THE INVOLVEMENT OF SECOND MESSENGERS IN HIPPOCAMPAL ACTIVITY AND SEIZURE

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September 1990

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment for the degree of Doctor of Philosophy.

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My ever loving parents

Hayk and Bercuhi Agopyan

whose dedication, example,

sacrifices,support....

made possible the realization

of the work reported here.

"Science demands much greater sacrifices. It does not permit any sharing. It demands that certain men devote to it their whole existence, their whole intelligence, their whole labour."

Charles Richet

ABSTRACT

This thesis focusses on the seizure-promoting effects of second messengers, in particular calcium and others activated by calcium in CA1 subfield of rat hippocampus in situ and in vitro. Interictal discharges were simulated in hippocampal slices using the low Ca^{2+} model where synaptic transmission is blocked. The antiepileptic drug valproic acid (VPA), which is postulated to potentiate GABAergic responses, reduced the low Ca^{2+} field bursts, thereby suggesting a non-synaptic mechanism of action of VPA. Extracellular and intracellular recordings from hippocampal neurones bathed in low Ca^{2+} solution revealed that even though evoked transmitter release was blocked, small spontaneous GABAergic release still persisted and that a voltage-dependent Cl^- conductance contributed to burst termination. To test the hypothesis that epileptiform activity induced by lowering extracellular Ca^{2+} was in part due to an alteration in the delicate balance between intracellular messengers, I investigated the effects of Ca^{2+} a) on the activity of hippocampal neurones, and b) on the second messengers activated by acetylcholine (ACh), a neuromodulator known for its seizure promoting effects. Inhibiting cyclic nucleotide-dependent kinase activity with H-8 did not have any effect either on excitability of CA1 neurones or the excitatory actions of ACh. Activation of protein kinase C (PKC), a Ca^{2+}-binding protein, by phorbol esters enhanced high threshold Ca^{2+} currents and subsequently facilitated neurotransmitter release (both excitatory and inhibitory) without significantly affecting ACh-induced effects. Inhibition of PKC by several dual PKC and Ca^{2+} /calmodulin-dependent kinase antagonists, such as H-7, sphingosine, and trifluoperazine reduced inhibitory transmission and prevented ACh-induced effects.

The evidence presented in this thesis was combined with that in the literature to propose a model accounting for the seizure-promoting effects of ACh: that gangliosides and/or sphingolipids are metabolized to sphingosine upon muscarinic receptor activation leading to inhibition of both PKC and Ca^{2+} / calmodulin-dependent kinases.

RÉSUMÉ

Cette thèse se concentre sur les effets épileptogènes des messagers seconds, en particulier le calcium et d'autres messagers activés par le calcium, dans le champs CA1 de l'hippocampe chez le rat *in situ* et *in vitro*. On a provoqué des crises d'épilepsie généralisées en utilisant le modèle de réduction de la concentration de calcium où la transmission synaptique se trouve ainsi bloquée. On a observé que l'administration d'acide valproique (AVP), substance antiépileptique, qui est censée potentialiser les réponses GABAergiques, réduit les bouffées d'activité induites en présence de faible Ca^{2+} extracellulaire, ce qui suggère que l'AVP agit selon un mécanisme non-synaptique. Des enregistrements extracellulaires et intracellulaires de neurones de l'hippocampe baignant dans des solutions à faible teneur en Ca^{2+} ont révèlé que, bien que la libération évoquée de transmetteurs se trouve bloquée, une faible libération GABAergique spontanée persiste et une conductance Cl^- voltage-dépendante contribue à mettre fin aux décharges en bouffée. Pour vérifier l'hypothèse selon laquelle l'activité épileptiforme induite suite à l'abaissement du Ca^{2+} extracellulaire est en partie due à une altération de l'équilibre précaire qui existe entre les messagers intracellulaires, nous avons étudié l'influence du Ca^{2+} a) sur l'activité des neurones de l'hippocampe et b) sur les messagers seconds activés par l'acétylcholine (ACh), un neuromodulateur connu pour ses effets épileptogènes. L'inhibition de la kinase sensible aux nucléotides cycliques avec du H-8 n'a eu aucun effet, ni sur l'activité des neurones du CA1, ni sur les effets excitants de l'ACh. L'activation de la protéine kinase C (PKC), une protéine qui se lie au Ca^{2+}, par les esters de phorbol a augmenté les courants calciques à seuil élevé et, par la suite, facilité la libération de neurotransmetteurs (à la fois excitants et inhibiteurs) sans pour autant affecter de manière significative les effets causés par l'ACh. On a observé une réduction de la transmission inhibitrice et des effets causés par l'ACh suite à l'inhibition de la PKC par plusieurs antagonistes, tels que le H-7, la sphingosine et la trifluoperazine qui antagonisent à la fois la PKC et les kinases activées par le complexe Ca^{2+}/calmoduline.

Nous combinons les données présentées dans cette thèse avec celles de la littérature pour proposer un modèle expliquant les effets épileptogènes de l'ACh, à savoir que les gangliosides et/ou les sphingolipides sont métabolisés en sphingosine suite à l'activation des récepteurs muscariniques, conduisant à une inhibition à la fois de la PKC et des kinases activées par le complexe Ca^{2+}/calmoduline.

PREFACE

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The work reported in a) PART I and b) PART II of this thesis was carried out by the author in:

a) Dr. M. AVOLI's laboratory at Montreal Neurological Institute.

b) Dr. K. KRNJEVIĆ's laboratory at McIntyre Medical Sciences building of McGill university.

ACKNOWLEDGEMENTS

I owe a special dept of gratitude to my research supervisors:

Drs. **Massimo Avoli** and **Kresimir I. Krnjević** for allowing me the opportunity to be in research. Their encouragement, guidance, support and example allowed me to continue my studies and taught me what it takes to be a scientist. I feel very fortunate to have studied with them.

I am grateful to Drs. N. Lake, R. Çapek, B. Esplin and A. Padjen for valuable discussions and encouragement during the course of my studies.

I am indebted to P. Krnjević, J. Leblond, Y. DeKoninck and P. Miu for their expertise with computers.

I would like to extend my gratitude to all the members of the Anaesthesia research and Neurophysiology department with whom I have worked these past years.

I gratefully acknowledge the Savoy Foundation, who provided me with financial support in the form of a studentship throughout the period of my graduate studies, Faculty of Medicine and Students Aid for personal funding during the last part of this work.

To my families and friends for their unending love, support and encouragement. Last but not least to Irma Agopyan and Peter Miu for being a great source of intellectual stimulation and emotional support.

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LIST OF ABBREVIATIONS

Artificial cerebrospinal fluid	ACSF
Afterhyperpolarization	AHP
Acetylcholine	ACh
Bicuculline methiodide	BMI
β-methyl choline	β-MCh
Guanosine 3'5'-monophosphate	cylic GMP
Extracellular calcium concentration	$[Ca^{2+}_o]$
Four aminopyridine	4-AP
Monosialoganglioside	GM1
1-(-5-isoquinoline sulfonyl) 2-methylpiperazine	H-7
N-2-(methylamine)ethyl-5-isoquinoline sulfonamide	H-8
Inositol trisphosphate	IP_3
Extracellular potassium concentration	$[K^+]_o$
Sodium methyl sulphate	NMS
Phorbol -12,13-diacetate	PDAC
4α-phorbol -12,13-diacetate	4α-PDAC
Protein kinase C	PKC
Tetraethylammonium	TEA
Trifluoperazine	TFP
Tetrodotoxin	TTX
Valproic aid	VPA

INTRODUCTION

DEFINITION

The word epilepsy derives from a greek word meaning "to seize" and refers to a patient being "seized" by an epileptic attack as if "possessed" by a demon. It is the clinical manifestation of excessive and/or hypersynchronous, usually self-limited, abnormal activity of neurones in the cerebral cortex. The behavioural features of an epileptic seizure reflect the functions of the cerebral cortical areas where the abnormal neuronal activity originates and spreads; it may consist of impaired higher mental function, altered consciousness, involuntary movements or cessation of movement, sensory or psychic experiences, or autonomic disturbances. However, until one hundred years ago epilepsy was perceived as the sacred disease and regarded as the punishment for sins. In this section I shall try to review the impact of advances in basic neurosciences research on our understanding of the mechanisms of the epilepsies.

HISTORICAL DEVELOPMENTS OF CONCEPTS RELATED TO EPILEPSY

The histories of research on the cerebral mechanisms of epilepsy and neurophysiology are so intimately interwoven that the former can only be dealt with after surveying the latter.

Antiquity

The history of epilepsy is probably as ancient as that of man. The great age of the disease is attested by the descriptions found from Mesopotamian civilizations, 2000 B.C. In the days when diseases were considered as acts of possessions by gods,

demons, or evil spirits and treated by the invocation of supernatural powers, epilepsy was regarded as an omen that had been cast upon the victim by a hostile demon (Temkin 1945; Margetts 1967).

The idea that epilepsy was a disease and not a curse was recorded by an anonymous physician and appeared in Hippocrates' collection of medical writings *On the Sacred Disease*. The author claimed that "epilepsy, like all diseases, is hereditary; its cause lies in the brain overflowing with a superfluity of phlegm. When the phlegm rushes into the blood vessels of the body it causes all the symptoms of the attack. The releasing factors of the attack are cold, sun, and winds, which change the consistency of the brain" (Temkin 1945). Hence, with this fundamental statement, the history of epilepsy entered a new era, where the cause of the disease was recognized to be in the brain, even though it was of humoral origin.

The scientists and physicians of the following centuries accepted the humoral theory, which was traced back to Plato and Hippocrates, with minor variations. Galen in the late second century systemized this theory, which later was simplified further by his followers. Galen believed that all epileptic attacks were due to affections of the brain. Hence he classified epilepsy as 1) true i.e. *idiopathic* epilepsy, which arose in the head without known trauma or disease; and 2) *sympathetic* epilepsy, i.e. convulsions induced secondarily by diseases of the body while the brain itself was healthy. According to Galen, in epilepsy, a thick humor, gathered in the cerebral ventricles and blocked the passage of psychic pneuma. The generalized convulsions were than produced by the shaking of the origin of the nerves. This shaking was a biological

reaction to the blockade in the brain, patterned after the human desire to rid itself of an irritation (Temkin 1964). In Galen's theory convulsions indicated the origin of irritation such that a) when the whole body, including the facial muscles, convulsed the brain was affected; b) when the whole body with the exception of the facial muscles convulsed all the nerves below the face were affected and; c) when convulsions occurred in an isolated part of the body, the corresponding nerve was affected. Convulsions preceded by a distinct aura or by local spasms without loss of consciousness were thought to be peripheral in origin. Thus the aura indicated the cause and location of the pathological process rather than the onset of an attack of cerebral origin. The writings and the teachings of this early Greek school became a dogma until the intellectual reawakening of the Renaissance, and the birth of scientific methods of study of the nervous system in the nineteenth and early twentieth centuries.

The Renaissance

During the sixteenth century clinical observations led to the establishment of theories, which, in spite of many errors and imperfections, were preserved until the middle of the seventeenth century. Most of the sixteenth century physicians were still under the influence of Greek school. They believed that only in *idiopathic* epilepsy was the brain primarily influenced. In *sympathetic* epilepsy, i.e. whenever an aura was located outside the head, the starting point of epilepsy was considered to be liver, spleen, kidneys and other organs.

In late sixteenth and early seventeenth century Charles le Pois (1563 - 1636)

came up with a doctrine that did away with the concept of *idiopathic* and *sympathetic* epilepsy. He was the first to state that all epilepsies are of cerebral origin, and that the auras were due to an affection of the central nervous system. However, his immediate contemporaries adhered to different views and his ideas were felt only later (Temkin 1971). Renaissance physicians accepted the hypothesis that epilepsy was due to an irritation of the brain by some poisonous substance.

The latter part of the seventeenth century developed some theories of epilepsy which took into account the new discoveries in chemistry and physics. By this time it had been demonstrated that stimulation of the spinal nerves with a knife excited the muscles and that irritation of the nerves caused slight and transient convulsions. The physicians and scientists were divided into two schools; those who believed in a) the chemical, and b) the physical nature of epilepsy. The differences between the two schools,though striking in many instances, were not fundamental in nature.

Thomas Willis (1622 - 1675), from the chemical school, made it clear that epilepsy originated in the brain. For him the muscular motion was brought about by an explosion. Thus he proposed that the animal spirits, which lay in the middle of the brain, exploded under the influence of a strong spasmodic copula, which was distilled from the blood into the brain. According to Willis this caused all the mental symptoms of the epileptic attack, and a series of similar explosions occurring along the rest of the nervous system gave rise to the convulsions of the body (Temkin 1971). Willis also pointed out that often the cause of the auras lay in the brain.

Malpighi (1628 - 1694), from the mechanical school, came up with the folowing

hypothesis: " cerebral cortex was a conglomeration of glands which under the influence of a constant contraction of the brain, excreted nervous spirits or juice, first into the fibers of the brain, and hence into the nerves, muscles, and membranes. The result was a moderate tension of the fibers of the brain, so that the waves arriving from external objects and communicated by the sense organs were presented to the soul. At the same time clefts were neld open in the muscles, into which humors could flow and effect the tension of the muscular fibers. In epilepsy, however, vitriolic and arsenical particles reached the brain, affected the nervous juice, and irritated the cerebral fibers, so that their normal tension was changed into a spasm. Consequently, the nerves drew back, the clefts in the muscles stayed open, the soul lost its power, and both senses and movements were impaired " (Temkin 1971).

The mechanical theory of epilepsy reached its extreme with Baglivi. Baglivi concluded that the fibers of the dura contracted autonomously to distribute the nervous fluid, which supplied tonus and a kind of oscillating movement, over all parts of the body (Temkin 1971). He hypothesized that epilepsy and any other nervous disease were induced by the interruption of the flow of the nervous fluid resulting from the irregular movement of the dura matter under the influence of some violent agent.

Thus far the theories put forth were very speculative and none of them proved satisfactory. Very little was known about the structure and physiology of the brain, meninges, nerves, muscles and chemistry.

Towards the end of the seventeenth century and during the eighteenth century the mechanical theory was replaced by theories emphasising the teleological principle

in the explanation of biological processes. The general idea was that " the causes of the epileptic seizures do not come from outside but lie inside the human organisms; its true architects are sufferings of the soul brought about by anxious and terrifying prefigurations " (Temkin 1971).

The Nineteenth Century and Hughlings Jackson

The concepts dominating the pathology of epilepsy around 1860 were subivided into a) reflex action, b) cerebral angiospasm, and c) changes in the molecular state of the brain through malnutrition or poisoning. In all these theories several parts of the central nervous system were believed to be affected either simultaneously or in succession. However, in none of them was the cause of epilepsy attributed to definite structural changes.

After Marshall Hall (1790 - 1857) introduced the reflex theory, it became a widely accepted hypothesis for the explanation of convulsions seen in epilepsy. It had been agreed by many physiologists that movements could be obtained from the basal ganglia and particularly the medulla oblongata, but not from the cerebral hemisphere. Hence, Hall explained epilepsy as being " of two kinds: the first has a centric origin in the medulla itself; the second is an affection of the reflex function, the exciting cause being eccentric, and acting chiefly upon the nerves of the stomach or intestines, which consequently form the first part of the reflex arc" (Hall 1841). Even though, the reflex theory seemed to explain the convulsions it could not account for the loss of consciousness manifested during a seizure. In an attempt to explain the loss of

consciousness it was proposed that blood supply to the part of the brain responsible for consciousness was changed during an epileptic attack. In 1836, Astley Cooper supported this hypothesis by showing that temporary anemia generated by occlusion of carotid and vertebral arteries induced loss of consciousness and convulsions (Cooper 1836). The reflex theory was established further with Brown-Séquard (1817 - 1894), who experimented on the spinal cord of animals, particularly of guinea pigs. He showed that transverse section of the spinal cord induced epileptiform convulsions (Brown-Séquard 1857). His findings led him to believe that certain parts of the cerebro-spinal axis had an increased reflex excitability, so that any slight irritation could lead to the beginning of an attack. Brown-Séquard explained unconsciousness by proposing that the blood vessels of the brain and the face were contracted as soon as the sympathetic nerves were irritated following the excitation of spinal cord and the base of the brain.

Kussmaul and Tenner (1859), who mainly studied the epileptic convulsions following profuse hemorrhage, reached the conclusion that general convulsions following hemorrhage had their origin within the cranium and were brought about by contraction of the blood vessels. Experiments performed after removal of certain parts of the brain convinced them that these convulsions proceeded from " the motor centers situated behind the thalamic optici " rather than the spinal cord. They also believed that sudden interruption in the nutrition of the brain underlies the convulsions.

A few British physicians, which formed the minority, strayed away from the accepted theory that medulla was the seat of convulsions. They reached their

conclusions after studying "hemiplegic epilepsy", which has come to be known as "Jacksonian epilepsy". "Hemiplegic epilepsy", which manifests itself with convulsions only of one side of the body, followed by paralysis of the side affected, was first observed by Bravais and Bright. Bright combined the clinical approach with the anatomical to conclude that the symptoms were associated with local lesions affecting the surface of the brain on the side opposite to the convulsions (Bright 1836). Todd, who also studied "hemeplegic epilepsy", observed that stimulation of the cerebral cortex caused facial muscles to twitch. In contrast to many of his contemporaries, he attributed a primary role (for the psychic component) to the cerebral hemispheres in the development of the epileptic paroxysms (Todd 1849). It was claimed that the fibers from the hemisphere ended in the corpus striatum and that from the corpus striatum a different set of fibers started out to the parts further below (Todd & Bowman 1857; Carpenter 1858). Observations from the pathological anatomy of paralysis led the scientists to accept the corpora striata as the origin of the motor tract, even though it was known that stimulation of these bodies did not provoke motor reactions. In 1861 Paul Broca after studying the case of an epileptic suffering from motor aphasia localized the speech defect to the left third frontal convolution of the brain (Broca 1861). Meanwhile, Samuel Wilks, by reporting that morbid changes in the cortex of the brain accounted for all cases of epilepsy, whether partial or general, extended Bright's and Todd's ideas (Wilks 1866).

Around this period Hughlings Jackson, whose clinical observations led him to anatomy, physiology, and pathology, reported his explanations for "hemiplegic

epilepsy". Knowing that the left middle cerebral artery supplied a) the roots of the olfactory bulb; b) the corpus striatum; and c) the hemispheres, he hypothesised that changes, such as spasms, in the left middle cerebral artery underlay the epileptiform seizures associated with an aura of disagreeable smell, defects of speech, and unilateral convulsions (Jackson 1864). While comparing hemiplegia with "hemiplegic epilepsy", he noted that paralysis in hemiplegia was due to destroyed nerve tissue, whereas in "hemiplegic epilepsy" it was due to exhaustion of the gray matter following the explosive discharges in the nerve tissue. Even though he did not disregard the corpus striatum in favor of the cortex, Jackson suggested that instability of the gray matter of the cerebral convolutions might account for the convulsions (Jackson 1870). Jackson's theory, which accounted for all forms of convulsive fits, rested heavily on speculation.

In 1870 Fritsch and Hitzig discovered the motor area of the hemispheres in dogs; they demonstrated that localized groups of muscles could be irritated by the application of weak electric currents upon a very small region of the hemisphere and that application of stonger currents or prolonged application would lead to convulsions as an after-effect (Fritsch & Hitzig 1870). Then Ferrier's experimental work and anatomical investigations of the conductive fibers supported Jackson's speculations that a) localized convulsions indicated localized injuries of the convolutions and b) convulsions might spread if the discharge involved more and more nervous tissue (Ferrier 1873).

Towards the end of the nineteenth century, the findings of many scientists, such as Sir Charles Sherrington's, provided anatomical precision to Jackson's doctrines. For Jackson the central nervous system consisted of three sensorimotor levels, each

representing "impressions and movements of all parts of the body" (Jackson 1870). The lowest level, which represented the simplest movements, consisted of the spinal cord, the medulla oblongata, and the pons. The middle level, which represented complex movements of all parts of the body, consisted of centres of the Rolandic region, and the ganglia of the corpus striatum. The highest level, which represented all of the most complex movements, was formed by the centres of the prefrontal lobes. Hence he classified seizures according to the level discharges originated from: lowest, middle, and highest level fits. He suggested that connections between cells of the same level, and between different levels, allowed any discharge, wherever it might start, to spread and involve neighboring as well as more distant parts of the nervous system. Thus the more excessive the discharge, the severer the fit. Excessive and swift discharge in the middle level would overcome the resistance of the neighboring cells and cause convulsions over a wide range of the body. A relatively slight discharge from the highest level would result in loss of consciousness and the attack would have the form of the petit mal. On the other hand , an excessive discharge from the highest level would result in a severe epileptic fit with loss of consciousness and violent convulsions. Paralysis and coma seen in post-epileptic states were produced by the diseased cells entering a negative state. The cells which were in a negative state, in addition, would lose their control over lower centers which would enable the latter to discharge excessively. Thus increased tendon reflexes would be observed during paralysis.

A better understanding of epilepsy as to its causes, symptoms, and treatment came with Gowers' book, which first appeared in 1881. He concluded that " all the

phenomena of the fits of idiopathic epilepsy may be explained by the discharge of grey matter. The hypothesis of vascular spasm is as unneeded as it is unproved. There are no facts to warrant us in seeking the seat of the disease elsewhere than in the grey matter in which the discharge commences. This is in most cases within the cerebral hemispheres, probably often in the cerebral cortex, although possibly in some instances lower down, even in the medulla oblongata. Epilepsy is thus a disease of grey matter, and has not any uniform seat. It is a disease of tissue, not of structure. " (Gowers 1881). Gowers also speculated that the epileptic discharge was the result of a sudden decrease in "resistance", which allowed a sudden release of "nerve force" (Gowers 1881). Thus with Jackson and Gowers, modern epileptology was founded.

The Twentieth Century

Following Jackson's and Gower's reasoning the task was to understand the mechanisms underlying the "explosive discharge" of neurones in the epileptic focus. However, the proof of the idea that epilepsy is an excessive discharge of nerve cells had to wait until the technique for recording electrical signals improved. The most significant developments were the introduction of a) electroencephalography (EEG) in early 1930s (Berger 1929) b) single unit recordings and c) intracellular recording in late 1940s (Graham & Gerard 1946; Ling & Gerard 1949; Nastuk & Hodgkin 1950). However, both practical and ethical difficulties prevented the detailed investigation of the electrophysiology of single neurones within the epileptic human brain. Hence several animal models (for a recent review see Schwartzkroin & Wheal 1984), were

devised for both generalized and partial (focal) epilepsy.

MODELS

Experimental models of epilepsy may be roughly characterized as acute and chronic. Acute models were induced by a) systemic administration or topical application of convulsants, b) electrical stimulation, and c) metabolic or ionic disturbances which produced transient epileptiform activity. These models were used to investigate mechanisms of development, maintenance and termination of epileptiform activities. Chronic models either occured spontaneously in genetically epileptic animals or were induced by either permanent structural lesions or repetitive electrical stimulation of the brain. A brief description of some of the commonly used experimental models (for a list see table I) will be given prior to reviewing the contributions made towards the understanding of mechanisms underlying epilepsies.

ACUTE MODELS:

An almost endless variety of agents, when applied directly to the surface of the brain, can produce convulsions (for a review see Craig & Colasanti 1987; Prince 1972; Ward 1972). Partial seizures are produced when such agents are applied to localized areas of the brain and generalized seizures when applied to bilateral or diffuse areas.

Partial Seizures:

Penicillin-induced epileptic focus:

Soon after the discovery of penicillin, it became evident from clinical observations that the antibiotic was a convulsant agent when applied directly to human brain (Johnson & Walker 1945). Walker & Johnson (1945) subsequently demonstrated in a systematic study that commercial penicillin applied topically to the neocortical surface of cats, dogs, monkeys and humans induced electrographic and behavioural manifestations of focal seizures. Since then it has become the most studied acute partial seizure model (Matsumoto & Ajmone-Marsan 1964a and 1964b; Prince 1968).

Generalized seizures:

Systemic or intracerebroventricular injections of most of the focal epileptogenic agents also produce generalized seizures. These generalized seizures were used as models of either human petit mal absences or generalized tonic-clonic convulsions. Generalized seizures are commonly produced in mice or rat by maximal electroshock administered to both ears or both eyes (Swinyard 1972). Seizures that were considered experimental petit-mal -consisting of behavioural arrest and occasional twitching in association with bilaterally synchronous spike and wave EEG discharges- were produced by systemic pentylenetetrazol (Woodbury 1972), γ-hydroxybutyrate (Snead 1978), intracerebroventricular enkephalin (Urca et al. 1977), and intramuscular penicillin injection (Prince and Farrell 1969).

