amplitude of stimulation was elicited with the stimulator probe attached directly to the muscle tendon. It seems unlikely, therefore, that muscle spindle afferents would have been activated at an amplitude of 0.002 mm in the present study because the stimulator probe was applied to skin which did not overlie tendons or muscles (see for example Fig. 11F). Additional evidence against involvement of afferents from muscle spindles comes from studies which have indicated that selective activation of Ia afferents, by electrical stimulation, fails to affect the discharge rate of dorsal horn neurones (Cervero et al., 1976; Hongo et al., 1968).

Process of elimination, then, leads us to the conclusion that the depressant effect of low-amplitude, high-frequency vibratory stimulation was most likely mediated by Pacinian corpuscle afferents. The time course of this depression was similar to that of the depression observed when the stimulator probe was located outside the low threshold receptive field (for example compare Fig. 11B with Fig. 4B in Chapter 3). In addition, in both cases depression appears to be evoked by mechanical transients. Therefore, we suggest further that the type of primary afferent mediating the vibration-induced depression produced when the stimulator probe was placed outside the low threshold receptive field is the same as the type mediating the depression caused by low-amplitude, high-frequency stimulation. Preliminary data from other experiments are consistent with

this suggestion as we have found that depression caused by low-amplitude, high-frequency stimulation can be blocked by administration of caffeine, the same pharmacological agent we have previously reported to block the depression elicited when the stimulator probe was placed outside the low threshold receptive field (see Chapter 4).

The suggestion that Pacinian corpuscle afferents mediate the depression seen in the present study is consistent with the observation that spinocervical tract neurones are hyperpolarized by cutaneously applied vibration using a 500 Hz tuning fork (Brown et al., 1987).

It is interesting to note that depressant effects of vibration have been previously reported in the trigeminal (Carmody and Rowe, 1974) and in the cuneate (Bystrzycka et al., 1977) nuclei. Bystrzycka et al. (1977) observed depression using 300 Hz stimulation at amplitudes less than 0.025 mm, which would be expected to preferentially activate Pacinian afferents. Therefore, the effect of these afferents may be similar in the cuneate nucleus and in the spinal cord.

b) *Biphasic response.* In contrast to depression, the biphasic response did not appear to be homogeneous across the population: approximately two-thirds of the neurones exhibited the biphasic response at all effective amplitudes or frequencies of stimulation; the remaining one-third of neurones was excited using stimulation amplitudes or frequencies lower than those which caused the biphasic response. On the basis of this difference in the amplitude- and frequency-response relationships we have divided the neurones which exhibited the biphasic response into 2 groups, termed BPH_1 and BPH_2, respectively. This part of the discussion will concentrate on the former subgroup; the latter group will be considered in a later section.

The amplitude- and frequency-response curves for group BPH_1 were monotonically decreasing which raises the possibility that this effect may be mediated by a single class of primary afferents, as described in the previous section in the case of the depressant response. Furthermore, the finding that the amplitude-response curve for group BPH_1 failed to differ at any point from that of group DPR is consistent with the possibility that these effects are mediated by the same type of primary afferents - Pacinian corpuscle afferents. Other evidence that the biphasic response may be mediated by Pacinian corpuscle afferents is that

this effect could be produced by low-amplitude, high-frequency stimulation.

Therefore, we suggest that both the depressant and the biphasic responses to vibration may be mediated by Pacinian corpuscle afferents. This suggestion is consistent with findings that the neurochemical basis of the depression and of the depressant component of the biphasic response are similar (see Chapter 4).

ii) *Possible postsynaptic mechanisms of effects of Pacinian afferents*

Two alternative models for production of both depressant and biphasic responses by Pacinian corpuscle afferents are illustrated in Fig. 12. Each of these models is consistent with the following observations concerning these responses: 1) the latency of the depression and of the depressant component of the biphasic response are longer than the latency of the excitatory component; 2) the time courses of the depression and of the depressant component are similar; 3) the amplitude-response relationships for groups DPR and BPH_1 are similar; 4) at effective frequencies of stimulation the percentage change for group DPR is greater than for group BPH_1 and 5) the pharmacological properties of the depression and the depressant component are similar. Both models use postsynaptic mechanisms to account for these observations because it has been found that inhibitory postsynaptic potentials (IPSPs) can be evoked in dorsal horn neurones by 500 Hz vibration of the skin (Brown et al., 1987) and by electrical stimulation of the interosseous nerve, which contains a large number of Pacinian corpuscle afferents (Hongo et al., 1968). In addition, activity evoked by glutamate can be blocked during the depression and during the depressant component of the biphasic response (see Chapter 3). These findings are most readily explained using postsynaptic mechanisms.

It is possible that Pacinian afferents may have presynaptic effects because activation of this type of afferent causes an increase in the excitability of the central terminals of primary afferents (Jänig et al., 1968a; Schmidt et al., 1967). The results of the present study have not eliminated the possibility that presynaptic effects might be involved in mediating the depressant effects of vibration on dorsal horn neurones. Therefore, it is possible to construct other models to explain the

observations made in this study and these other models might include presynaptic mechanisms in addition to postsynaptic mechanisms such as those shown in Fig. 12.

The 2 models shown in Fig. 12 use different methods to account for the longer latency of the depressant response as compared with the excitatory component of the biphasic response: on the one hand, model I incorporates an inhibitory interneurone; on the other hand, model II attributes the difference in latency to a slower onset for the inhibition compared with the excitation. Thus, in model I the depressant response and the depressant component of the biphasic response are mediated di-synaptically whereas in model II these responses are elicited monosynaptically. More details of the models are given in the legend to Fig. 12.

Evidence for monosynaptic IPSPs with a slow onset has been previously reported from studies in sympathetic ganglia of rabbits (Cole and Shinnick-Gallagher, 1984) and bullfrogs (Smith and Weight, 1986). In these cases, the slow IPSP appears to be caused by acetylcholine acting through muscarinic receptors as the IPSP is blocked by the muscarinic antagonist, atropine. Interestingly, acetylcholine also produces a fast excitatory postsynaptic potential in sympathetic ganglia by activation of nicotinic receptors (Dun, 1980). Therefore, biphasic synapses similar to that proposed in model II have been reported elsewhere. Thus, the presence of a biphasic synapse in model II may not make this model less probable than model I.

In the case of model II, it does not seem likely that acetylcholine is the compound producing the depressant effects on DPR and BPH_1 neurones because the methylxanthine, theophylline, has been found to potentiate inhibitory effects of activation of muscarinic receptors in sympathetic ganglia (Busis et al., 1978) whereas another methylxanthine, caffeine, blocks the depressant effects of vibration (see Chapter 4).

iii) *Effects of vibration on neurones in group BPH_2*

Vibration-induced effects in group BPH_2 appear to be more complex than the effects in the other groups. The average amplitude- and frequency-response curves for neurones in group BPH_2 were biphasic and hence both curves were different from the respective curves for group BPH_1 and for group DPR. The biphasic nature of the curves for group

BPH_2 raises the possibility that, in contrast to group BPH_1, the excitatory and depressant components of the responses in group BPH_2 might be mediated by different types of afferents.

Excitation of neurones in group BPH_2 occurred with stimulation frequencies of 80 Hz and below and with stimulation amplitudes less than 0.2 mm. These parameters of stimulation would be would be expected to preferentially activate rapidly adapting rather than Pacinian corpuscle afferents (Hämäläinen and Pertovaara, 1983; Johansson et al., 1982; Merzenich and Harrington, 1969; Talbot et al., 1968). It is possible, therefore, that the excitation of BPH_2 neurones may be mediated by rapidly adapting afferents. This suggestion is consistent with data from other studies which have indicated that input from rapidly adapting afferents excites dorsal horn neurones (Brown, 1969; Honda et al., 1986; Rowe and Sessle, 1972; Willis et al., 1975). The biphasic effects which occurred in the present study with stimulation using greater frequencies or amplitudes could be accounted for by inputs from Pacinian corpuscle afferents in addition to the inputs from rapidly adapting afferents.

iv) *Functional significance of vibration-induced effects on WDR neurones*

We have previously hypothesized that the differential effects of vibration on WDR vs non-nociceptive neurones may represent the basis, at the spinal level, for the analgesic effects of vibration (see Chapter 3). In many previous studies in humans the parameters of vibrational stimulation would be expected to have activated several types of primary afferents in addition to Pacinian afferents (Ekblom and Hansson, 1982; Lundeberg, 1983; Ottoson et al., 1981; Wall and Cronly-Dillon, 1960). However, Pertovaara (1979) found that vibration of 240 Hz at an amplitude of 0.02 mm was more effective in elevating pain threshold than was vibration of 20 Hz at an amplitude of 0.2 mm. This finding, together with the results of the present study, prompts us to extend our previous hypothesis and to suggest that vibration-induced analgesia might be mediated by the depression of WDR neurones in the dorsal horn caused by activation of Pacinian corpuscle afferents.

Ferrington et al. (1977) have reported that preferential activation of Pacinian afferents impairs the perception of innocuous mechanical stimuli. This phenomenon is known as "masking". While they suggested

that the neural basis of this effect may be inhibition of neurones in the dorsal column nuclei by Pacinian afferent input (Bystrzycka et al., 1977), they were careful to point out that their observations did not preclude similar inhibition occurring in the spinal cord even though it had not been reported. The present study provides the first parametric data indicating that activation of Pacinian afferents causes depression of spinal neurones. Thus, the possibility should be considered that the neural basis for "masking" may include depression of WDR neurones in the dorsal horn.

Von Békésy (1967) described a different sensory phenomenon which he termed "funneling". The differential response of WDR neurones to vibration applied inside vs outside the low threshold receptive field suggests that these neurones might be involved in this phenomenon as well.

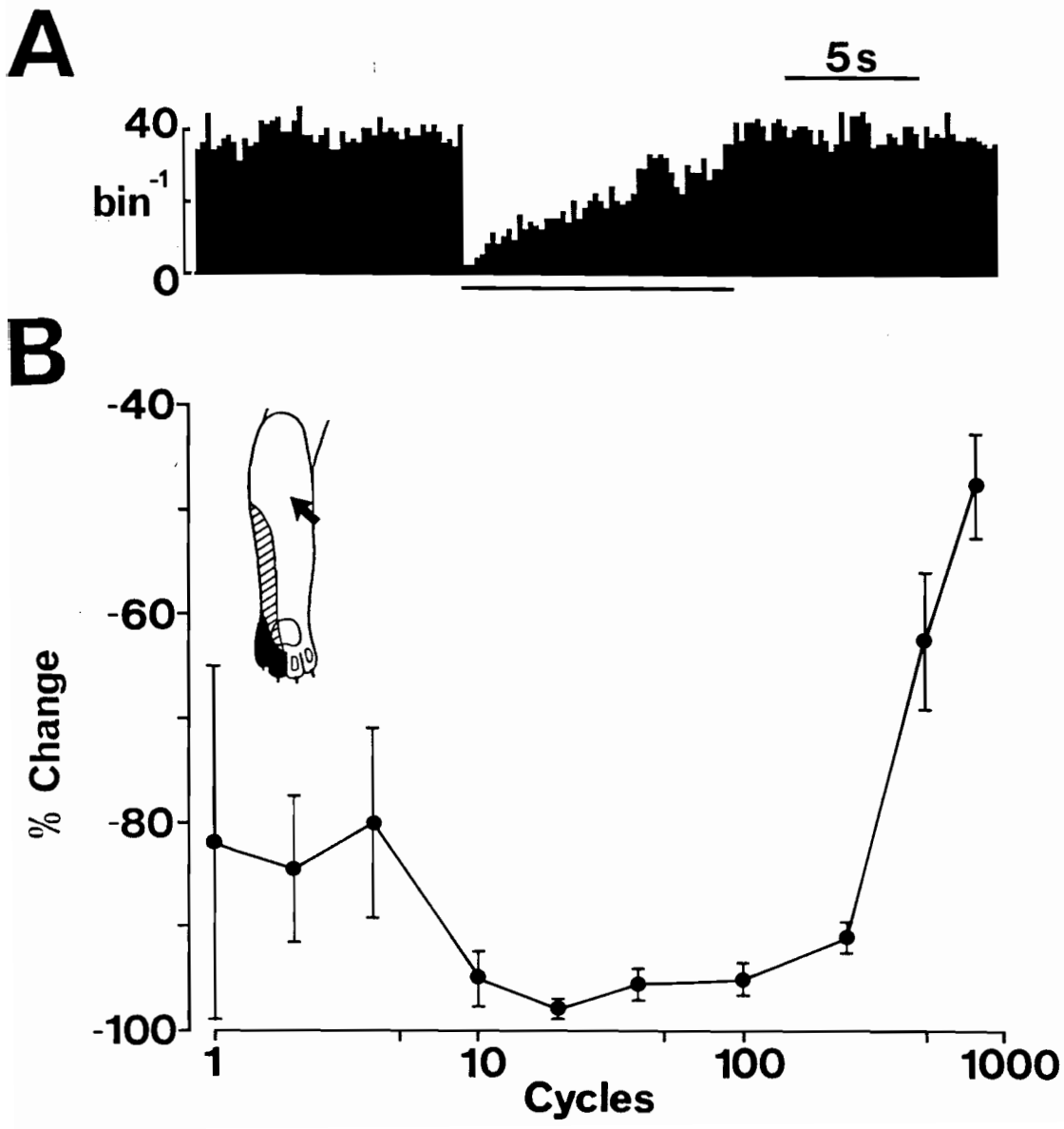
In conclusion, the results of the present study and of Chapter 3 indicate that WDR neurones may have a diverse role in the somatosensory system of the spinal cord.

Fig. 1 Comparative effects of various durations of vibrational stimulation.

A. The peristimulus time histogram (PSTH) was constructed using 10 consecutive applications of vibration (frequency of stimulation: 80 Hz). The bin width for the PSTH is 200 ms. The period of the vibration is indicated by the bar below the record. The stimulation was repeated every 60 s.

B. In the graph each point represents the average percentage change plotted against duration of stimulation in terms of the number of cycles; the duration of each cycle was 12.5 ms. The average was calculated using 30 trials. Each error bar in this graph, and in all others, shows ± one standard error of the mean.

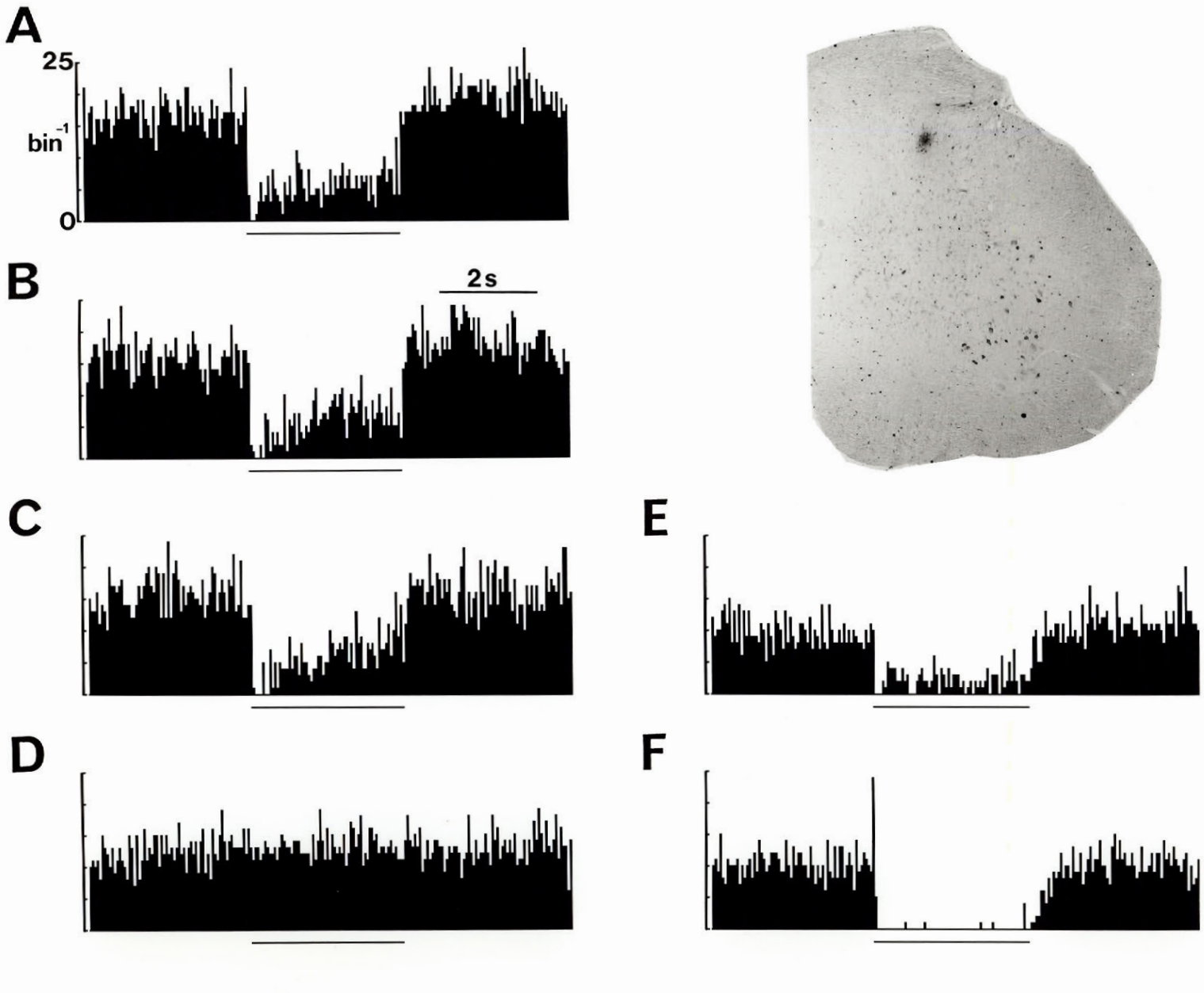
Inset- The receptive fields are illustrated in the diagram: the neurone could be excited by light touch in the blackened area and by noxious pinch in the hatched and blackened areas. In all figures, the arrow indicates the position of the stimulator probe and the orientation of its long axis.


A
5s
40
bin-1
0
B
-40
-60
-80
-100
% Change
1
10
100
1000
Cycles

'ig. 2 Vibration-induced depression is blocked by transection of peripheral nerves and is mimicked by electrical nerve stimulation.

Six PSTHs are shown; each was constructed using 5 consecutive pplications of vibration. The bin width is 50 ms. The period of ibration is indicated by the bars below the records in A-D (frequency - 0 Hz; amplitude - 0.4 mm). The PSTH in A shows the control response rior to any transections. The deep peroneal, superficial peroneal and ibial nerves were transected in sequence during the periods just before ,C and D, respectively. The PSTHs in E and F show responses to epetitive electrical stimulation of the central end of the cut tibial erve; the stimulation frequency was 80 Hz. The magnitude of the lectrical stimulus was 3.5 μA (1.1×threshold) for E and 5 μA for .

The dye deposit marking the site of recording in lamina III is shown n the histological section (magnification factor: 15×). In this ase the probe of the stimulator was applied 3 cm distal to the ankle on ne the plantar surface of the foot. The receptive fields for hair ovement and touch were located on the tip of the lateral toe and the eceptive field for pinch was the area distal to and including the central ad.


A
25
bin^{-1}
0
B
2s
C
D
E
F

ig. 3 Comparative effects of various amplitudes of stimulation for an individual neurone.

A. Each film record shows a single response to vibration (at 80 Hz) pplied during the period indicated by the bar above the upper trace. The timulation amplitude, in mm, is indicated by the number to the left of ach trace. Calibration bars apply to all film records: vertical - 00 μV; horizontal - 3 s.

B. Graph illustrating average percentage change as a function of mplitude of stimulation. Each point represents the percentage change in ne number of spikes during the entire period of stimulation, averaged for) trials.

Insets: Lower left. In the diagram the blackened area represents ne excitatory receptive field for hair movement and touch. The neurone as also excited by noxious pinch to the blackened and hatched areas. The row indicates the position and orientation of the stimulator probe.

Upper right. PSTH constructed using 10 trials with the stimulation mplitude of 0.3 mm; the period of vibration is indicated by the bar below ne histogram. The vertical scale is 10 spikes·bin^{-1} and the time bar epresents 2 s. The bin width for the PSTH is 10 ms.

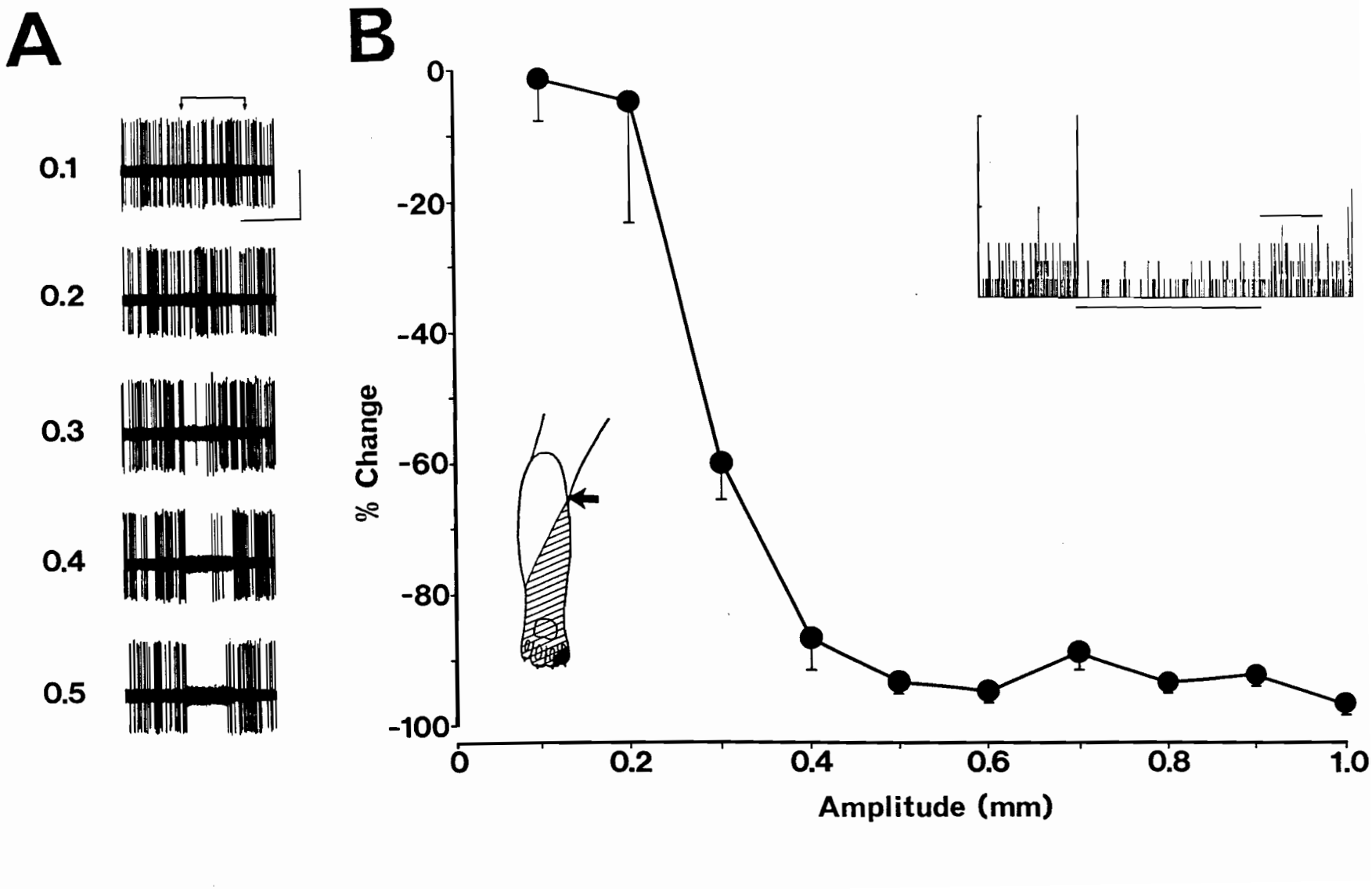

A
0.1
0.2
0.3
0.4
0.5
B
0
-20
-40
-60
-80
-100
% Change
0
0.2
0.4
0.6
0.8
1.0
Amplitude (mm)

Fig. 4 Amplitude-response curves for stimulation at 80 Hz.

Individual curves for 24 neurones are illustrated in the graph in A, which shows the percentage change and in the graph in B, which shows the average number of spikes during the period of vibration. In A and B the right-hand end of each line indicates the maximum amplitude tested. Averages of the individual curves for percentage change (circles) and for number of spikes (triangles) are represented in C. The upper limit of the graph in C is 0.8 mm because only 60% of the neurones were tested using greater amplitudes and this proportion was considered too small to allow reliable calculation of the average.

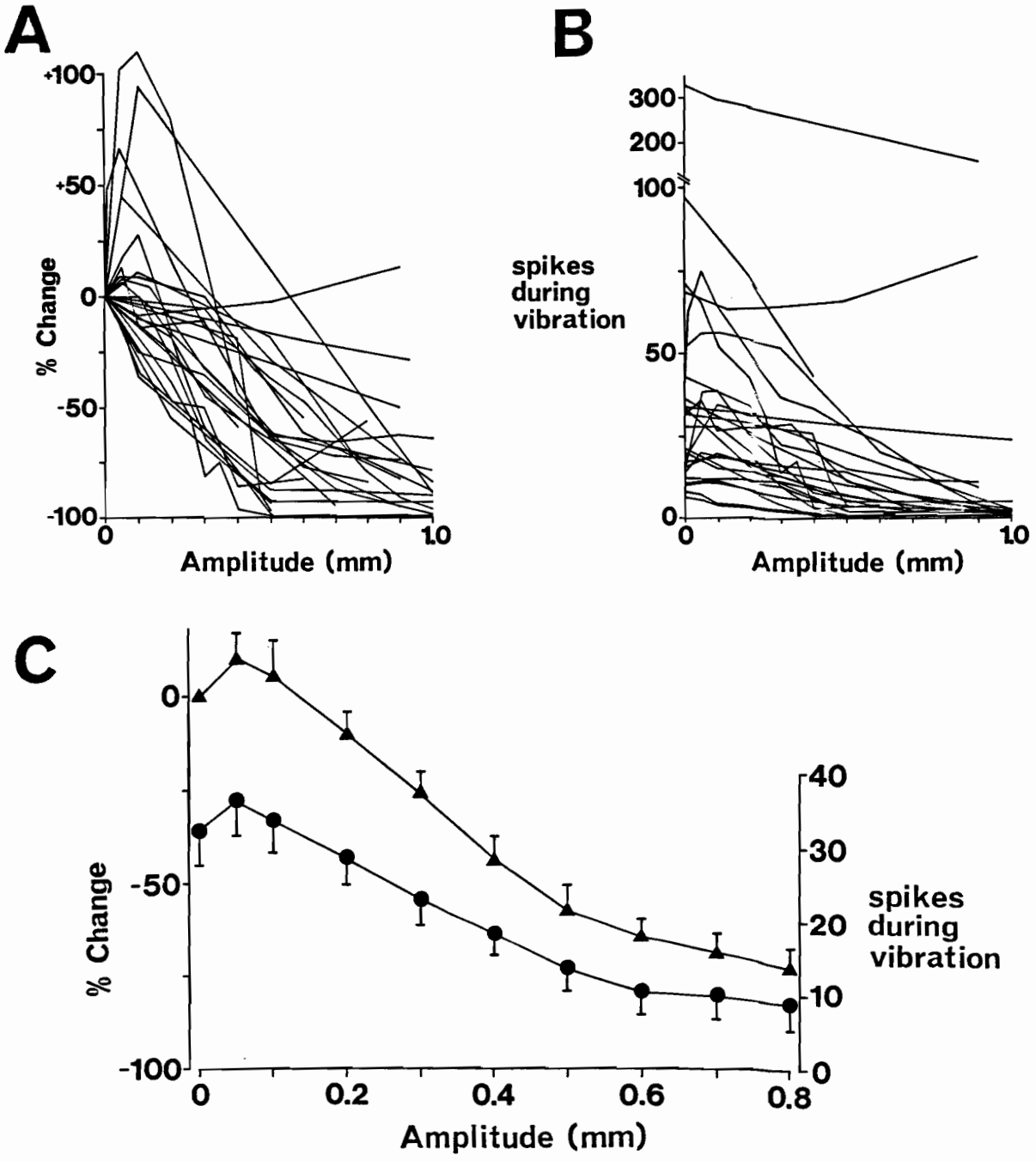

A
+100
+50
0
-50
-100
% Change
0
1.0
Amplitude (mm)
B
300
200
100
50
0
spikes
during
vibration
0
1.0
Amplitude (mm)
C
0
-50
-100
% Change
40
30
20
10
0
spikes
during
vibration
0
0.2
0.4
0.6
0.8
Amplitude (mm)

Fig. 5 Comparative effects of various frequencies of stimulation for one neurone.

