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AN IN VITRO STUDY ON

THE ROLE OF EXCITATORY AMINO-ACID RECEPTORS IN

NORMAL AND EPILEPTIC NEOCORTICES

by

Granger G.C. Hwa
Department of Neurology and Neurosurgery
McGill University, Montreal
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ABSTRACT

The putative neurotransmitters L-glutamate and L-aspartate can exert their excitatory action by activating the N-methyl-D-aspartate (NMDA) and non-NMDA receptors. The objective of this thesis is to investigate the physiological role of these excitatory amino-acid receptors in neocortical slices of normal rats and epileptic patients. My experiments in the rat neocortex indicate that excitatory synaptic transmission in normal artificial cerebrospinal fluid (ACSF) is primarily mediated by non-NMDA receptors, whereas the NMDA receptor is only activated when synaptic inhibition mediated by gamma-aminobutyric-acid-A receptors is reduced or blocked. Under such conditions, I found that the NMDA receptor participates in neuronal synchronization through polysynaptic circuits and contributes to the depolarization underlying the epileptiform discharge. In the epileptogenic human neocortex, I discovered that both NMDA and non-NMDA receptors are responsible for excitatory synaptic transmission in normal ACSF. Furthermore, their activation can lead to the appearance of epileptiform discharges. Together, the findings reported in this thesis suggest that the function of NMDA and non-NMDA receptors might be enhanced in the epileptogenic human neocortex, and lend further support to the hypothesis that excitatory amino acids are involved in epileptogenesis.

RÉSUMÉ

Les neurotransmetteurs L-glutamate et L-aspartate exercent leur action excitatrice en activant les récepteurs au N-méthyl-D-aspartate (NMDA), ainsi que les récepteurs non-NMDA. L'objectif de cette thèse est d'étudier le rôle physiologique de ces récepteurs sur des tranches de néocortex chez le rat et chez l'humain épileptique. Dans le néocortex du rat, la transmission synaptique excitatrice enregistrée dans du liquide céphalo-rachidien artificiel (LCRA) normal est principalement assurée par les récepteurs non-NMDA. Les récepteurs NMDA sont activés seulement lorsque l'inhibition synaptique assurée par les récepteurs à l'acide gamma-aminobutyrique de type A est réduite ou bloquée. Dans de telles conditions, j'ai trouvé que les récepteurs NMDA participent à la synchronisation neuronale à travers des circuits polysynaptiques, et contribuent à la dépolarisation qui sous-tend les décharges épileptiformes. Dans le néocortex épileptogène humain, dans du LCRA normal, j'ai découvert que les récepteurs NMDA et non-NMDA sont tous deux responsables de la transmission synaptique excitatrice. De plus, leur activation peut déclencher l'apparition de décharges épileptiformes. Considérées dans l'ensemble, les découvertes démontrent que la fonction des récepteurs NMDA et non-NMDA semble être amplifiée dans le néocortex épileptogène humain et apportent aussi un support additionnel à l'hypothèse voulant que les acides aminés excitateurs soient impliqués dans l'épileptogénèse.

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Officially, I am credited with the authorship of this thesis. However, I feel that its successful completion could not have been possible without the following individuals.

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I praise **God** for blessing me with this valuable learning opportunity. "Trust in the Lord with all your heart and lean not on your own understanding; in all your ways acknowledge him, and he will make your paths straight." (Proverbs 3:5-6; New International Version).

PREFACE

This thesis is comprised of seven chapters. Chapter 1 presents a comprehensive review on the roles of excitatory amino acids in the various experimental models of epilepsy, and thus provides a backdrop for stating the objectives of this thesis. Following this introductory chapter, the excitatory synaptic mechanism in rat neocortical slices, where synaptic inhibition is normal or impaired, is examined in chapters 2 to 4. Then the investigation of excitatory synaptic transmission in human neocortical slices resected from epileptic patients is presented in chapters 5 and 6. Finally, a general conclusion on these findings is given in chapter 7. This type of joint animal-human approach is designed to enable us to formulate hypotheses from the rat study and substantiate them in the human study.

A major portion of this work has been submitted to or published in scientific journals. They represent an original contribution to knowledge on excitatory amino acid-mediated synaptic transmission in the mammalian neocortex. Listed below are the principal contributions of these chapters.