Feline generalized penicillin epilepsy model:

In 1969, Prince and Farrell found that intramuscular injection of a large dose of

penicillin (200 000 to 400 000 IU/kg) induced bilaterally synchronous spike and wave discharges, in cat. This model has been used very extensively to study the mechanisms underlying petit mal epilepsy (Gloor et al. 1977; Quesney et al. 1977; Avoli & Gloor 1982).

CHRONIC MODELS:

The human epileptic disorders are more faithfully reproduced with chronic models rather than acute models. However, since too many variables are altered simultaneously, chronic models are more difficult to bring under adequate experimental control. Chronic models can also be subdivided into those that give rise to partial seizures and those that give rise to generalized seizures.

Partial seizures:

Chronic intermittent epileptic focus can be produced by a) partially isolating an area of neocortex -the cortical slab method- (Echlin 1959), b) freezing (Openchowski 1883), c) local application of metals such as alumina cream (Kopeloff et al. 1942), cobalt (Kopeloff 1960; Dow et al. 1962), tungsten (Blum and Liban 1960; Black et al. 1967), iron (Chusid and Kopeloff 1962), d) local application of anti-GM1 ganglioside antibodies (Karpiak et al. 1982), e) cortical or hippocampal injections of tetanus toxin (Mellanby & Hawkins 1986), f) local or systemic injections of kainic acid (Ben-Ari et al. 1981; Tanaka et al. 1982), and g) kindling (Goddard 1967).

Cryogenic-freeze lesions:

One of the first experimental models of epilepsy was described by Openchowski (1883), who applied cold locally to the cerebral cortex. Openchowski's technique was confirmed by Nims and his colleagues in 1941 (Nims et al. 1941) and subsequently modified by Morrell (Morrell 1959), who applied ethyl chloride spray topically to induce freezing of the cortex.

Alumina model:

In 1942, Kopeloff and his co-workers discovered that placement of alumina-filled discs on the pial surface produced seizures (Kopeloff et al. 1942). The original technique has since been modified to subpial alumina injections (Kopeloff et al. 1955) and to applications of alumina hydroxide to the sensorimotor subarachnoid space (Harris 1972, 1975).

Kindling:

Kindling is the phenomenon by which repeated stimulation of parts of the brain results in a progressive and permanent reduction in seizure threshold. A typical kindling stimulus, initially causes little or no response from the animal. As the stimulus is repeated every few hours to days, the response increases progressively through local myoclonus to general convulsions. Kindling brought under control the problem of secondary epileptogenesis, such as the mirror focus (Mayersdorf & Schmidt 1982; Morrell 1959/60) generated in the other hemisphere by a chronic focal epileptogenic lesion. Hence several models have been devised where the stimulus is either electrical (Goddard et al. 1969) or chemical, such as carbachol (Goddard et al. 1969; Vosu & Wise 1975), fluorothyl (Prichard et al. 1969) and pentylentetrazol (Mason & Cooper

1977).

Generalized seizures:

Animal models of chronic generalized seizures include a) mutant mouse models, such as the audiogenic mouse mutant (Seyfried et al. 1986), b) genetically epilepsy-prone rat (Jobe & Laird 1981), c) a rat strain with spontaneous generalized spike and wave discharges and absense seizures resembling those of human petit mal epilepsy (Vergnes et al. 1982), d) the seizure-prone gerbil (Loskata et al. 1974), e) the epileptic beagle (Edmonds et al. 1979), f) the photosensitive baboon species, especially Papio papio (Killam et al. 1966), and g) the photosensitive epileptic fowl (Johnson et al. 1979).

Among all the models, the penicillin-induced epileptogenesis has provided most of our current understanding of seizure mechanisms and the most extensive body of recent literature. However, there has been a remarkable similarity of many of the observations across models.

Table I

Commonly used experimental models of epileptic phenomena

I. Models of Partial Epileptic Phenomena

A: *Neocortical*
1: Electrical stimulation (acute)
2: Topical convulsant drugs (e.g., penicillin, bicuculline, picrotoxin, pentylenetetrazol)
3: Partially isolated cortical slab
4: Freeze lesion
5: Metals (e.g., alumina, cobalt, tungstic acid, ferric chloride
6: Neocortical kindling

B: *Limbic*
1: Electrical stimulation
2: Metals
3: Intracerebral and systemic kainic acid
4: Amygdala and hippocampal kindling

II. Models of Generalized Epileptic Phenomena

A: *Convulsive type*
1: Maximal electroshock seizure
2: Maximal pentylenetetrazol seizure
3: Maximal flurothyl-induced seizure
4: Maximal audiogenic seizure
5: Hyerthermia in immature animals

B: *Petit Mal type*
1: Systemic pentylenetetrazol
2: Feline generalized penicillin
3: Intracerebroventricular opioids
4: Systemic γ-hydroxybutyrate
5: Tetrahydroxyisoxosolopyridine model
6: Subcortical stimulation, lesions and convulsant drugs
7: CO_2 withdrawal seizures
8: Repetitive stimulation of cat spinal cord

C: *Genetic types*
1: Papio papio baboon
2: Audiogenic mice
3: Genetically epilepsy prone rat
4: Seizure-prone gerbil
5: Other mutant mouse models (e.g. totterer, reeler
6: Beagle dog
7: Epileptic fowl
8: Spontaneous spike and wave rat model

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THEORIES ON CELLULAR MECHANISMS OF EPILEPSY

Electroencephalography and the interictal spike

In 1929, motivated by an interest in psychic manifestations of brain function, Berger recorded uncontaminated rhythmic brain waves (Berger 1929) and found the characteristic high voltage and short duration discharges (the epileptic or interictal spike) in epileptics (Berger 1931). In 1935 Adrian, who probed the central nervous system in search for generators of bioelectric potentials, put forward the hypothesis that the electoencephalographic waves recorded from the surface of the cortex or the skull result from the summation of potential transients of excitable neuronal elements within or just below the cortex (Adrian & Yamagiva 1935). Also in 1935 Gibbs and his colleagues discovered in man, during absence attacks, the generalized spike and wave discharges, which consisted of an alternation between a brief polyphasic spike of usually less than 100 ms duration and a dome-shaped negative slow wave of about 300 ms duration recurring at a frequency of approximately 3 Hz (Gibbs et al. 1935). By recording simultaneously cortical field potentials and single unit discharges, during epileptiform activity, Adrian and Moruzzi (1939) demonstrated the high frequency burst discharges of single pyramidal tract neurones and provided evidence for the hypothesis that the interictal spike is associated with synchronized bursts of single unit discharges. Others studies on human foci (Rayport & Waller 1967; Calvin et al. 1973; Ishijima et al. 1975) and on different animal models (Jung 1953; Baumgartner 1954; Li 1955; Enomoto & Ajmone-Marsan 1959) provided further support for this hypothesis.

The interictal spike was recorded both directly from the exposed brain and in different experimental models (Jasper 1949). Penfield and Jasper also demonstrated that the randomly occurring surface negative spike discharge was a common manifestation of different epilepsies (for a review see Penfield & Jasper 1954). Having demonstrated that the epileptic process was fundamentally the same in most types of seizures, Penfield and his coworkers proposed that it was the localization of the process in the brain which explained the different symptoms (Penfield & Jasper 1946; Penfield & Kristiansen 1951). The location of the site or sites of abnormality that gave rise to interictal spikes became a much debated question (for a review see Gloor 1978).

The earlier experimental models of generalized epilepsy induced spike and wave discharges by manipulating either cortical or subcortical mechanisms. Hence, depending on the experimental protocol used, the conclusions about mechanisms underlying spike and wave discharges were divided into two rival hypotheses: the "diffuse cortical" hypothesis, proposed by Gibbs and Gibbs (1952) postulated that generalized spike and wave discharges originated exclusively in the cortex, whereas the "centrencephalic" hypothesis, proposed by Penfield and Jasper (1954), postulated that the spike and wave discharges were generated by the "centrencephalic system", a hypothetical integrating neuronal network centered in deep midline diencephalic and brainstem stuctures.

Experimental support for the "diffuse cortical" hypothesis came from the observations that widespread bilateral application of convulsant drugs to the cortex of cats or monkeys induced generalized spike and wave discharges (Gibbs & Gibbs 1952; Marcus & Watson 1966 & 1968), even when the cortex of both hemispheres was

disconnected from the subcortical inputs. Support for the "centrencephalic" hypothesis came from the observations that a) bilaterally synchronous spike and wave discharges were elicited in the cat by 3 Hz stimulation of the midline and intralaminar thalamic nuclei (Jasper & Droogleever-Fortuyn 1946), particularly when applied during very light barbiturate anaesthesia (Pollen et al. 1963); and b) stimulation of the mesencephalic reticular formation with brief, high frequency bursts delivered at 3 Hz was effective in eliciting generalized spike and wave discharges.

Studies carried out on the feline generalized penicillin model by Gloor and collaborators ended this dispute by showing that spike and wave discharges are generated in neocortex, but triggered by synchronizing afferent inputs from those thalamic nuclei that normally produce augmenting and recruiting responses (Gloor et al. 1977; Quesney et al. 1977). Furthermore, these discharges were also provoked by thalamic stimulation when penicillin was selectively applied to the neocortex but not to the thalamus. This indicated that the abnormality resulted from normal afferent input to epileptogenic cortex. These observations replaced the term "centrencephalic" epilepsy, coined by Penfield and Jasper, with "corticoreticular" epilepsy for clinical petit mal epilepsy (Gloor 1968). In the 1980's, Avoli & Gloor also showed that interactions between cortex and thalamus can cause thalamic disturbances which further enhance epileptogenicity.

Intracellular recording and paroxysmal depolarizing shift

The second most important development, contributing to the understanding of

mechanisms underlying epilepsies, was the introduction of microelectrodes as a means of probing the physiological properties of cells (Graham & Gerard 1946; Ling & Gerard 1949; Nastuk & Hodgkin 1950). The discovery of the end-plate potential by Fatt and Katz (1951) and the excitatory and inhibitory synaptic potentials of central neurones by Eccles and his collaborators (for a review see Eccles 1964) initiated the analysis of nervous system at a cellular level.

Intracellular studies from neurones exposed to convulsants such as strychnine (Li 1959), freezing (Goldensohn & Purpura 1963), alumina (Atkinson & Ward 1964), pentylenetetrazol (Creutzfeldt et al. 1966), electrical stimulation (Kandel & Spencer 1961), and penicillin (Matsumoto & Ajmone-Marsan 1964a) revealed that the paroxysmal burst discharge, corresponding to the interictal spike of EEG, arose from a long lasting depolarization of the neuronal membrane. This potential, which was first studied systematically by Matsumoto & Ajmone-Marsan (1964a), was called the paroxysmal depolarization shift (PDS). When fully developed PDS had an amplitude up to 30 mV and an average duration of 90 to 150 ms, which usually led to inactivation of the Na^+-spike. PDS was usually followed by a hyperpolarization (of 5-10 mV lasting up to 600 ms), which was reduced during the transition from the interictal to ictal state to give a continuous depolarization during the tonic part of the ictal state (Matsumoto & Ajmone-Marsan 1964b).

There has been considerable discussion concerning the mechanisms of PDS generation (for a review see Ajmone-Marsan 1969; Prince 1978; Schwartzkroin 1983). Major issues included whether the epileptic abnormality resided in either individual

neurones ("epileptic cell") or neuronal populations ("epileptic aggregate") (Atkinson & Ward 1964; Prince 1969; Dichter & Spencer 1969; Ayala et al. 1973). The main hypothesis of "epileptic aggregate" was that PDS was the sum of synchronous excitatory postsynaptic potentials generated by complex interactions in large groups of neurones in which augmentation of excitatory and/or depression of inhibitory synaptic activity has taken place (Ayala et al. 1973). The alternative hypothesis, supporting the "epileptic cell", suggested that PDS was generated by the intrinsic properties of a given cell and that synaptic events merely synchronized the discharge of populations of neurones (Prince 1968).

PDS And The Giant Synaptic Potential:

Support for the giant synaptic potential hypothesis came from the findings that a) PDS was readily evoked in neocortex and hippocampus by orthodromic but never intracellular stimuli (Dichter & Spencer 1969a; Matsumoto et al. 1969; Prince 1968), b) when evoked at short intervals (Matsumoto 1964; Prince 1966) or during development or waning of an epileptiform focus (Matsumoto & Ajmone-Marsan 1964) PDS was graded in amplitude, and c) in some cells PDS increased when triggered during hyperpolarizing and decreased during depolarizing current pulses (Prince 1968; Dichter & Spencer 1969b; Matsumoto et al. 1969). However, attempts to show a reversal potential for PDS were not successful.

For those who supported the giant synaptic potential hypothesis, an important task was to show the mechanism by which synaptic transmission would be increased to

generate the PDS. Hence, several studies were carried out to see whether or not presynaptic, postsynaptic or both processes were responsible for the increased excitation. The convulsive actions of barbiturates (Downes & Williams 1969) and of penicillin were attributed to an enhanced synaptic transmission, which at the time was thought to be a consequence of an increase in the size of the presynaptic fiber volley, as was observed in the stellate ganglion of the squid (Ayala et al. 1971) and the crayfish muscle preparation (Futamachi & Prince 1975).

PDS was also thought to be a manifestation of denervation supersensitivity, first reviewed by Cannon and Rosenbluth (1949). According to this hypothesis the sensitivity of the cell to various excitatory agents would increase after denervation (for a later review see Sharpless 1969). Support for denervation supersensitivity came from Echlin (1959), who showed that partially isolated cortex had an increased sensitivity to topically applied acetylcholine and a high susceptibility to epileptiform activity. However, the idea of hypersensitivity was not supported by Krnjević and colleagues, who carried out experiments with iontophoretic application of excitatory transmitters (Krnjević 1969; Krnjević et al. 1970).

Reduced removal of the excitatory transmitter was another hypothesis put forth to explain the giant synaptic potential. Support for this hypothesis came from the findings of Stone (1957), who induced epileptiform activity in intact dogs and Baker and Benedict (1968), who evoked paroxysmal discharges in hippocampus upon intravenous administration or microinjections of anticholinesterases respectively.

Studies from the penicillin foci also pointed to increased or released recurrent

excitation as another mechanism for the generation of PDS (Dichter & Spencer 1969b; Ayala et al. 1973). The experiments which formed the basis for the "recurrent excitation" hypothesis relied upon stimulation of the fornix, after cutting its afferent fibers (Dichter & Spencer 1969b) and the similarly "deafferented" corpus callosum (Ayala & Vasconetto 1972) to activate recurrent excitatory circuits. However, in both preparations PDSs were triggered with long latencies, and the activity of excitatory interneurones was not demonstrated. Furthermore, the problem of spread of stimulus current to orthodromic pathways was not ruled out (Ayala & Vasconetto 1972).

PDS And Reduced Synaptic Inhibition

Early intracellular recordings in neocortical and hippocampal penicillin foci, which showed large hyperpolarizing -presumably inhibitory- potentials, ruled out a total loss of in inhibitory transmission as a possible mechanism underlying PDS (Matsumoto & Ajmone-Marsan 1964; Prince 1968b; Dichter & Spencer 1969a). However, since the discoveries of a) pyridoxal phosphate (vitamin B_6) (Gyorgy 1934; Snell et al. 1942; Hagberg et al. 1966), which functions as a co-factor for glutamic decarboxylase (GAD) -the enzyme producing γ-aminobutyric acid (GABA)- (Lichstein et al. 1945; Schlenk & Snell 1945), b) GABA in the central nervous system (Awapara et al. 1950; Roberts & Frankel 1950), and c) the electrophysiological effect of exogenously applied GABA (Krnjević & Schwartz 1966), it is well established that reduced synthesis or release of inhibitory transmitters induces seizures (for a review see Tower 1969; Meldrum 1975; Wood 1975; Lloyd et al. 1981; Krnjević 1983). For example,

seizures were observed following administration of GAD inhibitors -and thus GABA synthesis- such as thiosemicarbazide (Killam & Bain 1957), hyperbaric oxygen (Wood et al. 1966), 3-mercaptopropionic acid and allylglycine (Horton & Meldrum 1973). The convulsant actions of tetanus toxin (Davies & Tongroach 1979) and 3-mercapto-propionic acid (Fan et al. 1981) were reported to be due to their ability to prevent GABA release. However, the demonstration of a lack of correlation between steady-state GABA levels and cerebral excitability (Baxter & Roberts 1960; Maynert & Kaji 1962; Kuriyama et al. 1966) and the presence of a large hyperpolarization in penicillin foci (Matsumoto & Ajmone-Marsan 1964; Prince 1968b; Dichter & Spencer 1969a) created controversy and confusion about the role of GABA in convulsions.

Hence several studies were carried out to understand the relationship between penicillin and GABAergic transmission. One of the hypothesis proposed to explain the epileptogenic property of penicillin was that penicillin, like bicuculline, blocked or reduced the sensitivity of the postsynaptic GABA receptor (Curtis et al. 1972). Another hypothesis was that penicillin, like picrotoxin, reduces transmitter-induced chloride conductance, rather than competing for the GABA receptor itself (Hochner et al. 1976; Pellmar & Wilson 1977). However, a more detailed analysis and thus understanding of the role of synaptic inhibition in epileptogenesis had to wait for the introduction of brain slices technique (see below).

Intrinsic i.e. nonsynaptic factors in PDS

The possibility that PDS could be due to changes in the intrinsic properties of

the neuronal membrane was raised by early intracellular studies in penicillin foci. During long trains or pairs of stimuli, it was noted that periods of refractoriness followed depolarization shifts but never synaptic potentials (Matsumoto 1964; Prince 1966; Prince 1968b), and that the depolarization shift had a very stereotyped appearance from stimulus to stimulus. Such stereotyped responses would be expected if the depolarization shift was a result of intrinsic neuronal activities evoked by a synaptic input. Increased excitability could be due to a depolarization, an increased resistance or a reduced threshold for spike generation. However, studies by Matsumoto et al. (1969) failed to show an alteration in membrane potential, firing level or membrane resistance in epileptic neurones.

Support for the concept that the depolarization shift represents an intrinsic cell response came from the finding that depolarization shift-like potentials may be triggered by intracellular current pulses in motoneurones of cat spinal cord exposed to penicillin (Kao & Crill 1972). Depolarization shift-like potentials have also been evoked by intracellular stimulation in hippocampal cells in culture (Zisper et al. 1973) and in convulsant-treated molluscan neurones isolated from their synaptic inputs (Ayala et al. 1970; 1971; Freeman 1973; Klee et al. 1973; Speckmann & Caspers 1973; Williamson & Crill 1976).

Proof that non-synaptic mechanisms -such as electrical interactions or effects of chemical substances and ions released by neuronal activities- are important for epileptogenesis in cortical foci was provided by studies on thalamocortical relay cells (whose axons project to cortex) during cortical penicillin discharges (Gutnick & Prince

1972; 1974; Rosen et al. 1973). Coincident with interictal EEG discharges, antidromic spike bursts were found in the thalamocortical relay cells. Following penicillin treatment, such antidromic bursts were also recorded in callosal axons (Schwarztkroin et al. 1975), dorsal root fibers (Lothman & Somjen 1976) of cat spinal cord and in the phrenic nerve-hemidiaphragm preparation (Nobels & Prince 1977) . Underlying repetitive spike generation in cortical axons, during epileptogenesis, was proposed to be due to changes in the extracellular ionic environment affecting the terminals (Pedley et al. 1976; Heinemann et al. 1977).

Alterations in ionic microenvironment

Since the resting membrane potential of a neurone is largely dependent upon the ratio of K^+ concentration across the membrane, any change in the extracellular K^+ concentration would alter the resting membrane potential. The suggestion that changes in extracellular ionic concentrations may give rise to epileptiform activities first came from Green (1964). Support for the hypothesis that K^+ released by neurones reach sufficient concentrations in the extracellular space to influence neuronal activities came from the findings that K^+ was released during epileptogenesis (Fertziger & Ranck 1970) and that seizures might be induced in hippocampus following superfusion with high K^+ containing solutions (Zuckermann & Glaser 1968). Recordings from glial cells, which behave as K^+ electrodes (Kuffler & Nicholls 1966), provided further support for increased extracellular K^+ concentration during focal epileptogenesis (Prince 1971; Sypert & Ward 1971; Dichter et al. 1972; Ransom 1974). The introduction of K^+ ion-

sensitive microelectrodes (for a review on the technology see Walker 1971; Vyskocil and Kriz 1972; Lux 1974; Krnjevic & Morris 1972; Thomas 1978; Nicholson 1980; Lubbers et al. 1981; Sykova et al. 1981; Zeuthen 1981; Krnjević & Morris 1981) provided direct evidence for the importance of the ionic microenvironment in the nonsynaptic modulation of neuronal excitability. Transient increases -lasting only seconds- and sustained increases in extracellular K^+ concentration up to 10 to 12 mM (Heinemann & Lux 1975; 1977) have been reported during interictal spikes (Prince et al. 1973; Fisher et al. 1976), and ictal episodes (Moody et al. 1974; Futamachi et al. 1974; Sypert & Ward 1974) in both neocortex and hippocampus.

Ca^{2+} is another ion involved in epileptogenesis. The importance of extracellular Ca^{2+} concentration in generation of spontaneous activity in muscle fibers was first realized by Ringer (1886). Later Kuffler (1945) showed that the excitability of nerve fibers were also enhanced by lack of extracellular Ca^{2+}. Since then it had been reported to be involved in transmitter release (Harvey & MacIntosh 1940; Katz 1966; Katz & Miledi 1969), in the generation of dendritic spikes (Llinas & Hess 1976), regulation of spike generation and membrane conductance following depolarization (Krnjević & Lisiewicz 1972; Meech 1972; Krnjević et al. 1975, 1978; Barrett & Barrett 1976), and modulation of membrane excitability (Shanes 1958; Frankenhaeuser & Hodgkin 1957). The importance of extracellular Ca^{2+} concentration in epileptogenesis has been demonstrated by the ability of Na^+-ethylenediaminetetracetic acid (EGTA), a Ca^{2+} chelator, to induce epileptiform activity (Harmony et al. 1973). The development of Ca^{2+} -sensitive microelectrode (Oehme et al. 1976) has allowed direct

measurements of Ca^{2+} activity during evoked cortical potentials and epileptogenesis (Heinemann et al. 1977). Substantial and long-lasting decreases in extracellular Ca^{2+} concentration to levels as low as 0.9 to 0.5 mM has been recorded during seizures, presumably due to Ca^{2+} entry into presynaptic terminals, dendrites, and neurones.

THE CONTRIBUTION OF BRAIN SLICES TO THE STUDY OF PDS

The first report about brain slices surviving for many hours in vitro, after being provided with the necessary ions, glucose and oxygen,was given by Warburg (1930). However, the brain slice preparation was first used for electrophysiological studies by Yamamoto and McIlwain (1966) and then much more extensively after the development of the transverse hippocampal slice (Skrede & Westgaard 1971), in which well defined neuropathways can be readily identified and studied selectively. It became popular among epileptologists (Ter Keurs et al. 1973; Voskuyl et al. 1975; Ogata 1975; Ogata et al. 1976; Schwartzkroin & Prince 1976; 1977; and others) only after the finding, by Yamamoto and Kawai (1967), that epileptiform discharges can be produced in hippocampal slices maintained in vitro (Yamamoto 1972).

During the past fifteen years, the in vitro brain slice technique has provided a wealth of information about the normal behaviour of cortical neurones as well as about the mechanisms of epileptogenesis. It has also resolved many of the arguments about the generation of the PDS induced by acute convulsants. Studies, using this technique, reported that epileptiform discharges in a population of neurones originate from the interaction of several factors: 1) the intrinsic membrane properties that lead to

pacemaker activity in specific subsets of neurones; 2) reduction of inhibitory control mechanisms; and 3) excitatory synaptic coupling among neurones of the epileptogenic region.

Intrinsic Membrane Properties

Brain slices exposed to convulsants such as penicillin and bicuculline (Schwartzkroin & Prince 1977; Schwartzkroin & Prince 1978; Johnston & Brown 1981; Gutnick et al. 1982) generate spontaneous synchronous epileptic bursts which have features exactly like those of interictal discharges from acute foci (Dichter & Spencer 1969; Matsumoto & Ajmone-Marsan 1964). Studies in hippocampal slices have shown that spontaneous epileptiform discharges are generated by the pyramidal cells in the CA2 and CA3 area, which normally generate intrinsic burst discharges (Schwarztkroin & Prince 1978; Wong & Traub 1983). The evidence that CA2-CA3 cells may be pacemakers came from studies where a) dual or multichannel extracellular recordings were used (Schwartzkroin & Prince 1978; Knowles et al. 1987); b) the connections between the pyramidal cells in CA3 and CA1 region -which are monosynaptically activated by the former-were cut (Schwartzkroin & Prince 1978; Miles & Wong 1983); and c) epileptogenic agents were applied, locally, to specific regions in the slice (Mesher & Schwartzkroin 1983). These experiments revealed that, while penicillin, picrotoxin etc., blocked inhibition in all regions of the slice, spontaneous bursts arose only in CA2-CA3 region and that almost all of the CA3 cells sustained epileptiform bursts even when isolated as small tissue prisms. In neocortical slices, even though

segregation of a group of pacemaker cells are not as clear, layers IV and upper V have been implicated in the initiation of synchronous epileptiform activity by a) local applications of convulsants or chemical excitants; b) current source density analysis; or c) extracellular Ca^{2+} measurements (Lockton & Holmes 1980; Chatt & Ebersole 1982; Pumain et al. 1983; Connors 1984; Ebersole & Chatt 1986).