A. A semilogarithmic graph is shown in which percentage change is plotted vs frequency of stimulation for amplitudes of 0.6 mm (dots) and 1.5 mm (triangles). The points indicate the average percentage change, calculated using 5 trials. For each point the average percentage change is compared with 0 using the *t*-test. ** - $p<0.01$; *** - $p<0.001$.

B. The film record shows a single response to vibration when the stimulus frequency was 240 Hz and the amplitude was 0.15 mm. Calibration bars - vertical - 300 μV; horizontal - 3 s.

C. The receptive fields and position of the stimulator probe are illustrated in the diagram using the same scheme as in Fig. 3.

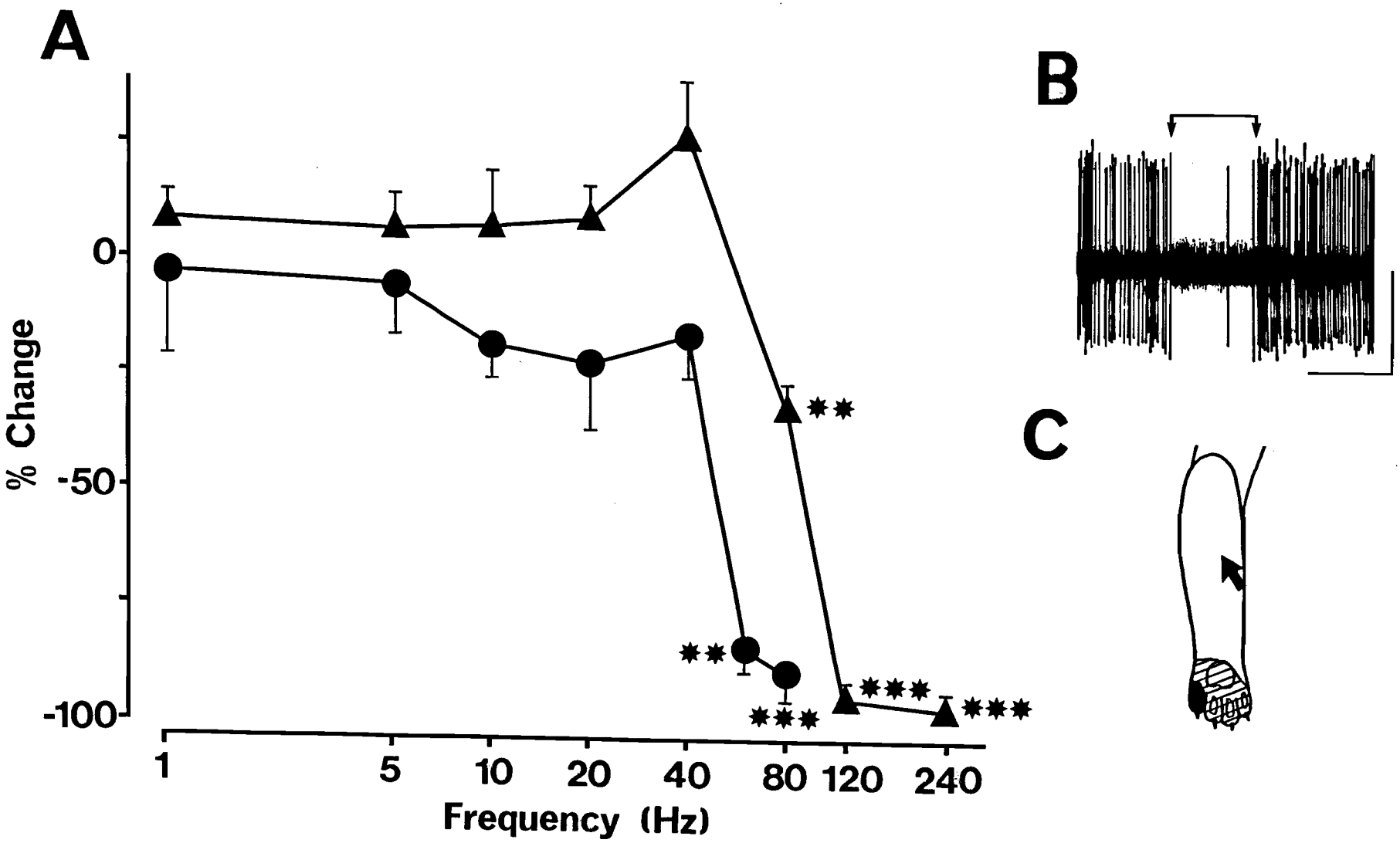
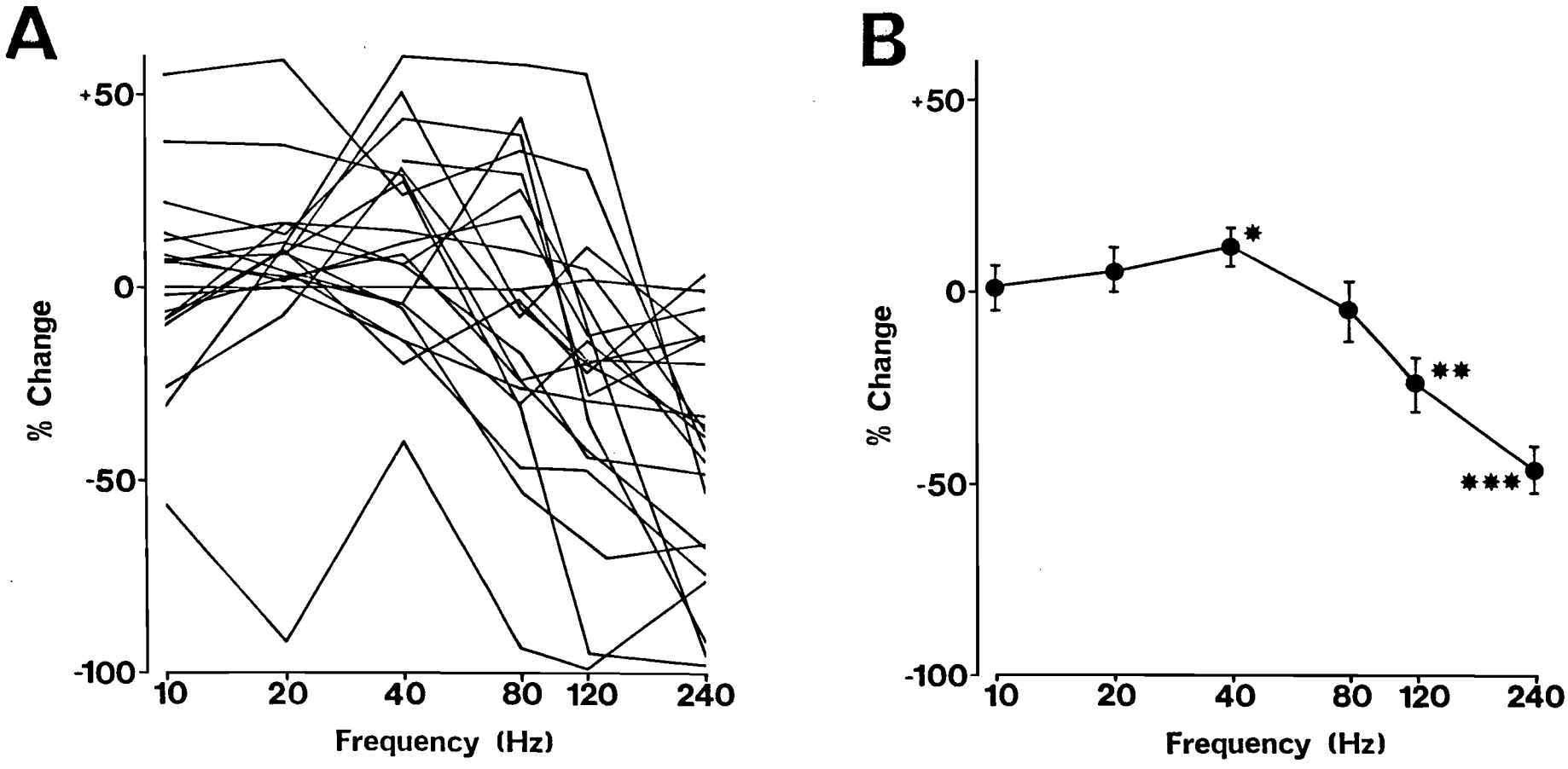

A
B
C
0
-50
-100
% Change
1
5
10
20
40
80
120
240
Frequency (Hz)

Fig. 6 Frequency-response curves for stimulation of 0.15 mm.

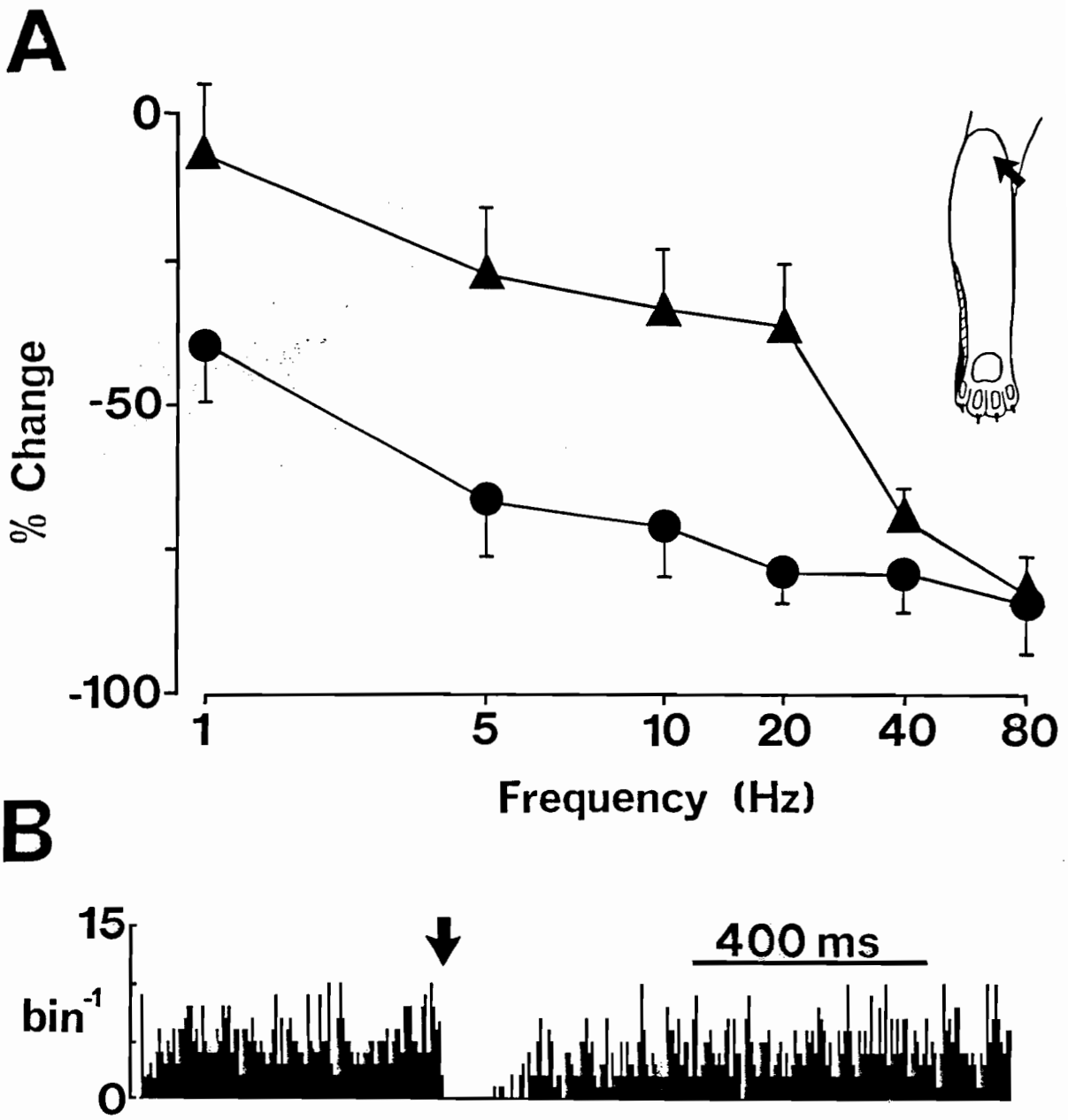
In both semilogarithmic graphs the percentage change is plotted vs the frequency of stimulation. **A** shows individual curves for 22 neurones; in 2 cases the lines start at frequencies above 10 Hz. The average of the individual curves is shown in **B**. The values for the points on the average curve were compared with 0 using the *t*-test. * - $p<0.05$; ** - $p<0.01$; *** - $p<0.001$.


A
+50
0
-50
-100
% Change
10
20
40
80
120
240
Frequency (Hz)
B
+50
0
-50
-100
% Change
*
**
***
10
20
40
80
120
240
Frequency (Hz)

'ig. 7 Comparison of effects of pulses vs sinusoids.

A. The percentage change is plotted vs the frequency of stimulation n a semilogarithmic graph. Each point shows the average percentage hange calculated for 5 trials. The triangles correspond to stimulation sing sinusoids. The dots correspond to stimulation using pulses as escribed in the text. The amplitude of stimulation was 1.0 mm. The iagram shows the receptive fields using the same scheme as Fig. 3.

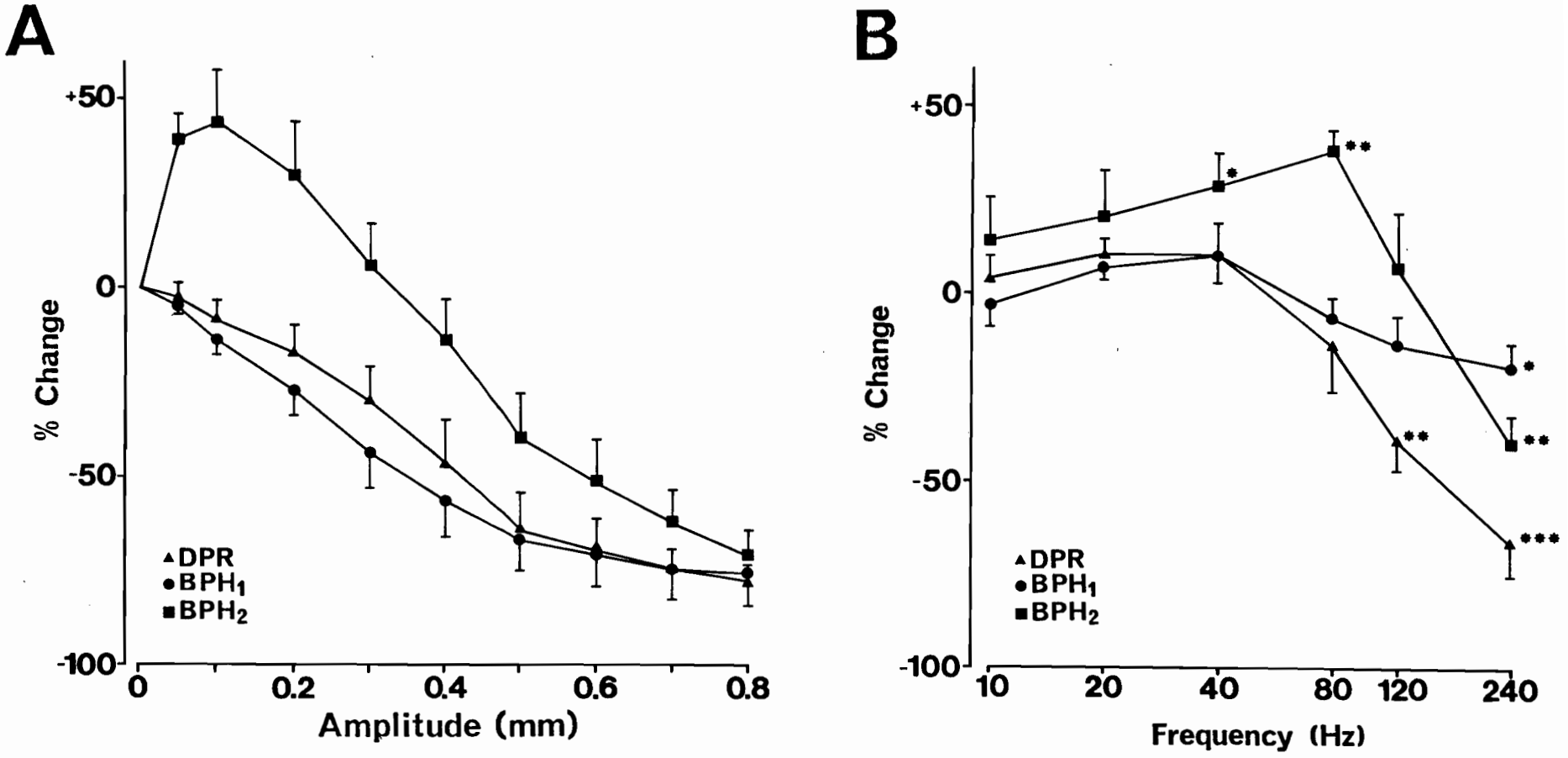
B. The trace shows a PSTH; the bin width is 5 ms. The arrow above he PSTH indicates the time of delivery of single pulses which had the ame amplitude and waveform as the pulses used in A. The pulses were iven every 2 s and 100 trials were used to construct the PSTH.


A
0
-50
-100
% Change
1
5
10
20
40
80
Frequency (Hz)
B
15
bin-1
0
400 ms

ig. 8 Amplitude- and frequency-response curves for the groups DPR, BPH_1 and BPH_2.

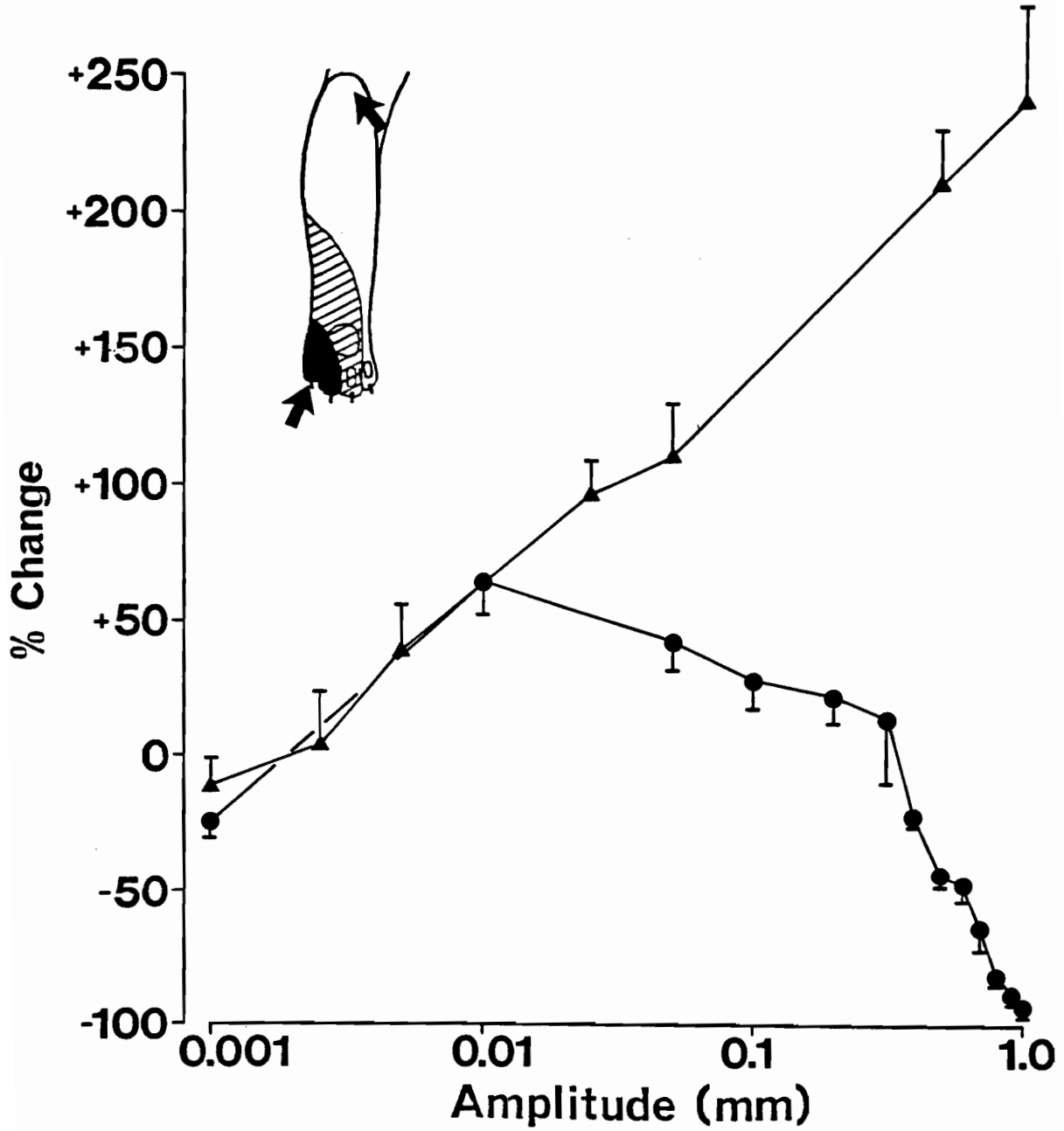
A. The percentage change is plotted against the amplitude of timulation; the stimulation frequency was 80 Hz. The points indicate the verage percentage change calculated in group DPR (triangles), group PH_1 (circles) and group BPH_2 (squares).

B. A semilogarithmic graph is shown in which percentage change is nown as a function of frequency of stimulation; stimulation amplitude was .15 mm. The points show the average percentage change in group DPR triangles, n=9), group BPH_1 (circles, n=9) and group BPH_2 squares, n=4). The value for each point was compared with 0 using a wo-tailed *t*-test. * - $p<0.05$; ** - $p<0.01$; *** - $p<0.001$.


A
+50
0
-50
-100
% Change
0
0.2
0.4
0.6
0.8
Amplitude (mm)
▲DPR
●BPH1
■BPH2
B
+50
0
-50
-100
% Change
10
20
40
80
120
240
Frequency (Hz)
*
**
*
**
**
***
▲DPR
●BPH1
■BPH2

ig. 9 Amplitude-response curves when the stimulator probe was located inside and outside the low threshold receptive field for a neurone in group BPH_2.

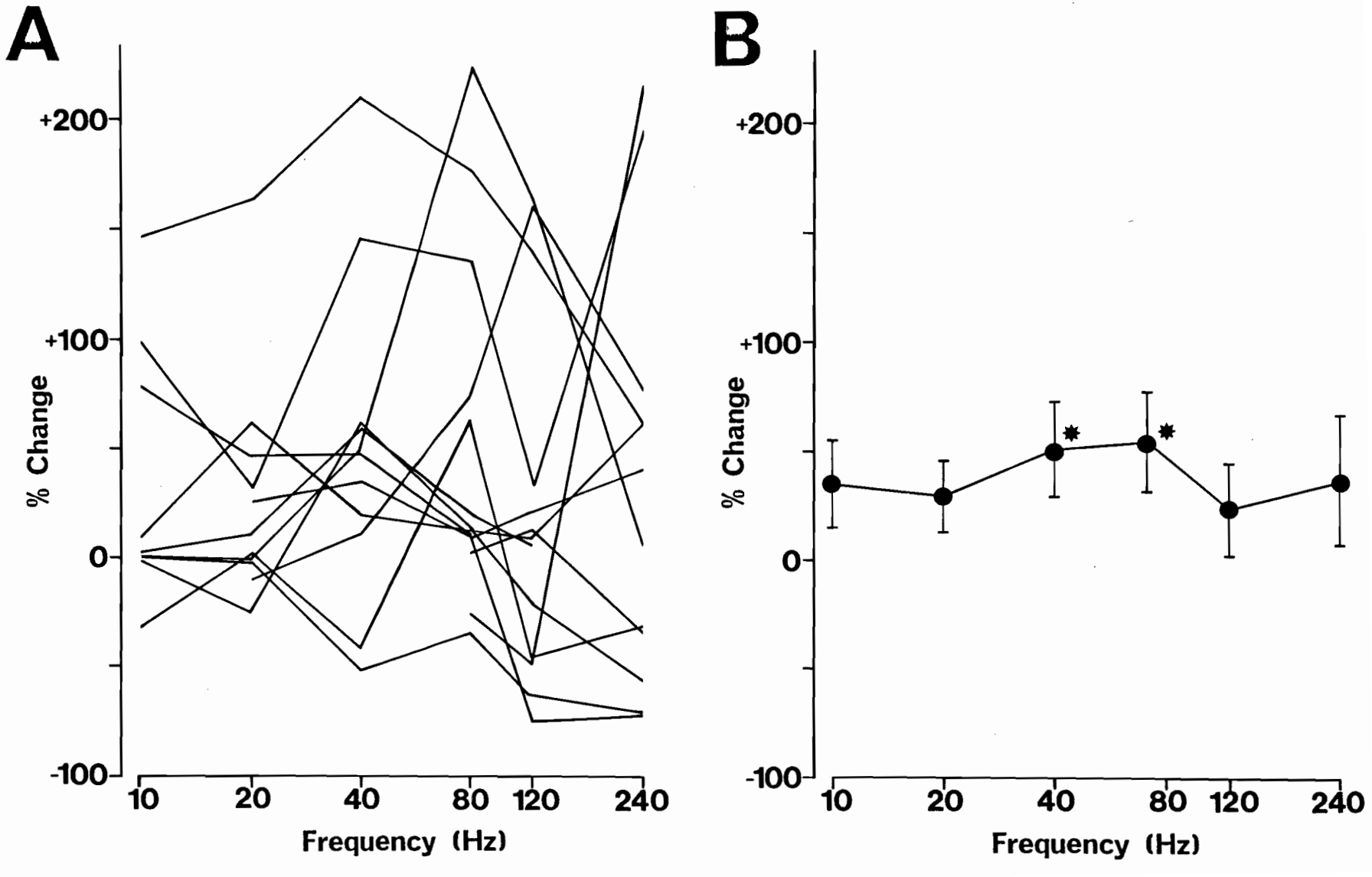
A semilogarithmic graph is shown in which percentage change is plotted s stimulation amplitude when the probe was positioned inside (triangles) r outside (circles) the receptive field for innocuous mechanical timuli. Each point represents the average of 5 trials; the stimulation requency was 80 Hz. The receptive fields are illustrated on the diagram sing the same scheme as in Fig. 1. The 2 positions of the stimulator robe are indicated by the arrows.