Chapter 2 entitled "Excitatory postsynaptic potentials recorded from regular-spiking cells in layers II/III of rat sensorimotor cortex" by GGC Hwa and M Avoli has appeared in *Journal of Neurophysiology* 67: 728-737, 1992. It provides an in depth analysis on the physiological and pharmacological aspects of excitatory synaptic transmission in the superficial layers of rat sensorimotor-cortical slices. We found that the voltage behavior of the excitatory postsynaptic potential (EPSP) is sensitive

to the influence of inward membrane rectifications. This indicates that the EPSP is partially contributed by voltage-dependent intrinsic conductances. With the aid of selective excitatory amino-acid antagonists, we demonstrated that excitatory synaptic transmission in normal artificial cerebrospinal fluid is primarily mediated by the non-(N-methyl-D-aspartate: NMDA) receptors. However, a slight reduction in gamma-aminobutyric acid $(GABA)_A$-mediated inhibition is sufficient to activate NMDA-mediated EPSPs. These EPSPs appeared to be polysynaptic in origin and could play an important role in epileptiform synchronization.

Chapter 3 entitled "The involvement of excitatory amino acids in neocortical epileptogenesis: NMDA and non-NMDA receptors" by GGC Hwa and M Avoli has appeared in *Experimental Brain Research* 86: 248-256, 1988. It demonstrates that when $GABA_A$-mediated inhibition in rat neocortical slices is blocked by bicuculline, the synaptic activation of excitatory amino-acid receptors is sufficient to elicit the paroxysmal depolarization shift (PDS). The non-NMDA receptors are crucial for PDS generation while the NMDA receptor is involved in its amplification. Since the NMDA-mediated phase of the PDS failed to display NMDA-like voltage dependency while the latency to onset of the PDS was augmented by NMDA antagonists, we proposed that the involvement of NMDA receptors in the PDS is polysynaptic in origin.

Chapter 4 entitled "Cesium potentiates epileptiform activities induced by bicuculline methiodide in rat neocortex maintained in vitro" by GGC Hwa and M Avoli has appeared in *Epilepsia* 32: 747-754, 1991. It reports that cesium can induce

the appearance of spontaneous epileptiform activities in rat neocortical slices treated with bicuculline. It also provides a electrophysiological and pharmacological characterization of this type of epileptiform activity.

Chapter 5 entitled "Excitatory synaptic transmission mediated by NMDA and non-NMDA receptors in the superficial/middle layers of the epileptogenic human neocortex maintained in vitro" by GGC Hwa and M Avoli has been submitted to *Neuroscience Letters*. It demonstrates that excitatory synaptic transmission in layers II-IV of the epileptogenic human neocortex is mediated by both the NMDA and the non-NMDA receptors. In addition, it shows that excitatory amino-acid receptors are involved in the epileptiform discharge recorded from this type of human biopsies. These results implicate that the function of NMDA and non-NMDA receptors might be enhanced in epileptogenic human foci.

Chapter 6 entitled "Bicuculline-induced epileptogenesis in the human neocortex maintained in vitro" by GGC Hwa, M Avoli, A Olivier and JG Villemure has appeared in *Experimental Brain Research* 83: 329-339, 1991. It provides a detailed characterization on the electrophysiological feature of the PDS evoked in human neocortical slices treated with bicuculline. Moreover, it documents the involvement of NMDA receptors in this type of epileptiform activity.

CHAPTER 1

GENERAL INTRODUCTION

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ABBREVIATIONS

ACPD	1-amino-cyclopentyl-1,3-dicarboxylate
ACSF	Artificial cerebrospinal fluid
AMPA	α-amino-3-hydroxyl-5-methyl-4-isoxazolepropionate
APV	2-amino-5-phosphonovalerate
L-AP4	L-2-amino-4-phosphonobutyrate
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CPP	3-((±)-2-carboxypiperazin-4-yl)-propyl-1-phosphonate
DNQX	6,7-dinitro-quinoxaline-2,3-dione
EAA	Excitatory amino acid
GABA	Gamma-aminobutyric acid
GEPR	Genetically epilepsy-prone rat
MK-801	(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate
NMDA	N-methyl-D-aspartate
PCP	Phencyclidine

1. INTRODUCTION

1.1. *Background*

The first documented link between excitatory amino acids (EAAs) and epilepsy can be dated back to the work by Hayashi (1954) that glutamate application onto the cerebral cortical surface induced powerful convulsant activities. Ironically, none of the medications that are currently useful to epileptic patients are believed to act through EAA control. In fact, much of our understanding of the role of EAAs in epilepsy is based on experimental studies. In the following sections, I will review the evidence for EAA involvement in the various models of epilepsy. Particular emphasis will be placed on the relationship between the EAA receptors and the mechanisms underlying neuronal hyperexcitability.