Having demonstrated that the capacity of a neuronal population for generating epileptiform activities was in part due to the intrinsic properties of its constituents, the scientists were faced with the task of dissecting the cellular properties that rendered these cells spontaneously active. The neuronal properties necessary for the generation of such intrinsic bursts have been studied in detail in molluscan neurones (for a review see Barker & Smith 1978; Boisson & Chalazonitis 1978; Gulrajani & Roberge 1978; Adams et al. 1980; Carnevale & Wachtel 1980; Gorman et al. 1981; Gorman & Hermann 1982; Wilson 1982) and seems that similar processes take place in the mammalian central nervous system (Llinas & Nicholson 1971; Llinas & Sugimori 1980a,b; Schwindt & Crill 1980a,b,c).

Molluscan neurones

Membrane potential of bursting pacemaker neurones oscillates between -60 and -30 mV, the depolarization phase of which may trigger action potentials (Strumwasser 1968; Mathieu & Roberge 1971). Voltage clamp data suggested a slow inward current for the depolarizing phase and a subsequent outward current for the hyperpolarizing phase of the cycle. The outward current that terminated the burst has been shown to be carried by potassium ions (Brodwick & Junge 1972; Carnevale & Wachtel 1980;

Johnston 1980; Gorman et al. 1981; 1982), which can be either a Ca^{2+} dependent K^+ current (Johnston 1976; Gorman & Thomas 1978, 1980; Gorman et al. 1982), or a purely voltage dependent K^+ current (Gola et al. 1977). The inward current, responsible of the slow depolarization, has been suggested to be either a sodium current (Smith et al. 1975; Carnevale & Wachtel 1980; Drake & Treistman 1981; Futamachi & Smith 1982), a calcium current (Eckert & Lux 1976; Akaike et al. 1978; Gorman & Thomas 1978, 1980; Gorman et al. 1981, 1982) or both (Gola 1976; Johnston 1976; Partridge et al. 1979).

Hippocampal neurones

CA3 neurones were reported to generate spontaneous activity, consisting of rhythmic bursts occurring at a frequency of one per second (Wong et al. 1979; Wong & Prince 1981). Bursting was regularly recorded both from soma and apical dendrites of CA3 neurones; however, bursting was only recorded in the apical dendrites of CA1 neurones (Schwartzkroin 1978; Wong et al. 1979). The lack of orthodromically induced bursts, in CA1, was reported to be due to the IPSP, following the stimulus-induced EPSP very closely (Wong & Prince 1979).

Ca^{2+} ions were reported to play an important role in the generation of bursts. In the presence of Ca^{2+} channel blockers the depolarizing after potentials following single action potentials in CA1 and CA3 cells, as well as the bursting of CA3 cells induced by depolarizing current pulses, were blocked (Wong & Prince 1978; Wong & Prince 1981). The long-duration after-hyperpolarization (AHP) following bursting in CA3 and repetitive firing in CA1 cells was also reported to be due to a Ca^{2+} dependent

K^+ current (Alger & Nicoll 1980; Hotson & Prince 1980; Schwartzkroin & Stafstrom 1980; Gustafsson & Wingström 1981; Hablitz 1981; Wong & Prince 1981) comparable to that reported in spinal motoneurones (Krnjević et al. 1975, 1978; Barrett & Barrett 1976).

Voltage-clamp studies revealed several voltage-dependent ion channels in the membranes of pyramidal cells in addition to the classical Na^+ and K^+ channels of the action potential. They were shown to possess voltage-dependent Ca^{2+} currents -low and high threshold- (Johnston et al. 1980; Brown & Griffith 1983; Bossu et al. 1985; Yaari et al. 1987), which were responsible for the slow Ca^{2+} spikes recorded after the fast Na^+ spikes have been blocked (Schwartzkroin & Slawsky 1977; Wong & Prince 1978; Schwarztkroin & Prince 1980).

Ca^{2+} currents may contribute to epileptogenesis by a) underlying bursting in pacemaker cells, b) enhancing postsynaptic excitatory responses, and c) providing postburst reexcitation. The high threshold Ca^{2+} currents are usually activated, when the membrane is depolarized by 30 mV or more from its resting potential (Carbone & Lux 1984). Since the inactivation of these currents is slow, they can produce prolonged plateau spikes during a burst. The low threshold Ca^{2+} currents, whose inactivation is removed during a prolonged hyperpolarization, would cause reexcitation following the inhibitory pause (Llinas & Yarom 1981; Carbone & Lux 1984). Thus increased intracellular Ca^{2+} will a) cause further depolarization, b) result in removal of magnesium blockade from NMDA receptors and thus further increase the influx of Ca^{2+}, and c) cause activation of an array of second messengers, which, in turn, will

modulate the function of ionic channels and neurotransmitter receptors (see sections on synaptic mechanisms and second messengers). Decreases in extracellular Ca^{2+} concentrations (due to the influx of Ca^{2+} from Ca^{2+}- and NMDA receptor- channels) which are especially pronounced around the cell bodies (Krnjević et al. 1980) where inhibitory synapses are concentrated leads to further increases in neuronal excitability probably by a) causing disinhibition and b) reducing the threshold for activation of inward currents (Frankenhaueser & Hodgkin 1957; Hille 1968; Lux 1980).

Neurones were reported to posess mechanisms to oppose sustained Ca^{2+}-dependent depolarizations to control the build up of excitation and free intracellular Ca^{2+} levels, which if increased too much destroys the cell (for a review see Meldrum 1981; 1986). The protective hyperpolarizing mechanisms, in hippocampus, which also terminates the burst, were shown to be a variety of K^+ currents either voltage dependent, such as the A, M, and K currents, or Ca^{2+} dependent such as the C current (for a review see Brown et al. 1985). These currents especially the M and C currents were reported to be responsible for the hyperpolarizations recorded after bursts of action potentials, and thereby contribute to the termination of many types of epileptic burst discharges (Alger & Nicoll 1980; Hotson & Prince 1981; Alger & Williamson 1988). A second mechanism terminating the bursts was reported to be the inactivation of Ca^{2+} currents by increased intracellular Ca^{2+} (Traub 1982; Eckert & Chad 1984).

When the balance between these ionic conductances is disturbed by agents such as tetraethylammonium, which blocks K^+ currents; 4-aminopyridine, which blocks the A current; or barium, which blocks the M and C currents and enhances the Ca^{2+}

current, spontaneous epileptiform bursts are induced (Schwartzkroin & Slawsky 1980; Schwindt & Crill 1980; Hotson & Prince 1981; Galvin et al. 1982; Voskuyl & Albus 1985). For example, in a genetic model, a mutant of the fruit fly *Drosophila* (the hyperkinetic strain), the absence of the C current was reported to be responsible for the motor disorders, such as vigorous leg-shaking (Jan et al. 1977). Even though, to date there is no evidence indicating such a dramatic loss of a specific channel in chronic mammalian or clinical epilepsy, it is very likely that an abnormality in their modulation would give rise to the disruption of the delicate balance. For example cholera toxin (Karpiak et al. 1978; Kuriyama & Kakita 1980) injections were reported to produce chronically recurring seizures. The mechanisms of action of cholera toxin was reported to be the result of either activation of GM1-ganglioside in the membrane by cholera toxin (Karpiak et al. 1978) or accumulation of cyclic adenosine monophosphate (cyclic AMP) (Kuriyama & Kakita 1980), which in turn would depress K^+ currents (Madison & Nicoll 1982).

It is obvious that bursting activity in an individual neurone would not constitute epileptic activity. The discharges of many neurones have to become synchronized to generate the EEG responses and other features characteristic of the epilepsies. The modulation of ion channels may generate bursts of action potentials in individual neurones but by themselves they cannot explain synchronization. The mechanisms for synchronization have been studied extensively for penicillin-induced epileptogenesis in the hippocampus, where synaptic connectivity was reported to be essential for synchronizing the epileptic bursts (see below).

However, other mechanisms are also involved in the synchronization of neuronal discharges, as illustrated by the low Ca^{2+} model (see part I). When hippocampal slices are exposed to low Ca^{2+} solution synaptic transmission and Ca^{2+} dependent processes are blocked, yet slices generate spontaneous synchronous discharges (Jefferys & Haas 1982; Taylor & Dudek 1982; Yaari et al. 1983). Electrotonic junctions, ephaptic interactions, and fluctuations in extracellular ionic concentrations have been proposed as possible mechanisms underlying the synchronization in the absence of synaptic transmission (Taylor & Dudek 1982; Dudek et al. 1983; Snow & Dudek 1984).

Electronic junctions, which are sites where two cells are joined (gap junctions under the electron microscope), provide low resistance connections between cells. Even though they exist at many sites in the mammalian brain (Korn & Faber 1979; Dudek et al. 1983), evidence does not support their involvement in synchronization of epileptiform activity, simply because these junctions join neurones in small clusters of only two to seven neighbouring cells, rather than the open syncytium which would be required to synchronize the population of neurones (Traub et al. 1985).

Ephaptic interactions occur when the electric currents generated in the extracellular space by the activity of one set of neurones affects the excitability of adjacent neurones in the absence of specialized contacts. In general, extracellular currents are too weak to alter significantly the membrane potentials and excitability of neighbouring neurones; however, occasionally when individual neuronal elements come close together -aided by an unusually high extracellular resistivity which would tend to increase extracellular voltage gradients and intracellular currents through the recipient

neurones (Arvanitaki 1942; Korn & Faber 1980; Haas & Jefferys 1984)- this does happen. In hippocampus, which is a laminated structure and where pyramidal cell bodies are very densely packed together, the geometry of the principal neurones causes the extracellular currents generated by their synchronous activity to sum in the axis perpendicular to the cell layers, resulting in large current densities and field potentials (Green 1964; Jefferys 1981) and functionally significant ephaptic interactions (Taylor & Dudek 1982; Yim et al. 1986).

The importance of electric field interactions in synchronizing low Ca^{2+} field bursts was shown by recording the transmembrane potentials during the bursts. With intracellular recordings action potentials were thought to arise either abruptly from the depolarizing plateau or from a negative inflection. However, transmembrane recordings revealed that ephaptic interactions preceeded the action potentials during low Ca^{2+} bursts (Taylor & Dudek 1982). Thus when the neurones are sufficiently excitable, as is the case in slices exposed to low Ca^{2+}, penicillin or picrotoxin, ephaptic interactions organize rapid, but asynchronous, trains of action potentials into synchronized population spikes (Taylor & Dudek 1982; Traub et al. 1985).

As mentioned earlier, *extracellular ions*, especially K^+ and Ca^{2+}, have been demonstrated to be crucial for neuronal activity. Unlike Ca^{2+}, extracellular K^+ concentration, even though demonstrated to change with ion sensitive microelectrodes, so far has not been shown to be responsible for the initiation of seizures. Earlier studies with ion sensitive electrodes had revealed that increases in extracellular K^+ concentrations occurred after electrical seizures had started, not before, and thus could

not have initiated them (Prince et al. 1973; Moody et al. 1974; Heinemann et al. 1978; Somjen & Giacchino 1985). However, recent studies on hippocampal slices, exposed to low Ca^{2+} solution, reported that initiation and spread of epileptiform activity is highly dependent on extracellular K^+ concentration (Yaari et al. 1986; Korn et al. 1987). Extracellular K^+ accumulation, which reached a ceiling value of 10 mM during epileptiform activity, was proposed to account for the termination of seizures in vivo (Sypert & Ward 1971), however, it has been reported not to be sufficient to block neuronal activity and thereby to abort epileptiform activity (Yaari et al. 1986).

Intrinsic burst generators may also become more effective, as suggested by some of the anatomical changes seen in chronic models, such as the alumina focus and Mongolian gerbil (Westrum et al. 1964; Scheibel et al. 1983). Shortening of dendrites and loss of dendritic spines were suggested to cause a reduction in the electrotonic length of neurones, which would enhance coupling of soma/axonic Na^+ spikes with the slow Ca^{2+} spikes of the dendrites. As a consequence, the absence of the attenuation and low-pass filtering of the intervening dendrites will enable a single fast somatic spike to trigger a Ca^{2+} spike and thereby sustained depolarization associated with burst discharges (Calvin 1980; Wyler & Ward 1986).

Reduction in synaptic inhibition

Recurrent inhibition, elicited in hippocampus by stimulation of the deafferented fornix, was first described by Kandel et al. (1961) and by Andersen et al. (1963, 1964). The interneurones mediating this response were thought to be the basket cells,

which synapse on the soma of pyramidal cells. A more detailed analysis of the electrophysiological and pharmacological properties of hippocampal inhibitory postsynaptic potentials came with the advent of the brain slices technique. Local inhibitory circuits have been directly demonstrated by simultaneous paired intracellular recordings made from the CA1 field in hippocampal slices (Knowles & Schwarztkroin 1981). In these studies it was shown that pyramidal cells can excite interneurones which in turn inhibit other nearby pyramidal cells. Later on, Alger and Nicoll described a feed-forward-inhibition localized on dendrites (1982). They showed that orthodromically induced IPSPs were biphasic -with an early and late component- whereas those induced by antidromic stimulation were monophasic. The late component was shown to be bicuculline-resistant and mediated by activation of a K^+ conductance (Alger 1984; Newberry & Nicoll 1984)

Yamamoto and Kawai (1968) were the first to show the occurrence of epileptic discharges upon disrupting inhibition by replacing Cl^- ions with impermeant anions. Later on, Ozawa and Okada (1976) demonstrated the correlation between the reduction in tissue GABA levels, induced by methoxypyridoxine, with the appearance of paroxysmal depolarizing shifts and bursts of firing.

Penicillin induced epileptiform activity was also demonstrated to be associated with a loss of inhibition (Wong & Prince 1979; Dingledine & Gjerstad 1980). These studies showed that, in presence of penicillin, the IPSP evoked by orthodromic stimulation disappeared, which allowed EPSPs to generate intrinsic bursts. Under normal conditions the IPSPs following orthodromic stimulation-evoked EPSPs prevented

the generation of dendritic burst dicharges by both orthodromic stimulation and direct depolarization. The hyperpolarization produced by IPSP, by an increase in Cl^- conductance, was proposed to shunt the inward currents carried by Ca^{2+} and Na^+ so that the threshold for burst generation was not reached. When the effects of bicuculline and penicillin were compared, both agents depressed IPSPs and the responses to GABA without affecting EPSPs (Swartzkroin & Prince 1980). However, unlike bicuculline, which was shown to bind to GABA receptors (Mohler & Okada 1977) and displace GABA (Curtis et al. 1972; Zuckin et al. 1974; Enna & Snyder 1975; MacDonald & Barker 1977), penicillin does not displace specific GABA-binding (Heyer et al. 1982). Hence, penicillin, like picrotoxin (Olsen & Leeb-Lundberg 1981; Johnston 1981) may interfere with the Cl^- ionophore opened by GABA (Hochner et al. 1976; Pellmar & Wilson 1977) to cause disinhibition.

GABA-mediated inhibition can also be reduced by repetitive stimulation (Andersen & Lomo 1968; Ben-Ari et al. 1979; Ben-Ari & Krnjević 1982; Krnjević et al. 1982; Wong & Watkins 1982). The disinhibition and thus generation of epileptiform activity was in part due to a "fading" - desensitization - of GABA action (Krnjević 1976; Ben-Ari et al. 1981; Numann & Wong 1984). Recently, McCarren & Alger (1985) and Korn et al. (1987) raised the possibility that altered ionic gradients, namely K^+ concentration, across neuronal membrane reduce the efficiency of GABAergic transmission.

The best example of disruption of inhibition causing chronic seizures came from studies on the convulsant action of tetanus toxin. Tetanus toxin was reported to block

the release of GABA from brain slices (Collingridge et al. 1981; Collingridge & Herron 1985). Recently, electrophysiological studies from hippocampal slices, injected with tetanus toxin prior to decapitation, confirmed this by exhibiting lack of IPSPs (Jefferys 1986).

Another mechanism of disinhibition is the selective vulnerability of inhibitory interneurones. The best example comes from the alumina focus, where studies showed a loss of a) tissue GAD activity (Bakay & Harris 1981), b) GAD-positive terminals (Ribak et al. 1979; Ribak 1985) and c) inhibitory synapses (Gray type II) (Ribak et al. 1982). A loss of Gray type II synapses was also described in seizures following hypoxia in juvenile monkeys (Sloper et al. 1980).

Excitatory Synaptic Mechanisms

The initial hypothesis that the PDS is a giant EPSP generated by enhanced excitatory transmitter release and recurrent excitatory activity (Dichter & Spencer 1969; Ayala et al. 1973) had given way to the hypothesis that both loss of inhibition and intrinsic burst generating mechanisms contribute to the generation of PDS. Much of the observed increase in EPSP amplitude can be attributed to a loss of simultaneously occurring IPSPs, which normally attenuate the EPSP (Dingledine & Gjerstad 1979). However, literature on kindling and long term potentiation provides ample evidence indicating an increase in EPSP amplitude and efficacy in the absence of loss of IPSPs (Lynch & Schubert 1980; though cf. Stelzer et al. 1987). An alternative hypothesis is an increased excitability of the postsynaptic neurones (Yamamoto & Chujo 1978;

Andersen et al. 1980; Lynch & Schubert 1980).

Excitatory transmission in many parts of the mammalian brain, including the hippocampus, uses excitatory amino acids, perticularly glutamate (Krnjević & Phillis 1963; Curtis & Johnston 1974; Krnjević 1974; Davidson 1976). Recent progress in the pharmacology of this system pointed out to three distinct glutamate receptors, which are named according to their preferential agonists: the N-methyl D-aspartate (NMDA), quisqualate, and kainate receptors (for a review see Watkins 1984; Fagg 1985; Mayer & Westbrook 1987). The NMDA receptor has attracted much attention, partly because of the existence of effective antagonists, such as D-2-amino-5-phosphonovaleric acid (APV) (Croucher et al. 1982) and partly because of its properties (for a review see Dingledine 1986). Activation of the NMDA receptor was shown to be dependent upon the presence of the transmitter as well as depolarization of the postsynaptic membrane, which removes the magnesium ions that normally block the NMDA channel (Mayer et al. 1984; Nowak et al. 1984; Dingledine 1986; Cotman et al. 1988). Normally the presence of inhibition prevents EPSPs from generating prolonged depolarization (Schwarztkroin & Prince 1980) and thus the activation of NMDA receptors. Once the inhibition breaks down, as in epilepsy, the NMDA receptor activation becomes possible and Ca^{2+} rushes into the cell (MacDermott et al. 1986; Collingridge & Bliss 1987; Jahr & Stevens 1987; Mayer et al. 1987; Cotman et al. 1988). In addition the dpolarization may activate intrinsic membrane conductances (Hotson et al. 1979; Johnston et al. 1980) which probably contribute to the prolonged depolarization observed in cells throughout the seizures. Support for this hypothesis came from studies which reported

that a) incubating slices in low magnesium solutions induces epileptiform activity (Herron et al. 1985; Thomson 1986; Avoli et al. 1987), and b) NMDA receptor antagonists, such as APV, are anticonvulsants for several experimental epilepsies, both in vivo and in vitro (Croucher et al. 1982; Herron et al. 1985; Ashwood & Wheal 1986; Avoli & Olivier 1987). Thus NMDA receptors amplify the excitatory synaptic drive provided by other synaptic inputs. In fact the anticonvulsant effect of APV is on the later components of the epileptiform discharge rather than on its onset.

Another role attributed to the EPSPs is synchronization of population firing. In the hippocampal slice, pyramidal cells in the CA3 region are connected through both recurrent inhibitory and monosynaptic excitatory synapses (MacVicar & Dudek 1980), whereas those in CA1 region have only recurrent inhibitory synapses (Knowles & Schwartzkroin 1981). Nevertheless, EPSPs synchronize the population and trigger intrinsic burst discharges in both regions.

The earliest evidence, supporting recurrent excitatory connections between CA3 cells, was provided by experiments where the major fiber tract to CA3 was cut (Dichter & Spencer 1969; Lebowitz et al. 1971; Ayala et al. 1973). In these experiments, contrary to the expectations, stimulation of the tract -after the afferents had degenerated- produced EPSPs in addition to antidromic action potentials and recurrent inhibitory potentials. More direct evidence for the existence of monosynaptic excitatory connections between the pyramidal cells in CA3 region, even though sparse (1.5 % of pairs of recordings), came from studies where simultaneous intracellular recordings were carried out from pairs of neurones (MacVicar & Dudek 1980; Miles & Wong

1981). To see how such a low incidence of recurrent excitatory connections would be sufficient to synchronize the pyramidal cells in a hippocampal slice, Traub and Wong (1982) designed a computer model by taking into account features such as intrinsic mechanisms and disinhibition. They showed that a population of neurones, which generates spontaneous burst discharges asynchronously and is interconnected by recurrent IPSPs and EPSPs, can be synchronized by EPSPs once the controlling IPSPs are blocked. Thus bursting of a few disinhibited neurones will trigger bursts in a synaptically-connected subset of cells, which in turn will excite other interconnected members of the population.

Hence, it appears that PDS, in a population, consists of potential changes produced by intrinsic and synaptic events, and that the development and spread of excessive excitation through the network of excitatory connections between pyramidal cells is normally prevented by inhibition.

MODULATION OF NEURONAL ACTIVITY

From what I have reviewed so far, it is apparent that neurones do have the potential to fire excessively, but are prevented from doing so by the activation of a) the intrinsic outward currents carried by K^+ and b) Cl^- (and perhaps K^+) currents evoked by GABA release. Several neurotransmitters and neuromodulators affect cell excitability by altering the normal functioning of these protective mechanisms. They exert their effects by opening or closing ion channels in the membrane, either directly or through the activation of intermediate proteins (G-proteins), as well as intracellular

second messengers. As an example, I shall review the cholinergic modulation, partly because most of my studies were focussed on the seizure-promoting effects of acetylcholine, and partly because a review of all effects of the neurotransmitters and modulators is beyond the scope of this thesis.

ACETYLCHOLINE

Even though acetylcholine (ACh) was the first substance discovered as a transmitter, both in the peripheral nervous system (Loewi 1921; Dale 1934) and the central nervous system (Eccles et al. 1954), it has been difficult to demonstrate cholinergic transmission in the mammalian brain. Since the late 1940's it was known that ACh is synthesised and released in the cerebral cortex (Feldberg & Vogt 1948; MacIntosh & Oborin 1953; Hebb & Silver 1956; Celesia & Jasper 1966). Similarly, numerous biochemical and histochemical studies showed the ubiquitous distribution of ACh and its metabolic enzymes, acetylcholinesterase and cholinacetyltransferase in the brain (Shute & Lewis 1963, 1967; Krnjević & Silver 1965, 1966). The fact that the cholinergic innervation of structures in the brain is diffuse and sparse -with the exception of the septohippocampal pathway- (Shute & Lewis 1963; Lewis & Shute 1967; Lewis et al. 1967) hampered the electrophysiological studies of the synaptic cholinergic effects. Iontophoresis of ACh showed that, in contrast to the peripheral nervous system, in the central nervous system, ACh acts predominantly via muscarinic receptors, which cause either excition (Krnjević & Phillis 1963; McLennan & York 1966), inhibition (Salmoiraghi & Steiner 1963; Andersen & Curtis 1964; Randić et al.

1964; Bradley et al. 1966; Crawford & Curtis 1966; Crawford et al. 1966; Curtis et al. 1966; Phillis & York 1967), or both excitation and inhibition (Tebecis 1972). The rest of this review will focus on the excitatory effects of ACh, however, those, who are interested in the mechanisms underlying the inhibitory actions of ACh, are referred to McCormick and Prince (1986,1987).