+250
+200
+150
+100
+50
0
-50
-100
% Change
0.001
0.01
0.1
1.0
Amplitude (mm)

:g. 10 Frequency-response curves when the stimulator probe was positioned inside the receptive field.

A. In a semilogarithmic graph individual curves are illustrated for 3 neurones. Stimulation amplitude: 0.15 mm.

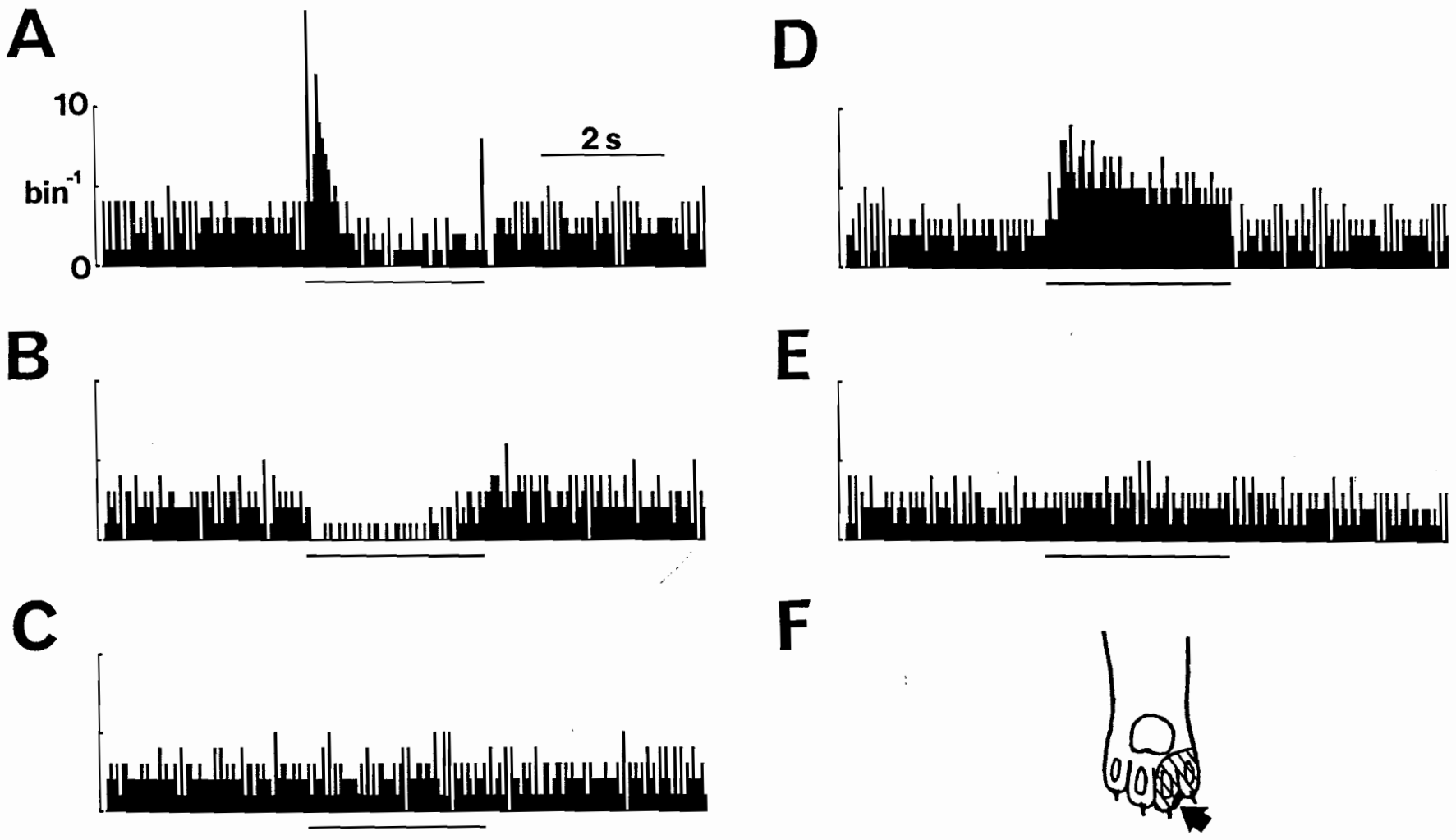
B. Average of the individual curves. The value for each point was ›mpared with 0 using a two-tailed *t*-test: * - $p<0.05$.


A
+200
+100
0
-100
% Change
10
20
40
80
120
240
Frequency (Hz)
B
+200
+100
0
-100
% Change
10
20
40
80
120
240
Frequency (Hz)

ig. 11 Effects of low-amplitude, high-frequency stimulation on a single neurone.

A-E are PSTHs, each was compiled from 5 consecutive applications of ibration. The period of vibration is indicated by the bar below each STH. The bin width for the PSTHs is 50 ms.

For **A,B** and **C** the frequency of stimulation was 240 Hz; 40 Hz timulation was used for **D** and **E**. The stimulation amplitude was 0.15 m for **A** and **D**, was 0.002 mm for **B** and **E** and was 0.001 mm for . The receptive field and the position of the stimulator probe are epresented the diagram in **F** using the same scheme as Fig. 3.

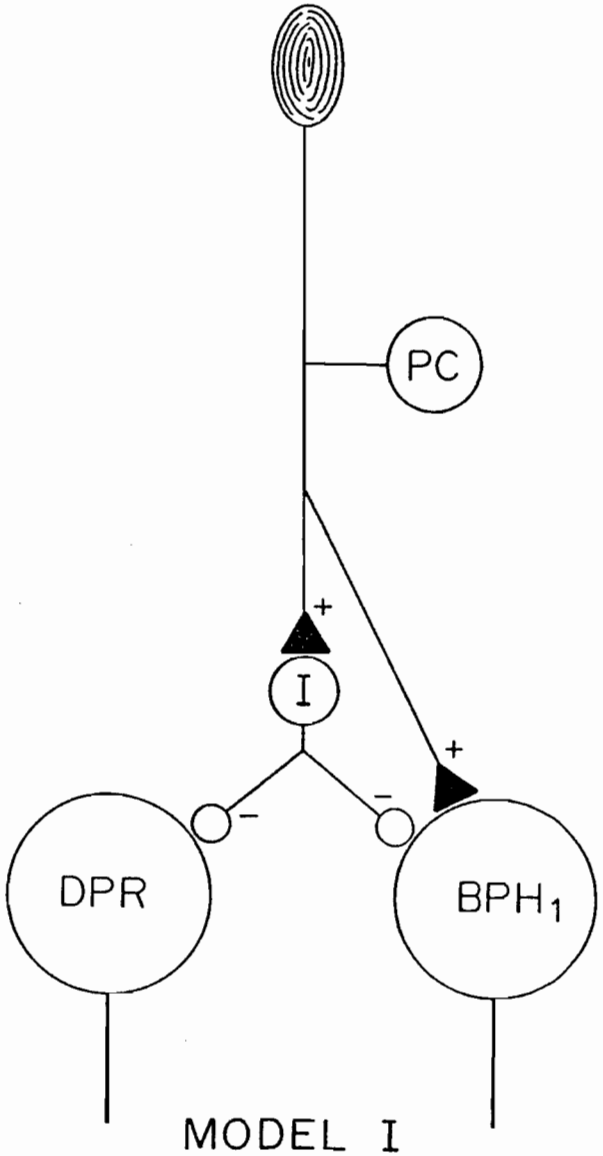

A
10
bin-1
0
2 s
B
C
D
E
F

ig. 12 Two possible models for the effects of Pacinian corpuscle afferents.

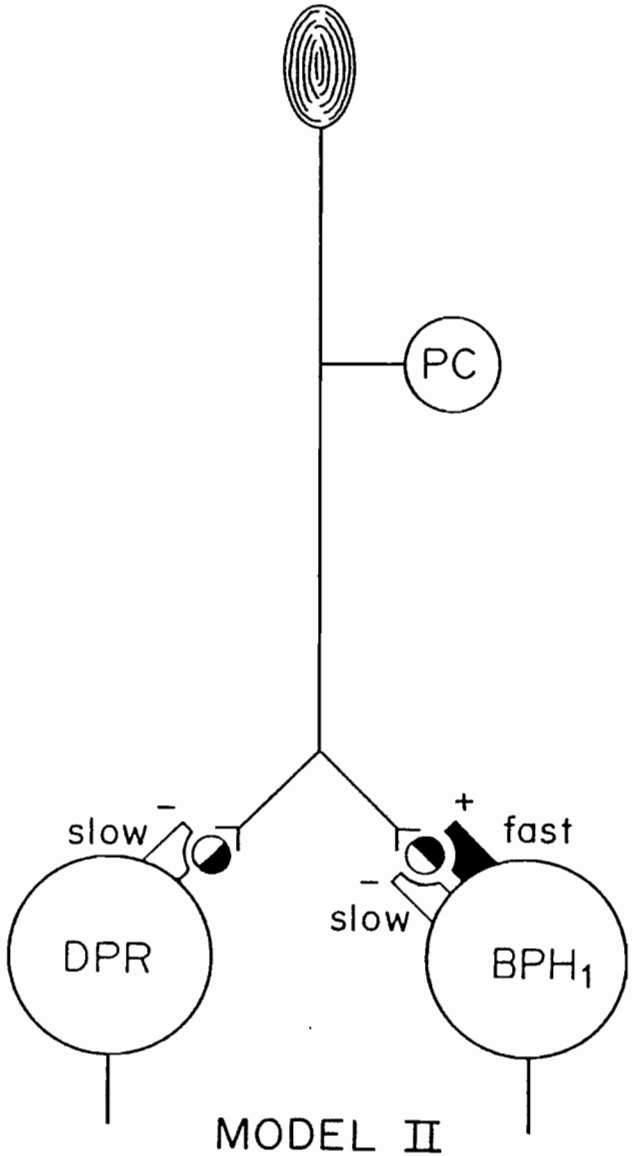
Each diagram illustrates a possible model to account for the effects f Pacinian corpuscle (PC) afferents on neurones in group DPR and PH_1.

In **model I**, a PC afferent makes excitatory synaptic contact indicated by the blackened presynaptic terminal) with a BPH_1 neurone nd an inhibitory neurone (I). The inhibitory neurone projects to the DPR nd the BPH_1 neurones.

In **model II**, the PC afferent makes synaptic contact with both the PR and the BPH_1 neurones. The PC afferent releases a single ransmitter with excitatory and inhibitory actions (◑). It is proposed hat the excitatory response (indicated by the blackened symbols) occurs ore rapidly than the inhibitory response (indicated by the open ymbols). It is also possible that the PC afferent might release 2 istinct transmitters to account for the different effects.



MODEL I



MODEL II

CHAPTER 6

PURINE-INDUCED DEPRESSION OF DORSAL HORN NEURONES IN THE CAT SPINAL CORD: ENHANCEMENT BY TACHYKININS

I. ABSTRACT

The neurokinins, physalaemin, substance P, neurokinin A and bradykinin, were tested on the responses of single spinal neurones to the purines, ATP and AMP, and to GABA. Experiments were done in anaesthetized cats, recording extracellularly from functionally identified sensory neurones in the lumbar dorsal horn. All compounds were administered by iontophoresis. Neurokinins caused a slow, prolonged excitation which outlasted the period of application. Physalaemin was tested on responses to ATP in 24 units. In each case application of ATP caused either depression, excitation or a biphasic response when the application was not pre-conditioned by ejection of physalaemin. For 11 units, with ATP applications subthreshold to alter the on-going firing rate, such applications caused depression when they were preceded by administration of physalaemin. Three units were tested with ATP applications which caused the excitatory response; when the applications of ATP were preceded by ejection of physalaemin, there was then a depressant component in the response. In these 14 cases, the magnitude of the depression or of the depressant component of the response, was measured using currents which failed to produce depression in the absence of physalaemin ejection; the mean magnitude of this depression was 34.7±1.6% (± SEM). With the 10 remaining units, responses to ATP were unaffected by application of physalaemin. The early components of the biphasic and excitatory responses were unaffected by physalaemin and hence it appeared to have a differential effect, enhancing only the depressant effects of ATP. The enhancement of depression was reversible, lasting up to 30 min following a single ejection. Neither control current nor glutamate mimicked the effect of physalaemin on the responses to application of ATP.

The depressant response to AMP was also enhanced by physalaemin: ejections of AMP subthreshold to affect the on-going firing rate caused depression after physalaemin application in 3 of 8 units (average depression: 35.0±3.3%). On the other hand, depression induced by GABA was unaffected by physalaemin in every case (n=8); in 4 of the cases GABA was tested on units for which purine-induced depression was enhanced by physalaemin. Thus, physalaemin preferentially affected depressant responses to ATP and to AMP. As depressant effects of purines appear to be mediated by P_1-purinergic receptors, physalaemin may specifically

enhance depression caused by activation of P_1-purinergic receptors.

Substance P enhanced purine-induced depression in 4/7 units tested. Neurokinin A had a similar effect in 5/9 units. Bradykinin failed to affect the depression with any of the 15 units studied. Thus, enhancement of purine-induced depression may be an effect specific to the tachykinins. The results of the present study prompt us to suggest that tachykinins endogenous to the spinal cord may enhance depression induced by activation of P_1-purinergic receptors.

II. INTRODUCTION

We have recently reported that iontophoretic application of adenosine 5'-triphosphate (ATP) induces three distinct types of response when applied to spinal dorsal horn neurones: depression, excitation and a biphasic effect consisting of excitation followed by depression (see Chapter 2). Similar responses to ATP have been reported with neurones in the medullary dorsal horn (Salt and Hill, 1983) and in the cerebral cortex (Phillis et al., 1979). In contrast to ATP, adenosine 5'-monophosphate (AMP) causes only depression of dorsal horn neurones (see Chapter 2). The depressant response induced by ATP and by AMP and the depressant phase of the biphasic response to ATP appear to be mediated by activation of P_1-purinergic receptors (Burnstock, 1978) as these effects, but not the excitatory effects of ATP, are blocked by P_1-purinergic receptor antagonists (Phillis et al., 1979; Phillis and Kostopoulos, 1975; Salter and Henry, 1985a; Stone and Taylor, 1980; Taylor and Stone, 1978).

Physalaemin is a tachykinin first isolated from amphibian skin (Erspamer et al., 1964) and immunoreactivity for a physalaemin-like peptide has been subsequently found in the spinal cord of rat, mouse and guinea-pig (Lazarus et al., 1980). Previous investigations in our laboratory have indicated that physalaemin causes a slow, prolonged excitation of dorsal horn neurones in the spinal cord of the cat (Salter and Henry, 1985b).

During the course of investigations of ATP and of physalaemin a serendipitous observation was made: the magnitude of the depressant response to application of ATP was greater when this purine was applied following administration of physalaemin. In the present paper we report the complete investigation which arose from this observation. Effects of physalaemin and of other tachykinins were studied on responses to application of ATP and AMP. To determine if depression induced by agents other than purines was enhanced, effects of tachykinins on depression induced by γ-aminobutyric acid (GABA) were studied. In addition, to investigate the possibility that non-tachykinins might enhance purine-induced depression experiments were done with bradykinin, another peptide in the neurokinin family.

Preliminary results of this study have been previously reported in

part (Salter and Henry, 1987b).

III. MATERIALS AND METHODS

i) *Animal preparation*

A detailed description of the experimental animal preparation and of the recording techniques is given elsewhere (see Chapter 2). Experiments were done on adult cats (2.5-4.5 kg) anaesthetized either with sodium pentobarbital (40 mg/kg i.p.; n=19) or with alpha-chloralose (60 mg/kg i.v. after induction with halothane/oxygen; n=9). Supplemental doses of anaesthetics were given during the experiments to maintain deep anaesthesia.

The spinal cord was exposed for recording in segments L5-L7. It was transected at the first lumbar level to remove any influence of supraspinal structures on the activity of dorsal horn neurones. The spinal cord was covered with a pool of warm mineral oil to prevent cooling.

Throughout the experiment mean arterial pressure was monitored continuously and maintained above 80 mm Hg with infusion of dextran (Macrodex, Pharmacia; 6% in normal saline) or noradrenaline bitartrate (Levophed, Winthrop; 0.002% in normal saline). End-tidal CO_2 concentration, monitored continuously with a Beckman LB2 Medical Gas Analyzer, was maintained at 3.5-5.0% Rectal temperature was kept at 38°C with a DC-driven infra-red bulb and a servo-controller.

ii) *Recording and Data Acquisition*

Seven-barrelled micropipettes (overall tip diameter 4-10 μm) were used for recording extracellular single unit spikes and for iontophoresis. The central recording barrel was filled with 2.7 M NaCl (impedance 4-10 MΩ). Recordings were amplified, displayed on oscilloscopes and recorded on film using a system described in detail elsewhere (see Chapter 2). The rate of discharge was displayed on a polygraph using the output of a gating unit. In addition, the raw data were recorded on magnetic cassette tape.

The output of an amplitude discriminator in the gating unit, as well

as trigger pulses from the iontophoresis unit, were fed into an IBM personal computer via a multiplexing system developed in our laboratory. Interspike and interstimulus intervals were measured to ±0.25 ms and stored for later analysis. Details of the computerized system for data sampling and analysis are given in Chapter 4.

iii) *Iontophoresis and Solutions*

Each of the outer barrels of the micropipettes contained one solution from among the following: sodium L-glutamate (1 M, pH 7.4; Sigma), disodium ATP (0.2 M, pH 7.0; Sigma, product A5394), sodium AMP (0.2 M, pH 7.0; Sigma), GABA (1 M, pH 6.5, Sigma), physalaemin (1 mM, Protein Research Foundation or Bachem), substance P (0.8 mM, Peninsula Laboratories or Institute Armand-Frappier), neurokinin A (1 mM, Institute Armand-Frappier), bradykinin (5 mM, Institute Armand-Frappier), control solution (165 mM NaCl, pH 5.5) and Pontamine Sky Blue 6BX (1% in 0.5 M sodium acetate, Gurr). The control solution was used to detect artefacts due to local changes in current or pH at the electrode tip. All solutions were prepared using double distilled water; the control solution was the vehicle used for peptides. Immediately after preparation, solutions were divided into aliquots and stored frozen. Electrodes were filled the day of the experiment; one aliquot of each solution was thawed immediately prior to use.

Glutamate, ATP and AMP were ejected with inward currents and peptides were passed with outward currents. To counteract leakage by diffusion, a current of polarity opposite to that used for ejection was passed between applications: for glutamate, ATP and AMP the magnitude of this retaining current was 10 nA and it was 2-5 nA for the peptides.

Ejections of Pontamine Sky Blue were used to mark sites of recording for later histological identification. Details of the histological methods can be found in Chapter 4.

iv) *Classification of units*

According to responses to natural stimulation each unit was classified as non-nociceptive, wide dynamic range, nociceptive specific or proprioceptive using the classification scheme described in Chapter 2.

v) *Data analysis*

Interspike and interstimulus intervals stored on computer were analyzed off-line with software developed in this laboratory; interspike interval distributions during selected time periods were compared using parametric or non-parametric means tests, as appropriate. Discharge rate, in relation to iontophoretic applications, was displayed as a peristimulus time histogram (PSTH; bin width 1 s). The mean number of spikes·bin^{-1} in 2 regions of appropriately selected histograms were compared using Student's *t* test or the Mann-Whitney U-test. Effects in different groups of neurones were compared with the χ^2 test or with Fisher's exact test. For statistical tests, $p<0.05$ was considered indicative of a significant difference between 2 samples. Numerical values given in the text are the mean ± 1 standard error of the mean.

vi) *Quantification of depression and analysis of effects of neurokinins*

The magnitude of the depression (D) caused by application of ATP, AMP or GABA, was calculated as the percentage decrease of the firing level in test versus in control intervals; the method for selecting these intervals is described below. For single trials the firing level was the mean firing rate in test and control intervals; for multiple trials the firing level was the mean number of spikes·bin^{-1} in control and test intervals of the PSTHs.

The time course of depression produced by application of the depressant compounds varied among the different units. Therefore, the test and control intervals were chosen on the basis of the time course of depression in each unit. Thus, as depression caused by ATP or AMP was delayed in onset and prolonged, the test interval began at or just after the end of purine ejection and terminated when the firing rate had returned to the level prior to the application. On the other hand, as GABA-induced depression was rapid in onset and persisted for less than 1 s after the end of ejection, in this case the test interval was taken as the duration of the ejection.

The control interval was a period equal in duration to the test interval. For applications of GABA the control interval was always the period immediately preceding the start of ejection. This relationship of control interval to the period of the ejection was also used for ATP and

AMP applications but only in cases where the effects of neurokinin administration was not being studied. In the cases where ATP and AMP ejection were preceded by neurokinin application, the firing rate in the period immediately prior to purine ejection was not the appropriate control because the neurokinins caused the firing rate to increase during this period. In these cases, then, the control interval was taken as the period after neurokinin application which was not followed by purine ejection; the relationship between the control and test intervals in these cases is illustrated diagrammatically in Fig. 1.

Comparing the magnitude of the depression in the 2 different conditions (i.e. with versus without pre-administration of neurokinin) would have been unreliable because the magnitude of depression might have varied depending upon the basal firing rate. This point is critical because of the increase in the activity caused by administration of the neurokinin. Two different strategies were used, therefore, to control for the possible influence of the basal firing rate on the magnitude of the depression induced by ATP, AMP or GABA.

The first strategy was to select an ejection current of the depressant agent which was just insufficient to cause depression and then to determine whether this same ejection current would induce a depression when applied following neurokinin administration. Thus, in the experiments short applications (8-20 s) of ATP, AMP or GABA were made at intervals of 60-150 s. The magnitude of the ejecting current was adjusted so that 3 or more consecutive applications failed to cause depression. A PSTH was constructed from these 3 or more applications and it was was carefully examined to avoid using ejections which caused statistically significant depression in the absence of administration of neurokinin. Applications with the selected parameters were then made after neurokinin ejection. To determine if depression now occurred, D was calculated as described above and statistical significance was determined by comparing firing levels in control versus test intervals. In cases where depression failed to occur after neurokinin administration a greater ejecting current for the depressant agent was chosen and the testing procedure repeated until depression was observed in the absence of neurokinin or until the ejecting current for the depressant agent was greater than 100 nA.

In the second strategy the influence of firing rate on the magnitude

of depression was investigated directly. Thus, reproducible depression was elicited with repeated applications of ATP, AMP or GABA and glutamate was applied continuously to change the basal firing rate. The magnitude of depression during glutamate application was compared with that during a period when the firing rate had been increased to a similar level after neurokinin administration. This comparison was made using an analysis of covariance (Snedecor and Cochran, 1967) when appropriate.

IV. RESULTS

Results obtained in cats anaesthetized with sodium pentobarbital were similar to those in cats with alpha-chloralose and therefore the data will be considered together. Each unit included here was required to satisfy 3 criteria before neurokinins were tested on responses to ATP, to AMP or to GABA. First, to eliminate recordings from fibres, each unit was tested with and excited by iontophoretic application of glutamate. Second, units were required to be spontaneously active to avoid the possibility of drug-induced changes in evoked responses Third, to be reasonably certain that current application through a peptide-containing barrel did indeed eject the peptide, every unit had to show a reproducible excitatory response to the peptide being tested. (In 3 units unaffected by current ejection through the barrel containing physalaemin, these ejections failed to affect responses to ATP application.)

Fifty-three units satisfied these criteria and hence were included in this report: one was nociceptive specific, 49 were wide dynamic range and 3 were proprioceptive. The spontaneous activity of non-nociceptive units was generally too low to allow reliable testing and therefore, non-nociceptive units were excluded from this study. Effects of iontophoretic applications were considered to be genuine responses only if they were reversible, reproducible and not mimicked by control current.

In all cases attempted, histological examination of the sites of recording revealed these to have been in the dorsal horn. In addition, responses of the units to natural stimulation of the periphery were similar to those previously reported in laminae I and III (Price and Dubner, 1977).