1.2. *Classification of EAA receptors and their pharmacology*

The classification and pharmacology of EAA receptors have been the subject of several excellent reviews (see Foster and Fagg, 1984; Sladeczek et al., 1988; Monaghan et al., 1989; Collingridge and Lester, 1989; Watkins et al., 1990). The recent trend favors a classification scheme that is based on the transduction mechanism of the receptor - ionotropic versus metabotropic (see Table 1). Under the ionotropic category, there exists the N-methyl-D-aspartate (NMDA), the kainate, the α-amino-3-hydroxyl-5-methyl-4-isoxazolepropionate (AMPA) and the L-2-amino-4-phosphonobutyrate (L-AP4) receptors. As the term ionotropic implies, the activation these receptors can lead to the opening of ion channels directly. In contrast, the

metabotropic receptor *trans*-1-amino-cyclopentyl-1,3-dicarboxylate (*trans*-ACPD) is coupled with guanine nucleotide-binding proteins. When activated, the *trans*-ACPD receptor stimulates phosphoinositide hydrolysis and mobilizes intracellular Ca^{2+}. The nomenclature for all five of the EAA receptors is derived from the names of the agonists (except for L-AP4 which is an antagonist) that selectively activate (inhibit) them.

A more traditional approach to classifying EAA receptors is based on the availability of selective antagonists. According to this method, receptors are known as either the NMDA type or the non-NMDA (kainate and AMPA) type. The NMDA receptor contains at least three different domains (see Table 1): (1) the agonist site to which EAAs bind. This site is blocked selectively by 3-((±)-2-carboxypiperazin-4-yl)-propyl-1-phosphonate (CPP) or α-amino-w-phosphocarboxylate like 2-amino-5-phosphonovalerate (APV); (2) the ionophore that is permeable to Na^+, K^+ and Ca^{2+}, and is blocked by Mg^{2+}, phencyclidine (PCP), (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-immine maleate (MK-801) or ketamine; and (3) the strychnine-insensitive glycine site which is sensitive to agonists like glycine or D-serine, and antagonist like 7-chlorokynurenic acid. The kainate and AMPA receptors are named collectively as the non-NMDA receptors because there is no pharmacological agent that can clearly distinguish between them. They are sensitive to selective antagonists such as 6-cyano-7-nitro-quinoxaline-2,3-dione (CNQX) or 6,7-dinitro-quinoxaline-2,3-dione (DNQX).

In the following discussion, we will adopt the "NMDA/non-NMDA receptors"

classification scheme since little is known about the involvement of the *trans*-ACPD and L-AP4 receptors in epilepsy.

2. LOW MAGNESIUM MODEL

2.1. *Extracellular Mg^{2+} gating of NMDA ionophore*

A correlation between hypomagnesia and seizure occurrence in man has been documented for many years (Hirschfelder, 1938; Suter and Klingman, 1955; Vallee et al., 1960; Canelas et al., 1965; Begna et al., 1985). The implication of this link, however, remained elusive until the demonstrations that the NMDA ionophore is gated by extracellular Mg^{2+} ($[Mg^{2+}]_o$) under physiological conditions (Mayer et al., 1984; Nowak et al., 1984) and the removal of this blockade is sufficient to induce, in the CA1 region of hippocampal slices, interictal-like epileptiform activities that are readily abolished by NMDA antagonists (Coan and Collingridge, 1987; Mody et al., 1987; Tancredi et al., 1990).

Likewise, in vitro studies in other brain regions such as area CA3 (Mody et al., 1987; Neuman et al., 1988,1989; Schneiderman and MacDonald, 1989; Tancredi et al., 1990) and dentate gyrus (Coan et al., 1987; Melchers and Pennartz, 1987) of the hippocampus, the neocortex (Thomson and West, 1986; Aram et al., 1989; Sutor and Hablitz, 1989; Wong and Prince, 1990; Avoli et al., 1991), the entorhinal cortex (Jones and Heinemann, 1988; Jones and Lambert, 1990), the olfactory cortex (Hoffman and Haberly, 1989) and the amygdala (Gean and Shinnick-Gallagher, 1988) all reported that the removal of $[Mg^{2+}]_o$ can induce interictal-like or ictal-like

epileptiform activities that are sensitive to the blockade of NMDA antagonists.