Seizure promoting effects of acetylcholine

The notion that ACh is a facilitator of seizures and may itself be a convulsant came from studies, which showed that direct application of ACh to the brain induced seizures (Feldberg 1945; Feldberg & Sherwood 1954; Haley & McCormick 1957; Guerrero-Figueroa et al. 1964). Anticholinesterases, which prevent the inactivation of the liberated ACh, by hyrdolysis (Wilson et al. 1950), were among the first agents used to induce epileptiform activity (Stone 1957). Stone and later on Baker and Benedict (1968) reported that the accumulation of unhydrolyzed ACh, in the presence of a potent cholinesterase inhibitor, such as tetraethylpyrophosphate or diisopropylfluorophosphate,induced convulsionsand paroxysmal discharges, which were abolished by atropine administration. Hence, the mechanism of action of anticholinesterases were thought to be due to enhanced activitation of muscarinic receptors (DuBois 1963; Niemegeers et al. 1982). Support for this came from kindling studies where local injections of carbamylcholine into the amygdala, caudate, and hippocampus at fourty eight hours intervals were effective in inducing seizures (Vosu & Wise 1975; Wasterlain & Jonec 1983). Therefore a question of great interest was

how acetylcholine promoted seizures.

Effects of acetylcholine on intrinsic membrane properties

Activation of muscarinic receptors in the cerebral cortex induces a slow and prolonged excitation (Krnjević & Phillis 1963). The depolarization produced by ACh had a very negative reversal potential -even in Cl^- loaded cells-and was associated with a decrease in membrane conductance of neocortical (Krnjević et al. 1971) and hippocampal (Benardo & Prince 1981; Dodd et al. 1981) neurones. These findings indicated that the decrease in membrane conductance was due to a reduction in resting K^+ conductance. Recent studies on the nature of the current responsible for the ACh-induced depolarization has pointed to the blockade of a leak K^+ current (Müller & Misgeld 1986; Madison et al. 1987; Misgeld et al. 1987; Benson et al. 1988). In addition to the slow depolarization, ACh was also reported to induce repetitve firing after depressing the postspike repolarization (see below). This effect, at the time, was attributed to the blockade of the delayed K^+ conductances associated with the action potential (Krnjević et al. 1971; Dodd et al. 1981; Herrling 1981; Segal 1982).

Brown and Adams (1980), and Halliwell and Adams (1982) analyzed the effects of muscarinic agonists under voltage clamp and attributed the muscarinic excitation (i.e. the depolarization) primarily to an inhibition of a specific K^+ current, which they named the M current. M current is mediated by a voltage- (activation range - 60 to - 10 mV) and time-dependent K^+ conductance, which was first reported in the bullfrog autonomic ganglia (Brown & Adams 1980). Being activated by membrane

depolarization it is an inhibitory K^+ current that limits cellular responses to depolarizing stimuli. Hence, by blocking this inhibitory influence, muscarinic agents enhance excitability.

Subsequently Benardo and Prince (1982a-c), and Cole and Nicoll (1984) reported that cholinergic agents blocked the slow AHP. The slow AHP is generated upon activation of a K^+ current (I_{AHP}) by an increase in the intracellular Ca^{2+} concentration, following a series of action potentials. It plays a major role in slowing the action potential discharge rate (accomodation) (Alger & Nicoll 1980; Hotson & Prince 1980; Gustafsson & Wingstrom 1981; Schwartzkroin & Staftstrom 1980; Lancaster & Adams 1986). Hence if the slow AHP is blocked either by preventing the rise in intracellular Ca^{2+} or by blocking Ca^{2+} entry through voltage-dependent Ca^{2+} channels, more action potentials will be evoked by a given depolarizing pulse. The entry of Ca^{2+} also activates another voltage dependent K^+ current called I_C (Adams et al. 1982; Brown & Griffith 1983; Lancaster & Adams 1986). I_C repolarizes the action potential and thus underlies the fast AHP (Storm 1987). Due to its voltage sensitivity it closes rapidly when the membrane repolarizes despite the continued presence of Ca^{2+}. Cholinergic agents were not shown to have any effect on the fast AHP and thus on action potential duration (Lancaster & Nicoll 1987).

Later on Nakajima and his colleagues (1986) investigated the action of cholinergic agonists on cultured hippocampal neurones by using the whole-cell patch clamp technique. They found that acetylcholine inhibited the fast transient K^+ current named the A current by Connor and Stevens (1971). The A current with its fast

kinetics -it reaches its peak in 6 to 11 msec and inactivates biexponentially with time constants of 100 msec and 4 sec (Kasai 1985)- is an important factor in determining the size of the action potential and especially the time course of repolarization (Belluzzi et al. 1985). Nakajima and his colleagues attributed the increase in the amplitude and the duration of the spike, caused by cholinergic agents, to the blockade of A current. If such an enhancement of spike height and shape takes place in the presynaptic nerve terminal, it will have a powerful excitatory action.

The finding that hippocampal Ca^{2+} spikes were not affected by ACh, supported the idea that cholinergic agents exerted their effects directly on K^+ currents (Cole & Nicoll 1984). However, in other preparations activation of muscarinic receptors were reported to alter the properties of voltage-dependent Ca^{2+} conductances (Misgeld et al. 1986; Wanke et al. 1987). Recently, whole-cell voltage-clamp studies revealed that hippocampal cells may also possess three different types of Ca^{2+} currents (Madison et al. 1987; Yaari et al. 1987), corresponding to T, N, and L type Ca^{2+} currents described in dorsal root ganglion (Fox et al. 1987). Subsequently, Gähwiler and Brown (1987) reported that muscarine reduces Ca^{2+} currents in hippocampal neurones. A more systematic study revealed that mucarinic agonists enhanced the low voltage-activated -the T type- Ca^{2+} current and depressed the high voltage-activated -N and/or L type- Ca^{2+} current (Toselli & Lux 1989). Such a reduction in the high threshold Ca^{2+} currents would result in blokade of Ca^{2+} dependent processes, such as I_{AHP} and I_C. High threshold Ca^{2+} currents also play a dominant role in neurotransmitter release (Miller 1987; Hirning et al. 1988). Hence their blockade may give rise to a reduction

in neurotransmitter release. The enhancement of the low threshold Ca^{2+} current, which was shown to contribute to the generation of pacemaker depolarizations (Llinas & Yarom 1981; Burlhis & Aghajanian 1988), may also underlie the slow depolarization produced by cholinergic agents, which also occur at voltages more negative than -60 mV.

Effects of acetylcholine on synaptic mechanisms

Even though several investigators have noticed that the release of ACh is reduced by muscarinic agonists and facilitated by antagonists (MacIntosh & Oborin 1953; Mitchell 1963; Celesia & Jasper 1966; Dudar & Szerb 1969), Yamamoto and Kawai (1967) were the first to postulate that ACh may modulate the release of other neurotransmitters as well. Support for this hypothesis came from the findings that ACh suppressed hippocampal IPSPs by a presynaptic action (Ben-Ari et al. 1981; Krnjević et al. 1981; Herrling 1981; Valentino & Dingledine 1981; Haas 1982; Gähwiler & Dreifuss 1982; Segal 1983). Recently Hasuo and his colleagues (1988) reported that ACh reduced IPSPs by acting on M_1 receptors located on GABAergic interneurones. This "disinhibitory" action of ACh was associated with a decrease in quantal content of IPSPs (Segal 1983) suggesting a reduction in Ca^{2+} influx into the GABAergic terminals.

Hippocampal EPSPs were also modulated by cholinergic agents: muscarinic agonists depressed (Hounsgaard 1978) whereas nicotinic agonists enhanced the EPSPs (Rovira et al. 1983). The opposite effects of muscarinic and nicotinic receptor

activation on neurotransmitter release in the same population of neurones has been well documented in other systems too (Hery et al. 1977; De Bellroche & Bradford 1978; Loffelholz 1979; Muscholl 1979; Starke 1981; Briggs & Cooper 1982).

The second messengers activated by acetylcholine

The electrophysiological findings that, unlike nicotinic receptors, activation of muscarinic receptors produces slow and long lasting effects (for a review see Purves 1976), and that their actions in cortical neurones were blocked by the metabolic inhibitor dinitrophenol (Godfraind et al. 1971), suggested the involvement of metabolic processes in the mediation of cholinergic effects.

Biochemical studies have demonstrated since early 1950's that muscarinic receptor activation is associated with an increase in metabolism of membrane phosphoinositides (Hokin & Hokin 1953, 1955; Weiss & Putney 1981; Poggioli et al. 1983; Fisher et al. 1983; Brown & Brown 1983), increase in cyclic guanosine monophosphate (cyclic GMP) levels (Ferrendelli et al. 1970; George et al. 1970; Lee et al. 1972; Kebabian et al. 1975; Matsuzawa & Nirenberg 1975; Hanley & Iversen 1978; Lenox et al. 1980) and decrease in cyclic adenosine monophosphate (cyclic AMP) levels (Walker & Walker 1973; Matsuzawa & Nirenberg 1975; Traber et al. 1975; Gross & Clark 1977; Nathanson et al. 1978; Lichtshtein et al. 1979; Lenox et al. 1980; Palmer et al. 1980; Olianas et al. 1982). However, it is only within the past few years that a coherent picture about the physiological function of phosphoinositide metabolism and its interaction with cyclic nucleotide system has

emerged (for a review see Downes 1982, 1983; Berridge 1984; Fisher & Agranoff 1986; Hawthorne 1986; Nahorski et al. 1986).

It is evident now that, by enhancing the hydrolysis of phosphoinositides, ACh initiates a multifuntional signal cascade that results in mobilization of calcium and thus activation of calcium / calmodulin-dependent kinase, activation of protein kinase C, release of arachidonic acid and stimulation of guanylate cyclase to form cyclic GMP (Berridge 1981, 1984; Irvine et al. 1982; Takai et al. 1982). The initial event in this cascade is the hydrolysis of phosphatidyl-inositol 4,5-bisphosphate (PIP_2) by phospholipase C to yield inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol. However, there is increasing evidence that, under certain conditions, phosphatidylinositol and phosphatidylinositol 4-phosphate, the precursors for PIP_2, can also be hydrolyzed by phospholipase C, leading to sustained production of diacylglycerol in the absence of IP_3 formation (for a review see Majerus et al. 1986, 1988). Phospholipase C exists mainly in a soluble form, although it has also been described and characterized as membrane bound (Lee et al. 1987; Ryu et al. 1987). Guanine nucleotides have been shown to activate the hydrolysis of PIP_2 by phospholipase C (Beckmyn et al. 1986; Dawson et al. 1986; Roth 1987), suggesting the involvement of guanine nucleotide binding proteins (G proteins) in the activation of this cascade (cf. Pfaffinger 1988; Brown et al. 1989).

IP_3 and Ca^{2+} mobilization

Michell's hypothesis (1975) that hydrolysis of phosphoinositides is responsible

for mobilizing Ca^{2+}, was first confirmed by Streb and colleagues (1983). They showed that IP_3 releases Ca^{2+} from a nonmitochondrial store (endoplasmic reticulum) in permeabilized pancreatic acinar cells. Since then, this observation has been confirmed in many other permeabilized cell systems (for a review see Berridge 1987). How IP_3 releases Ca^{2+} from internal stores has not been established yet. It may either act on an internal receptor to stimulate release or it may inhibit the uptake mechanisms responsible for sequestering intracellular Ca^{2+}. Irrespective of the mechanism, it is obvious that IP_3 is an important element in the control of intracellular Ca^{2+}, even when the cell is at rest. Thus neurotransmitters that increase IP_3 levels are inducing a large mobilization of the stored Ca^{2+}.

The biological activity of IP_3 can be terminated by several ways: a) by sequential dephosphorylation by a 5-phosphatase which produces first, inositol 1,4-bisphosphate and then inositol 4-monophosphate (Storey et al. 1984); and b) a specific IP_3 kinase, which has been shown to be a calcium / calmodulin dependent enzyme (Biden et al. 1987; Ryu et al. 1987; Yamaguchi et al. 1987), phosphorylates IP_3 to inositol 1,3,4,5-tetrakisphosphate (IP_4), which in turn is hydrolysed by a specific phosphatase to form the inactive isomer inositol 1,3,4-trisphosphate (Batty et al. 1985; Irvine et al. 1986). IP_4 has been shown to enhance the entry of Ca^{2+} into the cell and potentiate the response to IP_3 (Irvine & Moor 1986,1987; Putney 1986). Thus metabolism of IP_3 via phosphorylation provides a mechanism by which additional second messengers are generated.

Support for the idea that muscarinic receptors are coupled to phosphoinositide

hydrolysis came from numerous studies (for a review see Fisher & Agranoff 1985). This response was blocked by classical muscarinic antagonists such as atropine or scopolamine but not with nicotinic receptor antagonists such as mecamylamine or hexamethonium. The sensitivity of the response to the antagonist pirenzepine suggested that a pirenzepine-sensitive M_1 receptor is coupled to phospholipase C activation, while whereas a pirenzepine-insensitive M_2 receptor is linked to adenylate cyclase inhibition (Gil & Wolfe 1985; Watson et al. 1985). However, this classification appears to be an over-simplification as pharmacological and gene cloning studies are revealing the existence of more than two types of muscarinic receptors (Bonner et al. 1987; Doods et al. 1987; Waelbroeck et al. 1987). In the cerebral cortex, a group of agonists (also known as full agonists) such as ACh, carbamoylcholine, and oxotremorine-M, are more effective in enhancing phosphoinositide turnover than another group (of partial agonists), such as pilocarpine, bethanechol, and oxotremorine (Fisher et al. 1983, 1984; Gonzales & Crews 1984; Jacobson et al. 1985). It is of interest to note that this pattern of agonist efficacy is very similar to that described for stimulation of guanylate cyclase by cholinergic agents (McKinney et al. 1985). Recently, Kudo and his colleagues (1988) have reported increases in cytosolic free Ca^{2+} concentration upon stimulation of muscarinic receptors, in keeping with the previous biochemical studies.

Diacylglycerol and activation of protein kinase C

The other major product of phosphoinositide hydrolysis is the neutral lipid, diacylglycerol. It is formed transiently in the stimulated cell, and under resting

conditions, its concentration is too low to be detected. Low concentrations of diacylglycerol are due to the actions of two enzymes: a) it is phosphorylated to phosphatidic acid by a diacylglycerol kinase to initiate resynthesis of phosphoinositides (for a review see Abdel-Latif 1986; Taylor & Putney 1987); and b) it is hydrolysed by a diacylglycerol lipase to release arachidonic acid to the cytosol (Irvine 1982).

The importance of diacylglycerol as a second messenger emerged after Nishizuka and his colleagues purified protein kinase C (PKC), a protein kinase which required Ca^{2+}, phospholipid (such as phosphatidylserine), and diacylglycerol for maximum activity (Inoue et al. 1977; Takai et al. 1977; Kishomoto et al. 1980; Nishizuka 1983). Diacylglycerol activates PKC by lowering the latter's calcium requirement so that it is activated even at physiological intracellular calcium concentrations (0.1 μM) (Kaibuchi et al. 1981). Thus an agonist which increases diacylglycerol levels at the plasma membrane, by increasing the phosphoinositide turnover, could activate PKC without prior mobilization of Ca^{2+}. PKC can also be activated by low concentrations of arachidonic acid (McPhail et al. 1984; Naor et al. 1988), which is formed as a result of either receptor stimulated activation of phospholipase A_2 or the activity of diacylglycerol lipase (for a review see Axelrod et al. 1988), lipoxin A , an oxygenated product of arachidonic acid (Hansson et al. 1986), as well as lysophosphatidylcholine (Oishi et al. 1988).

PKC is both cytosolic and membranous and so far has been found in every mammalian tissue and in all phyla (Kuo et al. 1980). In unstimulated cells, the enzyme is localized in the cytosol and presumably inactive; however, when the cell is activated,

diacylglycerol is produced, and PKC binds to phosphatidylserine at the plasma membrane (Wooten & Wrenn 1984; Drust & Martin 1985: Hirota et al. 1985; May et al. 1985). Recently, several groups have reported the molecular cloning of multiple forms of PKC's cDNA from a variety of sources (Ono et al. 1986; Coussens et al. 1986; Knopf et al. 1986; Makowske et al. 1986; Ohno et al. 1987). Huang and his coworkers have purified three forms of rat brain PKC isozymes of 82kD, which they termed types I, II, and III (Huang et al. 1986). Their recent studies using isozyme-specific antibodies to determine the expression products of cells transfected with three forms of cDNA have shown that type I, II, and III PKC are products of PKC genes τ, β and α, respectively, collectively known as group A (Huang et al. 1987). Recently, Nishizuka and colleagues reported the presence of δ, ϵ and ζ genes, collectively known as group B (Ono et al. 1987). They also reported that as many as seven distinct types of PKC may exist in rat brain (Ono et al. 1987). Although the members of this PKC family share a high degree of sequence homology, they have different regulatory domains (Ono et al. 1987). Except for the Purkinje and granule cells in cerebellum (which contain mainly type I PKC) and mitral cells in the olfactory bulb (which contain mainly type III PKC), most neurones in brain were found to contain all three types of group A PKCs. Hippocampal pyramidal cells and granule cells of the dentate gyrus are reported to be rich in PKCs I, II, and III; whereas cells of the strata oriens, radiatum and lacunosum moleculare, and of the molecular layer of the dentate gyrus contain much less PKC (Huang et al. 1988). These studies also localized immunoreactivities of PKCs I and II on the plasma membrane whereas that of PKC III mainly in cytoplasm.

In Purkinje cells electron microscopic studies indicated that PKC I is localized in axoplasm and synaptic vesicles in addition to cell bodies and dendrites (Kose et al. 1988). The finding that PKC I is more sensitive to diacylglycerol and phorbol ester stimulation than PKCs II and III (Huang et al. 1988) indicates that this membrane bound form of PKC is more sensitive to input signals.

Most of the cellular substrates of PKC have not yet been defined. It catalyzes the phosphorylation of serine and threonine residues of various cellular proteins, and thus have a broad substrate specificity (for a review see Kuo et al. 1984; Majerus et al. 1984; Nishizuka 1984, 1986; Anderson et al. 1985; Ashendel 1985; Cockcroft & Gomperts 1985; Hirasawa & Nishizuka 1985; Marme & Matzen-Auer 1985; Nairn et al. 1985). To name a few PKC, when tested in vitro phosphorylates a) tyrosine hydroxylase (Albert et al. 1984); b) the voltage dependent sodium channel (Costa & Catterall 1984); c) nicotinic acetylcholine receptor (Huganir et al. 1984); d) microtubule-associated proteins MAP-2 and tau (Kaczmareck 1987); e) neuromodulin (also known as the growth cone proteins P-57, GAP-43, pp-46, B-50 or F-1) (Cimler et al. 1985; Benowitz & Routtenberg 1987); and f) the Na^+/H^+ exchange system (Burns & Rozengurt 1983; Moolenar et al. 1984).

Research on physiological effects of PKC accelerated once phorbol esters, such as phorbol 12, 13-diacetate (PDAc) and 12-O-tetradecanoyl-phorbol-13-acetate(TPA) - potent cell activators and cocarcinogens-were demonstrated to activate PKC directly, by substituting for diacylglycerol, both in vivo and in vitro (Castagna et al. 1982; Yamanishi et al. 1983; Niedel et al. 1983). Thus phorbol esters bypass the

physiological pathway of diacylglycerol formation via receptor activated phosphoinositide hydrolysis. To mimic the action of diacylglycerols, phorbol esters should be able to partition into membranes (Cabot & Jaken 1984). They function as a nonmetabolizable analogue of diacylglycerol and activate PKC by modifying its phospholipid microenvironment or through proteolytic modification. PKC has also been shown to be the intracellular receptor for phorbol esters (Castagna et al. 1982; Niedel et al. 1983), which cause a decrease in cytosolic PKC and a corresponding increase in the membrane bound one.

To confirm that phorbol ester-induced effects are indeed mediated by PKC, several specific inhibitors of PKC had to be identified. PKC inhibitors can be classified into two groups, depending upon whether their targets are the regulatory or the catalytic domain of PKC. The regulatory domain of PKC binds Ca^{2+}, phospholipid and diacylglycerol or phorbol ester and thus unmasks the active site in the catalytic domain. Several compounds that interfere with the binding of these activators to PKC were used as PKC inhibitors. However, most of the PKC inhibitors interacting with Ca^{2+} and acidic phospholipids, such as trifluoperazine, dibucane, adriamycin, polymixin B, melittin etc. also inhibit calmodulin-dependent protein kinases by competing with calmodulin. More recently discovered inhibitors such as calphostin (Kobayashi et al. 1989) and AMG (1-O-alkyl-2-O-methylglycerol)(Kramer et al. 1989) interfere with the binding of diacylglycerol or phorbol esters, and therefore appear to be more specific for PKC. Sphingosine, which competes with Ca^{2+}, phospholipid and diacylglycerol, has also been identified as a potent PKC inhibitor (Hannun et al. 1986). However, it has

been shown to be equally effective in inhibiting Ca^{2+}/calmodulin dependent enzymes (Jefferson & Schulman 1988). Thus sphingosine can be used as a general inhibitor for both branches of the phosphatidylinositol signaling pathway. Gangliosides, which are sialic acid-containing glycosphingolipids found in high concentrations on the outer leaflet of neural membrane, were also reported to inhibit PKC (Kreutter et al. 1987). When gangliosides are metabolized extracellularly, upon specific interaction with ligands, they generate the corresponding lysoganglioside or sphingosine (or both), which then crosses the membrane to act on the enzyme (Hannun & Bell 1989). However, unlike sphingosine, gangliosides at low concentrations (less than 125 μM) also activates the Ca^{2+}/calmodulin-dependent protein kinase II (Fukunaga et al. 1990).

A second group of PKC inhibitors act at the catalytic domain by interfering with either ATP or protein substrate. Several commonly used PKC inhibitors such as 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7) (Hidaka et al. 1984) and K-252 compounds (Kase et al. 1987) inhibit PKC by competing with ATP. However, since the ATP-binding site is conserved among all protein kinases, these compounds also interfere with the activation of other protein kinases. House and Kemp (1987) reported that synthetic peptides, that resemble the pseudosubstrate sequences of PKC, are potent and specific inhibitors of PKC. Staurosporine, which does not compete with ATP or other cofactors of PKC, inhibits both PKC and cyclic AMP dependent protein kinase (Tamaoki et al. 1986).

A number of different laboratories have examined the effects of PKC activation by using the above mentioned agents as tools. For a review of the electrophysiological

effects see part II of this thesis.

Increase in the cyclic GMP levels

Many of the Ca^{2+} mobilizing receptors that trigger an increase in the hydrolysis of phosphoinositides elevate the intracellular level of cyclic GMP by stimulating guanylate cyclase (Michell 1975; Berridge 1981). Removal of Ca^{2+} from the bathing medium has been reported to reduce the ability of agonists such as ACh to activate guanylate cyclase (Schultz et al. 1973; Ferrendelli et al. 1974). The fact that guanylate cyclase can be activated by a variety of fatty acids has led to the suggestion that this enzyme might be regulated by either arachidonic acid or its metabolites (Graff et al. 1978; Greutter & Ignaro 1979; Peach 1981; Takai et al. 1982; Gerzer et al. 1983). The calcium-mobilizing receptors may thus activate guanylate cyclase indirectly by causing the release of arachidonic acid (Berridge 1984).

The electrophysiological studies carried out to demonstrate the effects of cyclic GMP, and the arguments for and against the hypothesis that cyclic GMP mediates muscarinic actions are reviewed in part II (Chapters one and two) of this thesis.

Activation of calmodulin and Ca^{2+}/calmodulin dependent kinases

Many extracellular signals regulate intracellular events by causing an increase in the cytosolic Ca^{2+} concentration from less than 0.1 μM in the resting state to 1-10 μM in the stimulated state. As reviewed earlier, the increase in cytosolic Ca^{2+} arises from either increased influx through the activation of calcium channels or increased

release of Ca^{2+} from intracellular stores (which may be due to enhanced phosphoinositide hydrolysis or Ca^{2+} evoked release). To prevent the toxicity of Ca^{2+} (Carafoli & Lehninger 1971; Nayler et al. 1979; Schanne et al. 1979; Farber 1981; Rasmussen 1981), cells posess a number of Ca^{2+} regulatory mechanisms, including Ca^{2+} binding proteins, such as protein kinase C, calpain, calbindin and calmodulin. Calmodulin, a 15 kDA Ca^{2+}-binding regulatory protein (Cheung 1980; Klee et al. 1980), is present in brain cytosol at a concentration of 30 to 50 μM. Each molecule has four Ca^{2+} binding sites. As Ca^{2+} concentration rises the four binding sites become successively occupied which enables calmodulin to be a multifunctional activator.