Responses to applications of ATP and of AMP were similar in all

respects to those we have reported in Chapter 2.

i) *Responses to periodic application of neurokinins*

To reliably assess possible interactions between the neurokinins and the depressant agents, it was necessary that repeated application of neurokinins caused a response which was reproducible over periods of greater than one hour. An example of the reproducibility of the response to physalaemin is illustrated in Fig. 2. The latency and time course of this response are similar to those described previously (Salter and Henry, 1985b). As illustrated in Fig. 2B, the firing rate at the peak of the response varied by only ±5% on five consecutive trials and thus was considered stable over the first 72 min of testing. In addition, the time course of excitation was the same for each application. Responses of a given unit to substance P, neurokinin A and bradykinin showed greater variability than responses to physalaemin. Therefore, physalaemin was the neurokinin tested most frequently.

ii) *Effects of physalaemin on responses to application of ATP*

Physalaemin (50-100 nA; 25-60 s) was tested on the response to ATP (10-100 nA; 8-20 s) with 24 units. An example of the effects of administration of physalaemin on the response to application of ATP is illustrated in Fig. 3. Prior to administration of physalaemin application of ATP (50 nA, 18 s) failed to depress the on-going rate of firing of this unit; instead ATP ejection caused a brisk increase in the firing rate followed by a slowly-decaying afterdischarge (Fig. 3C). Application of physalaemin (65 nA, 35 s) caused a reproducible 40% increase in the rate of firing of this unit; one response is illustrated in Fig. 3C. The excitation began 20 s after the start of the ejection and the firing rate had reached its maximum level 20 s after the end of the application. Over the next 5 min the firing rate gradually returned to the pre-application level.

a) *Depression of the firing rate occurred when ATP was applied after ejection of physalaemin.* On the basis of the excitatory responses to application of ATP and of physalaemin one might have expected an increase in the rate of firing when these compounds were applied consecutively. However, when ATP was applied 28 s after administration of physalaemin,

the initial excitation was followed by a profound depression of the firing rate (Fig. 3C). The nadir of this depression occurred about 110 s after the end of ATP ejection and the rate of firing gradually recovered approximately 90 s after the nadir. The mean firing rate in the 20-s period around the nadir of the depression was significantly less than that in the 2-min period prior to application of physalaemin (2.3±1.4 vs 13.0±1.9 spikes·s^{-1}, respectively; $p=10^{-9}$). Spike amplitude and spike configuration remained unchanged during the responses to physalaemin and to ATP (Fig. 3A).

b) *The depressant component in the response to application of ATP persists long after administration of physalaemin.* In the lower graph of Fig. 3B the magnitude of depression was calculated for 12 consecutive ATP applications. In the first case where application of ATP was closely preceded by ejection of physalaemin (physalaemin-ATP trial; indicated by the down-going arrow on the left side), the rate of firing in the test interval was depressed by 56%. The 3 subsequent applications of ATP also caused a depression of the firing rate in the test interval; but, the magnitude of the depression caused by each of these was less than that immediately following the physalaemin-ATP trial. Thus, the change in the response to application of ATP persisted after the physalaemin-induced excitation has ended.

c) *The change in the response to application of ATP is reversible.* For the second physalaemin-ATP trial (middle down-going arrow in Fig. 3B) the magnitude of depression was unchanged from the level after the previous ATP ejection. The depression for the subsequent ATP application was less than that for the previous application of ATP and with the next ATP application, made 50 min after the second physalaemin-ATP trial, depression failed to occur. Thus, it was concluded that there was complete recovery of the response to ATP application.

d) *Response to physalaemin is unaffected between physalaemin-ATP trials.* During the period in which there was a depressant component in the response to ATP, application of physalaemin, not followed by ATP ejection, caused only an increase in the rate of firing similar to that shown in Fig. 3C. Thus, the response to application of ATP rather than the response to application of physalaemin was affected after the physalaemin-ATP trials.

e) *Reproducibility of the effect of physalaemin.* After complete recovery of the response to ATP application, a third physalaemin-ATP trial was again followed by depression: the magnitude of this depression was 44% (Fig. 3B). Thus, this effect of application of physalaemin was considered to be reproducible as well as reversible and as such satisfied criteria stated above for a genuine response.

f) *Time course for the induction of the effects of physalaemin.* It was found with other units that depression caused by application of ATP was most profound when this was made in the period after the end of physalaemin ejection, when the firing rate had peaked (i.e. 5-45 s after the end of ejection). ATP applications made immediately after the end of physalaemin ejection, when the firing rate was increasing, were much less effective. When ATP was applied just before or during ejection of physalaemin, the response to application of this purine was unaffected. Therefore, effects of physalaemin on AMP- and GABA-induced depression were studied using ejections of these compounds made after application of physalaemin.

g) *Different time courses for induction of this effect of physalaemin and for recovery.* As mentioned above applications of ATP were followed by depression for up to 28 min after a physalaemin-ATP trial (Fig. 3B). On the other hand, when ATP was applied 9 min after ejection of physalaemin alone there was no depression (Fig. 3C). It was found, with other units, that to observe a change in the ATP-induced response it was necessary to apply ATP within 3 min after ejection of physalaemin. Responses to application of ATP were then modified for up to 30 min.

h) *The change in the response to ATP application is not due to the increase in baseline firing rate caused by physalaemin.* At the time indicated by the arrow below the upper line in Fig. 3B the hindlimb was repositioned to produce a higher, stable baseline rate of firing which was approximately equal to the rate shown by the right-hand down-going arrow. The magnitudes of depression for the subsequent applications of ATP, alone, were much less than that for the first physalaemin-ATP trial. These magnitudes decreased progressively in spite of constancy of the baseline firing rate, and the fourth ATP application after increasing the baseline rate of firing caused no depression. The interpretation given to these results is that the change in the response to ATP caused by

physalaemin cannot be accounted for by the increase in rate of discharge caused by this peptide.

i) *Initial excitatory component of the response to ATP is unaffected by physalaemin ejection.* When ATP ejection was preceded by application of physalaemin, the mean firing rate during the 15-s period around the peak of ATP-induced excitation was 80% greater than that during the 10-s period just prior to ATP ejection (Fig. 3C). On the basis of the previous responses, it was predicted that the mean firing rate around the peak of excitation would have increased 86%. The similarity between the predicted and observed increases in rate of firing indicates that physalaemin failed to affect the early component of the response to ATP.

j) *Applications of ATP which fail to affect the on-going firing rate cause depression after administration of physalaemin.* An additional important observation about the effect of application of physalaemin on the response to ATP ejection is illustrated by the wide dynamic range unit shown in Fig. 4. This unit was reproducibly excited by administration of physalaemin (80 nA, 28 s; Fig. 4A) but was not excited by application of ATP. Applications of ATP of longer duration than those shown in Fig. 4B caused depression. The ejection of ATP (100 nA, 8 s) shown in Fig. 4B failed to alter the on-going rate of discharge. Depression occurred when similar applications were made after ejection of physalaemin (Fig. 4C): the mean firing rate during the 20-s period immediately after ATP ejection was significantly less than that during the corresponding period after application of physalaemin in Fig. 4A (7.6±0.16 vs 10.8±0.15 spikes·bin^{-1}, respectively; $p=0.0035$). The depression after ATP application appears not to have been due to possible variability in the response to physalaemin because the mean firing rates during the periods before application of ATP and after the ATP-induced depression in Fig. 4C failed to be significantly different from the mean rates during the corresponding periods in Fig. 4A. As ejections of ATP which failed to alter the on-going rate of firing caused depression when preceded by physalaemin and as greater ejections of ATP evoked depression in the absence of this peptide, the depressant response to application of ATP was considered to have been enhanced by ejection of physalaemin.

k) *Proportion of units in which responses to ATP application were affected by ejection of physalaemin.* For 3 units, responses to

application of ATP which were excitatory before administration of physalaemin had a depressant component similar to that shown in Fig. 3 after physalaemin ejection. With 11 units, ejections of ATP which failed to alter the on-going firing rate in the absence of physalaemin produced depression when closely preceded by physalaemin application. In these cases ejections of ATP not preceded by physalaemin caused depressant or biphasic responses when higher ejecting currents or longer applications were administered. The response to application of ATP for the 10 remaining units was unaffected by administration of physalaemin. Importantly, in no case did ejections of ATP which failed to alter the on-going firing rate ever cause excitation when preceded by physalaemin.

1) *Average magnitude of depression after ejection of physalaemin.* For the 14 units in which ATP induced a depression after application of physalaemin, the magnitude of the depression or of the depressant component of the response was measured using currents which failed to depress the on-going rate of firing. In these cases, the mean magnitude of depression was 34.7±1.6% after application of physalaemin.

iii) *Effects of physalaemin on the depression evoked by AMP*

In contrast to ATP, application of AMP had only one effect on spinal dorsal horn neurones: depression of the firing rate (Chapter 2). Physalaemin was tested on the responses of 8 units to application of AMP (10-80 nA, 8-20 s); data from one of these are illustrated in Fig. 4. Application of AMP (25 nA, 8 s) failed to affect the firing rate of this unit, although ejections with greater currents caused depression (not shown). When physalaemin administration preceded ejection of AMP, the firing rate in the 20-s after AMP application (Fig. 4E) was decreased compared with the firing rate in the corresponding period during the control response shown in Fig. 4A. Furthermore, the mean number of spikes·bin^{-1} was also significantly less in the 10-s period around the nadir of AMP-induced depression than in the 30-s period immediately preceding physalaemin application (1.6±0.1 vs 2.6±0.6 spikes·bin^{-1}, respectively; $p=0.03$). Thus, AMP-induced depression was enhanced by physalaemin.

With 3 units AMP caused depression after physalaemin application (average depression: 35.0±3.3%) at ejecting currents which, in the absence

of ejection of physalaemin, failed to affect the discharge rate. The time course of the effect of physalaemin on AMP-induced depression was similar to that of the effect on depressant responses to application of ATP. Application of physalaemin had no effect on the response to AMP of the 5 remaining units.

iv) *Comparison of effects of physalaemin on responses to ATP and to AMP*

In 7 cases, physalaemin was tested on responses to both application of ATP and of AMP. For the unit illustrated in Fig. 4, applications of ATP and AMP which were initially subthreshold caused depression when the purines were applied 15 s after ejection of physalaemin; the magnitude of the AMP-induced depression was 44% and that due to ATP was 30%. Physalaemin ejection affected depressant responses of 2 units to both ATP and AMP and in the remaining cases no effect was observed on depression caused by application of either purine ($p=0.048$, Fisher's exact test).

v) *Experiments to control for possible effects of increased rate of discharge on the depression caused by purine application.*

With the unit illustrated in Fig. 3, increasing the firing rate by re-positioning the hindlimb failed to mimic the effect of physalaemin on the response to ATP application. To control further for the possibility that the effects of neurokinin administration could be accounted for on the basis only of an increase in the rate of firing, the effects of application of glutamate were tested on purine-induced responses of 11 units. Eight additional cases were examined in which purines caused depression of excitatory responses to peripheral stimulation.

For 17 units applications of purines were used which caused depression. When the on-going rate of discharge was increased with glutamate the rate of firing at the nadir of the depression also increased in every case tested ($n=9$). Furthermore, in all 8 cases examined, increasing the magnitude of the control response always resulted in a larger minimum response at the nadir of the depression. These findings suggest that increasing the rate of discharge, *per se*, leads to an increase in the rate of discharge at the nadir of purine-induced depression rather than to a decrease as occurred after administration of physalaemin (see Figs. 3 and 4E).

For the 2 remaining units applications of ATP were used which were subthreshold prior to administration of physalaemin; one case is illustrated in Fig 5. The firing rate of this unit began to increase 15-20 s after the beginning of application of physalaemin (70 nA, 28 s) and there was the typical prolonged afterdischarge. Ejection of inward current through a barrel containing the control solution (Cl^-,25 nA, 8 s) failed to affect the response to physalaemin (Fig. 5A). On the other hand, when ATP (25 nA, 8 s) was applied 8 s after the end of physalaemin ejection the afterdischarge was abolished about 20 s after the end of application of ATP (Fig. 5B). In contrast to the situation with ejection of physalaemin, during application of glutamate the rate of discharge was increased after application of ATP (Fig. 5C). As the rate of firing after application of ATP was lower when the rate of firing was increased with physalaemin than when it was increased with glutamate, it was concluded that the effect of physalaemin on the response to ATP cannot be accounted for by the increase in firing rate evoked by this peptide.

With another unit, applications of ATP which were initially subthreshold caused depression after ejection of physalaemin. Increasing the rate of discharge with glutamate failed to mimic the effect of physalaemin on this unit as well.

vi) *Effect of physalaemin on depression induced by GABA*

GABA (1-50 nA, 6-30 s) depressed all units tested. In all cases automatically controlled applications of GABA made at regular intervals caused a reproducible depression of the rate of firing.

Two of the 8 units for which application of physalaemin was tested on GABA-induced depression are shown in Fig. 6. In both cases inspection of the continuous-time histogram suggests that neither the magnitude nor the duration of the GABA-induced depression was altered by ejection of physalaemin. Analysis of covariance was used to study, quantitatively, the relationship between the number of spikes during the control and test intervals. This analysis indicated that physalaemin had no significant effect on this relationship. With both units, ejections of GABA which failed to depress the on-going firing rate never caused depression after application of physalaemin. Therefore, it was concluded that physalaemin had no effect on GABA-induced depression apart from the effect of

increasing the firing rate. However, the depression induced in both units by ATP ejection was enhanced by application of physalaemin.

In a total of 4 cases physalaemin was tested on units for which the depressant response to ATP was enhanced by this peptide. In these cases and in all others physalaemin failed to affect the depressant response to GABA. A comparison of the numbers of units in which physalaemin affected depressant responses to application of the purines or of GABA is shown in Table 1. This comparison shows that administration of physalaemin preferentially affected the depression induced by application of ATP and AMP rather than that evoked by GABA.

vii) *Effects of substance P on depression induced by purines*

To investigate the possibility that the effect of physalaemin on responses purines were a general effect of tachykinins, substance P and the recently discovered tachykinin, neurokinin A (Kangawa et al., 1983; Kimura et al., 1983), were tested in a similar way as was physalaemin. Substance P (65-100 nA, 30-75 s) was tested on responses to ATP (4 units) or to AMP (3 units); data from one unit are illustrated in Fig. 7.

Ejection of substance P excited this wide dynamic range unit; excitation was delayed in onset and prolonged after the end of ejection and thus was similar to that previously reported (Henry, 1976; Randić and Miletić, 1977; Sastry, 1979; Zieglgänsberger and Tulloch, 1979a). Three applications of ATP (60 nA, 8 s) caused a small depression (8±2%) before ejection of substance P (80 nA, 40 s). The magnitude of the depression increased after substance P application and following substance P, the mean firing rate at the nadir of the depression (i.e. 25-50 s after ATP ejection) was 5.1±0.4 spikes$\cdot s^{-1}$ compared with 10.3±0.7 spikes$\cdot s^{-1}$ in the same period after the ATP application before substance P ($z=4.6$, $p=2\cdot10^{-6}$).

Three subsequent ejections of ATP depressed the firing rate but the magnitudes of these responses were less than that of the response to the ATP ejection which immediately followed application of substance P. Then, following the second ejection of substance P, the magnitude of ATP-induced depression again increased. In this case application of ATP was made 3 min after the end of substance P ejection. The magnitude of the depression caused by ATP applications progressively recovered with the 3

subsequent ejections. It was concluded, therefore, that the depression induced by application of ATP was reproducibly and reversibly enhanced by administration of substance P.

Substance P similarly affected purine-induced responses of 4 of the 7 units; in the remaining cases, the responses were unaffected. For the units affected, ejections of purines subthreshold to depress the on-going rate of discharge caused depression with an average magnitude 35.0±4.8% when preceded by application of substance P.

viii) *Effects of neurokinin A on responses to application of ATP*

Neurokinin A (60-125 nA, 30-60 s) had a slow, persistent excitatory effect on dorsal horn neurones; details of this effect are given elsewhere (Henry and Salter, 1987). One of 9 units in which neurokinin A was tested on responses to application of ATP is illustrated in Fig. 7; neurokinin A was never tested on AMP-induced responses.

Neurokinin A (80 nA, 40 s) was applied when the depressant response to ejection of ATP had returned approximately to the level before the preceding application of substance P. The mean firing rate at the nadir of the depression after ATP application was less when ATP followed ejection of neurokinin A versus when ATP was applied before neurokinin A (5.0±0.3 vs 10.4±0.7 spikes·s^{-1}, $z=4.7$, $p=10^{-6}$). In addition, the duration of depression was prolonged following the ATP application made after neurokinin A ejection compared with that of the depression following the previous ATP ejection (150 vs 60 s).

The magnitudes of the next 5 depressant responses decreased progressively. Then, ejection of outward current through the barrel containing the control solution failed to affect the response to application of ATP. On the other hand, the magnitude of the depressant response to ATP application was increased by the subsequent ejection of neurokinin A. Thus, it was concluded that neurokinin A caused a reversible and reproducible enhancement of the depression.

Responses of 5 units to application of ATP were similarly affected by ejection of neurokinin A and those of the remaining 4 units were unaffected. The average magnitude of ATP-induced depression after neurokinin A application was 32.2±3.8% using ATP ejections which failed to cause depression without application of this tachykinin.

ix) *Effects of bradykinin on depression caused by application of purines*

The neurokinin, bradykinin, was tested to investigate the possibility that non-tachykinins might affect responses to ejection of purines in a similar way as did tachykinins. Like the tachykinins, bradykinin caused a delayed, prolonged excitation of dorsal horn units, an effect reported previously (Henry, 1976; Randić and Yu, 1976). During experiments in which bradykinin was tested on responses to purines it was the only peptide contained in the micropipette to avoid possible artefacts due to leakage of tachykinins. For each unit, responses were examined either to application of ATP or of AMP.

Bradykinin had no observable effect on depressions caused by ATP (11 units) or by AMP (4 units) despite extensive testing with currents of up to 135 nA for periods of application up to 2 min.

The effects of physalaemin, substance P, neurokinin A and bradykinin on responses to purine ejection are compared in Table 2. This comparison shows that purine-induced depression was preferentially affected by the tachykinins.

V. DISCUSSION

i) *Purine-induced depression is enhanced by tachykinins*

This study indicates that responses to ATP and to AMP are modified when application of purines is preceded by administration of tachykinins. Two principal observations have been made: first, ejections of ATP or of AMP subthreshold to affect the rate of discharge caused depression when identical ejections were made following tachykinin application. This observation indicates that ejection of tachykinins enhanced the depressant response to purine application. The second principal observation is that ATP applications which increased the firing rate in the absence of tachykinins elicited a biphasic excitatory/depressant response after tachykinin ejection. Interpretation of this observation is not as simple as the first because in this case ejection of ATP failed to cause depression without application of tachykinins. In view of the fact that excitatory responses to ATP never spontaneously converted to biphasic ones

(see Chapter 2), it is possible that application of tachykinins may have produced, *de novo*, a depressant component in the response to ATP or may have enhanced a pre-existing depression which had been masked by a more potent excitation. Our previous finding that units which were excited by ATP could be depressed by application of AMP (see Chapter 2) suggests the possibility that in these units a depression following ATP application may have been unseen because of more powerful excitation. In view of this possibility together with the clear evidence of enhancement of depression provided by the first observation, we suggest that in both cases purine-induced depression was enhanced by tachykinins.

The time courses of the post-tachykinin depressant and biphasic responses were similar to those previously reported for the respective responses in the absence of tachykinin application: depression and the depressant phase of the biphasic response are delayed in onset and the nadir occurs 15-60 s after the end of ejection. On the other hand, excitation and the excitatory component of the biphasic effect begin more rapidly and the maximum excitation occurred within 10 s after the end of ejection. The initial component of ATP-induced excitation was unaffected by tachykinins and subthreshold ejections of ATP failed to cause excitation after tachykinin application. Thus, compared with excitatory effects, depressant effects of purine application appear to be enhanced preferentially. Depressant, but not excitatory, effects of purine application appear to be mediated by P_1-purinergic receptors as the former are blocked by P_1-purinergic receptor antagonists (Phillis et al., 1979; Phillis and Kostopoulos, 1975; Salter and Henry, 1985a; Stone and Taylor, 1980; Taylor and Stone, 1978). Thus, the P_1-purinergic-mediated effects of purines appear to be those which are enhanced by application of tachykinins. In view of the preferential effect of tachykinins on purine-induced rather than on GABA-induced depression, tachykinins may enhance, specifically, the depression mediated by P_1-purinergic receptors.

Contrary to the tachykinins, bradykinin failed to affect the purine-induced depression. Hence, enhancement of P_1-purinergic-induced depression may be an effect specifically of tachykinins. These results, obtained with exogenously applied tachykinins, prompt us to suggest that tachykinins endogenous to the spinal cord may enhance depression of dorsal

horn neurones caused by activation of P_1-purinergic receptors.

ii) *Mechanism by which tachykinins enhance purine-induced depression*

The preferential effect of tachykinins on purine- versus GABA-induced depression indicates that dorsal horn neurones are not simply more depressible after administration of tachykinins. Thus, it appears likely that a specific mechanism is activated.

Some possible mechanisms have been eliminated by experiments in this study. The increased rate of discharge caused by tachykinins cannot account for the enhancement of depression because increasing the firing rate with glutamate, bradykinin or a change in peripheral input, failed to mimic the effect of the tachykinins. In addition, purine-induced depression was enhanced even after the excitatory effect of tachykinins had ended. Finally, the firing rate at the nadir of the depression was less after tachykinin administration whereas excitation with glutamate or with increased peripheral inputs resulted in an increase in the discharge rate at the nadir.

The finding that the firing rate was less at the nadir of the purine-induced depression than during the period prior to tachykinin application also excludes the possibility that purines simply antagonize the excitatory effect of tachykinins.

Considering the observation that a biphasic response to ATP ejection followed tachykinin application in units where, beforehand, the response to ATP alone was excitation, the possibility is raised that the depressant phase was a post-excitatory rebound depression. This possibility can be eliminated because depression was enhanced even in units where purine ejection failed to cause excitation. In addition, the possibility is excluded that the depression was caused by neuronal inactivation due to excessive depolarization, because the spike amplitude remained constant.

Artefactually-induced enhancement due to a change in the amount of purines released is unlikely to be a factor because applications of the tachykinins were temporally separated by many seconds and because ejection of outward current through barrels containing either the control solution or bradykinin failed to have an effect similar to that of tachykinins. This last finding also eliminates other possible artefacts such as changes in tip potential or current, and local changes in the pH, electrolyte

composition or osmolality of the extracellular fluid around the electrode tip. Thus, tachykinins released from the micropipette appear to be the primary causative agents in the enhancement of purine-induced depression.

Many possible specific mechanisms could be proposed to account for the enhancement of P_1-purinergic mediated depression in terms of changes in ionic conductances or otherwise. In view of the fact that the mechanisms of the depressant effects of purines in the dorsal horn have yet to be worked out in detail, speculation as to specific loci for the interaction between tachykinins and purines does not seem reasonable. Therefore, we will suggest possible points for interaction only in general, in terms of effects of tachykinins which might occur prior to P_1-purinergic receptor activation, at the level of the receptor or after receptor activation. It is important to note that the enhancement of depression may occur through direct effects on the neurones studied or may occur through indirect effects on other neurones.

As ATP and AMP may be converted enzymatically to adenosine before activation of P_1-purinergic receptors occurs (see Discussion in Chapter 2) tachykinins might increase the conversion of nucleotides to adenosine (in preliminary experiments ejection of adenosine was unsuccessful presumably due to its low aqueous solubility; therefore, this possibility could be neither confirmed nor rejected experimentally). Alternatively, or in addition, tachykinins might increase the sensitivity of the P_1-purinergic receptor or they might decrease uptake of adenosine; the latter mechanism has been proposed for the enhancement by estradiol of adenosine-induced depression of neurones in the cerebral cortex (Phillis et al., 1985). Also, tachykinins might increase the coupling between activated P_1-purinergic receptors and the intracellular processes which lead to the depression. For example, it is possible that tachykinins may enhance effects of P_1-purinergic receptors via an action through second messengers. On the other hand, the effects of tachykinins might be direct at the level of the ionophores. These possible mechanisms are not mutually exclusive.

None of these mechanisms appears more or less likely than another in view of previous work by others indicating that tachykinins can interact in various ways with processes involving other neuroactive chemicals. For example, substance P directly activates acetylcholinesterase *in vitro*

(Catalan et al., 1984) and thus can increase metabolic degradation and inactivation of acetylcholine. Substance P has been suggested to alter the sensitivity of nicotinic, cholinergic receptors because it blocks responses to nicotinic agents of Renshaw cells, *in vivo* (Belcher and Ryall, 1977; Krnjevic and Lekic, 1977; Ryall, 1982), and of sympathetic post-ganglionic neurones (Akasu et al., 1983b; Ryall, 1982) and adrenal chromaffin cells, *in vitro* (Clapham and Neher, 1984; Livett et al., 1979). In addition, receptors for serotonin may be affected by substance P as it increases the affinity and number of binding sites for serotonin in synaptosomes derived from the spinal cord dorsal horn (Hitzemann et al., 1985). Finally, substance P appears to modulate the intracellular interaction between nicotinic receptors and sodium ionophores in a clonal nerve cell line, *in vitro* (Stallcup and Patrick, 1980).

iii) *Possible significance of the enhancement of purine-induced depression by tachykinins*

In view of the fact that tachykinins and purines modify the activity of nociceptive spinal neurones, the results of the present study may be relevant to the modulation of nociception in the dorsal horn. Nociceptive dorsal horn neurones are depressed by non-nociceptive primary afferent inputs activated by peripheral vibration and this depression appears to be mediated by P_1-purinergic receptors (see Chapter 3 and 4). We suggest, therefore, that tachykinins may enhance this type of depression. This suggestion raises the further possibility that the antinociceptive effects of peripheral vibration may also be enhanced by tachykinins.

Table 1. Comparison of effects of physalaemin on responses to application of ATP, of AMP and of GABA. Numbers represent numbers of neurones.

	Effect of Physalaemin on Responses to Depressant Agents	
	Enhancement of Depression	No effect
ATP	14	10
AMP	3	5
GABA	0	8

$\chi^2 = 8.5$, 2 df, p=0.01

Table 2. Comparison of effects of physalaemin (PHYS), substance P (SP), neurokinin A (NKA) and bradykinin (BK) on purine-induced responses. Numbers represent numbers of neurones.

	PHYS	SP	NKA	BK
Enhancement of Purine-induced Depression	15	4	5	0
No effect	10	3	4	15

χ^2=15.4, 3 df, p=0.002

Fig. 1 Definition of control and test intervals used when investigating effects of administration of neurokinin on responses to purine application.

Two simulated PSTHs are shown. The control interval is shown by the bar above the upper record in which neurokinin (N) was applied alone. The bar above the lower record indicates the test interval, this followed application of purine (P).

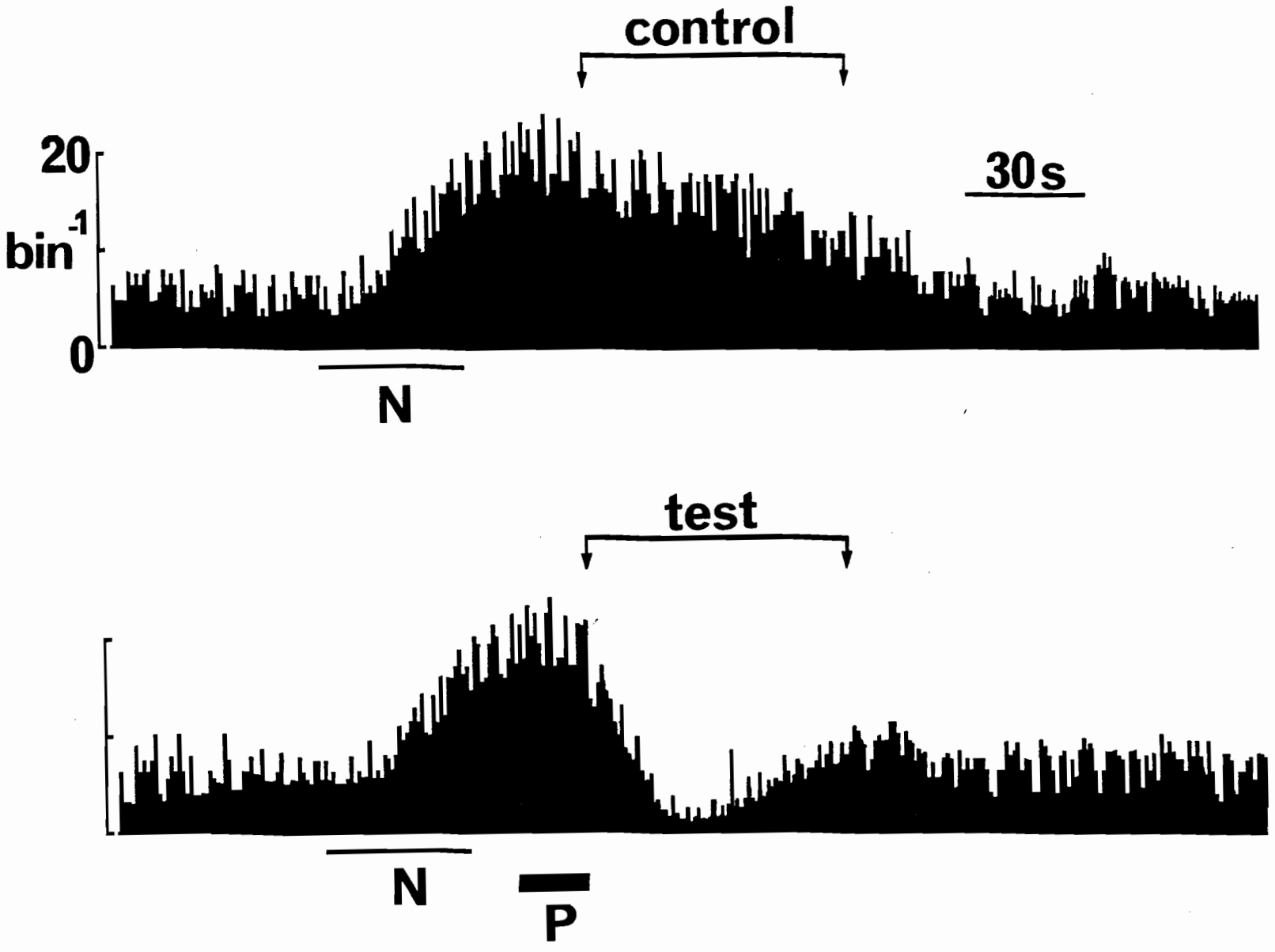

control
20
bin-1
0
30s
N
test
N
P

Fig. 2 Consistency of responses to repeated application of physalaemin.