2.2. *Mechanisms underlying hyperexcitability*

The appearance of epileptiform activities in low-Mg^{2+} medium might be due to a reduction in the surface screening effect on the extracellular membrane (McLaughlin et al., 1971) as well as an enhancement in the intrinsic bursting capability of neurons (Neuman et al., 1989). The general consensus, however, is that the activation of NMDA receptors is the pivotal factor since this type of epileptiform activity is readily blocked by NMDA antagonists (see section 2.1).

The condition of low $[Mg^{2+}]_o$ can also augment the release of EAAs by reducing both the Mg^{2+}-antagonism on presynaptic Ca^{2+} entry (Llinás and Walton, 1980) as well as the presynaptic inhibitory effect of adenosine (O'Shaughnessy et al., 1988; Stone et al., 1990). Indeed following the removal of $[Mg^{2+}]_o$, Smith et al. (1989) were able to observe a selective increase in the level of endogenous glutamate prior to the appearance of epileptiform activities. This rise in glutamate level seems to be targeted towards the NMDA receptor since the non-NMDA antagonists CNQX or DNQX could exert little influence on low-Mg^{2+}-induced epileptiform activities (Fletcher et al., 1988; Jones and Lambert, 1990; Avoli et al., 1991).

With the aid of 7-chlorokynurenic acid, which is a selective blocker for the glycine site of the NMDA receptor complex, Kleckner and Dingledine (1989) examined the involvement of this site in low-$[Mg^{2+}]_o$ epileptogenesis. They found in the CA1 region of hippocampal slices that 7-chlorokynurenic acid could eliminate the

appearance of multiple population spikes while leaving the initial population spike intact. In agreement with this finding, Fletcher et al. (1989) reported that the exogenous application glycine can increase the frequency of low-Mg^{2+}-induced epileptiform discharge and its duration in neocortical slices. Therefore, it appears that this type of epileptiform is positively modulated by the NMDA-glycine site, possibly by preventing the NMDA receptor from undergoing prolonged desensitization (Mayer et al., 1989).

Recently, El-Beheiry and Puil (1990) reported that the lowering of $[Mg^{2+}]_o$ can produce a depression effect on the action of gamma-aminobutyric acid (GABA) receptors. This finding is in line with the observation that NMDA-receptor activation is accompanied by GABA-receptor desensitization (Stelzer et al., 1987). It also suggests that neuronal hyperexcitability observed in hypomagnesia might be related to an impairment of the inhibitory mechanism. The significance of this idea, however, remains unclear since the GABA-mediated inhibitory postsynaptic potentials still play an active role in controlling neuronal excitability in low-Mg^{2+} medium (Swatzwelder et al., 1987,1988; Lewis et al., 1989; Jones, 1989; Tancredi et al., 1990).

2.3. *NMDA receptor and long-lasting changes*

The activation of NMDA receptors in low-Mg^{2+} medium can lead to long-lasting enhancement of synaptic efficacy. It has been reported that brief reduction in $[Mg^{2+}]_o$ for 20-30 min is sufficient to trigger long-term potentiation of the population spike

in area CA1 of the hippocampus (Avoli et al., 1988; Psarropoulou and Kostopoulos, 1991). Likewise, transient epileptiform bursting in low-Mg^{2+} medium for 2-30 min can either long-term potentiate the early excitatory postsynaptic potential in CA1 neurons (Neuman et al., 1987), the extracellular spike in the dentate gyrus (Melchers and Pennartz, 1987) or induce the appearance of an all-or-none, late excitatory postsynaptic potential in olfactory cortical neurons (Hoffman and Haberly, 1989). A possible mechanism for explaining these long-lasting synaptic changes is the activation of intracellular messenger systems by Ca^{2+} influx through the NMDA ionophore (see Kennedy, 1989; Malenka et al., 1989). In agreement, Churn et al. (1991) found that the exposure of hippocampal slices to low $[Mg^{2+}]_o$ medium can alter the activity of Ca^{2+}-dependent protein kinase. From a clinical point of view, the massive influx of Ca^{2+} through the NMDA ionophore might represent a cellular mechanism that underlies some of the long-lasting postictal dysfunction or damage observed in patients suffering from partial, particularly temporal lobe epileptic seizures (see Gloor, 1989).