In its Ca^{2+} -bound form calmodulin binds to specific proteins and alters their functions. The first two Ca^{2+}/calmodulin - dependent enzymes to be identified were an isozyme of cyclic nucleotide phosphodiesterase (Cheung 1970; Kakiuchi & Yamasuki 1970) and an adenylate cyclase (Brostrom et al. 1975). Thus in cells that posess these enzymes, the Ca^{2+}/calmodulin complex will modulate the phosphorylation states of proteins indirectly, by altering the activites of cyclic nucleotide - dependent kinases. Since then several other Ca^{2+}/calmodulin - dependent kinases were identified, the properties of which are summarized in several reviews (DeLorenzo 1982; Kennedy et al. 1987; Kennedy 1989). One of these, which is the predominant Ca^{2+}/calmodulin - dependent kinase in cortical and hippocampal neurones, is the Ca^{2+}/calmodulin - dependent kinase II (Erondu & Kennedy 1985). It is present throughout the cytosol in cell bodies, dendrites, axons, and terminals (Ouimet et al. 1984), as well as the postsynaptic densities (Kennedy et al. 1983; Kelly et al. 1984). Because of its high

level of expression in the hippocampus, and its concentration beneath postsynaptic membranes, it is thought to be a target for the postsynaptic Ca^{2+} current generated by the activation of NMDA receptors (Nicoll 1988). An important feature of Ca^{2+}/calmodulin - dependent kinase II is that it undergoes autophosphorylation (Miller & Kennedy 1986) so that the phosphorylated form no longer requires Ca^{2+}/calmodulin for its persistence. This suggested a role for its participation in long-term potentiation (Malenka et al. 1989). However, the postsynaptic substrates for this kinase have still not been identified. The substrates for the Ca^{2+}/calmodulin - dependent kinase II present in nerve terminals, where it facilitates synaptic transmission, are better defined. Synapsin I, a protein that is associated with synaptic vessicles (Huttner et al. 1983) and also binds to the cytoskeleton (Petrucci & Morrow 1987), is phosphorylated by Ca^{2+}/calmodulin - dependent kinase II at site II (Bennett et al. 1983; McGuinness et al. 1983,1985; Miller & Kennedy 1985). Phosphorylation by Ca^{2+}/calmodulin dependent kinase II reduces the affinity of synapsin I for synaptic vesicles, thereby making them available for fusion upon depolarization, and thus enhancing the amount of transmitter released per nerve impulse (Llinas et al. 1985).

Calcineurin (also known as protein phosphatase-2B), a protein phosphatase abundant in brain, is also activated by Ca^{2+}/calmodulin (Armstrong 1989). Recently, it has been implicated in the Ca^{2+}-dependent inactivation of the L-type Ca^{2+} channels in neurones (Chad & Eckert 1986; Armstrong 1989). Calcineurin also dephosphorylates and inactivates DARRP-43, a protein inhibitor of the brain protein phosphatase, phosphatase-I (Hemmings et al. 1984).

Several compounds, such as phenothiazines especially trifluoperazine (for a review see Weiss et al. 1980) and naphthalenesulfonamides (Hidaka et al. 1978), have been reported to inhibit the activation of Ca^{2+}/calmodulin dependent kinases. Chapter five of part II deals with the effects of trifluoperazine and its interaction with ACh.

In summary, biochemical studies have associated muscarinic (M_1) receptor activation with increased cyclic GMP levels and phosphoinositide metabolism. Increased phosphoinositide metabolism results in IP_3 and diacylglycerol formation, which in turn enhance intracellular Ca^{2+} levels and activate PKC respectively. Increased intracellular Ca^{2+} in turn activates an array of Ca^{2+}/calmodulin dependent kinases. However, so far, the physiological significance of these changes is still not known.

Hence, in part two of this thesis, in an attempt to identify the second messenger(s) mediating the seizure promoting effects of acetylcholine I investigated the effects of a) cylcic GMP; b) PKC activators and inhibitors; and c) Ca^{2+}/calmodulin dependent kinase inhibitors on hippocampal activity and their interactions with acetylcholine. In chapter one, I provide evidence disproving the hypothesis that cyclic GMP is the intracellular mediator of acetylcholine. In chapters two and three, I present data suggesting that inhibition of PKC rather than its activation mimics the disinhibitory action of acetylcholine, and in chapter four I provide evidence for the involvement of calmodulin dependent kinases in acetylcholine's excitatory actions.

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"Is it so bad, then, to be misunderstood ? Pythagoras was misunderstood, and Socrates, and Jesus, and Luther, and Copernicus, and Galileo, and Newton, and every pure and wise spirit that ever took flesh. To be great is to be misunderstood."

Ralph Waldo Emerson

PART ONE

Chapter One

Depression Of Hippocampal Low Calcium Field Bursts By The Antiepileptic Drug Valproic Acid.

by AGOPYAN N., AVOLI M., RIEB L., and TANCREDI V.

Neuroscience Letters (1985) 60: 57-62.

ABSTRACT

The antiepileptic drug valproic acid (VPA) reduces the occurrence of the rhythmic and synchronous bursts produced by hippocampal neurons maintained *in vitro* and bathed in Ringer-containing low-Ca^{2+} (0.2 mM), high-Mg^{2+} (4.0 mM). In this medium, synaptic transmission is blocked, thus demonstrating an action of VPA unrelated to potentiation of GABAergic phenomena. This conclusion is reenforced by the persistence of VPA effects in the presence of bicuculline. Also, the VPA doses effective in reducing the low-calcium synchronous burst in the hippocampal slice are similar to the free plasma levels of VPA observed to exert anticonvulsant effects in kindled rats.

INTRODUCTION

Valproic acid (VPA) has been used as an antiepileptic drug for almost 20 years (for reviews see refs. 5 and 25), yet its mechanism of action is still unclear. Biochemical studies have suggested that VPA selectively increases the levels of γ-aminobutyric acid (GABA) in the brain via an interaction with some of the enzymes involved in the synthesis and degradation of GABA [3,9,15,20,24]. However, other experiments have argued against an elevation of GABA in the brain as an explanation for VPA effects since its anticonvulsant action has been demonstrated at dosage levels below those required to raise brain GABA levels [6,19,29,30].

Alternative mechanisms of action for VPA derive from electrophysiological experiments. These have shown that GABA-induced responses are enhanced by VPA application in both *in vivo* and *in vitro* preparations [4,8,12,17,21],although it has been suggested that the interaction of VPA with the GABA receptor-ionophore complex occurs only at concentrations much higher (e.g. 10mM) than those having a therapeutic effect in humans, e.g. 0.1mM [13]. Also, high doses of VPA have been shown to increase a K^+ outward current in *Aplysia* neurons [26]. At odds with these findings is the evidence that this antiepileptic drug depresses inhibitory pathways in the spinal trigeminal nucleus [7].

In the experiments reported here, we studied the effects induced by VPA on the rhythmic and synchronous bursts which occur in the CA1 subfield of the hippocampal slice bathed in a solution containing low Ca^{2+} and increased Mg^{2+} [10,16,31].Synaptic transmission is assumed to be blocked in this type of medium, and non-synaptic

mechanisms, such as field interactions, can account for the burst synchronization observed in this preparation [27,28].

METHODS

Hippocampal slices from brains of adult, male, Sprague-Dawley rats (150 - 300 g) were cut (450 μm thick) with a McIlwain tissue chopper. They were maintained at 32°C in a tissue chamber which was similar either to the type employed by Haas et al. (11) or to that used by Langmoen and Andersen (18). In both cases hippocampal slices lay at the interface between artificial cerebrospinal fluid (ACSF) and humidified gas (95% O_2 - 5% CO_2) at a pH of 7.4. The composition of the ACSF was (mM): NaCl 124, KCl 5, KH_2PO_4 1.25, $MgSO_4$ 2 or 4, $CaCl_2$ 2 or 0.2, $NaHCO_3$ 26, and glucose 10. In some experiments KCl was increased upto 7 mM or bicuculline methiodide (20 μM) was added to the ACSF. VPA was freshly prepared by adding 1 N NaOH to achieve water solubility. A complete exchange of the ACSF in the chamber well was achieved in 2 - 8 min depending upon the perfusion rate and the type of chamber used. Glass micropipettes filled with 3 M NaCl (resistance: 5 - 10 MΩ) were used for extracellular recordings in the stratum pyramidale of the CA1 subfield. The recorded signal was fed to a high - impedance negative capacitance DC amplifier and displayed on a Gould pen recorder. Antidromic activation of CA1 hippocampal pyramidal neurons was achieved by stimulating (90 μs, 0.1 - 0.5 mA) the alveus with a sharpened and insulated monopolar tungsten electrode.

RESULTS

Spontaneous rhythmic and synchronous field discharges (hereafter called "low - Ca^{2+} bursts") were recorded in stratum pyramidale 3 - 5 h after exchanging normal ACSF with one containing low - Ca^{2+} (0.2 mM), high - Mg^{2+} (4 mM) concentrations. At this time the low - Ca^{2+} bursts, characterized by a group of population spikes superimposed on a negative potential shift, usually recurred with remarkable regularity at a frequency which was typical for each slice or could be evoked by alveus stimulation.

The application of VPA at concentrations ranging between 0.4 and 2 mM for periods of 10 - 30 min reduced the occurrence of low - Ca^{2+} bursts in 30 of 42 cases (Fig. 1A). The VPA effect became evident after a latent period of 5 - 10 min, was fully reversible and was dose related. Thus, lower concentrations of VPA (0.4 - 0.6 mM) were capable of decreasing the low - Ca^{2+} bursts in 15 of 23 slices while 1 mM VPA was effective in 12 out of 15 and 2 mM in all the slices tested. Concentrations of VPA smaller than 0.4 mM appeared to be ineffective in reducing the occurrence of low - Ca^{2+}. In 4 slices, 0.4 - 0.6 mM VPA was also capable of clearly reducing the synchronous burst evoked by single shock stimulation of the alveus (Fig. 2). In 6 experiments, VPA - induced effects were studied in the same slice in presence of both 6.25 mM and 7.25 or 8.25 mM $[K^+]_o$. As shown in Fig. 1A, VPA retained its ability to reduce the occurrence of the low - Ca^{2+} bursts even in the presence of higher $[K^+]_o$. The frequent occurrence of spreading depression like phenomena, associated with $[K^+]_o$ increase, did not allow us to study the effects of VPA at $[K^+]_o$ higher than 8.25 mM.

Although synaptic transmission is virtually blocked in this model, the application of the disinhibitory drug bicuculline can further increase the frequency of occurrence of low - calcium bursts suggesting that a residual GABAergic mechanism is still operant at either pre- or postsynaptic level (1). Consequently, in 7 experiments bicuculline was added to the ACSF. In 4 instances VPA was tested in the same slice both before and during bicuculline perfusion, while in 3 slices VPA effects were studied only during bicuculline. In either case, VPA (0.5 - 2 mM) was still able to decrease the occurrence of low - Ca^{2+} bursts (Fig. 1B) suggesting a mechanism of action which is unrelated to synaptic transmission, i.e. to a potentiation of inhibitory phenomena.

DISCUSSION

The main findings of this study is that VPA reduces the occurrence of rhythmic and synchronous field bursts produced by hippocampal neurons maintained *in vitro* and bathed in ACSF containing low - Ca^{2+}, high - Mg^{2+}. In this medium, synaptic transmission is blocked, thus demonstrating an action of VPA unrelated to a GABAergic mechanism as suggested by previous biochemical and electrophysiological studies (3, 4, 8, 9, 12, 15, 17, 20, 21, 24). This conclusion is reenforced by the persistence of VPA - induced effects even when the disinhibitory drug bicuculline was added to the ASCF in order to block any residual GABAergic action.

Non - synaptic mechanisms through which VPA might exert its effect in this model could possibly be: (i) an increased efficacy of outward (repolarizing) K^+ conductances or (ii) a decrease of inward currents which in this model would most likely be carried by Na^+ ions. The first mechanism is in line with previous experiments indicating that in *Aplysia* neurons VPA increases a K^+ conductance (26). Although we could not observe any clear change in VPA efficacy when $[K^+]_o$ was increased by 1 - 2 mM, it should be noted that such a change in $[K^+]_o$ might not be large enough to alter the effect produced by VPA. The second mechanism is supported by recent data showing that in cultured mouse neurons VPA selectively limits sustained high - frequency repetitive firing (22).

In our study the doses of VPA found to be effective in reducing the occurrence of low - Ca^{2+} bursts were higher than the therapeutical free plasma levels found in man (23). However, there is evidence suggesting that in kindled rats consistent anti -

convulsant effects are achieved only with plasma levels of 3 mM (2) which should correspond to a free plasma level of 0.6 mM (13). This value is within the range of VPA doses used in the present experiments.

In conclusion these data demonstrate an anticonvulsant effect of VPA which is not dependent upon synaptic phenomena. Similar findings have been recently reported by Heinemann et al. (14) who, however, were able to observe consistent effects only at concentrations of 2 - 5 mM.

This study was supported by the Hospital for Sick Children Foundation and the Medical Research Council of Canada (MA 8109). M. A. is a FRSQ Scholar. We acknowledge the gift of valproic acid from Abbott Labs. Ltd., the continuous encouragement of Dr. P. Gloor and the technical assistance of T. De la Fosse, C. Fink, G. Robillard and S. Schiller.

Fig. 1. A: reduction of occurrence of spontaneous low - Ca^{2+} bursts induced by 0.5 mM VPA in presence of 6.25 mM (a) and 7.25 mM (b and c) $[K^+]_o$. B: persistence of VPA (2 mM) effect in the presence 20 μM of bicuculline. Note that in this and the following figure each burst is composed of a negative DC shift on which are superimposed several population spikes, not clearly distinguishable because of the low speed of the pen recorder.

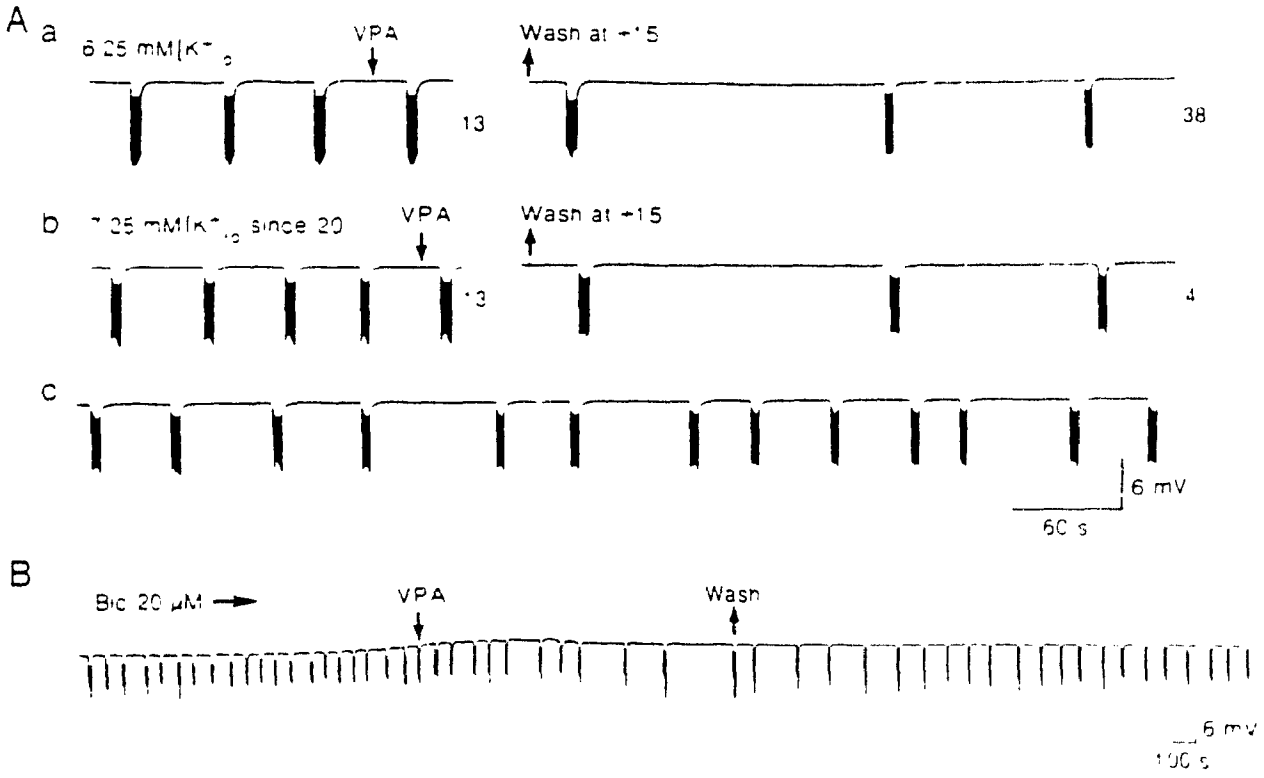
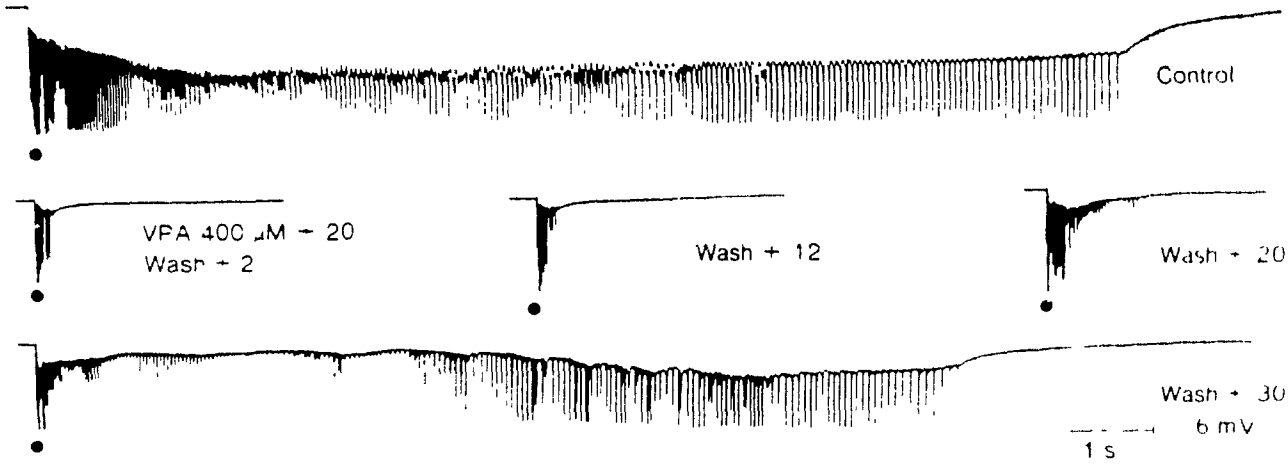

A
a
6.25 mM[K+]o
VPA
Wash at +15
13
38
b
7.25 mM[K+]o since 20
VPA
Wash at +15
13
4
c
6 mV
60 s
B
Bic 20 μM
VPA
Wash
6 mV
100 s

Fig. 2. VPA effects upon the low - Ca^{2+} burst induced by single - shock stimulation of the alveus. The stimulus intensity (0.3 mA, 90 μs) was maintained constant throughout the experiment.


Control
VPA 400 µM + 20
Wash + 2
Wash + 12
Wash + 20
Wash + 30
6 mV
1 s

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Chapter Two

MECHANISMS FOR LOW CALCIUM, HIGH MAGNESIUM SYNCHRONOUS BURST IN THE IN VITRO HIPPOCAMPAL SLICE.

by M. AVOLI and N. AGOPYAN

In: Epilepsy and Calcium. Speckmann E.J., Schulze H. and Walden J. (eds), (1986), Urban & Schwarzenberg, Munchen, Wien, Baltimore, pp.63-83.

Synaptic transmission, which is dependent upon the availability of extracellular Ca^{2+}, plays an important role in neuronal synchronization (Andersen and Andersson, 1968). This is a feature common to epileptic discharges experimentally induced in both in vivo and in vitro preparations (Ayala et al., 1973; Traub and Wong, 1983; Johnston and Brown, 1984a; Prince and Connors, 1984; Prince, this volume). However, pyramidal cells in the CA1 subfield of the in vitro hippocampal slice can generate synchronous field bursts when the $[Ca^{2+}]_o$, usually kept between 1.5 and 2 mM, is lowered below 0.2 mM, while $[Mg^{2+}]_o$ is increased up to 4 mM (Jeffery and Haas, 1982; Yaari et al., 1983; Haas and Jefferys, 1984; Konnerth et al., 1984). In this type of medium synaptic transmission is assumed to be blocked suggesting that mechanisms other than those related to the operation of chemical synapses could account for both the synchronization of the firing generated by hippocampal pyramidal cells and for the spread of this activity throughout the CA1 subfield.

The synchronous field bursts generated in the presence of low-Ca^{2+}, high-Mg^{2+} (hereafter called "low-Ca^{2+} bursts") may represent an experimental in vitro model for epileptiform discharges displaying features of seizure-like activity. In in vivo preparations, measurements with ion-sensitive electrodes have shown that during sustained neuronal discharges evoked by chemical convulsants or electrical stimulation, $[Ca^{2+}]_o$ decreases to levels similar to those required for the appearence of low-Ca^{2+} bursts (Heinemann and Lux, 1977; Heinemann et al., 1977; Krnjević et al., 1980; Dietzel et al., 1982; Pumain et al., 1985). Also the low-Ca^{2+} model can be used for studying the pharmacology of hippocampal pyramidal neurons (Haas et al., 1984; Lee

et al., 1984), as well as for testing the efficacy and mode of action of antiepileptic drugs (Hood et al., 1983; Agopyan et al., 1985; Heinemann et al., 1985; Rose et al., 1986).

Development of the low-Ca^{2+} synchronous burst in CA1 hippocampal subfield

In normal Ringer solution, single shock stimulation of the stratum (s.) radiatum evokes an orthodromic response of CA1 hippocampal pyramidal cells characterized in extracellular recordings in s. pyramidale by a negative population spike (signaling the number of action potentials fired synchronously by the pyramidal neurons) which rises from a slower positive wave (representing both the dendritic EPSP and the somatic IPSP; Fig. 1B). At the same time, alveus stimulation induces one antidromic population spike at fixed and short latency (Fig. 1B). Both these responses are gradually modified upon changing the normal Ringer solution with one containing low-Ca^{2+} (0.2 mM), high-Mg^{2+} (4mM). Thus, within 20-40 min, the orthodromic response disappears while antidromic stimulation evokes a burst of population spikes which can last up to 100 ms, and display an interspike interval varying between 5 and 10 ms (Fig. 1B). In the following 30-60 min this short latency burst becomes clearly associated with a pronounced negative potential shift on which further population spikes are superimposed (so-called late burst). As shown in Fig. 1C it appears that the maturation of the late burst (more and more population spikes being triggered by a single stimulus delivered in the alveus at constant strength) is related to a further increase in amplitude and duration (up to several seconds) of the negative potential shift.

The development of alveus-induced low-Ca^{2+} bursts is accompanied by a decreased efficacy of both recurrent and feed-forward inhibitory mechanisms. This can be shown by intracellular recordings performed with KCl-filled microelectrodes. Thus, as shown in Figs. 2B and 3, the depolarizing envelope which follows stimulation of the alveus (representing an inverted IPSP) decreases a few tens of min after bathing the slice in low-Ca^{2+} Ringer, while later on a long lasting depolarizing potential associated with synchronous firing is evoked by alvear shock. Also, in low-Ca^{2+} Ringer both the intracellular orthodromic response to s. radiatum (whose tail is associated with a feed-forward IPSP; Fig 2Aa) and spontaneous depolarizing IPSPs decrease (Fig. 3).

The low-Ca^{2+} Ringer, in addition to having these effects upon synaptic inhibitory mechanisms, also decreases the efficacy of repolarizing Ca^{2+} -dependent K^+ conductances (Hotson and Prince, 1980; Brown and Griffith, 1983a). The evidence for the impairment of this mechanism comes from the findings that after replacing normal with low-Ca^{2+} Ringer (i) hippocampal neurons often tend to depolarize steadily by 10 - 20mV; (ii) the number of action potentials evoked by a constant pulse of depolarizing current increases even when the membrane resting level is set at the same value as in control Ringer (Fig. 2Ab); (iii) both fast (Fig. 2Ab, 4B) and slow afterhyperpolarizations decrease in amplitude (see also Fig. 4 in Haas and Jefferys, 1984). Thus, the ability of the alvear stimulus to evoke a burst of population spikes in the low-Ca^{2+} Ringer can be explained by a diminished efficacy of the repolarizing events representing a combination of a recurrent IPSP (Andersen et al., 1964; Allen et al., 1977; Dingledine and Langmoen, 1980) and Ca^{2+}-dependent K^+ conductances

(Hotson and Prince, 1980; Brown and Griffith, 1983a), all of which depend highly upon Ca^{2+} availability.

The appearence of spontaneous synchronous field bursts, which show in both extra- and intracellular recordings a morphology similar to bursts induced by alvear stimulation (Fig. 6A), is linked to the final maturation of the late burst.