A. Continuous oscilloscope record. The bar above the trace indicates the period of application of physalaemin.

B. Continuous-time histogram generated by the computer shows the response to physalaemin (PHYS). This response is the same as that shown in the oscilloscope record in A. The period of physalaemin ejection is indicated by the bar below the histogram (ejecting current is in nA). The ordinate shows the firing rate in spikes·s^{-1}.

C. Graph illustrating the mean firing rate at the peak of six consecutive applications of physalaemin. Each dot represents the response to a single ejection of physalaemin. Identical ejecting current and duration of application were used for all ejections. The first application of physalaemin in the graph corresponds to the response illustrated in A and B.

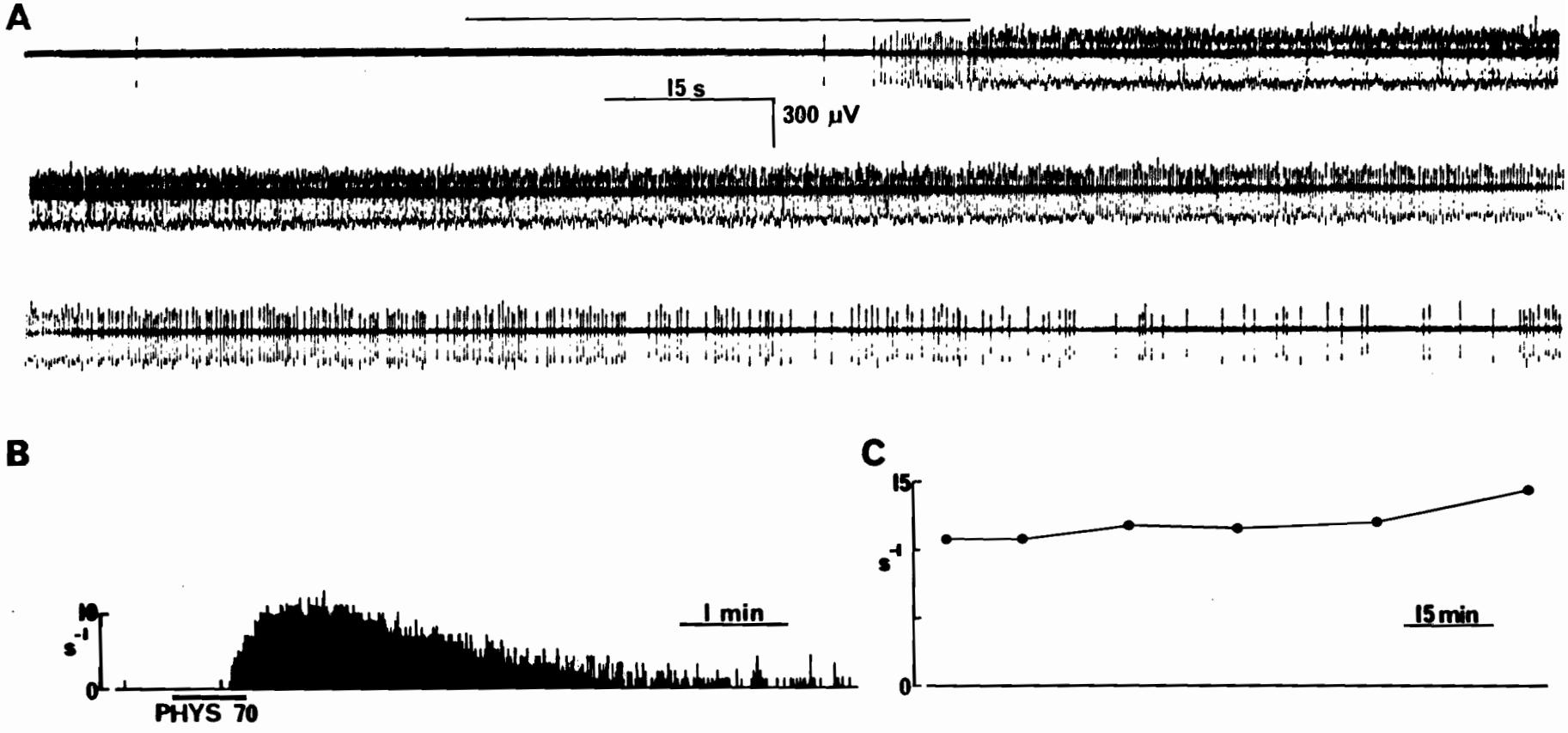

A
15 s
300 µV
B
10
s⁻¹
0
1 min
PHYS 70
C
15
s⁻¹
0
15 min

Fig. 3 Application of physalaemin modifies the response of a proprioceptive unit to application of ATP.

A. Continuous oscilloscope record of the discharge of this unit which responded only to passive movement of the hindlimb. Bars below the trace indicate the periods of application of physalaemin (PHYS) and of ATP; ejecting currents are in nA. The same ATP application is shown on the right in C.

B. The upper graph shows the mean rate of firing (squares) in spikes·s^{-1} during the 10-s period before each of 12 ATP applications. The mean firing rate in the 10-s period before ATP application was considered an accurate reflection of the level of excitability because regardless of whether or not the ATP application was preceded by physalaemin the rate of firing was stable in this period. In the lower graph, the circles indicate the percentage depression calculated for each of the applications; the test interval was the period 15-200 s after each ATP application. The first 3 applications of ATP caused only excitation and so the magnitude of depression for these applications was defined as being zero. The 3 down-going arrows indicate the points representing the applications of ATP which were made 28 s after an ejection of physalaemin. The mean rate of firing during the period 15-200 s following each ejection of ATP was used as the test level of firing to calculate the magnitude of depression. At the time indicated by the up-going arrow the position of the hind limb was adjusted to increase the tonic rate of firing to approximately the level caused by the first physalaemin injection.

C. Continuous-time histogram of the discharge rate of this unit. Physalaemin and ATP were applied during the periods indicated by the long thin and short heavy bars, respectively. The ATP application on the right corresponds to the points below the first down-going arrow in the graph in

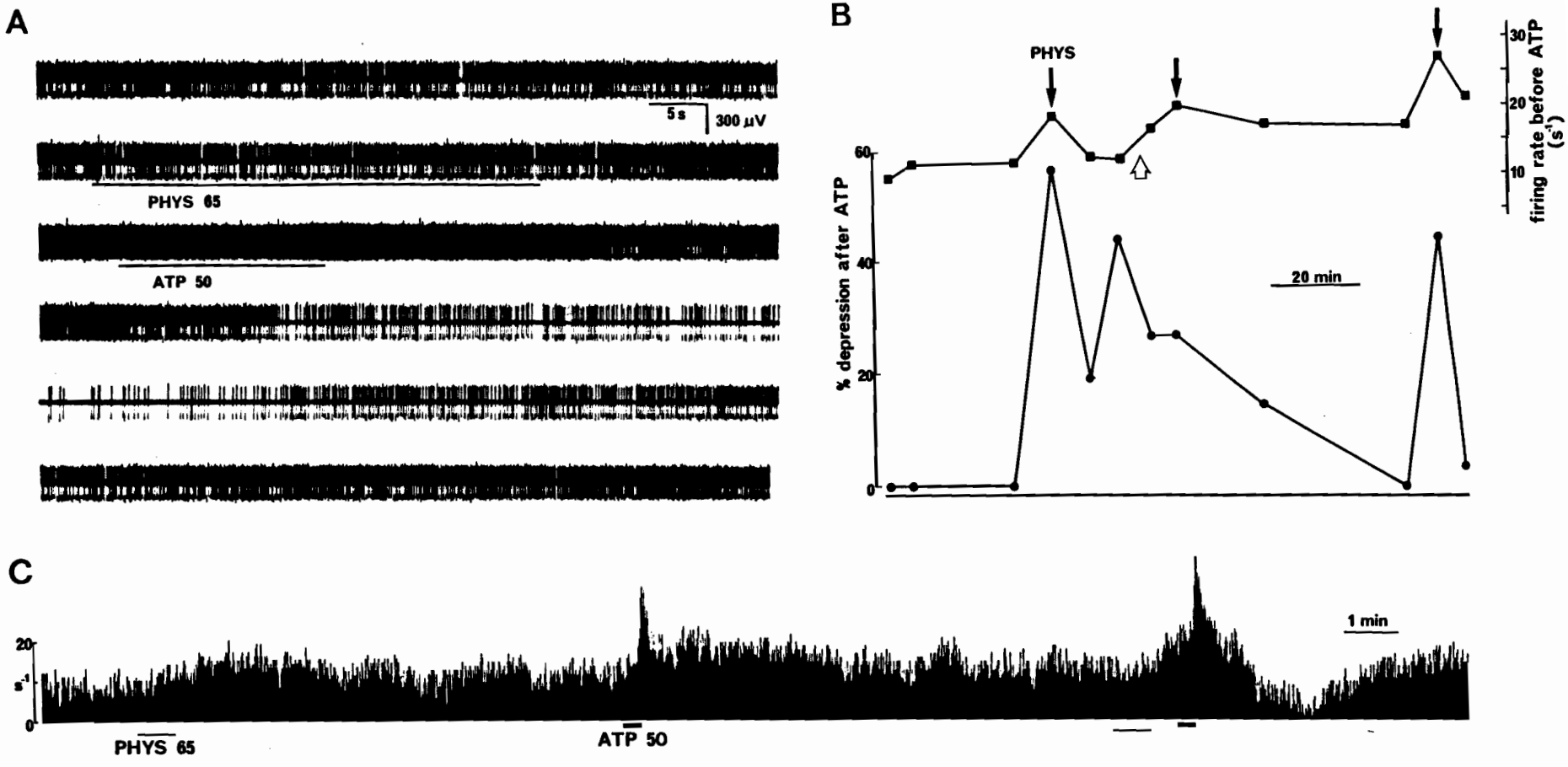

A
5 s
300 µV
PHYS 65
ATP 50
B
PHYS
% depression after ATP
60
40
20
0
20 min
30
20
10
firing rate before ATP (s⁻¹)
C
20
s⁻¹
0
1 min
PHYS 65
ATP 50

Fig. 4 Ejections of ATP and AMP subthreshold to alter on-going activity cause depression when preceded by application of physalaemin.

Each record illustrates an averaged PSTH of the discharge of this wide dynamic range unit: **A,C** and **E** were compiled from 2 trials and **B** and **D** were constructed from 3 trials. ATP, AMP and physalaemin (PHYS) were applied during the periods indicated by the open short bars, by the filled short bars and by the long bars, respectively (ejecting currents are in nA). Ordinates of the histograms show the firing rate in spikes per 1-s bin; the same scale applies to all records.

Inset: diagrammatic representation of the receptive field to mechanical stimulation. The unit was excited by hair movement, light touch and innocuous pressure to the blackened area only and by pinching the skin with a serrated forceps in both the blackened and the hatched areas.

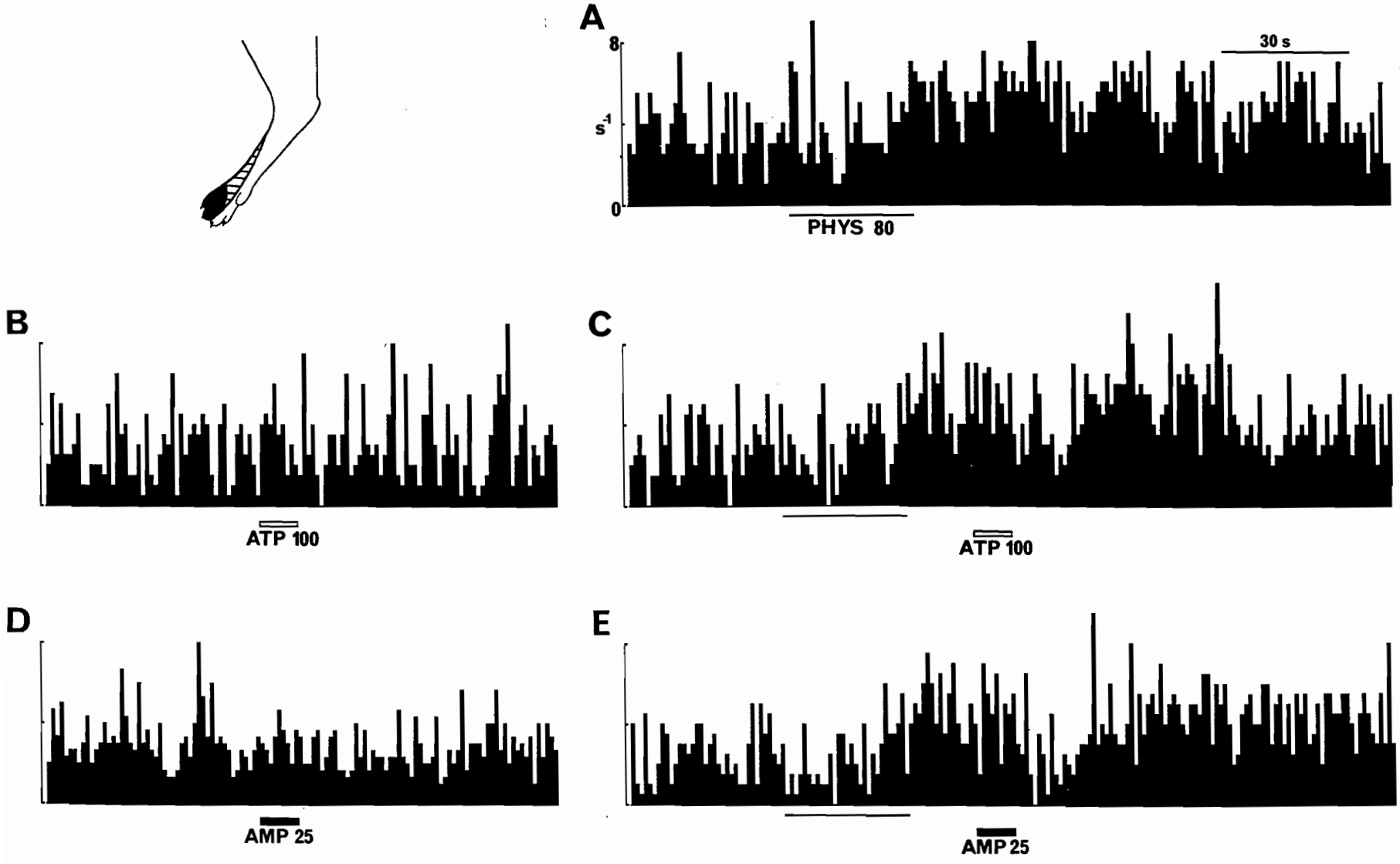

A
8
s-1
0
30 s
PHYS 80
B
ATP 100
C
ATP 100
D
AMP 25
E
AMP 25

Fig. 5 Effects of application of physalaemin and of glutamate on responses to ejection of ATP.

Each record is an averaged PSTH constructed from 2 trials which were run consecutively. The ordinates show the firing rate in spikes per 1-s bin; note that the scale in C is different from that in A and B. Physalaemin (PHYS) was applied during the period indicated by the long bar below the records in A and B. Periods of ATP application are shown by the short bars in B and C. The short bar below the record in A indicates the period of ejection of inward current through a barrel containing the control solution (Cl^-) which was done to control for possible artefacts of current ejection.

The arrow below the record in C indicates the time at which the current ejecting glutamate was increased to a new constant level in an attempt to mimic the increase in firing rate caused by physalaemin.

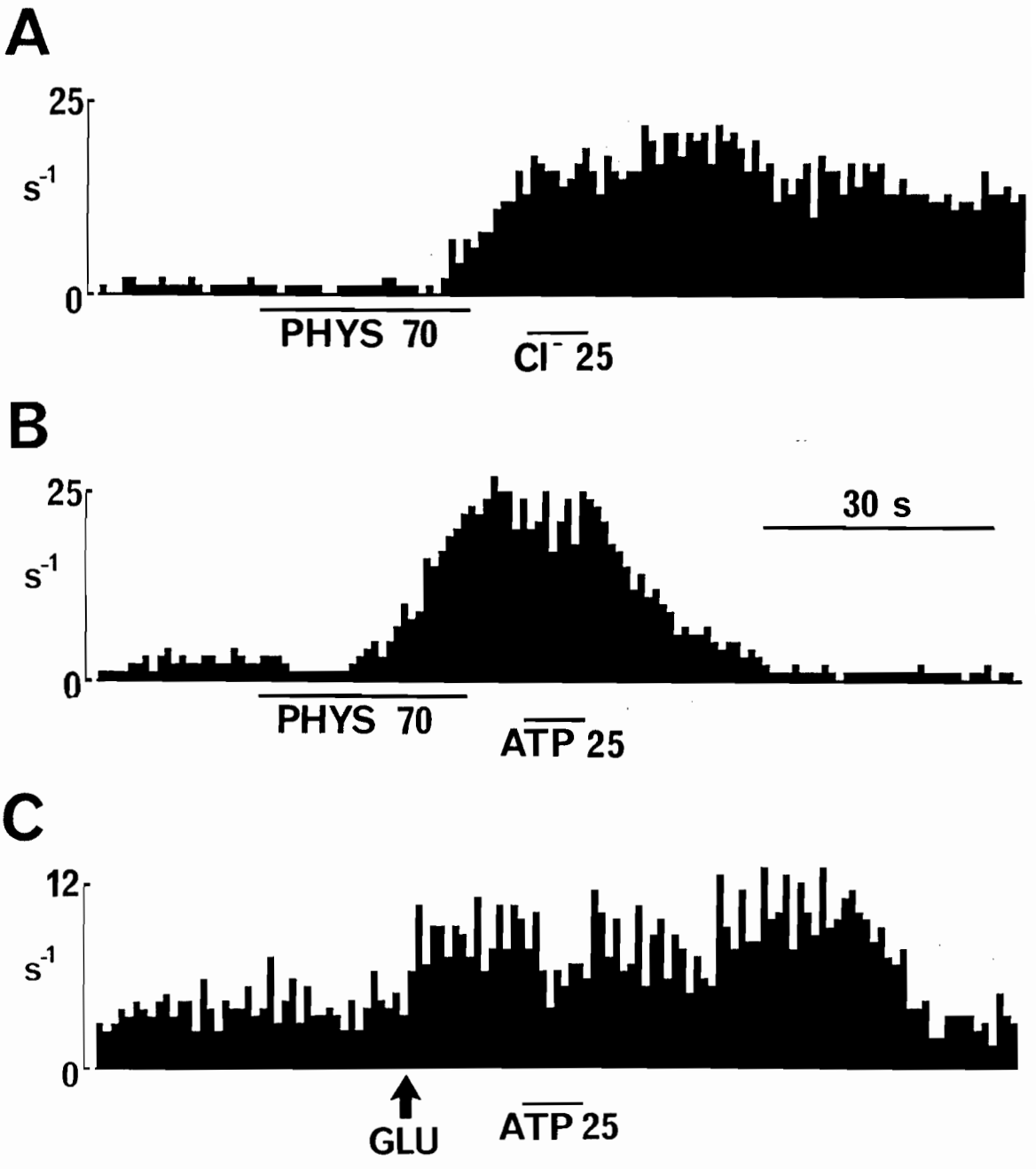
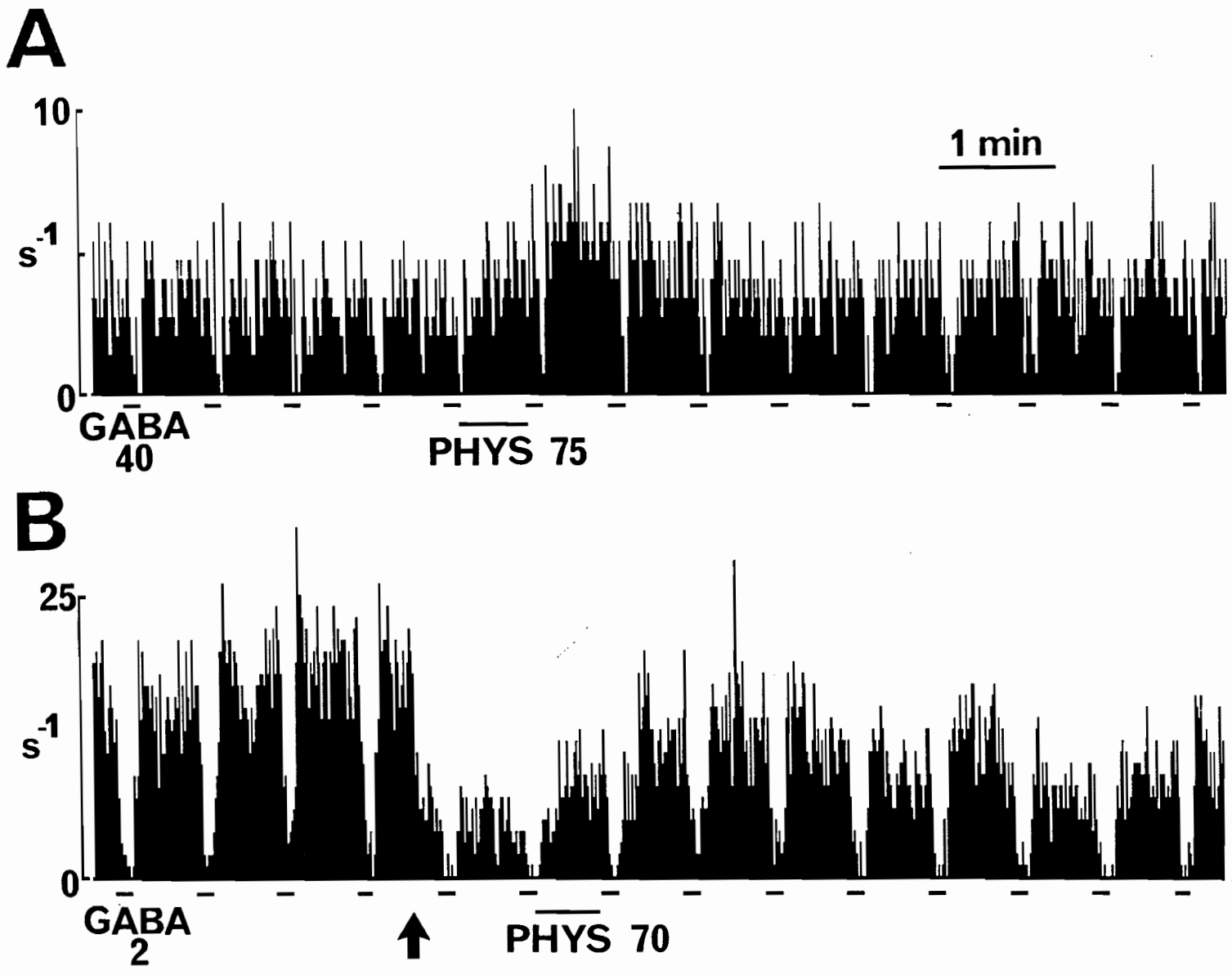

A
25
s-1
0
PHYS 70
Cl- 25
B
25
s-1
0
30 s
PHYS 70
ATP 25
C
12
s-1
0
GLU
ATP 25

Fig. 6 Application of physalaemin fails to affect GABA-induced depression.

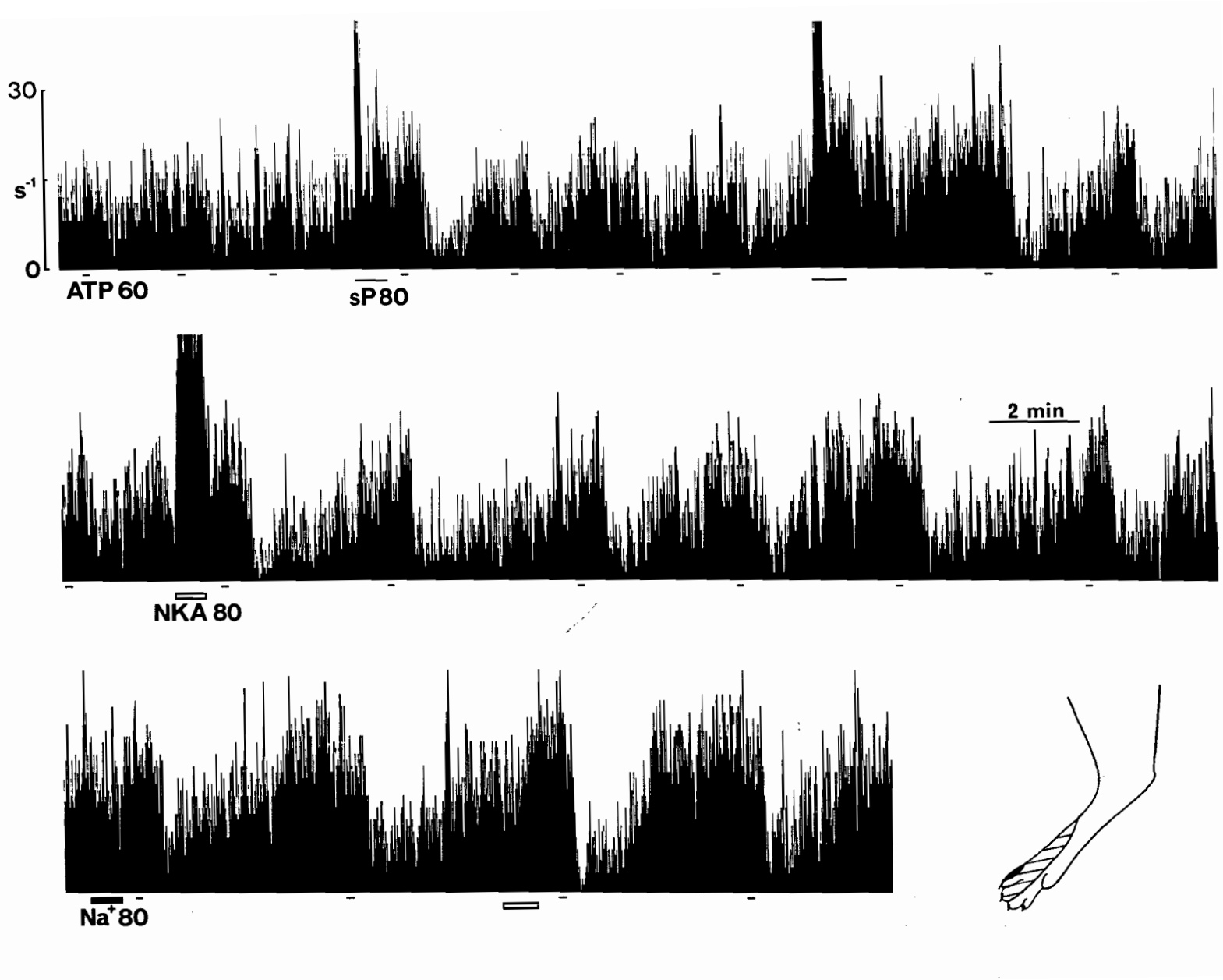
A and B illustrate continuous-time histograms from 2 different units. GABA and physalaemin were applied during the periods indicated by the short and long bars, respectively. Glutamate was applied throughout the duration of the record in B; at the time shown by the arrow below the record the ejecting current was decreased from 35 to 24 nA and was maintained at this level. For the unit shown in A, glutamate was not ejected during the period illustrated by the record. The same time scale applies to both records.