3. HIGH POTASSIUM MODEL

During the appearance of each epileptiform discharge, the level of extracellular K^+ ($[K^+]_o$) increases transiently to between 9 and 13 mM (see Somjen, 1984; Heinemann et al., 1986). This rise in $[K^+]_o$ is presumably a consequence rather than a cause of neuronal hyperexcitability. However, there is little doubt that an increase in $[K^+]_o$ can still play an important role in epileptogenesis. For example, Ogata

(1975) first demonstrated that exposure of hippocampal slices to a medium that contains 10 mM $[K^+]_o$ can induce interictal-like discharge in the CA3 region. Since then, other investigators have explored the possible involvement of EAA receptors in this type of epileptiform discharge.

According to pharmacological studies, the high-K^+-induced interictal-like discharge evoked in area CA3 is primarily mediated by non-NMDA receptors since it is not significantly influenced by NMDA antagonists (Neuman et al., 1988; Traynelis and Dingledine, 1988; Chamberlin et al., 1990) but can be abolished by CNQX (Chamberlin et al., 1990). Based on the anatomical distribution of EAA receptors, these findings might not seem too surprising since area CA3 is known to contain a much higher level of non-NMDA binding sites than NMDA binding sites (Monaghan and Cotman, 1985; Miller et al., 1990). What is interesting however, is that when the $[K^+]_o$ is raised to between 7 and 9 mM, the CA3 interictal-like discharge can precipitate the neighboring CA1 subfield into a state that resembles the one observed during electrographic seizure (Traynelis and Dingledine, 1988). Moreover, since this process is readily blocked by NMDA antagonist, it points to a crucial role played by the NMDA receptor (Traynelis and Dingledine, 1988). These authors proposed that under the condition of high $[K^+]_o$, the occurrence of each interictal-like discharge can create a further build-up of baseline $[K^+]_o$ which in turn can enhance the activation of NMDA receptor and subsequently lead to the generation of seizure-like activities in the CA1 subfield. In agreement with this view, Poolos and Kocsis (1990) reported that elevation of $[K^+]_o$ to 7.5 mM can selectively augment the

activation of NMDA receptors in CA1 hippocampal neurons.

Although it might appear that the linkage between the rise in extracellular K^+ and the activation of NMDA receptors is a positive feedback one that can lead to self-perpetuating activity, it should be pointed out that extracellular K^+ can also exert a direct negative influence on the NMDA channel. By patch-clamping from hippocampal neurons, Ozawa et al. (1990) demonstrated that when the $[K^+]_o$ is elevated to between 20 and 50 mM, it can selectively attenuate the NMDA-mediated conductance by 36 to 78%, without affecting the non-NMDA-mediated conductance. Interestingly, this range of $[K^+]_o$ is similar to that observed during spreading depression (e.g. Vyskocil et al., 1972; Nicholson et al., 1978; Balestrino et al., 1989). From a functional point of view, these findings imply that any excessive build-up of $[K^+]_o$ during intensive neuronal firings as in the case of spreading depression can actually serve as a down-regulator of excitability by interfering with the NMDA ionophore. As a result, this can also provide some kind of neuroprotective effect against cell death by preventing the massive intracellular build-up of Ca^{2+}.

4. KINDLING MODEL

The term "kindling" was first used by Goddard et al. (1969) to describe the process whereby daily electrical stimulation of a certain brain region with an initially subconvulsive stimulus train can progressively prime a naive animal into developing electrographic afterdischarges and stage 1 to 5 motor seizures subsequently. More recently, the definition of kindling is often expanded to include in vitro studies where

epileptiform activities are induced in cortical slices by kindling-like stimulation (i.e. repetitive application of stimulus trains). A feature that is common to both in vivo and in vitro kindling is that once seizure or epileptiform activities have been established, the changes last for a long time. In this respect, the study of kindling is often separated into the developmental stage (i.e. during kindling) and the fully-established stage (after kindling).