Synchronization and spread of bursting generated by CA1 pyramidal neurons in low-Ca^{2+} Ringer

During replacement of normal with low-Ca^{2+} Ringer, bursts of action potentials can be recorded in the CA1 subfield between synchronous field bursts (Fig. 4). These bursts are not a population phenomenon, as they are not recorded by an extracellular electrode at a distance of 50-100 μm (Fig. 4C); they appear intracellularly as composed of 3-10 presumably Na^+ action potentials arising from a depolarizing envelope and lacking both fast and slow afterhyperpolarizations (Fig. 4B). These bursts can be elicited by depolarizing pulses or upon termination of a hyperpolarizing pulse of adequate intensity; they must be endogenous since they can be aborted by hyperpolarizing the neuron with a steady DC current. Since in this model the $[Ca^{2+}]_o$ is quite low, the tendency of CA1 cells to burst is probably caused by an inward Na^+ current (MacVicar, 1985; Crill, this volume), which is not sufficiently counteracted by the depressed Ca^{2+}-dependent K^+ conductance. Also this bursting tendency, which in CA1 subfield pyramidal neurons is brought out by the low-Ca^{2+}-high-Mg^{2+} extracellular environment, contributes to the synchronous field bursts whenever field (so-called

ephaptic) interactions allow neurons to fire synchronously (Haas and Jefferys, 1984; Taylor and Dudek, 1984a, b).

The ephaptic effects occur when the extracellular electrical field generated by the activity of some neurons modifies the behaviour of nearby non-active cells, namely the current flowing through the extracellular space also passes through the membrane of inactive neurons, and by doing so depolarizes them. Ephaptic interactions have been described in different experimental conditions even when synaptic transmission is operant (Rasminski, 1980; Snow and Dudek, 1984; Taylor and Dudek, 1984a,b; Taylor et al., 1984). Two conditions are essential for exerting maximal synchronizing effect through ephaptic interactions: (i) The neurons in question must be densely packed and oriented along the same axis; (ii) they should be at a membrane potential close to the threshold for action potential generation. The first prerequisite is fulfilled in every subfield of the hippocampus (most markedly so in CA1 and in the dentate gyrus), and the second one in hippocampal neurons bathed in low-Ca^{2+}-high-Mg^{2+} Ringer (see above).

Ephaptic mechanisms might also account for the spread of both alveus-induced and spontaneous low-Ca^{2+} bursts throughout the CA1 subfield (Haas and Jefferys, 1984). Konnerth et al. (1984), however, have suggested that the propagation of low-Ca^{2+} bursts might be due to a mechanism related to K^+ accumulation. Accordingly: (i) The velocity of spread is comparable to K^+ redistribution by a spatial buffer mechanism (Heinemann et al., 1983); (ii) a rise in extracellular K^+ precedes and accompanies the spread of the neuronal burst (Haas and Jefferys, 1984; Konnerth et al.,

1984); (iii) the velocity of the spread is accelerated or slowed by increasing or decreasing the $[K^+]_o$ by 1-2 mM. Both of the mechanisms discussed above appear to be unrelated to synaptic transmission, thus implying that epileptiform activity can synchronize and propagate through the brain with modalities which are not dependent upon synaptic connectivity (Pumain et al., 1985).

Features of the spontaneous low-Ca^{2+} bursts

In our experiments spontaneous low-Ca^{2+} bursts are usually observed 2-3 hours after replacing normal with low-Ca^{2+} Ringer. However, the time of appearence can be shortened by one of the following procedures: (i) A higher (> 0.2 ml/min) rate of perfusion of the slices with low-Ca^{2+} Ringer; (ii) intense and frequent (e.g. > 0.2 Hz) alvear stimulation; (iii) increase of $[K^+]$ or further decrease of $[Ca^{2+}]$ in the Ringer. After their appearence, spontaneous bursts mature further and reach (within the next 20-30 min) a steady state during which their morphology, duration and frequency of occurence in a given slice remain constant for several hours. As shown by Haas and Jefferys (1984) the occurence of spontaneous low-Ca^{2+} bursts can be increased by decreasing the PO_2 or lowering the temperature in the tissue bath below 32°C. The low-Ca^{2+} bursts are also sensitive to changes of pH: When it is lowered the frequency of occurrence decreases, when it is raised the frequency of occurrence increases (Haas and Jefferys, 1984).

In the s. pyramidale each spontaneous burst is associated with a negative extracellular potential shift and population spikes (Figs. 5-8). In most of the cases,

population spikes occurring in the initial part of the burst exhibit a higher frequency and lower amplitude than those in the final part of the burst, an electrographic feature which resembles a miniature tonic-clonic seizure as observed in in vivo preparations. Spontaneous low-Ca^{2+} bursts "usually, but by no means always" (Haas and Jefferys, 1984) start near the subiculum and then progress towards a preferential region (e.g. Fig. 6Ab), thus suggesting a preferential involvement of pyramidal neurons in the subicular part of the CA1 subfield in the genesis of low-Ca^{2+} bursts.

Field potential analysis of the spontaneous bursts at different levels along the axis perpendicular to the s. radiatum shows that both the slow shift and the superimposed population spikes are negative in the cell body layer and positive in the apical dendrites (Fig. 5). This finding suggests that similar to what has been shown for the alveus-induced bursts (Yaari et al., 1983; Taylor and Dudek, 1984b), the spontaneous low-Ca^{2+} bursts are characterized by an active sink in the soma and a passive source located in the apical dendrites. This type of experiment, by displaying a complete absence of active sinks in the dendrites where a large portion of the excitatory synaptic input for hippocampal pyramidal neurons resides, further demonstrates a lack of synaptic currents in the genesis of low-Ca^{2+} bursts. In addition these data suggest the existence of a depolarizing potential which is located at the level of the somatic region (Fig. 6A). A large component of this somatic depolarization appears to be caused by voltage- dependent conductances, since it is clearly decreased by hyperpolarizing the cell membrane by 10-20 mV with either steady current (Taylor and Dudek, 1984a, b) or pulses of current (Fig. 6B).

The mechanisms underlying the initiation of the low-Ca^{2+} bursts are still far from being completely defined. An important role, however, is probably played by fluctuation in $[K^+]_o$. Thus, in extracellular recordings from the s. pyramidale a small negative shift can often be observed to precede the full-blown synchronous bursts. The negative shift, whose intracellular counterpart consists of a depolarizing potential of a few mV, seems to represent an increase in $[K^+]o$, as it has been shown by using K^+-sensitive microelectrodes (Konnerth et al., 1984).

Further evidence for a role of K^+ in the initiation of the low-Ca^{2+} bursts comes from those experiments in which $[K^+]_o$ is modified by increasing the KCl concentration in the Ringer solution by 1-2 mM (i.e. 7.25-8.25 mM). If this is done early in the transition, it accelerates the maturation of the alveus-induced bursts and causes the appearence of spontaneous discharge (Fig.7). The fluctuations of $[K^+]_o$ responsible for initiating the low-Ca^{2+} bursts might be related to cyclic fluctuations in the spontaneous release of excitatory neurotransmitters from synaptic terminals (Johnston and Brown, 1984b). However, when considering the possible involvement of K^+ in the initiation of synchronous low-Ca^{2+} bursts, one should not overlook the findings of Haas and Jefferys (1984), who have reported that fluctuations in $[K^+]_o$ do not necessarily trigger the field bursts, as the K^+ activity in their experiments often increases during, but not before the bursts.

Repolarizing K^+ and energy-dependent phenomena

Both K^+-outward currents and energy-dependent repolarizing mechanisms (such

as the electrogenic pump) play a role in modulating low-Ca^{2+} bursts (Haas and Jefferys, 1984; Haas et al., 1984). Hence, biogenic amines known to modify the afterhyperpolarization in hippocampal neurons (Benardo and Prince, 1982; Madison and Nicoll, 1982; Haas and Konnerth, 1983) are capable of changing the frequency of occurrence of spontaneous low-Ca^{2+} bursts: Acetylcholine, histamine, or noradrenaline, which depress the K^+ conductance, can shorten the interburst period and increase the frequency of occurrence. However, these effects are never associated with an increase in the duration of the bursts. A similar lack of effect upon the duration is also observed after addition of the Ca^{2+} chelator EGTA. Thus, K^+ currents appear to modify the frequency of occurrence rather than the duration of each single burst.

A similar conclusion can be drawn from those experiments in which the efficacy of energy-dependent mechanisms is reduced by the application of ouabain. Here again the main effect consists of an increase in the occurrence of low-Ca^{2+} bursts. It should, however, be noted that hypoxia, which should also decrease the efficiency of the electrogenic pump, clearly increases the duration of the burst (Haas and Jefferys, 1984).

Residual GABA-ergic conductances in low-Ca^{2+} bursts

The experiments reviewed in this paper show that stimulus-induced postsynaptic responses are abolished in low-Ca^{2+} Ringer at a time when stimulation of the alveus is effective in eliciting a full-blown synchronous burst. In addition at this stage, spontaneously occurring postsynaptic potentials are almost undetectable in intracellular recordings performed with KCl filled microelectrodes (Fig.3). However, some residual

inhibitory (i.e. Cl^-, GABAergic) mechanism might still be operant even several hours after bathing the hippocampal slice with low-Ca^{2+} Ringer, due to a probable release of Ca^{2+} ions from intracellular storage into the extracellular space. This may be an important point to take into consideration, mainly when low-Ca^{2+} bursts are used as a model for screening pharmacologically active compounds such as antiepileptic drugs on the assumption that synaptic inhibitory mechanisms are blocked (Agopyan et al., 1985).

In keeping with a partial preservation of inhibitory processes, the disinhibitory drug bicuculline at relatively low concentrations (i.e. 20 μM) exerts clear effects which vary depending upon the stage during which it is applied. At a time when only the early burst is evoked by alvear stimulation, bicuculline is capable of evoking the appearence of a late burst; if applied at a later stage this disinhibitory drug is capable of inducing the appearence of spontaneous low-Ca^{2+} bursts or of accelerating them if they are already present (Fig. 8). The action of bicuculline upon the low-Ca^{2+} bursts is related to a reduction of residual GABAergic potentials rather than to an effect on K^+-repolarizing conductances (Heyer et al., 1982), since bicuculline fails to induce its effects in this model when NaCl in the Ringer is replaced with Na-methylsulphate or Na-isothionate (Avoli and Agopyan, in preparation).

Low-Ca^{2+} synchronous bursts as a model of epileptic discharges

The work reviewed in this paper demonstrates that synaptic mechanisms are not essential for neuronal synchronization during epileptiform activity. Rather, in hippocampal slices bathed with low-Ca^{2+} Ringer, synchronization and spread of the

bursts appear to be related to ephaptic mechanisms and K^+ redistribution (Haas and Jefferys, 1984; Konnerth et al., 1984; Taylor and Dudek, 1984a, b). These findings are relevant in epileptogenesis since $[Ca^{2+}]_o$ decreases dramatically during paroxysmal activity induced by electrical or chemical activation (Heinemann and Lux, 1977; Heinemann et al., 1977; Krnjević et al., 1980; Dietzel et al., 1982). Recently Pumain et al. (1985) have shown that in the allylglycine treated photosensitive baboon, prolonged light-induced generalized seizures are accompanied by large decreases in $[Ca^{2+}]_o$, reaching values at which chemical synaptic transmission was certainly reduced and even blocked. Although one should take into consideration differences which exist between the decrease in $[Ca^{2+}]_o$ during epileptiform activity and the low-Ca^{2+} bursts (e.g. in the former situation Ca^{2+} enters neuronal or glial compartments, whereas in the latter it leaks out), knowledge of the mechanisms underlying low-Ca^{2+} bursts are by all means relevant for understanding the persistence of epileptic discharge of ictal type as well as their termination in a situation where synaptic inhibitory and Ca^{2+}-dependent K^+ repolarizing conductances are depressed.

On the other hand, work carried out in different laboratories indicates that low-Ca^{2+} bursts can, in fact, be used as a tool for screening pharmacologically active molecules as well as for gaining insight into their mechanisms of action (Agopyan et al., 1985; Heinemann et al., 1985). In this respect Rose et al. (1986) have recently reported that several antiepileptic drugs display a different degree of eficacy when tested upon low-Ca^{2+} or penicillin-induced bursts.

Acknowledgement: This work was supported by the Medical Research Council of Canada (Grant MA-8109) and the Hospital for Sick Children Foundation. MA is a FRSQ Chercheur Boursier. We thank Ms. L. Rieb and Dr. V. Tancredi for participating to some preliminary experiments and Drs. U. Heinemann and H. L. Haas for helpful and constructive discussion. The technical and secreterial assistance of Ms. T. De la Fosse and G. Robillard are acknowledged.

Fig. 1. A: Schematic representation of the experimental model. The recording microelectrode is aimed to the s. pyramidale in the CA1 subfield of the hippocampal slice while stimulating electrodes are placed in s. radiatum and alveus for ortho- and antidromic activation, respectively. B: Extracellular field potentials evoked by ortho- (Radiatum) and antidromic (Alveus) stimulation in normal and low-Ca^{2+}, high-Mg^{2+} Ringer. Stimuli were delivered at 3 different strengths which were kept constant throughout the experiment. Note the blockage of orthodromic response and the appearence of repetitive population spikes following alvear stimulation in the low-Ca^{2+}, high-Mg^{2+} medium. Also, the first antidromic population spike appears to increase in amplitude, a phenomenon observed in nearly half of the experiments. C: Progressive appearence of the late burst evoked by stimulation of the alveus which was kept at constant strength. Leftward numbers indicate the time of perfusion with low-Ca^{2+}, high-Mg^{2+} Ringer. Note how the increase in number of population spikes correlates with an increase in amplitude and duration of the negative shift.

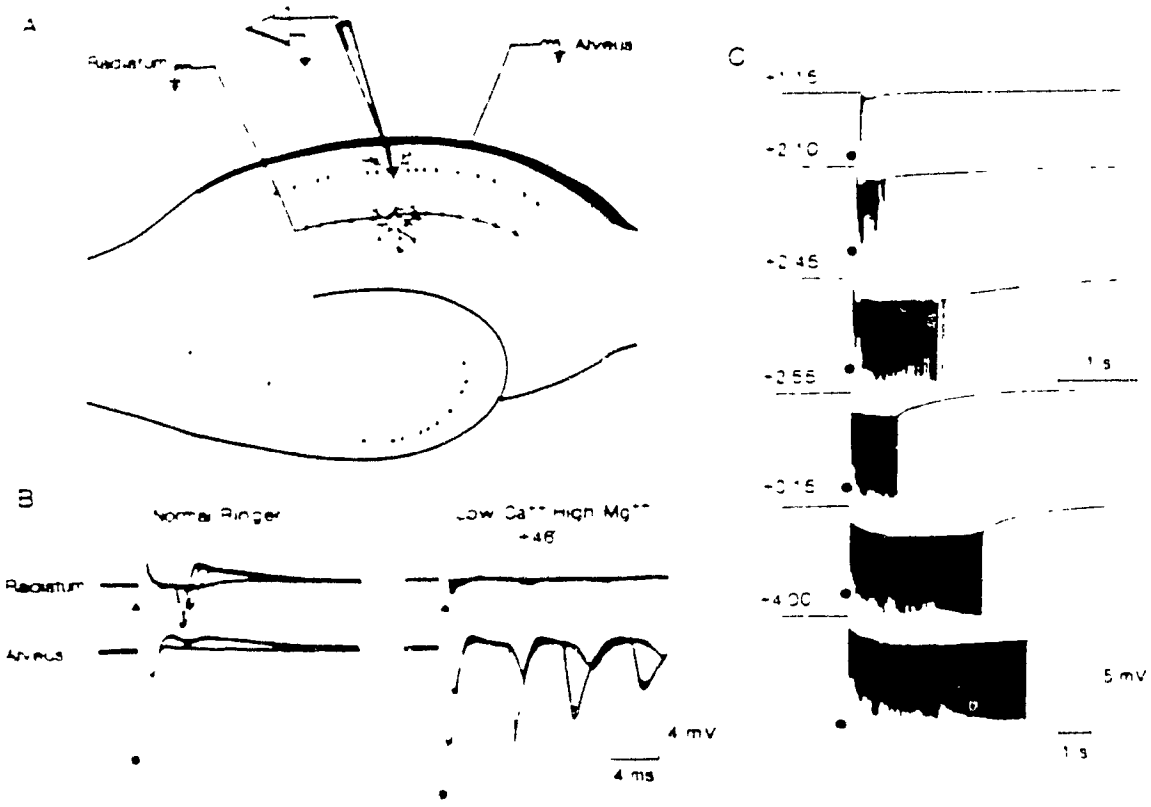
A
Alveus
B
Normal Ringer
+46
4 mV
4 ms
C
+2.10
+2.45
+2.55
+3.15
+4.00
5 mV
1 s

Fig. 2. Intracellular recordings (KCl filled microelectrodes) from hippocampal neurons in control and at different times after replacement of low-Ca^{2+}, high-Mg^{2+} Ringer. A and B are two different experiments. A: Orthodromic stimuli (triangles, a) and intracellular pulses of constant intensity (b and c) were delivered before and 40' after low-Ca^{2+}, high-Mg^{2+} replacement. B: Stimulation of the alveus (dot) was constant throughout the experiment; note the decreased amplitude of the depolarizing envelope representing an inverted IPSP at 45' low-Ca^{2+} as well as the synchronous prolonged burst at 60' low-Ca^{2+}. Upper trace in a, b and c is an extracellular recording in s. pyramidale (calibration: 24 mV), lower trace is the intracellular signal (calibration: 96 mV) simultaneously recorded.

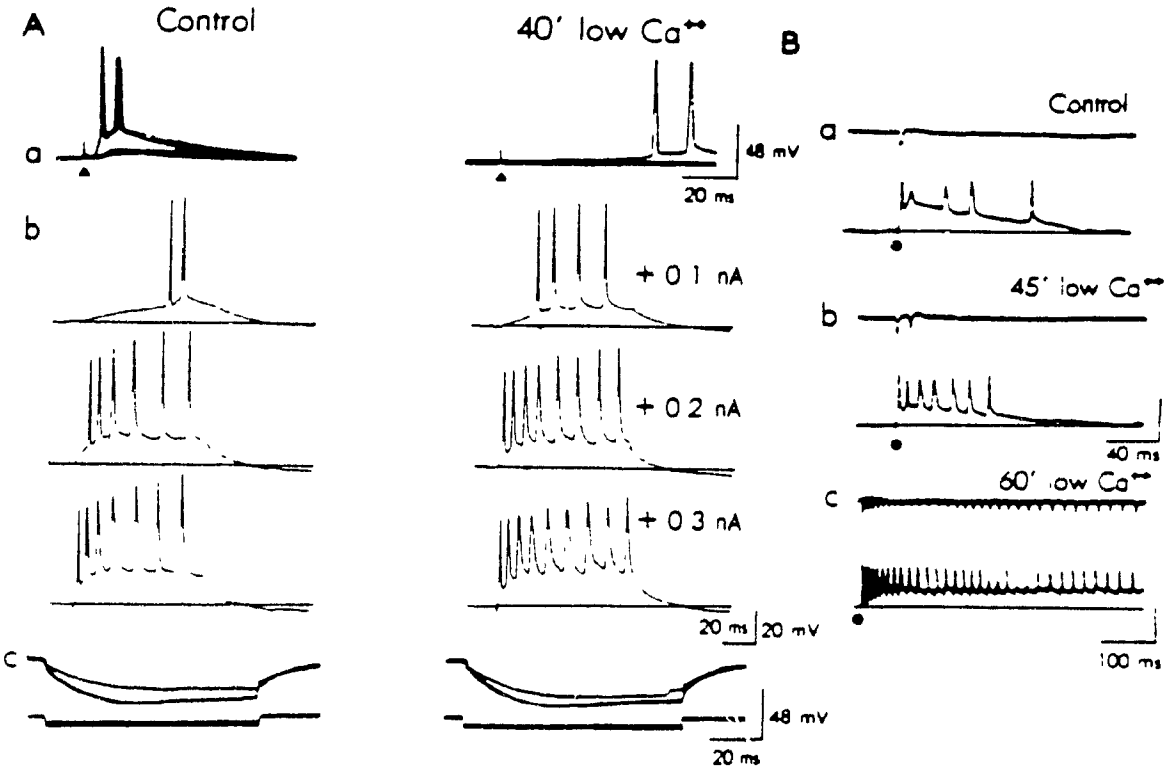
A
Control
a
b
c
40' low Ca++
B
48 mV
20 ms
+ 0.1 nA
+ 0.2 nA
+ 0.3 nA
20 ms
20 mV
48 mV
20 ms
Control
a
45' low Ca++
b
40 ms
60' low Ca++
c
100 ms

Fig. 3. Decrease of alveus induced (dots) and spontaneous depolarizing postsynaptic potentials 25' after perfusing the slice with low-Ca^{2+} Ringer. At 134', when an alvear stimulus delivered at higher strength is capable of inducing a low-Ca^{2+} burst, spontaneous depolarizing postsynaptic potentials have almost completely disappeared. The intracellular microelectrode was KCl-filled.

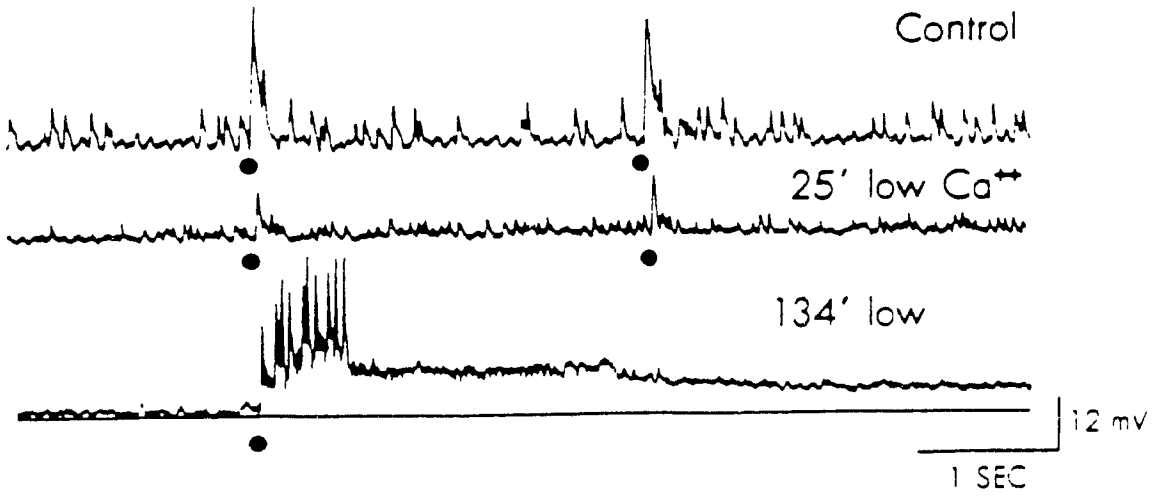
Control
25' low Ca++
134' low
12 mV
1 SEC

Fig. 4. A: Extracellular single unit recording 3 hours after exchange with low-Ca^{2+} Ringer. In a, the bursts of action potentials (arrows) which are not associated with any synchronous field burst are shown at low speed to display the regularity of the phenomenon. In b, several bursts have been superimposed on a scope triggered by the first action potential of the burst. B: Intracellular features of the burst recorded with K-acetate-filled microelectrode. In small a, spontaneous bursts are superimposed, while in b and c the bursts are evoked by a depolarizing pulse and upon termination of a hyperpolarizing pulse, respectively. Note the consistent lack of afterhyperpolarizing potentials. C: Simultaneous extra- (upper trace) and intracellular (lower trace; KCl-filled microelectrode) recordings in the CA1 subfield showing that these spontaneous bursts of action potentials are not a population phenomenon.