A
10
s-1
0
1 min
GABA
40
PHYS 75
B
25
s-1
0
GABA
2
PHYS 70

'ig. 7 Ejections of substance P and neurokinin A enhance the depressant response to application of ATP.

The records show a single continuous-time histogram of the discharge ate of this wide dynamic range unit. The periods of application of ATP nd substance P (sP) are shown, respectively, by the short and long lines elow the records. Neurokinin A (NKA) was applied during the periods ndicated by the open bars. Note that current ejection through the barrel ontaining substance P caused noise which occasionally triggered the ating unit; noise also occurred during the first application of eurokinin A. The noise terminated immediately on cessation of ejection. herefore, the records are unreliable during the periods of both pplications of substance P and the first application of neurokinin A. owever, the records are accurate during the periods of interest for this tudy (i.e. immediately before and after the ATP ejections). Because of ne noise, the records were truncated at 40 counts$\cdot s^{-1}$. Outward current nrough a barrel containing control solution (Na^+, filled bar below the ower record) failed to mimic the effect of the tachykinins.

Inset: illustration of the receptive field to mechanical stimuli. The nit was unaffected by hair movement but was excited by light touch and nnocuous pressure to the blackened area and by noxious pinch to the Lackened and hatched regions.


30
s-1
0
ATP 60
sP 80
2 min
NKA 80
Na+ 80

CHAPTER 7

TACHYKININS ENHANCE THE DEPRESSION OF SPINAL NOCICEPTIVE NEURONES CAUSED BY CUTANEOUSLY APPLIED VIBRATION IN THE CAT

I. ABSTRACT

In previous chapters it has been reported that vibration-induced depression of nociceptive dorsal horn neurones may be mediated by adenosine and that depressant effects of purines, which are likely mediated by adenosine, are enhanced by tachykinins. These observations prompted the present investigation of the possible effects of tachykinins on vibration-induced depression. Extracellular recordings were made from single nociceptive neurones in the lower lumbar segments of anaesthetized cats. Vibration (80 Hz; 2.5-3.5 s every 20-25 s) was applied to the hindlimb using a feedback-controlled mechanical stimulator. The probe of the stimulator was placed so that a decrease in the number of spikes occurred during the period of vibration. The tachykinins physalaemin, substance P and neurokinin A, were administered by iontophoresis and they caused excitation. To attempt to control for possible changes in the response to vibration caused by an increase in firing rate, *per se*, input-output relationships for the response to vibration were compared during excitation by glutamate and during excitation by tachykinins.

It was possible to investigate effects of tachykinins for 34 of the 54 neurones for which input-output lines were constructed (average linear correlation coefficient: 0.64 ± 0.04 [SEM]). Physalaemin was tested on vibration-induced responses in 19 cases: with 10 of these the slope of the input-output line made after administration of physalaemin was significantly lower than that of the line made during ejection of glutamate. Importantly, for the 9 remaining neurones the lines constructed with administration of physalaemin or of glutamate were not significantly different and in no case was the slope of the input-output line with glutamate less than that with physalaemin. Thus, while physalaemin and glutamate increased the on-going rate of discharge, they appeared to have different effects on the vibration-induced responses. It is consistent with our previous observations to suggest that physalaemin may have enhanced the vibration-induced depression.

Vibration-induced depression also appeared to be enhanced by neurokinin A (3/8 cases) and by substance P (1/7 cases).

The results of the present study prompt us to raise the possibility that tachykinins released endogenously may increase the sensitivity of nociceptive neurones to the depressant effects of vibration.

II. INTRODUCTION

In Chapter 4 it was suggested that depression of nociceptive neurones caused by cutaneously applied vibration may be mediated by adenosine because this depression is blocked by caffeine and 8-sulphophenyltheophylline, P_1-purinergic (adenosine) receptor antagonists (Bruns et al., 1980; Daly et al., 1985; Snyder et al., 1981), and is potentiated by dipyridamole, an inhibitor of adenosine uptake (Phillis et al., 1979; Wu & Phillis, 1984). Chapter 6 reported that the depressant effects of adenosine 5'-triphosphate (ATP) and adenosine 5'-monophosphate (AMP) are enhanced by the tachykinins, physalaemin, substance P and neurokinin A. Depression caused by ATP and AMP appears likely to be due to activation of P_1-purinergic receptors by adenosine formed by conversion of the nucleotides (Phillis et al., 1979; Phillis & Kostopoulos, 1975; Salter & Henry, 1985a; Stone & Taylor, 1980; Taylor & Stone, 1978).

The possibility which follows logically from these previous findings is that vibration-induced depression might be enhanced by tachykinins. Therefore, in the present study an investigation has been made of the effects of physalaemin, substance P and neurokinin A on vibration-induced depression.

III. MATERIALS AND METHODS

The methodology used is described in detail Chapter 3. In brief, experiments were done on adult cats (n=28) anaesthetized with sodium pentobarbital (40 $mg \cdot kg^{-1}$, i.p.; supplemental doses 5 $mg \cdot kg^{-1}$ i.v. every 3 hours). The animals were ventilated artificially after bilateral pneumothorax and were paralyzed using pancuronium bromide (Pavulon, Organon; 1 $mg \cdot kg^{-1}$ i.v.). Arterial blood pressure, end tidal CO_2 concentration and core temperature were monitored continuously and maintained within physiologically normal limits.

Spinal segments L5-L7 were exposed for recording and the spinal cord was transected at the first lumbar level. Prior to the transection 0.1 mL of lidocaine hydrochloride were injected into the L1 segment to minimize

spinal shock.

Extracellular recordings were made from single, functionally identified units in the dorsal horn. Details of the system used to functionally classify the units are given in Chapter 2. Multibarrelled micropipettes (overall tip diameter 4-8 μm) were used for recording and for iontophoresis; the central, recording barrel was filled with a solution of 2.7 M NaCl. Recordings were amplified, displayed on oscilloscopes and stored on magnetic cassette tape. The rate of discharge was displayed continuously on a Grass Model 5 polygraph. Interspike and interstimulation intervals were stored on a fixed disk using an IBM personal computer and hardware and software developed in our laboratory.

Each of the outer barrels of the micropipettes was filled with one of the following solutions: sodium L-glutamate (1M, pH 7.4; Sigma), physalaemin (1 mM, Protein Research Foundation), substance P (0.8 mM, Institute Armand-Frappier), neurokinin A (1 mM, Institute Armand-Frappier), control solution (165 mM NaCl, pH 5.5) and Pontamine Sky Blue 6BX (1% in 0.5 M sodium acetate; Gurr). The control solution was the vehicle used for peptides. Ejection of Pontamine Sky Blue was used to mark the site of recording. Leakage of compounds was minimized by using a retaining current during the periods between applications; for glutamate and Pontamine Sky Blue an outward current (10 nA) was used and for the peptides the retaining current was inward (5 nA).

Vibration was produced using a feedback-controlled mechanical stimulator (Chubbuck, 1966). The vibrational stimulation was 2.5-3.5 s in duration and was repeated at regular intervals every 20-25 s. The duration and repetition rate of stimulation were constant for each unit and the interval between applications was sufficient to allow complete recovery. The frequency of stimulation was 80 Hz. The stimulation amplitude was constant during testing and was between 0.3 and 1.0 mm. The probe of the stimulator was placed outside the excitatory receptive field for low intensity stimuli (see for example Figs. 1-4) because depressant effects of vibration with the parameters used here occurred when the probe was in this position (see Chapter 3).

Administration of tachykinins causes excitation of dorsal horn neurones (for example see Henry, 1976). Therefore, an experimental method was devised to attempt to take into account the possibility that any

changes in responses to vibration during tachykinin-induced excitation might be due to the increase in rate of discharge, *per se*. With this method the vibration-induced responses during excitation by tachykinins were compared to the vibration-induced responses when the firing rate was elevated to similar levels by ejection of glutamate. Glutamate was chosen as the standard for comparison because it produces a potent excitation of dorsal horn neurones and because the firing rate can easily be maintained at a constant level during the ejection. Thus, during the experiments vibration was applied at regular, automatically controlled intervals and the rate of firing was systematically elevated by periodically increasing the ejection current for glutamate. The increases in the glutamate ejection current were made at least 10 s prior to the next application of vibration to allow the firing rate to stabilize before the subsequent response. The response to vibration during ejection of glutamate was then compared to the response during the excitation caused by tachykinin administration.

Quantitative data analysis was done off-line. Peristimulus time histograms (PSTHs) were constructed to show the rate of discharge in relation to the vibrational stimulation. In addition, a scatter graph showing the input-output relationship was made: the number of spikes during the period of stimulation was plotted against the number of spikes in the control period, which was the period of the same duration immediately preceding the stimulation. A least-squares linear regression analysis was done using data collected during application of glutamate. A separate analysis was done for data during the response to tachykinins. The regression lines determined by these analyses will be termed the input-output lines. The 2 input-output lines were compared using an analysis of covariance (Snedecor and Cochran, 1967); a difference between the lines was considered statistically significant for $p<0.05$.

IV. RESULTS

Recordings from 54 units are included in these results. Fifty-three units were wide dynamic range (WDR); that is they were excited by innocuous as well as noxious cutaneous stimuli. The remaining unit was

excited only by noxious cutaneous stimuli. All units were excited by iontophoretic application of glutamate and hence, the recordings were considered to have been made from an excitable area (likely the somato-dendritic region) of neurones intrinsic to the dorsal horn.

All neurones had features similar to those previously reported for those in laminae I and III-VI (Price and Dubner, 1977). The locations of selected sites of recording were identified in histological sections and were found to be in one of these laminae.

Responses to vibration were identical to those reported previously (see Chapter 3 and 5): each neurone showed either depression, excitation or a biphasic response consisting of excitation followed by depression. When effects of the excitatory agents were investigated submaximum responses to vibration were used.

i) *Input-output relationship during ejection of glutamate*

Glutamate-evoked excitation was the standard used for comparison when investigating effects of tachykinins on vibration-induced responses. The input-output relationship during ejection of glutamate was studied for each of the 54 neurones. In all cases the number of spikes during the period of stimulation increased when the number of spikes during the control period was increased with glutamate (for example see Fig. 1). The correlation coefficient for the linear regression was statistically significant in 40 cases and the average value in the entire sample was 0.64 $\pm$0.04 ($\pm$SEM). Thus, it seemed reasonable to use a method of linear analysis when examining the effects of tachykinins on vibration-induced responses.

To verify that differences in the magnitude of depression could be detected using the method of comparing input-output lines, comparison of input-output lines was done when effects of different magnitudes were produced by different amplitudes of stimulation. For example, with the neurone shown in Fig. 1, two amplitudes of stimulation, 0.2 and 0.5 mm, were used as these were in the range of those found previously to cause significant depression (see Chapter 5). As can be seen in the PSTHs the magnitude of depression was greater using an amplitude of 0.5 mm than using 0.2 mm stimulation. The scatter graph illustrates that the data points for the larger stimulation amplitude fell below those for the

smaller amplitude. In addition, the slope of the regression line made using an amplitude of 0.5 mm was less than the slope of the line using the 0.2 mm stimulation ($F(1,36)=7.3$, $p=0.01$). In all cases tested (n=10), the slopes or the elevations of input-output lines were different when depressions of differing magnitudes were evoked by varying the amplitude of stimulation. Therefore, it was concluded that comparison of input-output lines provides a valid statistical method to distinguish between vibration-induced depressions of different magnitudes.

ii) *Effects of physalaemin on input-output relationship*

Physalaemin (50-100 nA; 25-60 s) was tested on vibration-induced responses for 19 neurones. In all of these cases physalaemin caused a delayed excitation which outlasted the period of application (Salter and Henry, 1985b).

An example of the effect of physalaemin on vibration-induced depression is shown in Fig. 2. The average PSTHs in Fig. 2B show that after application of physalaemin the magnitude of depression was increased. The scatter graph of Fig. 2C illustrates that the slope of the input-output line made after administration of physalaemin (100 nA, 30 s) was less than the slope of the line during ejection of glutamate ($F(1,14)=9.8$, $p=0.007$). Therefore, although physalaemin and glutamate caused similar increases in the rate of discharge, they had different effects on the response to vibration. The film records in Fig. 2A show that spike amplitude and spike configuration were unchanged during the response to physalaemin compared with during ejection of glutamate, indicating that the difference seen in the graph was not likely produced artefactually.

For the neurone shown in Fig. 3, vibration caused a biphasic response: excitation preceded the depression (Fig. 3A). The scatter graph (Fig. 3B) illustrates that the slope of the input-output line made after physalaemin had been applied was less than the slope of the line made during glutamate ejection ($F(1,40)=21.9$, $p=0.0001$). Thus, the effect of physalaemin on the biphasic response was similar to the effect on the vibration-induced depression.

For Fig. 3, glutamate was applied before and 6 min after ejection of physalaemin. There was no significant difference between the input-output

lines made with these two applications and hence, the data points were combined on the scatter graph. This lack of difference indicates that the effect of physalaemin on the vibration-induced response was completely reversible.

In 10 cases the slope of the input-output line for vibration was significantly less after administration of physalaemin compared with during ejection of glutamate; with the remaining 9 neurones the 2 input-output lines were not significantly different either in slope or in elevation (the other parameter which controls the position of the line). In addition, in one of the 10 cases it was found that vibration which was subthreshold before application of physalaemin caused depression afterwards. In this case the vibration-induced depression which occurred after administration of physalaemin was reversibly blocked by intravenous administration of caffeine (10 $mg \cdot kg^{-1}$).

The input-output relationships for physalaemin and for glutamate were significantly different with 6 of 11 neurones showing vibration-induced depression and with 3 of 7 neurones exhibiting the biphasic response. The remaining neurone for which the input-output relationships differed is illustrated in Fig. 4. In this case, prior to application of physalaemin there was an increase in the number of spikes during the period of vibration; after application of the peptide the number of spikes during the vibrational stimulation was decreased relative to the control period. This neurone is noteworthy because a change such as this never occurred spontaneously or with application of glutamate.

iii) *Effects of substance P on input-output relationship*

Substance P (65-100 nA, 30-75 s) was tested on vibration-induced responses with 7 neurones which were excited by this peptide. The slope of the input-output line for vibration-induced depression was decreased after application of substance P in one case. In the remaining cases, input-output relationships were unaffected by this peptide with neurones in which vibration had depressant (n=3) and biphasic (n=3) effects.

iv) *Effects of neurokinin A on input-output relationship*

The recently discovered mammalian tachykinin, neurokinin A (Kangawa et al., 1983; Kimura et al., 1983), was tested in 8 cases; ejection

parameters were 60-125 nA, 30-60 s. In 3 cases, the slope of the input-output line made after application of neurokinin A was significantly less than that of the line made during ejection of glutamate; vibration caused depression in one of these cases and the biphasic response in the other two. Neurokinin A failed to affect the input-output relationship in the other cases (depression, n=4; biphasic response n=1).

V. DISCUSSION

i) *Effects of tachykinins on depressant response to vibration*

The results of the present study indicate that the magnitude of vibration-induced depression was greater after application of tachykinins than during application of glutamate. Thus, physalaemin and glutamate have different effects on the vibration-induced depression. As the on-going rate of discharge was increased to similar levels by both substances (tachykinins and glutamate) the greater magnitude of depression after application of tachykinins cannot be fully accounted for by the excitation caused by these peptides. The additional observation was made that vibration which was subthreshold caused depression after application of tachykinins. Therefore, the possibility is suggested that vibration-induced depression may be enhanced by tachykinins.

ii) *Similarity of effects of tachykinins on vibration- and purine-induced depression*

As mentioned in the Introduction, tachykinins enhance the depressant effects of purines. Purine-induced depression is likely mediated by activation of P_1-purinergic receptors (Phillis et al., 1979; Phillis & Kostopoulos, 1975; Salter & Henry, 1985a; Stone & Taylor, 1980; Taylor & Stone, 1978) and thus tachykinins appear to have similar effects on P_1-receptor-mediated depression and on depression produced by vibration. This similarity provides novel pharmacological evidence consistent with the previous suggestion that vibration-induced depression may be mediated by P_1-purinergic receptors (see Chapter 4). Support for this suggestion comes from the observation that the P_1-purinergic antagonist, caffeine, attenuated the vibration-induced depression which occurred following application of physalaemin.

We have reported previously that depression of dorsal horn neurones by γ-aminobutyric acid (GABA) is unaffected by tachykinins (see Chapter 6). Therefore, the enhancement of vibration-induced depression by these peptides is inconsistent with the possibility that GABA might mediate this depression. The present results, therefore, extend the work reported in Chapter 4 showing that vibration-induced depression is unaffected by the bicuculline, an antagonist at the A subtype of GABA receptor.

iii) *Effects of tachykinins on biphasic responses to vibration*

Physalaemin and neurokinin A had an effect on the input-output relationship of neurones exhibiting the biphasic effect similar to the effect of these peptides on the input-output relationship of neurones exhibiting vibration-induced depression. This similarity is interpreted as indicating that depression during biphasic response was enhanced by the tachykinins, for the reasons described above in the case of the depressant response. Thus, the present results are consistent with the possibility that the depression during the biphasic response may be mediated by P_1-purinergic receptors (see Chapter 4).

iv) *Effects of tachykinins on the excitatory response to vibration*

After application of physalaemin, vibration caused a biphasic response in the neurone which showed excitation beforehand. In light of the evidence that physalaemin appears to enhance vibration-induced depression, this finding raises the possibility that physalaemin may have enhanced and thus unmasked a depression underlying the excitation.

v) *Possible functional significance of effects of tachykinins on vibration-induced responses*

I consider that the results of the present study are likely relevant to modulation of pain and nociception at the spinal level because the effects of vibration and tachykinins were observed with neurones excited by noxious cutaneous stimuli. As tachykinins appear to be present in the dorsal horn (Hökfelt et al., 1975) and to be released specifically by noxious peripheral stimuli (Kuraishi et al., 1984, 1985), the possibility is suggested that noxious stimulation may render nociceptive dorsal horn neurones more sensitive than they would otherwise be to the depressant

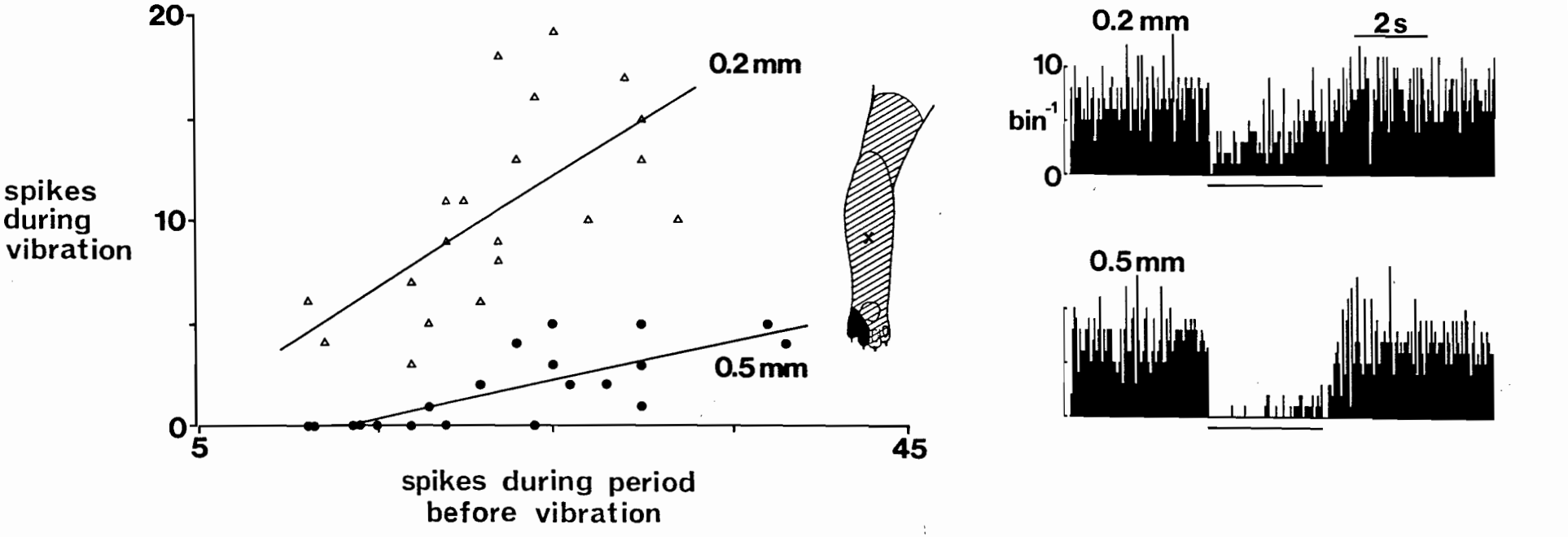
effects of vibration.

In humans vibration causes analgesia (Lundeberg, 1983), the neural basis of which may be depression of nociceptive neurones in the dorsal horn (see Chapter 3). Logically, it seems possible that tachykinins, if administered locally to the spinal cord, might be useful as adjuvants to increase the analgesic efficacy of vibration. However, when given at the spinal level in experimental animals tachykinins themselves facilitate nociceptive reflexes (Yashpal et al., 1982) and cause behavioural changes similar to those seen with reactions to noxious stimulation (Yashpal et al., 1982; Seybold et al., 1982). Tachykinins would thus be expected to evoke pain if administered in the same way to humans. Therefore, for tachykinins to be useful as adjuvants with vibration it will likely be necessary to find new ones which enhance vibration-induced depression but which do not evoke nociceptive reactions. Recently, many synthetic tachykinin analogues have been developed some of which mimic and some of which antagonize effects of tachykinins (see Henry et al., 1987). Thus, it may be possible in the future to develop tachykinin analogues with properties which allow them to be used clinically to increase vibration-induced analgesia.

'ig. 1 Input-output relationships using stimulation amplitudes of 0.2 and 0.5 mm.

In the scatter graph, data points collected when the amplitude of timulation was 0.2 mm are shown by the triangles (n=20); the input-output ine for these points has the equation y= 0.54x - 1.5 (r=0.64; p<0.01). he dots (n=20) show data points when the stimulation amplitude was 0.5 m; the equation for the input-output line calculated using these points s y= 0.17x - 2.3 (r=0.80, p<0.01). The excitatory receptive fields for air movement and for light touch are represented by the blackened area on he diagram in the inset; the neurone was excited by noxious pinch in the oth the blackened and hatched areas. The location of the stimulator robe is shown by the "x" which also signifies that the long axis of the robe was perpendicular to the plantar surface of the foot.

Two PSTHs are shown to the right of the scatter graph. The period of ibration is indicated by the bar below each histogram. For the upper STH the amplitude of stimulation was 0.2 mm and for the lower it was 0.5 m. In each case the same 20 stimulations used for the data points in catter graph were used to compile the histogram. The bin width for the STHs is 50 ms.

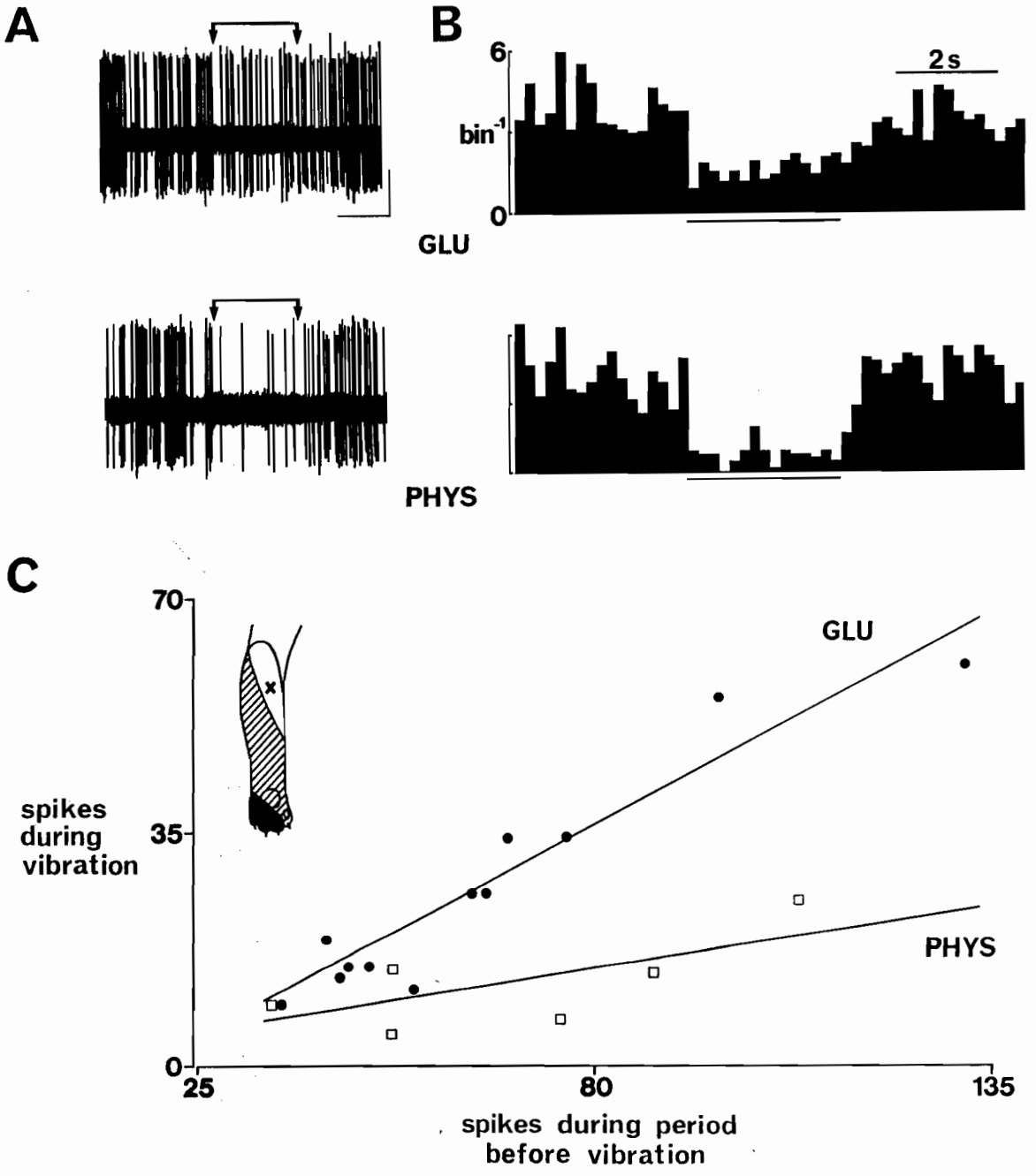

20
10
0
spikes during vibration
0.2 mm
0.5 mm
5
45
spikes during period before vibration
0.2 mm
2 s
10
bin-1
0
0.5 mm

ig. 2 Comparison of vibration-induced depression during ejection of glutamate and during response to physalaemin.