4.1. *Anti-epileptic effect of NMDA antagonists*

Numerous in vivo studies have examined the involvement of the NMDA receptor during kindling. Using blockers of either the ionophore (Callaghan and Schwark, 1980; Bowyer, 1982; Gilbert, 1988; Sato et al., 1988; McNamara et al., 1988; Trommer and Pasternak, 1990), the transmitter binding site (Cain et al., 1988; Croucher et al., 1988; Vezzani et al., 1988; Holmes et al., 1990), or the strychnine-insensitive glycine site (Croucher and Bradford, 1990) of the NMDA receptor complex, the authors cited here demonstrated that all three types of antagonists can retard the development of electrographic afterdischarges if they are injected daily prior to each stimulation. Consequently, the number of stimulations required to fully kindle an animal (i.e. to achieve a stage 5 motor seizure) is significantly augmented. In some instances, the blockade of NMDA receptors can even prevent an animal from progressing beyond seizure stages 2 and 3 (Bowyer, 1982; Gilbert, 1988; McNamara et al., 1988; Croucher et al., 1988; Croucher and Bradford, 1990; Holmes et al., 1990).

Based on these findings, it might be suggested that the NMDA receptor is directly involved in mechanisms underlying the development of kindling. Alternatively, the effects of NMDA antagonists described above may merely reflect an inhibition of seizure expression. In order to rule out this possibility, several investigators employed the re-kindling protocol following the cessation of drug treatment (Croucher et al., 1988; McNamara et al., 1988; Croucher and Bradford, 1990). They reasoned that if the NMDA antagonists were only masking the appearance of seizures, then the first stimulus train after drug washout should elicit a stage 5 seizure. Interestingly, all of them found that the appearance of stage 5 seizure with re-kindling can only occur progressively at a rate similar to that observed in the control group. This indicates that the NMDA receptor is involved in the kindling process directly.

A similar conclusion was also derived from in vitro studies. Using the hippocampal slice preparation, it was reported that the presence of NMDA antagonists in the extracellular medium during the stimulus trains was sufficient to prevent the appearance of epileptiform activities (Slater et al., 1985; Anderson et al., 1987; Stasheff et al., 1989; Bawin et al., 1991). However, once the epileptiform activities were induced, the NMDA antagonists showed little effect. This demonstrates that the NMDA receptor is directly involved in the induction mechanism underlying kindling-like stimulation, and led Stasheff et al. (1989) to coin the term "antiepileptic" (as opposed to "anticonvulsant") for describing the possible usefulness of NMDA receptor antagonists.

4.2. *Role of NMDA receptor during kindling*

The opening of the NMDA ionophore can lead to an increase in intracellular Ca^{2+} concentration (MacDermott et al., 1986). One consequence of this process is the activation of intracellular messenger systems (see Kennedy, 1989) that may underlie some of the permanent changes observed in fully-kindled animals (see section 4.3). Inevitably, this influx of Ca^{2+} might also influence the development of kindled seizure. For example, the activation of NMDA receptors can trigger the release of arachidonic-acid metabolites (Dumis et al., 1988) which can act as retrograde messengers and augment the presynaptic release of EAAs (Lynch and Voss, 1990). In agreement, biochemical studies had found that the levels of endogenous glutamate/aspartate are significantly increased during kindling (Peterson et al., 1983; Pless et al., 1989). This presumably reflects an enhancement in the release of EAAs since the uptake mechanism of EAAs is not altered with kindling (Slevin and Ferrara, 1985). An increase in the level of EAAs can greatly facilitate the development of kindled seizure (Mori and Wada, 1987).

The NMDA-mediated build-up of intracellular Ca^{2+} (MacDermott et al., 1986) can also reduce the affinity of the GABA receptor (Inoue et al., 1986). This might explain why the activation of NMDA receptor during kindling is accompanied by a reduction in inhibition. According to Stelzer et al. (1987), kindling-like stimulation can progressively attenuate the inhibitory postsynaptic potential by desensitizing the GABA receptor in hippocampal slices. These changes are presumably mediated by the NMDA receptor since they were not observed in the presence of its antagonist

(Stelzer et al., 1987). Likewise, in vivo study had shown that NMDA antagonist can prevent the loss of GABAergic inhibition induced by kindling (Kapur and Lothman, 1990)

The enhancement in the release of EAAs and the reduction in inhibition might also influence the non-NMDA receptors. Using the quasi-selective non-NMDA antagonist gamma-D-glutamylaminomethyl sulphonate, Croucher et al. (1988) reported that this drug can retard the development of kindled seizure, though less potently than NMDA antagonists. According to Cain et al. (1988), blockers of the NMDA receptor and the cholinergic receptor can create an additive effect on kindling. Together these findings indicate that the non-NMDA receptors and other non-EAA transmitter systems are also involved in the development stage, during which the NMDA receptor might function synergistically with them. This would explain why some investigators had found that the injection of NMDA antagonists alone was not sufficient to completely suppress the development of kindled seizure (see section 4.1).