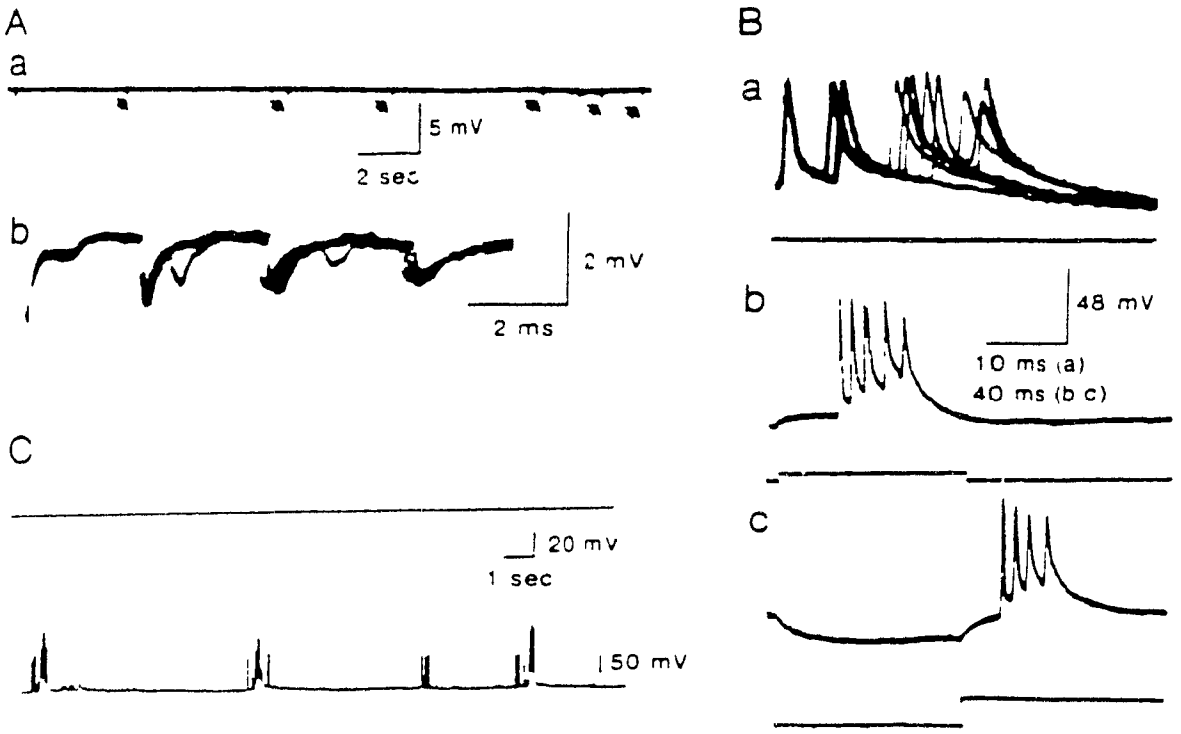
A
a
5 mV
2 sec
b
2 mV
2 ms
C
20 mv
1 sec
150 mV
B
a
b
48 mV
10 ms (a)
40 ms (b c)
c

Fig. 5. Profile analysis (left panel) of the spontaneously occurring low-Ca^{2+} bursts recorded extracellularly at the different locations (right panel) along an axis parallel to hippocampal pyramidal neurons in the CA1 subfield. Upper traces: fixed microelectrode located in the stratum pyramidale, lower traces: moving microelectrode placed in the positions shown in the schematic drawing of the CA1 subfield of the hippocampal slice. Vertical calibration: 10 mV.

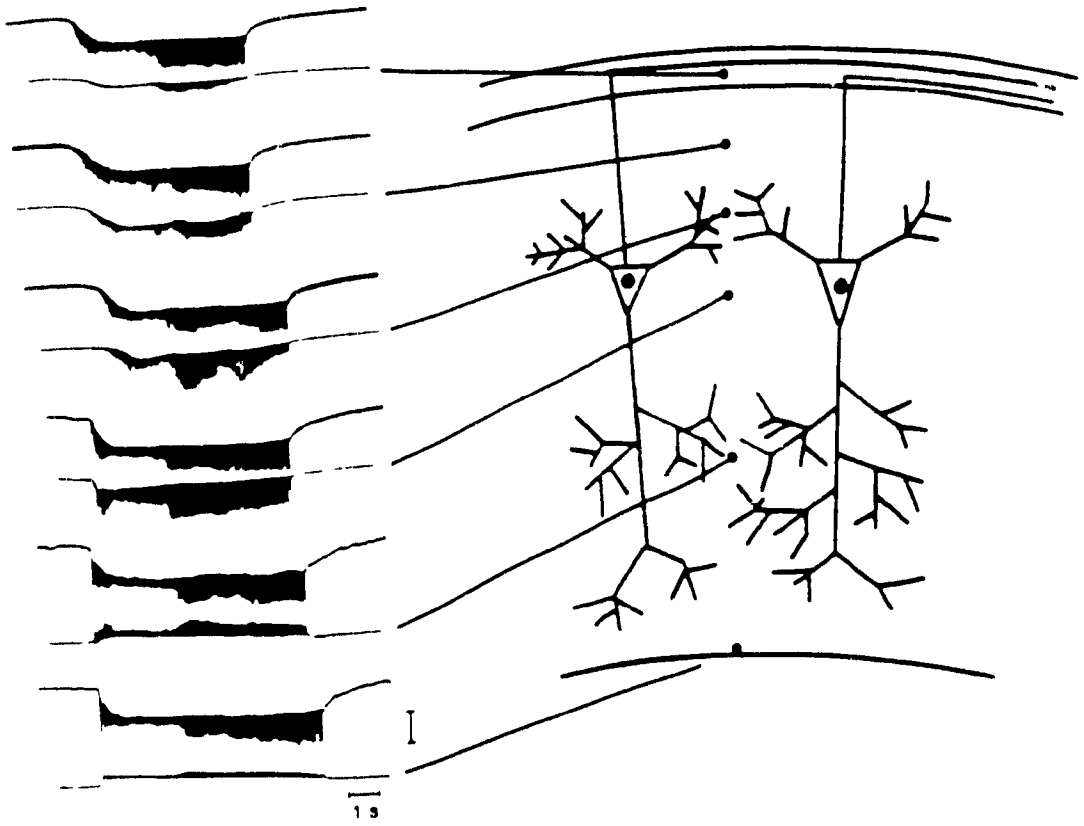
I
1 s

Fig. 6. A: Extra- and intracellular recordings from the CA1 subfield of hippocampal slice bathed for 3 hours in low-Ca^{2+}, high-Mg^{2+} Ringer. The electrodes were 1 mm apart, the intracellular one being located near the subiculum. Note the similarity between the alveus-induced and the spontaneous low-Ca^{2+} synchronous burst. B: Effects of hyperpolarizing pulses delivered at two different times after alvear shock in a different experiment. Note the ability to abort both action potential and large part of the depolarizing component of the alveus-induced low-Ca^{2+} burst.

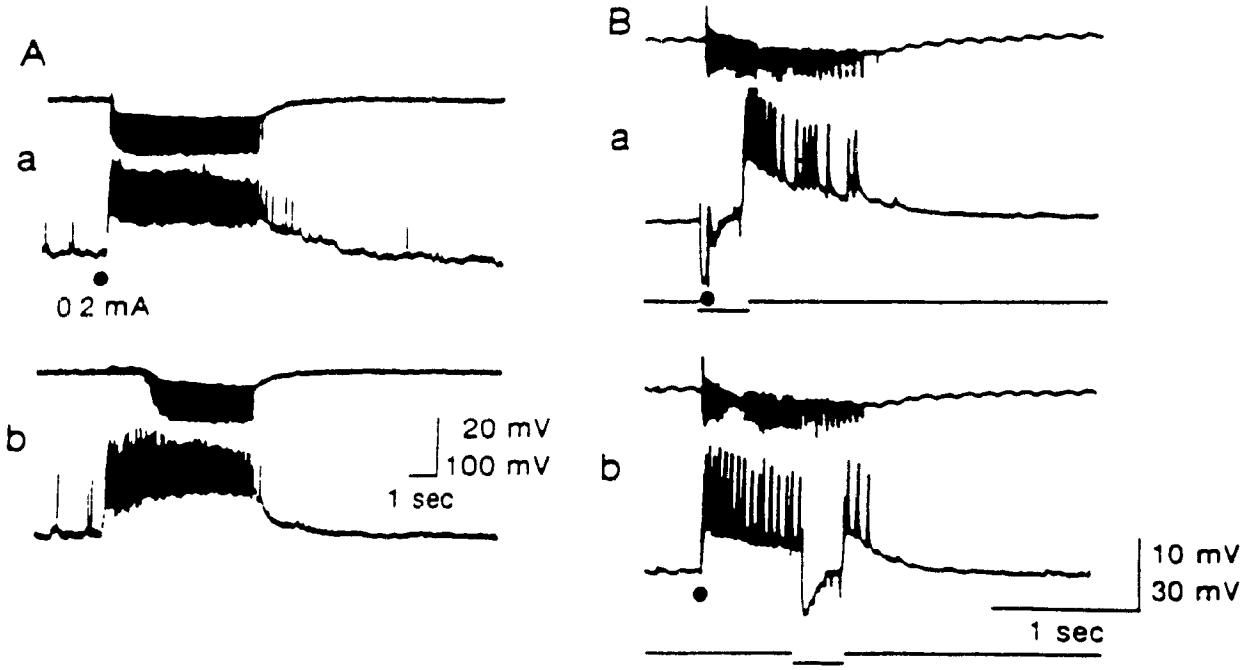
A
a
0 2 mA
b
20 mV
100 mV
1 sec
B
a
b
10 mV
30 mV
1 sec

Fig. 7. Effects induced by increasing $[K^+]_o$. Note that this procedure easily induces spontaneous, regularly occurring low-Ca^{2+} bursts. Top numbers indicate times of perfusion with low-Ca^{2+}, high-Mg^{2+} Ringer.

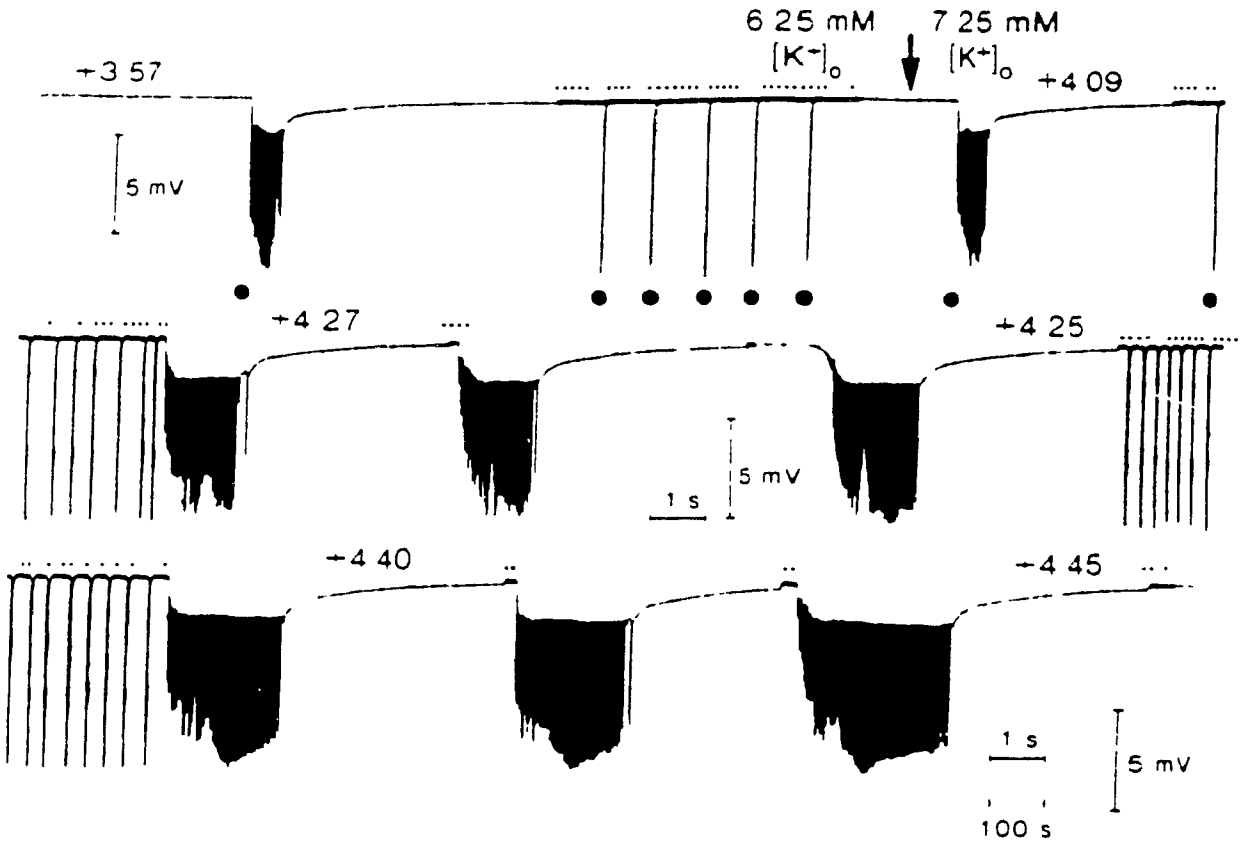
6 25 mM
$[K^+]_o$
7 25 mM
$[K^+]_o$
+3 57
+4 09
5 mV
+4 27
+4 25
5 mV
1 s
+4 40
+4 45
1 s
5 mV
100 s

Fig. 8. Effects induced by the disinhibitory agent bicuculline (BMI, 20 μM) at a time when spontaneous low-Ca^{2+} bursts occur regularly.


Control
BMI 20 μM, 10'
5 mV
100 sec

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Chapter Three

SYNAPTIC AND NON-SYNAPTIC MECHANISMS UNDERLYING LOW CALCIUM BURSTS IN THE IN VITRO HIPPOCAMPAL SLICE.

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Experimental Brain Research (1988) 73: 533-540.

ABSTRACT

1. The epileptiform activity generated by lowering extracellular Ca^{2+} was studied in the CA1 subfield of rat hippocampal slices maintained in vitro at 32° C. Extracellular and intracellular recordings were performed with NaCl and KCl filled microelectrodes. 2. Synaptic potentials evoked by stimulation of the stratum radiatum and alveus were blocked upon perfusion with artificial cerebrospinal fluid (ACSF) containing 0.2 mM Ca^{2+}, 4 mM Mg^{2+}. Blockade of synaptic potentials was accompanied by the appearance of synchronous field bursts which either occurred spontaneously or could be induced by stimulation of the alveus. 3. Both spontaneous and stimulus-induced low Ca^{2+} bursts recorded extracellularly in stratum pyramidale consisted of a negative potential shift with superimposed population spikes. This extracellular event was closely associated with intracellularly recorded action potentials rising from a prolonged depolarization shift. Steady hyperpolarization of the cell membrane potential decreased the amplitude of the depolarizing shift suggesting that synaptic conductance were not involved in the genesis of the low Ca^{2+} burst. 4. Spontaneous depolarizing inhibitory potentials recorded in normal ACSF with KCl filled microelectrodes were reduced in size in low Ca^{2+} ACSF. However, small amplitude potentials could still be observed at a time when low Ca^{2+} bursts were generated by hippocampal CA1 pyramidal neurons. 5. Bicuculline methiodide, an antagonist of γ-aminobutyric acid (GABA), was capable of modifying the frequency of occurrence and the shape of synchronous field bursts. The effects evoked by bicuculline methiodide were, however, not observed when 81-100% of NaCl was replaced with Na-Methylsulphate. Hence, it was concluded

that in low Ca^{2+} ACSF even though large release of transmitter such as those following electrical activation of stratum radiatum or alveus cannot be observed, small spontaneous release of the inhibitory transmitter GABA seems to persist. 6. Substitution of NaCl with Na-Methylsulphate also caused changes in the synchronous field bursts which were different from those observed following application of bicuculline methiodide. These findings suggest that in low Ca^{2+} ACSF, in addition to residual GABAergic Cl^- mechanisms, non-synaptic Cl^- conductances might play a role in controlling the excitability of hippocampal neurons.

INTRODUCTION

The generation and spread of focal epileptiform discharges is generally attributed to synaptic excitation (Gjerstad et al. 1981; Traub and Wong 1983) for which Ca^{2+} influx into presynaptic terminals is a prerequisite (Dingledine and Somjen 1981). However, synaptic activity does not appear to be essential for the generation of the rhythmic bursts recorded in the CA1 subfield of the hippocampal slice when extracellular Ca^{2+} concentration is lowered to levels between 0.1 - 0.2 mM (Jefferys and Haas 1982; Taylor and Dudek 1982; Haas and Jefferys 1984; Yaari et al. 1983, 1986; Konnerth et al. 1984, 1986). Furthermore, studies performed in situ with ion sensitive electrodes (Heinemann et al. 1977; Pumain et al. 1985) have shown that extracellular Ca^{2+} concentration during sustained neuronal discharges decreases to levels similar to those required of the appearence of low Ca^{2+} bursts. Consequently, the synchronous bursts generated in the low Ca^{2+} medium may represent an experimental in vitro model for analysing some of the cellular mechanisms associated with prolonged epileptiform discharges analogous to those occuring in the course of seizures in situ. It has been demonstrated that in low Ca^{2+} medium synaptic transmission is blocked and ephaptic interactions are the main mechanism for synchronizing the firing generated by hippocampal cells (Jefferys and Haas 1982; Taylor and Dudek 1982; Haas and Jefferys 1984; Yaari et al. 1983, 1986; Konnerth et al. 1984, 1986). However, the degree of this blockade has not been assessed so far. Spontaneous transmitter relays in the central nervous system (Kuno 1971; Kuno and Weakly 1972; Alger and Nicoll 1980; Miles and Wong 1984) may still be operant in the low Ca^{2+} medium. Such spontaneous release

may be due to either potentiated asynchronous release of quanta or tonic activity of neurons (Erulkar and Rahamimoff 1978). In the present study we examined the degree of synaptic blockade evoked by bathing hippocampal slices with low Ca^{2+}, high Mg^{2+} medium. Particular emphasis was given to those potentials which are inhibitory. We also present some evidence for non-synaptic mechanisms which could account for the termination of the epileptiform bursts generated by CA1 hippocampal pyramidal cells. Preliminary reports of part of this work were published in abstact form (Agopyan and Avoli 1985) and incorporated in a review article (Avoli and Agopyan 1986).

METHODS

Experiments were performed on hippocampal slices from Sprague Dawley male rats which were prepared and maintained at 32° C according to conventional techniques (Tancredi and Avoli 1987). Slices were perfused with an oxygenated (95% O_2/5% CO_2) artificial cerebrospinal fluid (ACSF) at pH 7.4. The composition of ACSF was in mM: NaCl: 124, KCl: 5, KH_2PO_4: 1.25, $MgCl_2$: 2, NaH_2CO_3: 26, $CaCl_2$: 2 and glucose: 10. Low Ca^{2+}, high Mg^{2+} ACSF (hereafter called low Ca^{2+} ACSF) was prepared by decreasing the concentration of $CaCl_2$ to 0.2 mM and increasing that of $MgCl_2$ to 4 mM. Na-methylsulphate (ICN) was used to replace NaCl for low Cl^- ACSF. Bicuculline methiodide (BMI, Sigma) was applied in the perfusing ACSF.

Neuronal activity was recorded in the stratum pyramidale of the CA1 subfield . Glass microelectrodes filled with 3 M NaCl (5-10 MΩ) and/or 3M KCl (60-100 MΩ) were used for extracellular and intracellular recordings respectively. Intracellular recordings were obtained from 54 pyramidal neurons which displayed input resistance greater than 30 MΩ, overshooting action potentials with amplitudes larger than 70 mV and membrane potential (Vm) more negative than -50 mV. Constant current stimuli (90 μs, 0.02-0.2 mA) were delivered through sharpened and insulated monopolar tungsten electrodes which were placed in the alveus for antidromic and stratum radiatum for orthodromic stimulation. Recorded signals were simultaneously displayed on a Gould pen recorder and on an oscilloscope. In some instances data were stored on a Gould magnetic tape recorder for subsequent analysis.

RESULTS

Development of epileptiform activity during perfusion with low Ca^{2+} ACSF

Responses to orthodromic and antidromic stimuli were modified by perfusion with low Ca^{2+} ACSF. Thus in the experiment illustrated in Fig. 1A stimulation of the stratum radiatum after 46 min of low Ca^{2+} perfusion, failed to induce any orthodromic response (indicating the blockade of synaptic transmission), while alvear stimulation evoked a burst of 4-5 population spikes. Over time this antidromically evoked burst became associated with a negative potential shift, on which population spikes were superimposed (hereafter referred as the low Ca^{2+} burst, Fig. 1B).

A similar pattern of changes could be observed intracellularly. In Cl^- loaded CA1 pyramidal cells the depolarizing envelope which represents the recurrent, inhibitory postsynaptic potential (IPSP) following antidromic stimulation decreased in amplitude and duration 30 min after perfusion with low Ca^{2+} ACSF (Fig. 1C), suggesting a reduction in Cl^- mediated GABAergic potentials at the soma (Andersen et al. 1964; Kandel et al. 1964). Later, the remaining depolarizing potential evoked by stimulation of the alveus increased in duration and became associated with synchronous firing (Figs. 1C, 68' and Da).

In control ACSF intracellular recordings with KCl-filled microelectrodes also revealed the occurrence of spontaneous, transient depolarizations of variable amplitude, some as large as 40 mV (Fig. 2A). Since these depolarizing events were seen with KCl filled electrodes, they likely represented spontaneously occurring IPSPs. As low Ca^{2+} ACSF equilibrated in the tissue chamber the amplitude of these spontaneous IPSPs

decreased (Fig. 2A). The frequency histogram of amplitudes of the recurring depolarizations, constructed after one hour and half of perfusion with low Ca^{2+} ACSF showed a marked reduction in those with a large amplitude but almost no change in the occurrence of the smaller ones (Fig. 2B).

As previously reported (Haas and Jefferys 1984; Avoli and Agopyan 1986; Jones and Heinemann 1987), pyramidal neurons could depolarize by 10-20 mV upon perfusion with low Ca^{2+} ACSF. However, in spite of this steady depolarization, afterhyperpolarizations were always of smaller amplitude than under control conditions (cf. Haas and Jefferys 1984). Therefore, the low Ca^{2+} ACSF in addition to blocking stimulus induced synaptic transmission, decreased the efficacy of repolarizing, presumably Ca^{2+} dependent K^+ conductances (Hotson and Prince 1980).

Fullblown low Ca^{2+} bursts

Two to three hours after replacing normal with low Ca^{2+} ACSF, spontaneous low Ca^{2+} bursts were also observed. These bursts were similar to those evoked by antidromic stimuli; they consisted of an extracellularly recorded negative potential shift with superimposed population spikes. In intracellular recordings both stimulus-induced and spontaneous low Ca^{2+} bursts were characterized by fast, presumably Na^+ action potentials rising from a prolonged depolarizing shift (Fig. 1Db). After their appearance, spontaneous low Ca^{2+} bursts matured further within the next 20 to 30 min and reached a steady state during which their morphology, duration and frequency of occurrence remained constant in any given slice throughout the experiment.

Steady injection of hyperpolarizing current decreased the depolarizing shift and

the action potentials associated with both spontaneous and stimulus-induced low Ca^{2+} bursts suggesting that a large component of the depolarization shift was caused by voltage dependent conductances (Fig. 3A). However, at least within the range of V_m tested (i.e. up to 30 mV negative to rest), some portion of the depolarizing envelope still persisted. Furthermore hyperpolarizing pulses delivered during stimulus-induced low Ca^{2+} bursts were capable of reducing the depolarizing shift and the action potentials superimposed on it, although they displayed a clear sag toward resting V_m (Fig. 3B, C).

Cl^- mediated mechanisms and low Ca^{2+} bursts

The findings reported so far suggest that synaptic transmission, at least as tested by stimulating the Schaffer collaterals and the alveus, is blocked in low Ca^{2+} ACSF. However, the application of BMI (20 - 100 μM) modified the frequency of occurrence of spontaneous low Ca^{2+} bursts. As shown in Fig. 4A, BMI accelerated the burst frequency by 3 - 4 fold with either a decrease or no change in each burst duration (n = 12 experiments). In slices where only stimulus-induced low Ca^{2+} bursts could be observed 4 - 6 h after low Ca^{2+} ACSF perfusion (n = 5 experiments), BMI application induced the appearance of spontaneous low Ca^{2+} burst (Fig. 6B).

BMI blocks inhibitory potentials by antagonizing GABA rather than Cl conductances. However, BMI can also decrease K^+ repolarizing conductances (Heyer et al. 1982). In order to determine whether the effects induced by BMI in low Ca^{2+} ACSF represent a true blockade of residual GABAergic conductances or a reduction of K^+ repolarizing conductances we tried to eliminate any residual inhibitory conductance by reducing the Cl^- concentration in the ACSF and tested BMI on low Ca^{2+} burst

generated by hippocampal neurons perfused with low Cl^- medium.

Replacing 81 - 100 % of the NaCl in the low Ca^{2+} ACSF with Na-methylsulphate modified the shape of spontaneous and stimulus-induced low Ca^{2+} bursts (n = 6 experiments) which increased by 6 - 10 folds in duration. In addition, the amplitude of the negative potential shift was augmented (Fig. 5B). During replacement of NaCl the frequency of occurrence of spontaneous low Ca^{2+} bursts, following an initial transient increase, decreased by an average of 40 %.