A. Film records illustrate single responses to vibration (indicated y the bar above each trace) during continuous ejection of glutamate ıpper) and 30 s after administration of physalaemin (100 nA, 30 s). ılibration bars: vertical, 100 μV; horizontal, 2 s.

B. The records are averaged PSTHs compiled using 12 vibrational :imulations during ejection of glutamate (upper) and using 6 stimulations ıe first of which was 10 s after the application of physalaemin (lower). ıe period of vibration is indicated by the bar below each histogram. The .n width for the PSTHs is 200 ms.

C. Input-output relationships determined using the same stimulations ; in B are shown in the scatter graph. Data points collected during ection of glutamate are indicated by the filled dots and those taken :ter the application of physalaemin are shown as the open circles. The ıuation of the input-output line compiled from data obtained during ection of glutamate is $y= 0.59x - 12.4$ ($r=0.93$, $p<0.01$) and that of the .ne made following application of physalaemin is $y= 0.18x + 0.2$ ($r=0.68$, ·0.05). The excitatory receptive fields and the location of the :imulator probe are illustrated as in Fig. 1.


A
B
6
bin⁻¹
0
2 s
GLU
PHYS
C
70
35
0
spikes during vibration
GLU
PHYS
25
80
135
spikes during period before vibration

'ig. 3 Comparison of the biphasic response to vibration during ejection of glutamate vs after application of physalaemin.

A. The average PSTH illustrates that excitation preceded depression f this neurone indicating that this neurone had a biphasic response to ibration. The PSTH was compiled using 30 applications of vibration uring ejection of glutamate; the bin width is 2 ms. The trace below the istogram shows a representation of the amplitude output of the mechanical timulator. The amplitude of stimulation was 0.4 mm. In the diagram to he right the excitatory receptive fields are illustrated as in Fig. 1. he arrow shows the location of the stimulator probe and indicates the rientation of its long axis.

B. Data points from 30 stimulations during ejection of glutamate are nown by the filled circles. The equation for the input-output line onstructed using these points is $y = 0.80x - 6.8$ ($r=0.97$, $p<0.01$). The pen squares show 14 data points, collected in series of 7, immediately fter each of 2 applications of physalaemin. One application was made sing a current of 50 nA for 75 s; the parameters for the second pplication were 60 nA for 100 s.

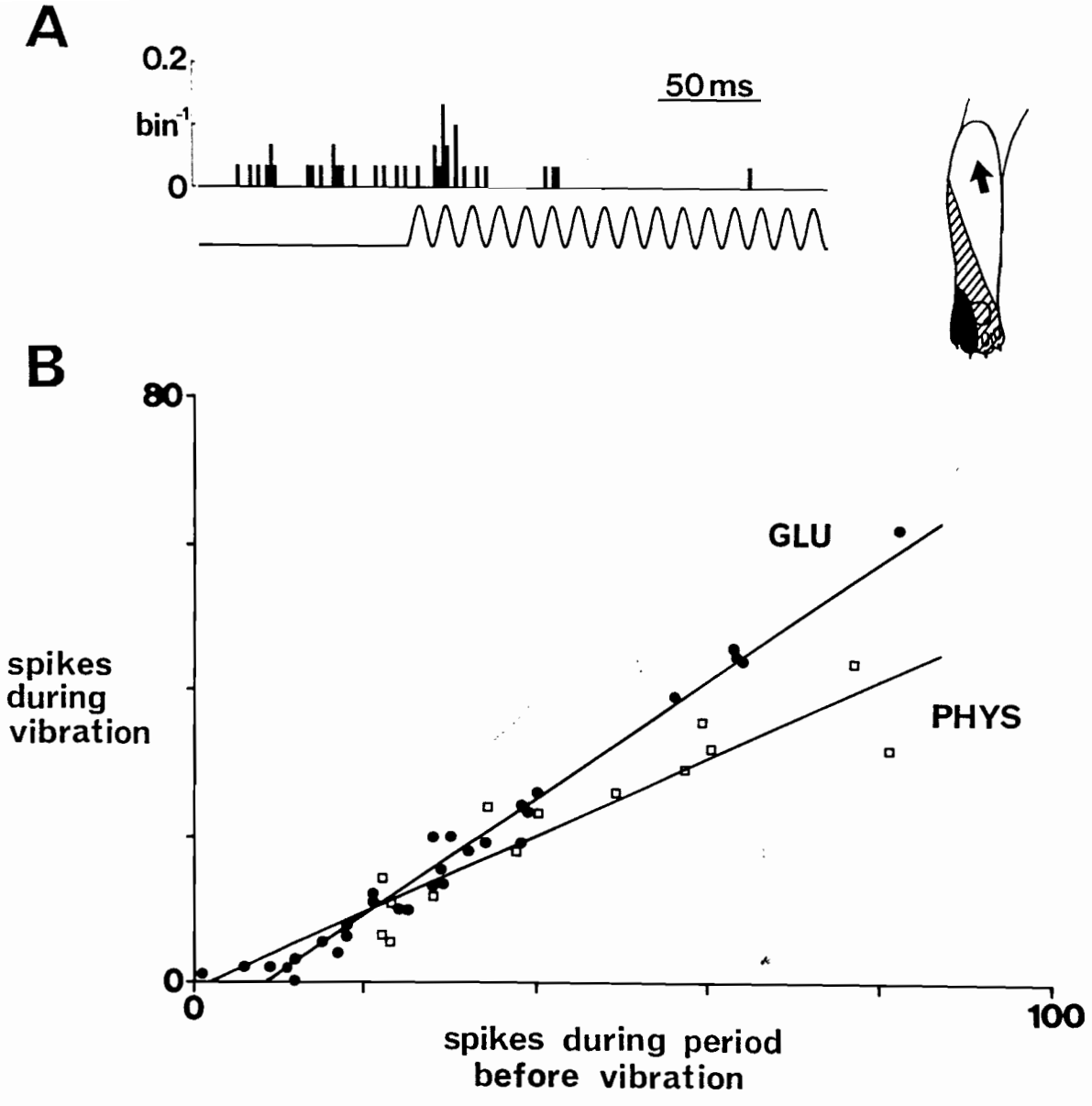
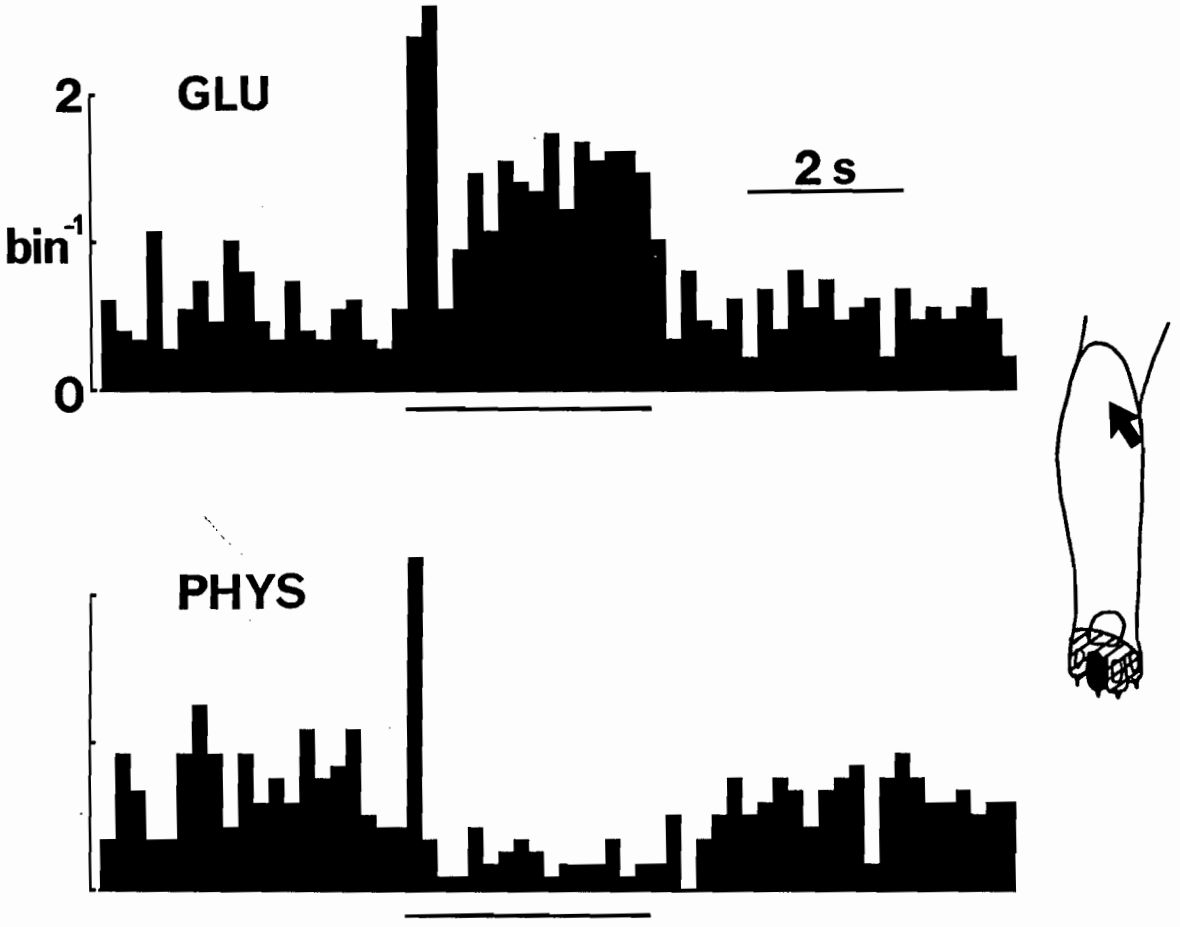

A
0.2
bin⁻¹
0
50 ms
B
80
0
0
100
GLU
PHYS
spikes during vibration
spikes during period before vibration

Fig. 4 Vibration causes excitation during ejection of glutamate and a biphasic (excitatory/depressant) response after administration of physalaemin.

Two average PSTHs illustrate the response to vibration; the period of stimulation is indicated bar below each record. The upper histogram was compiled using 15 stimulations during ejection of glutamate. The lower histogram was constructed from 12 stimulations; 6 were taken immediately after each of 2 applications of physalaemin (70 nA, 75 s). The excitatory receptive fields and the position of the probe of the stimulator are shown on the diagram. The blackened and hatched areas have the same significance as in Fig. 1.


2
GLU
2 s
bin-1
0
PHYS

CHAPTER 8

DISCUSSION

"Hypothesis has its right place, it forms a working basis; but it is an acknowledged makeshift, and, as a final expression of opinion, an open confession of failure, or, at the best, of purpose unaccomplished."

(Lewis, 1920)

I. ON INVOLVEMENT OF ATP AND ADENOSINE IN RESPONSES OF DORSAL HORN NEURONES TO CUTANEOUSLY APPLIED VIBRATION

i) *Introduction*

In reflecting on how the observations made in this thesis might shed some light on the possible roles of purines in the dorsal horn, it seemed worthwhile to bring together the findings made herein concerning the responses of dorsal horn neurones to adenine nucleotides and to vibration, and the findings made by others concerning the biochemistry, physiology and pharmacology of ATP and adenosine. Thus, I undertook to synthesize from these findings a conceptual framework which can be used to account for the main results of this thesis. This conceptual framework was developed in the form of a model for the vibration-induced response of wide dynamic range neurones in group BPH_1. In my view the model which I shall present provides the best explanation for this response based on the available information. The model, however, should be seen as a working hypothesis rather than as a statement of fact.

The core of the model is represented diagrammatically in Fig. 1 which shows a possible synaptic interaction between a Pacinian afferent and a BPH_1 neurone. [BPH_1 neurones are those which exhibit a biphasic response to vibration at all effective amplitudes and frequencies of stimulation (see Chapter 5).] I shall first describe the model and the advantages I see of this particular model as opposed to other possible models. Next, the evidence which gave rise to the model will be reviewed. I shall then discuss aspects of the model which require further experimental investigation. Finally, I will indicate how, through simple modifications, the model may be used as a possible explanation for the different effects of vibration on the wide dynamic range neurones in groups BPH_1 and DPR, on nociceptive specific neurones and on non-nociceptive neurones.

It is possible to incorporate the apparent enhancement of purine- and vibration-induced depressions by tachykinins (see Chapters 6 and 7) into the model using the suggestions made in Chapter 6. However, for the sake of simplicity the tachykinin-induced enhancement will not be discussed here.

ii) *A proposed model for the vibration-induced response of BPH_1 neurones*

Any model of the response of dorsal horn neurones to vibration must, at the least, account for the following observations made during the course of these thesis studies:

1) both the excitatory and the depressant components of the biphasic effect of vibration on BPH_1 neurones appear to be mediated by activation of Pacinian corpuscle afferents;

2) antagonists of P_1-purinergic receptors, caffeine and 8-SPT, block vibration-induced depression and in cases where vibration has a biphasic effect, they appear to block specifically the depressant component;

3) dipyridamole, which blocks adenosine uptake, increases the magnitude and the duration of vibration-induced depression;

4) exogenously applied ATP has a biphasic (excitatory/depressant) effect on a subgroup of dorsal horn neurones and the P_1-receptor antagonist, theophylline, appears to block the depressant component.

The model developed to account for these observations has the following aspects:

a) central terminals of a Pacinian corpuscle afferent make synaptic contact onto the dendrites of a BPH_1 neurone (one such synaptic contact is represented in Fig. 1);

b) cutaneously applied vibration evokes an action potential in the Pacinian afferent which results in the release of ATP from the central terminals;

c) ATP activates a receptor on the postsynaptic membrane which leads to depolarization by an increased conductance to sodium;

d) ATP is enzymatically converted to adenosine in the extracellular space;

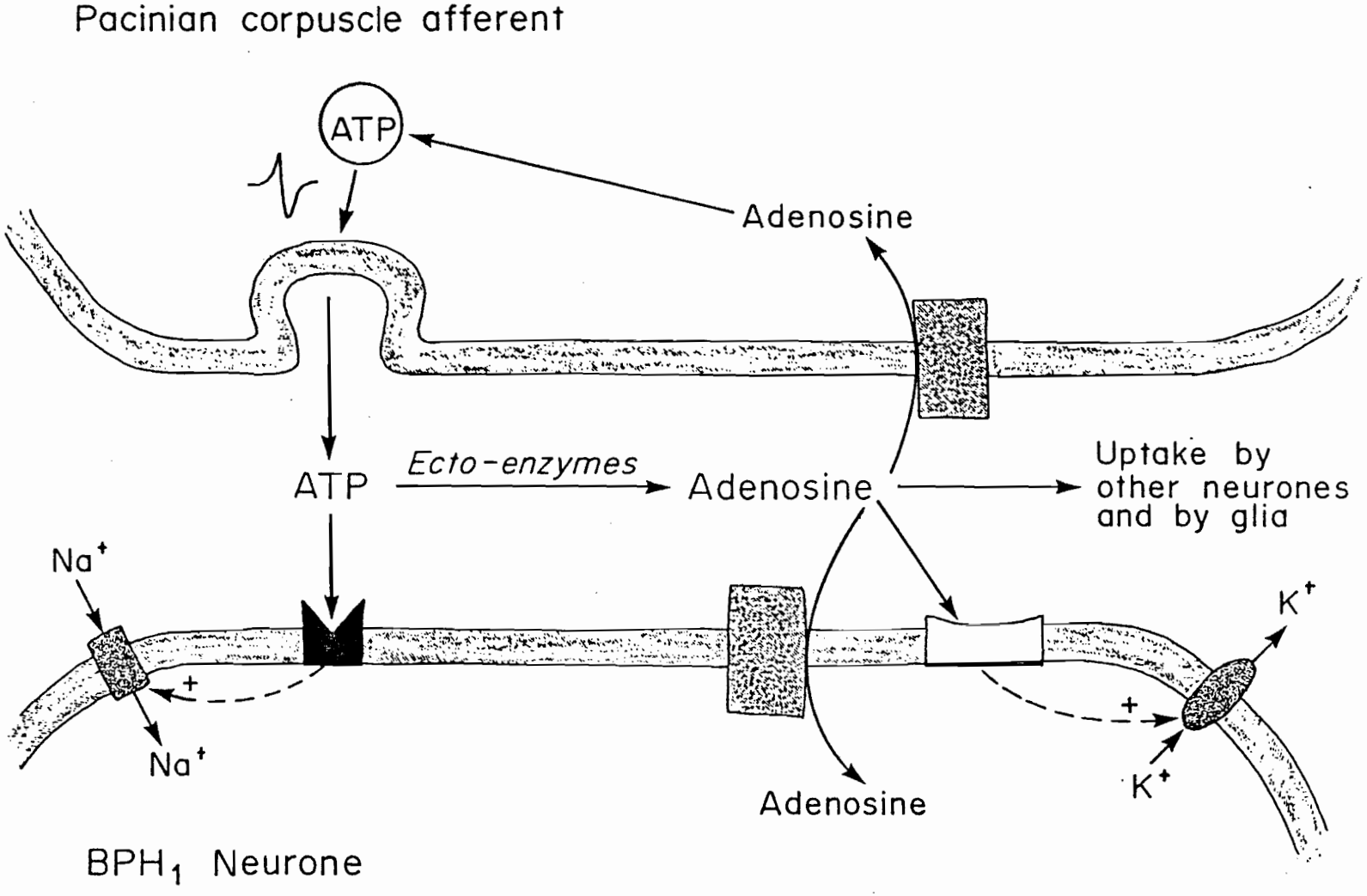
e) adenosine then acts on a postsynaptic receptor which is different from the type acted upon by ATP;

f) activation of the receptor by adenosine leads to hyperpolarization by increasing conductance to potassium;

g) the action of adenosine is terminated by uptake into neurones and/or glia.

Fig. 1 Diagrammatic representation of a possible model for the response of a BPH_1 to an action potential in a Pacinian afferent.

The workings of the model are described in the text.


Pacinian corpuscle afferent
ATP
Adenosine
ATP
Ecto-enzymes
Adenosine
Uptake by other neurones and by glia
Na+
Na+
+
+
K+
K+
Adenosine
BPH1 Neurone

iii) *Advantages of this model*

The main advantages I see of the proposed model over alternative models which can be readily imagined to explain the data (for example, in model I of Fig. 12 in Chapter 5 another excitatory agent might be released from the Pacinian afferent and adenosine or ATP might be released by the inhibitory neurone "I"), is that this model is simple in two respects: first, there is only one synapse and second, only one substance[1] is released. Other possible models would require more than one synapse and/or release of more than one substance and hence, in these respects, other possible models would be more complicated.

While the proposed model is simple in terms of the number of synapses and released substances, the response to vibration is relatively complex (i.e. there is excitation followed by depression). In the model the complexity of the response to vibration arises not from complex neuronal wiring or release of multiple substances, but rather from an orderly, albeit somewhat complex, cascade of biochemical events following the release of one substance, ATP.

iv) *Evidence which gave rise to the model*

The proposed model was developed on the basis of evidence from two sources: that presented in this thesis and that found in an extensive survey of the literature. In this section the relevant evidence will be discussed in terms of the different aspects of the model.

a) *Central terminals of a Pacinian corpuscle afferent make synaptic contact onto the dendrites of a BPH_1 neurone.* Anatomical studies using injection of tracers into identified Pacinian afferent fibres have indicated that these fibres arborize extensively in laminae III-VI of the dorsal horn (Brown, 1981). At the electron microscopic level, the contacts of the boutons of Pacinian afferents are observed predominantly with dendrites or dendritic spines of dorsal horn neurones; few of the

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[1] I have avoided using the term "transmitter" because while, in the model, ATP is functioning as a transmitter in the sense that it is released and has a postsynaptic effect, ATP also has the additional function of being a precursor of another active agent, adenosine. Adenosine, as well, cannot be seen strictly as a transmitter or even as a co-tranmitter because it is not released from the presynaptic neurone. Thus, a new terminology seems to be required in this case and possibly in others if post-release modification resulting in neuroactive substances is found to be a widespread phenomenon.

contacts are axosomatic or axoaxonic (Semba et al., 1984). These morphological features together with a central delay of 1.2 ms for the vibration-induced excitation (Chapter 3) raises the possibility that Pacinian afferents make monosynaptic connections with dendrites of BPH_1 neurones.

b) *Cutaneously applied vibration evokes an action potential in the Pacinian afferent which results in the release of ATP from the central terminals.* Evidence related to the physiological responses of Pacinian corpuscle afferents has been extensively reviewed by Hunt (1974); this evidence leaves little doubt that Pacinian afferents are sensitive to vibration applied with the parameters of stimulation which evoke the biphasic response from BPH_1 neurones. However, there is much less certainty as to whether ATP can be released from the central terminals of Pacinian afferents. In this regard, the Holtons (Holton and Holton, 1954; Holton, 1959) gave the first evidence that ATP might be released from the peripheral endings of primary afferents. This evidence raised the possibility that ATP might also be released from the central terminals of primary afferent neurones. Subsequently, White et al. (1985) have provided data that upon stimulation with elevated concentrations of potassium, ATP is released in a calcium-dependent manner from synaptosomal preparations derived from the dorsal horn. ATP can also be released from this type of preparation by depolarizing concentrations of veratridine (White et al., 1985; Yoshioka and Jessell, 1984). Thus, it is conceivable that when excited by vibration, Pacinian corpuscle afferents might release ATP from their central terminals.

However, in none of the studies of release was it possible to determine the precise source of the released ATP, nor was it determined whether ATP might be released under physiological conditions. As the studies discussed here represent the only ones to investigate the possibility of release of ATP from primary afferents or from dorsal horn tissue, this aspect of the model clearly requires further investigation.

c) *ATP activates a receptor on the postsynaptic membrane which leads to depolarization by an increased conductance to sodium.* Experiments on peripheral tissue have revealed that effects of ATP are dose-dependent and can be antagonized by pharmacological agents (for reviews see Burnstock, 1978; Burnstock and Kennedy, 1985) which suggests that these effects are

mediated by a receptor. Fedan et al. (1985) have reported that in guinea-pig vas deferens, a tissue in which ATP has an excitatory effect (Hogaboom et al., 1980), the photoaffinity analogue of ATP, $ANAPP_3$, binds to distinct membrane components with apparent molecular weights of 54-66 and 43-57 kilodaltons. These findings raise the possibility that the effects of ATP might be mediated by its binding to a specific membrane component (i.e. to a receptor complex). Therefore, in the model the effect of ATP is considered to be mediated by a membrane receptor.

In an electrophysiological study, Jahr and Jessell (1983) found that ATP causes depolarization of dorsal horn neurones in culture and that this effect persists when synaptic transmission is blocked by 100 μM $CdSO_4$. Therefore, it seems reasonable to suggest that ATP might have a postsynaptic effect.

Experiments done *in vivo* at locations where Pacinian afferents terminate have indicated that exogenously applied ATP has an excitatory effect: ATP increases the rate of firing of neurones in both the medullary (Salt and Hill, 1983) and the spinal dorsal horn (Fyffe and Perl, 1984; Chapter 2) and in the cuneate nucleus (Galindo et al., 1967). These findings are consistent with the depolarizing effect of ATP noted above in cultured dorsal horn neurones (Jahr and Jessell, 1983). Thus, in the model it is suggested that ATP causes depolarization.

In the Introduction it was noted that several different ionic mechanisms for ATP-induced depolarization have been reported and that different mechanisms seem to occur in different tissues. It is of particular relevance, therefore, that with dorsal horn neurones in culture the depolarizing effect of ATP was blocked by replacement of 90% of the sodium in the medium with choline (Jahr and Jessell, 1983), indicating that the effect is mainly due to an increase in sodium conductance. The same authors have subsequently mentioned, in a chapter in a book, unpublished data from experiments using patch-clamp recordings in which effects of ATP were said likely to be due to increased conductance to sodium and potassium (Jessell and Jahr, 1985). In light of reports that adenosine increases potassium conductance in hippocampal neurones (Segal, 1982; Greene and Haas, 1985), it seems possible that the increase in conductance to potassium seen by Jessell and Jahr may have been due to conversion of ATP to adenosine. Hence, in the model an increase in

membrane conductance to sodium has been suggested as the principal ionic mechanism by which ATP causes excitation.

d) *ATP is enzymatically converted to adenosine in the extracellular space*. There are two points which indicate it is possible that released ATP might be converted extracellularly to form adenosine. The first point is that the dorsal horn has been shown to contain two of the three requisite ecto-enzymes: Ca^{++}-Mg^{++} dependent ATPase (Dodd et al., 1983) and 5'-nucleotidase (Scott, 1967; Suran 1974 a,b). Although existence of an ecto-ADPase has not been described in the dorsal horn this enzyme has been well characterized on vascular endothelial cells (Gordon, 1986). In view of evidence, presented next, indicating that ATP can be converted to adenosine in the dorsal horn, it seems reasonable to expect the presence of an ecto-ADPase in the dorsal horn.

The second point is that iontophoretic application of ATP, in addition to causing excitation, causes a delayed depression of neuronal firing rate in the medullary (Salt and Hill, 1983) and spinal dorsal horns (see Chapter 2). For reasons described fully in the Discussion in Chapter 2 this depression appears to be mediated by conversion of the nucleotide to adenosine. Therefore, it seems likely that the enzymes which convert ATP to adenosine are functional in the dorsal horn *in vivo*.

Extracellular conversion of ATP to adenosine is a crucial step in the model for it is suggested that this conversion introduces a time delay. It possible that an additional time delay occurs following activation of the postsynaptic receptor by adenosine because, as will be described subsequently, there may be several steps between receptor activation and the change in membrane conductance. Thus, it is proposed that the time delays caused by formation and/or the action of adenosine are the reason that the biphasic effect, both of vibration and of administration of exogenous ATP, consists of excitation (putative effect of ATP) followed by depression (putative effect of adenosine)[2]. These time delays will be

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[2]It might be questioned whether in fact the proposed steps can occur rapidly enough to account for the onset of the depressant component which appears to occur within less than 5 ms in some cases (Chapter 3). The kinetic studies required to answer this question have not been done. However, in the retina, hydrolysis of cyclic GMP, which is due to a multi-step cascade after photoexcitation of rhodopsin, appears to proceed in about 1 ms (Vuong et al., 1984). Thus, it is clearly possible for biochemical reactions to occur rapidly enough to account for the onset of the depressant component.

seen as important, as well, when considering cases in which vibration causes only depression.

Evidence from other studies indicates that 5'-nucleotidase is present at or near to morphologically defined synapses in a number of regions of the brain (Kreutzberg et al., 1986). Thus, it seems not unreasonable that this might also be the case in the dorsal horn. However, in view of the fact that the model does not require an immediate action by adenosine, it is not critical that the conversion of ATP to adenosine occurs within a synaptic cleft. It is only necessary that the conversion takes place in a location such that adenosine can diffuse to receptors which, for reasons described in the next section, are believed to be present on the postsynaptic neurone.

e) *Adenosine then acts on a postsynaptic receptor which is different from the type acted upon by ATP.* Studies in many tissues have suggested that effects of adenosine are receptor-mediated as they are dose-dependent and are antagonized by methylxanthines and their derivatives (for reviews see Burnstock, 1978; Daly, 1982; Patel and Marangos, 1984). In the model, it is suggested that the effect of adenosine is mediated by activation of a specific membrane component. This suggestion is made in light of the fact that several groups working with peripheral and central nervous system tissues have isolated a single membrane glycoprotein which is labelled by photoaffinity analogues of adenosine (Choca et al., 1985; Green et al., 1986; Klotz et al., 1986; Stiles et al., 1985).

That the effect of adenosine may be postsynaptic is suggested on the basis of a pharmacological study done using the *in vitro* preparation of the rat spinal cord slice. The results of this study indicate that adenosine causes hyperpolarization of dorsal horn neurones under conditions when synaptic transmission has been blocked by lowering the extracellular calcium concentration (Kangrga et al., 1987). This finding is consistent with reports of postsynaptic hyperpolarizing effects of adenosine on neurones in the hippocampus (Segal, 1982), in the locus coeruleus (Shefner and Chiu, 1986) and in parasympathetic ganglia (Akasu et al., 1984).

A piece of evidence consistent with a postsynaptic localization of receptors acted upon by adenosine in the dorsal horn, is that binding sites for [^{3}H]cyclohexyladenosine, a putative ligand for A_1 subtype of

adenosine binding sites (Bruns et al., 1980), fail to decrease in number following dorsal rhizotomy or spinal transection but do fall in number after injection of kainic acid into the dorsal horn (Geiger et al., 1984). Thus, it may be that these binding sites are located on intrinsic neurones in the dorsal horn. However, whether the binding sites are on dendrites, as suggested in the model, remains to be determined.

The suggestion that the receptor acted upon by adenosine is different from that acted upon by ATP is in keeping with the proposal made by Burnstock (1978) that there are two independent purinergic receptors. This proposal was based on differences in the rank order of potency for adenosine and adenine nucleotides and on the effectiveness of methylxanthines in blocking effects caused by adenosine but not those produced by ATP.

As far as the dorsal horn is concerned, evidence consistent with the suggestion of independent receptors is that the depressant effect of administration of AMP and of ATP, but not the excitatory effect of ATP, appears to be blocked *in vivo* by the P_1-receptor antagonist, theophylline (see Chapter 2). In addition, with dorsal horn neurones *in vitro*, excitatory effects of administration of ATP and slowly-degraded analogues are not blocked by P_1-receptor antagonists (Jahr and Jessell, 1983).

Evidence from binding studies indicates that the dorsal horn may contain more than one subtype of adenosine binding site (Choca et al., 1986) and thus, possibly more than one subtype of receptor. However, the precise subtype of receptor mediating the effect of adenosine in the model is not specified because of the lack of pharmacological evidence concerning the effects of adenosine analogues in the dorsal horn. This lack of evidence makes premature any speculation as to the possible subtype involved in the present case.

f) *Activation of the receptor by adenosine leads to hyperpolarization by increasing conductance to potassium.* Intracellular recordings from dorsal horn neurones in the rat spinal cord slice preparation have indicated that adenosine produces hyperpolarization accompanied by an increase in membrane conductance, the apparent reversal potential being less than -90 mV (Kangrga et al., 1987). This evidence is consistent with that from other studies where adenosine has been found to cause

hyperpolarization by increasing potassium conductance in neurones of the hippocampus (Segal, 1982; Greene and Haas, 1985) and of the parasympathetic ganglion (Akasu et al., 1984), and in atrial muscle of the heart (Jochem and Nawrath, 1983). Therefore, a similar effect of adenosine is postulated in the model.

As an aside the possibility will be discussed that the proposed adenosine-mediated effect might be produced through a second messenger system. Adenosine has well-known stimulatory and inhibitory effects on adenylate cyclase (Londos et al., 1980; van Calker, 1979) which may be mediated by stimulatory and inhibitory G proteins, G_s and G_i, respectively (Patel and Marangos, 1984). It has been found that pertussis toxin, which causes inactivation of G_i and of another G protein, G_o, but not of G_s, reverses the adenosine-induced decrease in evoked release of glutamate from cerebellar neurones in culture (Dolphin and Prestwich, 1985). However, this decrease in release of glutamate does not seem to be the result of a decrease in cyclic AMP (Fredholm et al., 1986). It seems possible, therefore, that the adenosine-induced effect proposed in the model, i.e. increased potassium conductance, might be mediated by a G protein but whether the effect is caused by changes in cyclic AMP levels is uncertain.

It is relevant to this possibility that in other systems receptor-mediated activation of potassium conductance appears to occur via a step involving a G protein: for example pertussis toxin blocks the increase in potassium conductance caused by activation of opiate receptors in locus coeruleus neurones (Aghajanian and Wang, 1986), by activation of serotonin and $GABA_B$ receptors in hippocampal neurones (Andrade et al., 1986) and by activation of muscarinic cholinergic receptors in cardiac cells (Pfaffinger et al., 1985; Sasaki and Sato, 1987; Logothetis et al., 1987). Thus, the proposition that the effect of adenosine may be mediated via a G protein is consistent with data from these other systems.

g) *The action of adenosine is terminated by uptake into neurones and/or glia.* For the central nervous system there is an extensive literature suggesting that adenosine is taken up by neurones and by glia (for review see Wu and Phillis, 1984). Most studies of the central nervous system have concentrated on areas other than the spinal cord, but Geiger and Nagy (1984) have reported that the dorsal horn contains binding sites for [^{3}H]nitrobenzylthioinosine, an inhibitor of adenosine uptake. They suggested that the presence of these binding sites in the dorsal horn indicates that there are uptake sites for adenosine. Hence, carrier-mediated uptake of adenosine has been included in the model.

As the action of adenosine postulated in the model requires that the

nucleoside be located extracellularly, uptake of adenosine would thus lead to termination of its action.

v) *Responses to repeated activation of the Pacinian afferent*

The model illustrated in Fig. 1 applies to the situation of a single action potential in a Pacinian afferent. In the experiments reported in this thesis the parameters of vibrational stimulation were such that repetitive activation of Pacinian afferents would be expected (Hunt, 1961; Talbot et al., 1968). Using the model the response of BPH_1 neurones to the vibrational stimulation (for example see Fig. 3 in Chapter 5) can be explained as follows: the first action potential in the Pacinian afferent causes release of ATP with the ensuing excitation; the excitation is large because too little adenosine is present to have an observable effect. The Pacinian afferent continues to be activated by vibration and hence ATP is repeatedly released. At the same time, however, adenosine is being formed by conversion of ATP and so a relatively steady state may develop where the excitation caused by ATP is offset by the inhibition caused by adenosine. In the case of BPH_1 neurones, it is suggested that the inhibition by adenosine is able to more than compensate for the ATP-induced excitation and thus the rate of firing during the steady state is less than the baseline level of discharge.

vi) *Lack of presynaptic effects in the model*

The model focuses specifically on postsynaptic effects because these can fully account for the observations made concerning the response of BPH_1 neurones to vibration. Therefore, addition of presynaptic effects to the model seems superfluous. This is not to say, however, that there is no evidence that vibration might also produce presynaptic effects. Indeed, it has been found that Pacinian afferents cause an increase in the excitability of the terminals of primary afferent neurones (Jänig et al., 1968). This observation suggests that vibration may be causing primary afferent depolarization which is associated with presynaptic inhibition (Eccles, 1964). However, on the basis that adenosine fails to change the dorsal root potential in the toad spinal cord (Phillis and Kirkpatrick, 1978) and fails to alter the membrane potential of mammalian dorsal root ganglion neurones (Dolphin et al.,

1986; MacDonald et al., 1986), it seems unlikely that the increased excitability of the terminals is caused by adenosine. On the other hand, the evidence that P_1-purinergic receptors block and that an adenosine uptake inhibitor enhances vibration-induced depression suggests that this depression is caused by adenosine (Chapter 4). Thus, there appears to be a different chemical basis for vibration-induced depression and for primary afferent depolarization. Accordingly, it seems unnecessary to include primary afferent depolarization in the model.

The results presented in this thesis have not ruled out the possibilities that vibration might cause presynaptic inhibition of primary afferents through a mechanism different from primary afferent depolarization or that it may cause presynaptic inhibition of excitatory, non-primary afferents inputs.

vii) *Areas of the model which require further investigation*

One purpose of developing a model for the vibration-induced response is that it can be used to make predictions which may then be tested experimentally. Some predictions reveal how the model accounts for observations reported in this thesis. For example, on the basis of the model, one would predict that the depression of firing rate caused by vibration will be blocked by antagonists of P_1-purinergic receptors, as was reported in Chapter 4. On the other hand, there are clearly many predictions which have not been examined in this thesis or in other studies. Certain of these predictions appear to be amenable to investigation using presently available techniques. For example, one would predict that the depressant component of the response to a single action potential will be mediated by membrane hyperpolarization due to an increase in potassium conductance. It might be possible to test this prediction by recording intracellularly from dorsal horn neurones *in vivo* and determining whether or not vibration-induced hyperpolarization, such as that observed by Brown et al. (1987), is associated with changes in membrane properties consistent with an increase in conductance to potassium. Other predictions which might be tested are that vibration-induced excitation will be due to an increase in membrane conductance to sodium, and that the vibration-induced depression but not the excitation will be blocked by agents which inactivate G proteins.

Unfortunately, one of the most difficult predictions to test is also one of the cornerstones of the model: ATP will be released from the central terminals of Pacinian afferents. Three reasons prompt the suggestion that this prediction will be difficult to test. First, even when the most sensitive and accurate techniques presently available for measuring release of ATP are used, the exact source of the released nucleotide is virtually unknown; the most that can be determined is whether or not the release depends on the presence of calcium. [It will be recalled that the release of ATP from dorsal horn synaptosomal preparations is calcium-dependent (White et al., 1985) and so this minimum criterion has been satisfied.]

Second, it is possible that the primary method presently used to rule out release of substances from terminals of primary afferents (i.e. dorsal rhizotomy of a small number of segments with time allowed for the terminals to degenerate before testing for release) may not adequately eliminate terminals of Pacinian afferents. This possibility is raised because a single Pacinian afferent has arbors which extend up to 1.5 mm in the rostro-caudal direction in the spinal cord (Semba et al., 1984). Furthermore, arbors come off along the course of both of the main branches of the afferent, the one which runs rostrally to the dorsal column nuclei and the one which runs caudally for many segments from the site at which the afferent enters the spinal cord (Brown, 1981).

Third, it will likely be necessary to do the most relevant experiments *in vivo* because it is important to activate specifically Pacinian and not other afferents and because specific activation of functionally defined groups of primary afferents cannot be accomplished using the presently available *in vitro* preparations. However, at present, reliable measurement of release of ATP can only be performed *in vitro*. Thus, it may be necessary to develop new experimental approaches before one can reasonably decide whether or not Pacinian afferents release ATP from their central terminals.

viii) *How to account for the other types of vibration-induced responses*

The model represented in Fig. 1 applies only to the response of BPH_1 neurones. An important question, then, is whether it is possible to use the model to account for the three other types of vibration-induced

responses observed during the course of this thesis. These three additional types are as follows: a) the biphasic response of neurones in group BPH_2, b) depression and c) excitation. The applicability of the model to these types of responses is discussed in the following sections.

a) *Biphasic response of BPH_2* neurones. Neurones in group BPH_2 showed a biphasic response to vibration but, as discussed in Chapter 5, in these cases vibration may have been activating an excitatory input to these neurones in addition to the input from Pacinian afferents. As Pacinian afferents appear to mediate both the biphasic response of BPH_1 neurones and the depressant response of DPR neurones, it is possible that, had Pacinian inputs to neurones in group BPH_2 been specifically activated, these neurones would have shown one of the responses seen in the other two groups. Therefore, group BPH_2 will not be discussed any further.

b) *Vibration-induced depression.* Depression was observed with wide dynamic range neurones in group DPR and with nociceptive specific neurones. In the case of the neurones in group DPR, evidence was presented in Chapter 5 consistent with the possibility that the vibration-induced depression of these neurones, like the biphasic effect seen with BPH_1 neurones, is mediated by Pacinian afferents. In addition, the pharmacological evidence presented in Chapter 4 suggests that vibration-induced depression and the depressant component of the biphasic response are both mediated by adenosine. The similarities between depressant and biphasic responses to vibration make it seem reasonable to use, if possible, the model developed for BPH_1 neurones to explain the response of DPR neurones.

A major difference between the depressant response of DPR neurones and the biphasic response of BPH_1 is that the excitation at the onset of vibration which occurs with BPH_1 neurones is absent from the response of DPR neurones. Thus, it is possible that DPR neurones lack an excitatory input from Pacinian afferents, which is present with BPH_1 neurones. This possibility is also suggested by the finding that as stimulation frequency increases there is a greater decrease in the number of spikes during vibration with the DPR group than with the BPH_1 group (see Fig. 8 in Chapter 5).

The following simple modification of the model for the response of

BPH_1 neurones can account for the apparent lack of excitatory input from Pacinian afferents with DPR neurones: DPR neurones may lack functional receptors which can be activated by ATP, or at least these receptors are not present where they can be activated by ATP released from the Pacinian afferents. Using this modification of the model one would predict that the synaptic delay for the inhibition will be longer than for excitation; this prediction is in agreement with previous results (Hongo et al., 1968; Chapter 3 of this thesis). The longer synaptic delay for the inhibitory response is predicted on the basis of the time required for the formation and/or the action of adenosine. Thus, it is possible that DPR neurones may have monosynaptic inhibitory connections with Pacinian afferents.

The possibility that DPR neurones may lack functional receptors which can be activated by ATP is consistent with the observation made repeatedly, both *in vivo* (Fyffe and Perl, 1984; Chapter 2) and *in vitro* (Jahr and Jessell, 1983), that there are spinal dorsal horn neurones which fail to be excited by application of ATP. Importantly, it has been possible to demonstrate that there are wide dynamic range neurones in the dorsal horn which are only depressed by ATP applications (see Chapter 2).

Nociceptive specific neurones also showed only depressant responses to vibration and to application of ATP. Thus, it is possible that these neurones lack receptors mediating ATP-induced excitation. Further parametric studies will be required, however, to examine the possibility that the vibration-induced depression of nociceptive neurones is due to activation of Pacinian afferents because this possibility was not specifically investigated during the course of this thesis. If this possibility is verified then the same modifications of the model used to explain the depressant response of DPR neurones may be applicable to nociceptive specific neurones.

c) *Vibration-induced excitation.* All non-nociceptive neurones were excited by vibration. However, it is not possible to decide which class or classes of primary afferents mediate this excitation because the necessary parametric studies have yet to be done. Nonetheless, it is possible that, because of their sensitivity to vibration, Pacinian afferents might mediate at least part of the excitatory response. In

light of this possibility and the possibility that Pacinian afferents may release ATP, it seems significant that excitation was the predominant response to application of ATP (Chapter 2). The paucity of depressant or biphasic responses of non-nociceptive neurones to ATP was not likely due, however, to lack of P_1-receptors because these neurones were depressed by application of AMP. Thus, a possible explanation for the predominance of excitatory effects both of exogenously applied ATP and of vibration is that the ecto-enzymes required for conversion of ATP to AMP might not be associated with non-nociceptive neurones in such a way as to allow for activation of the P_1-receptors following release of ATP: for example, the enzymes might not be present in the vicinity of the non-nociceptive neurones. Consistent with this possibility are anatomical studies which have indicated that the distribution of ecto-Ca^{++}-Mg^{++} ATPase follows that of terminals of small diameter primary afferents in the superficial dorsal horn (Dodd et al., 1983; 1984); these terminals are not likely closely associated with non-nociceptive neurones.

Excitation of wide dynamic range neurones was observed in a small number of cases when the probe of the stimulator was placed outside the low threshold area of the receptive field and in a larger number of cases when the probe was inside this area (see Chapter 5). As discussed in Chapter 5, this excitation may have been caused by non-Pacinian excitatory inputs which were greater than depressant or biphasic inputs, if present, from Pacinian afferents. Thus, these cases may have been similar to that of the BPH_2 neurones except that the additional excitatory input may have had a smaller magnitude in the BPH_2 group.

In summary, it appears that with minor modifications, the model presented for the biphasic response to Pacinian input can be used to account for depressant and for possible excitatory responses to this type of input. Further experiments are required, however, to determine if excitatory responses to vibration, in particular those of non-nociceptive neurones, are mediated by Pacinian afferents.

II. POSSIBLE CLINICAL SIGNIFICANCE OF THE RESULTS OF THIS THESIS

The studies which led to this thesis were undertaken with the hope that new information about the properties and behaviour of dorsal horn neurones might provide a scientific basis for future advancements in the rational therapy for pain. The results of these studies provide, I believe, relevant new information which may be useful in developing novel strategies for alleviation of pain in humans.

For me, the most important aspect of this thesis in terms of possible clinical significance, is the discovery that spinal nociceptive neurones are depressed by purines, in particular adenosine. This depression is observed with the apparent release of endogenous purines, as in the case of vibration-induced depression, and with the administration of exogenous purines, as in the case of depression caused by iontophoretic application of ATP or AMP. Thus, it is possible that release of endogenous purines at the spinal level or administration of exogenous agents which act to mimic or to enhance the action of the endogenous substances, may cause analgesia in humans[3].

In the following sections specific examples of possible analgesic effects due to adenosine will be examined.

i) *Vibration-induced analgesia may be mediated by adenosine*

The results presented in Chapter 4 suggest that adenosine may mediate the depression of the rate of discharge caused by vibration. In view of the finding that nociceptive neurones rather than non-nociceptive neurones are depressed by vibrational stimulation (Chapter 3) it is suggested that the analgesic effects of vibration (Ekblom and Hansson, 1982; Lundeberg, 1984; Lundeberg et al., 1984a,b; Pertovaara, 1979; Sullivan, 1968; Wall and Cronly-Dillon, 1960) may be mediated at the spinal level by adenosine.

This possibility may be amenable to testing in clinical trials as it appears possible to safely administer the P_1-receptor antagonist,

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[3] These conclusions follow from the observations concerning the pharmacological properties of the vibration-induced depression and the depression caused by iontophoretic application of ATP and AMP. Thus, these conclusions do not depend on validity of the model presented in section I.

caffeine, at doses in the lower end of the range which cause significant attenuation of the vibration-induced depression of neuronal rate of firing, i.e. 10-20 mg/kg. This dose of caffeine, which represents the equivalent of 8-15 cups of coffee (Barone and Roberts, 1984), is well below the minimum fatal dose of 57 $mg \cdot kg^{-1}$ reported by Peters (1967) in an extensive review of the literature. In addition, at this dose the level of caffeine would be expected to be in the range where caffeine acts selectively as a P_1-antagonist (see Discussion in Chapter 4).

If caffeine is found to block the analgesic effects of vibration in humans then two clinically relevant suggestions may be made. First, that patients receiving vibration for pain relief should likely refrain from consumption of caffeine and other methylxanthines. [In this context it is important to note that methylxanthines are the most commonly consumed pharmacological agents (Rall, 1980).] Second, that drugs which block adenosine uptake or degradation or which otherwise potentiate the action of adenosine might be useful for increasing the efficacy of this type of treatment.

With regard to the latter suggestion, it may be possible in the future to develop tachykinins or analogues of tachykinins which can be used as adjuvants to promote the efficacy of vibration as an analgesic. This possibility is raised because tachykinins appear to enhance the depressant effect of vibration (Chapter 7). It is unlikely that the presently known tachykinins would be useful for this purpose as they have been found to increase the activity of nociceptive neurones (for example Henry, 1976) and to facilitate nociceptive reflexes (Yashpal et al., 1982). Thus, these tachykinins would likely cause pain or allodynia if administered, for example, at the spinal level in humans. It seems that it would be necessary, therefore, to develop other tachykinins or tachykinin analogues which do not excite nociceptive neurones but which do enhance vibration-induced depression.

The additional possibilities that the analgesic effects of electrical stimulation of peripheral nerves and of dorsal columns may be mediated by adenosine are suggested for reasons described fully in Chapter 4. A clinical protocol to investigate the former possibility is presently being established.

ii) *Analgesics which exploit the adenosine system*

The conclusion made in Chapter 2 that activation of P_1-receptors decreases the rate of discharge of nociceptive neurones raises the possibility that drugs which activate or cause activation of this type of receptor or which enhance the action of adenosine, that is drugs which act through the "adenosine system", may be useful as analgesics. The possibility that these types of drugs might be used as analgesics is consistent with behavioural data indicating that in experimental animals, nociceptive reflexes are depressed by administration of adenosine analogues which activate P_1-receptors (Ahlijanian et al., 1985; Holmgren et al., 1983; Post, 1984; Yarbrough and McGuffin-Clineschmidt, 1981). Therefore, it seems possible that in the future drugs which act through the adenosine system may be used as analgesics and, in addition, that the analgesic effects of some drugs used at present may be the result of their action on this system.

An example of a currently used drug whose analgesic effects may be caused by actions on the adenosine system is the tricyclic antidepressant, amitryptiline. The major metabolite of amitryptiline is another antidepressant, nortryptiline. These two drugs are used in the treatment of chronic pain (Feinmann, 1985; Gomez-Perez et al., 1985) and they appear to be especially useful for the pain of post-herpetic neuralgia (Taub, 1973; Watson et al., 1982; Woodforde et al., 1965). However, the analgesic action of amitryptiline, at least in post-herpetic neuralgia, appears to be independent from its antidepressant effect (Taub, 1973; Watson et al., 1982). Tricyclic antidepressants are known to cause inhibition of the re-uptake of serotonin (for review see Baldessarini, 1985) but their analgesic action likely does not depend on this property because the antidepressant, zimelidine, which is a more potent blocker of serotonin re-uptake than amitryptiline, does not produce analgesia (Watson and Evans, 1985). In contrast to this finding, nortryptiline has been found to be a potent inhibitor of adenosine uptake whereas zimelidine is a weak adenosine uptake inhibitor (Phillis and Wu, 1982). These data are consistent with the possibility that the analgesic but not necessarily the antidepressant effects of amitryptiline and nortryptiline may be mediated by inhibition of adenosine. Interestingly, the pain of post-herpetic neuralgia appears to be particularly amenable to treatment by cutaneous

vibration (Haas, 1977; Loeser, 1986; Russell et al., 1957; Todd et al., 1965) and by transcutaneous stimulation of peripheral nerves (Long and Hagfors, 1975).

The possibility that drugs which are known to act on the adenosine system may be analgesic has been considered in the case of dipyridamole, which is a potent inhibitor of adenosine uptake (Wu and Phillis, 1984). Currently a preliminary, uncontrolled study is being made on the basis of the ideas put forward in this thesis. Results have been obtained thus far for 11 patients, all of whom have had prolonged pain problems refractory to standard forms of therapy. These results are encouraging because 7 of the patients reported significant pain relief when taking dipyridamole (H. Merskey, personal communication). Quite clearly, further controlled studies are necessary to verify these preliminary results and to determine whether dipyridamole will be useful as an analgesic.

In conclusion, it is hoped that the results of this thesis may lead to development of drugs which exploit the adenosine system for the treatment of pain.

CLAIM OF ORIGINALITY

The results and conclusions presented in this thesis are original and have not appeared elsewhere except as specifically mentioned in the text and the Preface. The principal original contributions arising from the present studies are briefly outlined in the following paragraphs. The specific details appear in the respective chapters.

In Chapter 2 an investigation was made of the effects of iontophoretic application of the purines, ATP and AMP, on functionally identified, dorsal horn neurones. A pattern of differential effects of ATP, but not of AMP, was found which prompted the suggestion that ATP might be a chemical mediator released by low threshold primary afferent neurones.

This suggestion led to an examination, described in Chapter 3, of the effects of cutaneously applied vibration on functionally identified, dorsal horn neurones. Non-nociceptive neurones were excited by vibration regardless of the location of the stimulus. In contrast, vibration excited WDR neurones when it was applied to their low threshold receptive field but elicited depression or a biphasic (excitatory/depressant) response when applied outside this field. Nociceptive specific neurones showed only depression.

In Chapter 4 the chemical basis of the vibration-induced depression and of the depressant component of the biphasic response of WDR neurones was studied. Through the use of various pharmacological tools the conclusion was made that these two types of depressant response are caused by the same chemical mediator, adenosine, acting through P_1-purinergic receptors.

The physiological properties of the vibration-induced responses of WDR neurones were investigated in Chapter 5. The parametric studies reported in this chapter led to the conclusion that the vibration-induced depression and, in some cases, the biphasic response are mediated by Pacinian corpuscle afferents.

During the course of the studies reported in Chapter 2 and other studies which were being done concurrently, the observation was made that the depressant effects of administration of ATP are enhanced by a peptide, the tachykinin physalaemin. As reported in Chapter 6, a thorough investigation of this phenomenon was made which indicated that depression

caused by administration of ATP or of AMP is enhanced by physalaemin and by the two other tachykinins studied, substance P and neurokinin A. The effect of the tachykinins may be specific for purine-induced depressions as the depression caused by GABA was unaffected. A peptide related to the tachykinins, bradykinin, failed to have the same effect.

Reasoning by syllogism from the results of Chapters 4 and 6, the possibility was considered that tachykinins might enhance vibration-induced depression. This possibility was confirmed by the results reported in Chapter 7.

From evidence presented in this thesis and that available in the literature, a model was synthesized to account for effects of vibration. This model is discussed in Chapter 8. This chapter also includes discussion of the possible clinical implications of the material presented in this thesis.

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