BMI (20 - 50 μM) did not induce any change in either the frequency or the shape of the low Ca^{2+} bursts generated by slices bathed in low Ca^{2+} ACSF where NaCl had been substituted (Fig. 5). However, with higher concentrations of BMI (100 μM) subtle changes could be observed, namely the number of population spikes associated with the low Ca^{2+} bursts and their frequency slightly increased (Fig. 5). These findings suggest that the effects evoked upon the low Ca^{2+} bursts by BMI up to concentrations of 50 μM, are probably related to an action on residual GABAergic synaptic mechanisms rather than to a decrease in K^+ repolarizing conductances.

As shown in Fig. 5, NaCl replacement evoked changes in the negative potential shift which were different from these following BMI application (compare with Fig. 4). Furthermore, the duration of the low Ca^{2+} bursts was greatly prolonged by replacement of NaCl with Na-methylsulphate. These differences would suggest that NaCl replacement affected Cl^- conductances non-synaptic in origin rather than a sole blockade of residual GABAergic conductances. This conclusion was further supported by experiments where NaCl was replaced following at least 30 min of BMI (100 μM)

perfusion. If the effects induced by Na-Methylsulphate were related to a synaptic Cl^- conductance, by using this experimental procedure one would expect that an almost complete blockade of residual GABAergic mechanisms had been induced by BMI and thus one would predict no further change following Cl^- replacement. However, Cl^- substitution consistently evoked an increase in the duration and amplitude of the negative potential shift.

DISCUSSION

The results presented in this paper confirm previous reports (Jefferys and Haas 1982; Yaari et al. 1983; Haas and Jefferys 1984; Konnerth et al. 1986; Yaari et al. 1986) that lowering extracellular Ca^{2+} allows CA1 pyramidal neurons to generate synchronous epileptiform bursts. Normally, sustained neuronal discharges are opposed by recurrent and feedforward inhibition (Kandel et al. 1961; Andersen et al. 1964) as well as by Ca^{2+} dependent K^+ mediated afterhyperpolarizations (Hotson and Prince 1980). In low Ca^{2+} medium these processes are reduced as shown by the decrease in amplitude of the depolarizing envelope evoked by orthodromic or antidromic stimulation as well as by the appearance of a burst of action potentials following antidromic stimulation. In addition, in low Ca^{2+} ACSF, hippocampal cells are also rendered more excitable by the loss of stabilizing action of Ca^{2+} on fixed negative charges of the membrane (Frankenhaeuser 1957; Kelly et al. 1959).

The finding that orthodromic stimulation fails to induce a response at a time when bursts are developing suggests that synaptic currents are not actively involved in the genesis of both spontaneous and alveus induced low Ca^{2+} bursts. In accordance with this conclusion is the absence of an active sink in the dendrites where a large number of excitatory synapses reside (Avoli and Agopyan 1986). Furthermore, hyperpolarizing the V_m did not increase the amplitude of the depolarizing shift underlying the low Ca^{2+} burst. As it is expected from an endogenous, voltage-dependent burst, the amplitude of the depolarizing shift, after reaching a maximum, decreased as the V_m was varied in either hyperpolarizing or depolarizing direction.

Within the range of V_m studied we could not completely abort the depolarizing envelope. Moreover, hyperpolarizing square pulses delivered during the burst displayed a sag which is suggestive for the activation of an inward current. The fact that a sag of similar amplitude was not seen when the pulse was given during either an immature burst or the refractory period rules out the possibility that this sag was caused by the activation of the anomalous rectifier, named Q current in hippocampal neurons (Adams and Halliwell 1982). The conductance increase revealed by short hyperpolarizing pulses given during a burst might be caused by the activation of inward Ca^{2+} (cf. Francheschetti et al. 1986) or Na^+ conductances or to K^+ accumulation in the extracellular space.

Spontaneous synaptic potentials have previously been observed in hippocampal neurons (Alger and Nicoll 1980; Miles and Wong 1984). In control ACSF we observed spontaneous post-synaptic potentials only with KCl-filled electrodes suggesting that they were due to the outward movement of Cl^- ions and that they represented spontaneously occurring IPSPs (cf. Alger and Nicoll 1980). As the hippocampal CA1 neurons stabilized in low Ca^{2+} medium, the spontaneous IPSPs diminished in size and frequency. Thus in low Ca^{2+} medium, the spontaneous release of inhibitory transmitter is reduced but not abolished. This finding may be explained by the fact that if these residual spontaneously occurring IPSPs are quantal in nature, the extracellular Ca^{2+} gradient is probably not sufficiently reversed to abolish quantal release (cf. Krnjević et al. 1986) or if they represent activity dependent release, then in a low Ca^{2+} medium tonic activity in inhibitory interneurons is still preserved. In keeping with either

mechanisms, BMI (20-100 μM), which blocked spontaneous inhibitory potentials, accelerated the frequency of low Ca^{2+} bursts. Thus in low Ca^{2+} ACSF even though large releases of transmitter such as those following electrical activation of stratum radiatum or alveus cannot be observed, small spontaneous release of inhibitory transmitter GABA seems to persist. This residual GABAergic transmission is capable of modulating the frequency of low Ca^{2+} bursts.

It has been shown that increasing the extracellular K^+ concentrations causes an increase in the frequency of spontaneous low Ca^{2+} bursts while decreasing their duration (Haas and Jefferys 1984; Yaari et al. 1986). Similar effects of increasing extracellular K^+ have also characterized epileptiform discharges induced by the addition of convulsant drugs to normal ACSF (cf. Tancredi and Avoli 1987). Thus the termination of epileptiform discharges appears to be not dependent upon a mechanism related to extracellular K^+. In keeping with this conclusion, extracellular K^+ at the moment of seizure termination is not any higher than during the seizure itself, that is to say it does not cause a "cathodal blockade" of excitability (Yaari et al. 1986). Moreover, in low Ca^{2+} medium acetylcholine, histamine or noradrenaline, which depress K^+ conductance and thus the afterhyperpolarization of hippocampal neurons (Benardo and Prince 1982; Madison and Nicoll 1982; Haas and Konnerth 1983), only shorten the interburst interval, but never increase the duration of the burst (Haas et al. 1984). Neither does the addition of Ca^{2+} chelator EGTA nor hypoxia and ouabain (Yaari et al. 1983; Haas and Jefferys 1984; Konnerth et al. 1986; Yaari et al. 1986).

However, substitution of NaCl increases the duration of the low Ca^{2+} burst.

Recently Ca^{2+} and/or voltage dependent Cl^- conductances have been demonstrated in hippocampal and other vertebrate neurons (Owen et al. 1984; Madison et al. 1986). The Ca^{2+} sensitive Cl^- conductance (Owen et al. 1984) was probably reduced in our low Ca^{2+} model as were Ca^{2+} dependent K^+ conductances. On the other hand the voltage sensitive Cl^- conductance is active at the resting potential and inactive at membrane potentials positive to -10 mV (Madison et al. 1986). Therefore the depolarization induced by lowering extracellular Ca^{2+} in some hippocampal cells would not be sufficient to completely block this non-synaptic, Cl^- mechanism. In fact replacement of NaCl, after residual inhibitory mechanisms had been blocked by BMI, prolonged the low Ca^{2+} burst, suggesting the presence of a non-synaptic Cl^- conductance in low Ca^{2+} ACSF.

In experiments where NaCl was replaced while the concentrations of Ca^{2+} and K^+ were kept at their normal values (2 and 3.5 mM respectively) sustained bursts similar to those induced by low Ca^{2+} have been observed (Yamamoto and Kawai 1968; Chamberlain et al. 1986). The effect seen with NaCl substitution might be attributed to a Donnan reequilibration, i.e. K^+ concentration may increase extracellularly to compensate for the reduction in Cl^- concentration. However, studies performed in our laboratory with ion sensitive electrodes have recently shown that replacement of NaCl with Na-Methylsuphate does not induce any change in extracellular K^+ concentration (M. Avoli, C. Drapeau, P. Perreault, J. Louvel and R. Pumain, in preparation). The observation that the duration of low Ca^{2+} bursts increases even further upon substitution of NaCl may suggest a role for non-synaptic Cl^- conductances in terminating the bursts.

In conclusion our data suggest that in low Ca^{2+} medium inhibitory potentials are still partially operant and BMI in low doses blocks these residual GABAergic mechanisms. Furthermore non-synaptic Cl^- conductances appear to play a role in controlling the excitability of hippocampal neurons.

Acknowledgements. We thank Drs. P. Gloor and K. Krnjević for reading an earlier draft of this manuscript, and Ms. G. Robillard for secretarial assistance. We also acknowledge the participation of Ms. L. Rieb and Dr. V. Tancredi in some early experiments.

Fig. 1. A: Extracellular field potentials evoked by orthodromic (a) and antidromic (b) stimulation in normal and low Ca^{2+} ACSF. Stimuli were delivered at 3 different strengths which were kept constant throughout the experiment. B: Progressive maturation of low Ca^{2+} burst induced by alvear stimulation kept at a constant intensity. Leftward numbers indicate the time elapsed since the start of of low Ca^{2+} ACSF. C: Responses to alveus stimulation recorded intracellularly with a KCl-filled microelectrodes in control and at different times after replacement with low Ca^{2+} ACSF. The intensity of the alveus stimulation was kept constant throughout the experiment. D: Simultaneous extra- and intracellular recordings from CA1 subfield show a close similarity between a stimulus-induced (a) and a spontaneous (b) low Ca^{2+} burst. These samples were taken 3 h after perfusion with low Ca^{2+} ACSF. The recording electrodes were 1 mm apart, the intracellular one being located near the subiculum.

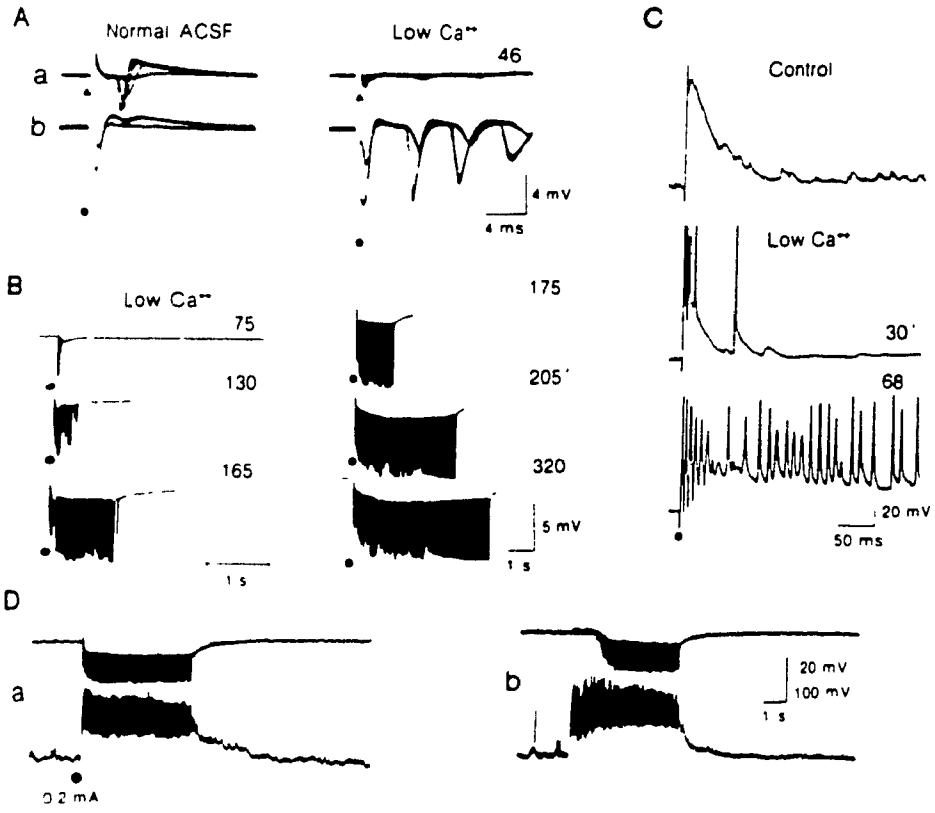

A
Normal ACSF
Low Ca++
a
b
46
4 mV
4 ms
B
Low Ca++
75
130
165
175
205
320
5 mV
1 s
C
Control
Low Ca++
30
68
20 mV
50 ms
D
a
0.2 mA
b
20 mV
100 mV
1 s

Fig. 2. A: Spontaneous post-synaptic potentials recorded intracellularly with KCl-filled microelectrodes in control, at 25 and 134 min of low Ca^{2+} ACSF. Dots represent stimulation of the alveus. The strength of alveus stimulation was doubled at 134 min to show that at a time when stimulus-induced low Ca^{2+} burst can be induced the high amplitude spontaneous post-synaptic potentials are abolished. B: Frequency histograms of the amplitudes of the spontaneously occuring synaptic potentials constructed during control (a) and after 1 h and half of low Ca^{2+} perfusion (b). Inserts are representative examples of raw data.

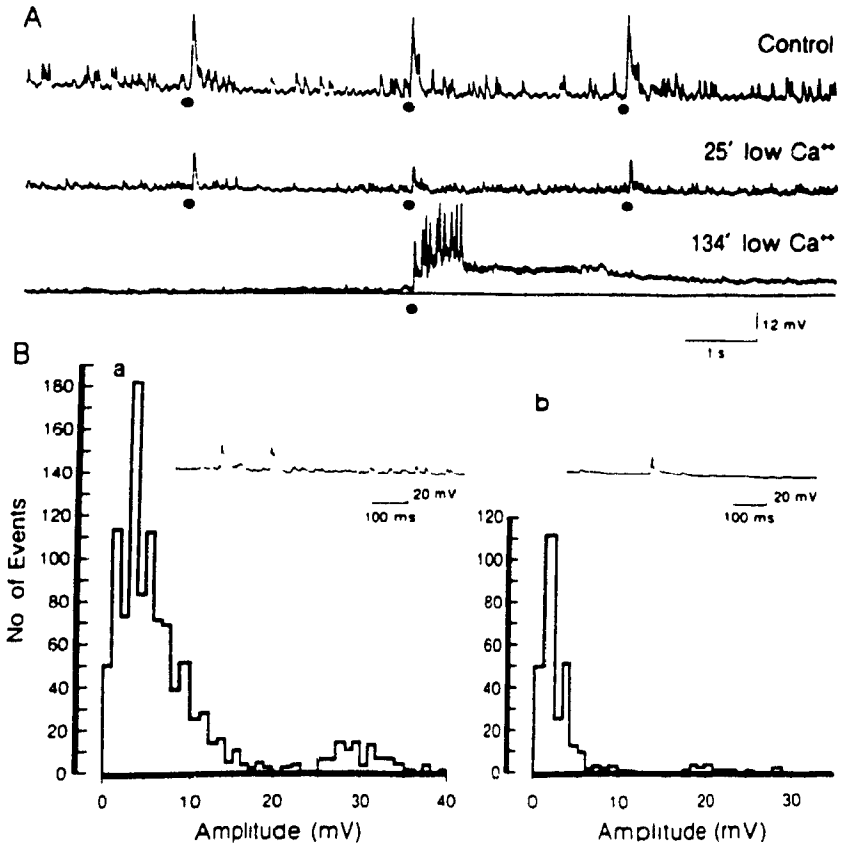

A
Control
25' low Ca++
134' low Ca++
12 mV
1 s
B
a
b
20 mV
100 ms
No of Events
Amplitude (mV)

Fig. 3. A: Effects of steady hyperpolarization on stimulus-induced low Ca^{2+} burst. The range of hyperpolarization used was not sufficient to completly block the depolarizing envelope and the superimposed action potentials. B: Hyperpolarizing pulses (0.37 nA) delivered during the initial (a) and terminal (b) part of an alveus-induced low Ca^{2+} burst. Note that even though the action potentials are aborted the depolarizing envelope appears as a sag. The numbers 1,2 and 3 represent extracellular and intracellular potentials and current traces respectively. C: Effect of hyperpolarizing pulses (0.32 nA) delivered during a silent period (a), in conjuction with a low Ca^{2+} burst evoked during the refractory period (b) and during a fullblown low Ca^{2+} burst (c). Note that the sag is only seen when there is an underlying fullblown burst.

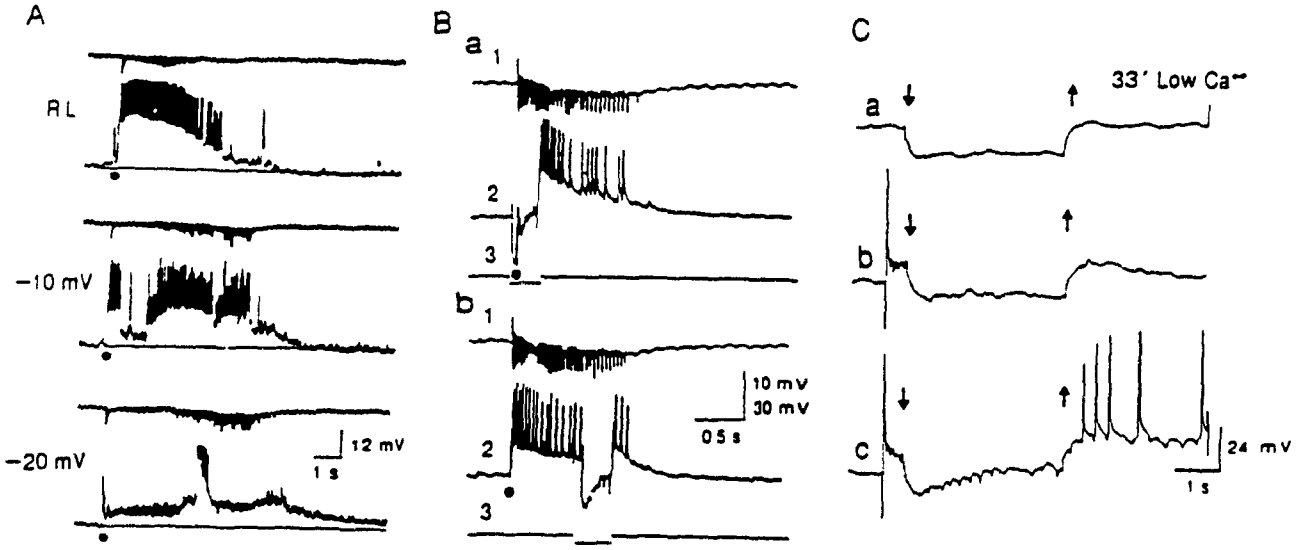

A
RL
−10 mV
−20 mV
12 mV
1 s
B
a 1
2
3
b 1
2
3
10 mV
30 mV
0.5 s
C
33' Low Ca++
a
b
c
24 mV
1 s

Fig. 4. A: Effects evoked by BMI upon spontaneously occurring low Ca^{2+} burst. 1 and 2 in b are expanded traces of spontaneous low Ca^{2+} burst before and after application of BMI. B: BMI applied at a time when only alveus-induced (dots) low Ca^{2+} burst could be observed, causes the appearance of spontaneous low Ca^{2+} bursts. The asteriks indicates a lapse of 30 s.

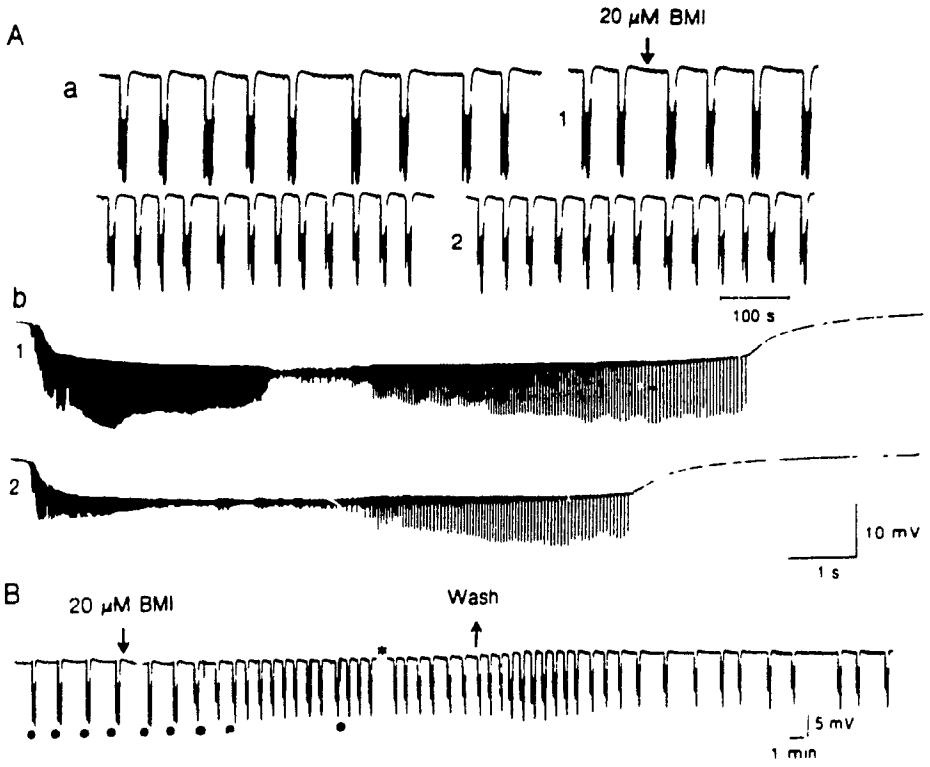
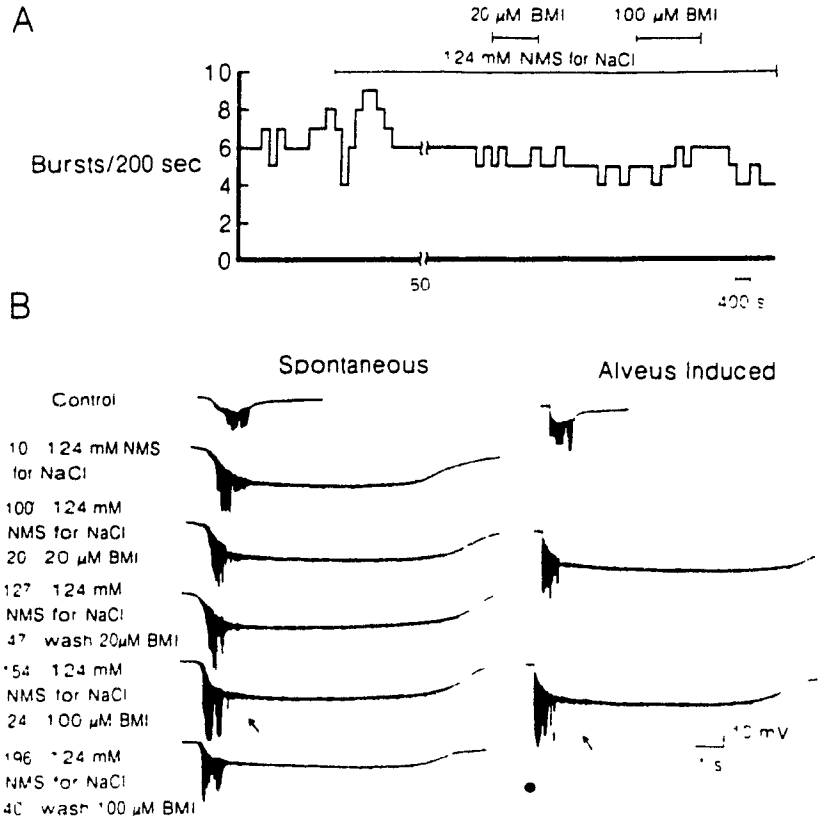

A
20 μM BMI
a
1
2
100 s
b
1
2
10 mV
1 s
B
20 μM BMI
Wash
5 mV
1 min

Fig. 5. A,B: Effects induced by 100% substitution of NaCl in the ACSF with Na-Methylsulphate (NMS) and changes evoked by BMI upon low Ca^{2+} burst generated by slices bathed with this type of ACSF. A and B show a frequency histogram and raw data from the same experiment. Note in A that initially the bursts occur more frequently following replacement of NaCl with NMS. This phenomenon is associated with a prolongation of the low Ca^{2+} bursts (10', 124 mM NMS for NaCl) as shown in B. Later, the frequency of occurrence returns to the control level, but the low Ca^{2+} bursts remain prolonged. Note that BMI (20 μM) fails to induce any change, while a slight increase in frequency of occurrence (A) and increase in number of associated population spikes (B arrows) is observed with 100 μM.


A
20 μM BMI
100 μM BMI
124 mM NMS for NaCl
Bursts/200 sec
10
8
6
4
2
0
50
400 s
B
Spontaneous
Alveus Induced
Control
10 124 mM NMS for NaCl
100 124 mM NMS for NaCl
20 20 μM BMI
127 124 mM NMS for NaCl
47 wash 20μM BMI
154 124 mM NMS for NaCl
24 100 μM BMI
196 124 mM NMS for NaCl
40 wash 100 μM BMI
10 mV
5

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