4.3. *Up-regulation of NMDA receptor after kindling*

The NMDA receptor is also involved in the long-lasting seizure susceptibility of already kindled animals. For example, the injection of NMDA antagonists into fully-kindled animals can reduce the duration of electrographic afterdischarge, elevate the threshold of motor seizure (Callaghan and Schwark, 1980; Bowyer and Winters, 1981; Freeman et al., 1982; Bowyer et al., 1983; Peterson et al., 1983,1984;

McNamara et al., 1988; Vezzani et al., 1988; Young et al., 1989; Holmes et al., 1990; Croucher and Bradford, 1991) or abolish the expression of stage 5 seizure completely (Gilbert, 1988; Sato et al., 1988; Trommer and Pasternak, 1990). Based on these "anticonvulsant" properties of NMDA antagonists, many investigators had explored the possibility that the NMDA receptor might be up-regulated following kindling.

With the aid of ion-selective electrodes, Wadman et al. (1985) demonstrated that the EAA-stimulated decrease of extracellular Ca^{2+} is greater in hippocampal slices of fully-kindled animals when compared with the control group. This difference might reflect an up-regulation of the NMDA receptor since its ionophore is permeable to Ca^{2+}. In line with this idea, several investigators had reported that the NMDA-mediated excitatory transmission is enhanced in cortical slices excised from fully-kindled rats. For example in the hippocampus, kindling can disclose a NMDA-mediated excitatory postsynaptic potential in dentate granule cells and make them more prone to the generation of epileptiform activities under low $[Mg^{2+}]_o$ medium (Mody and Heinemann, 1987; Mody et al., 1988). Similarly, kindling can induce the appearance of stimulus-induced or spontaneous epileptiform discharge that are mediated by NMDA receptors in the amygdala (Gean et al., 1989).

What are the mechanisms that might underlie this long-lasting up-regulation of the NMDA receptor by kindling? According to biochemical studies, the K^+ or veratrine stimulated-release of endogenous glutamate/aspartate in cortical slices of fully-kindled animals is significantly higher than in slices of sham animals (Leach et

al., 1985; Geula et al., 1988; Jarvie et al., 1990). Since this increase is a Ca^{2+}-dependent process (Geula et al., 1988; Jarvie et al., 1990) while the uptake of EAAs into synaptosomes is not altered with kindling (Slevin and Ferrara, 1985), it appears that the presynaptic release of endogenous EAAs is permanently enhanced by kindling. According to autoradiographic studies, the density of radioligand-binding sites for the NMDA receptor complex is also higher in the brains of fully-kindled animals (Yeh et al., 1989; Wu et al., 1990; Cincotta et al., 1991). Taken together, the increase in the number of NMDA receptors and the release of EAAs can complement each others, and account for the long-lasting up-regulation of the NMDA receptor after kindling.

5. GENETIC MODELS

A growing body of evidence indicates that genetic mutation can contribute to seizure predisposition in human (see Anderson et al., 1986). Indeed, there exists several strains of mutant animals that have an inherited susceptibility to seizure. These so-called genetic models of epilepsy include the genetically epilepsy-prone rat, the WAG/Rij rat, the El mouse, the DBA/2 mouse and the *Papio Papio* baboon.

5.1 *Genetically epilepsy-prone rat*

Genetically epilepsy-prone rats (GEPRs) are known to be hypersensitive to sensory stimuli such as sound or atmospheric pressure. The motor manifestation of this hypersensitivity (i.e. seizure) is dependent on the activation of NMDA receptors.

For example, the focal injection of NMDA antagonist into auditory nuclei or midbrain structures of the GEPR can significantly reduce the severity of convulsions triggered by acoustic stimulation (Faingold et al., 1988; Meldrum et al., 1988; Millan et al., 1988). Likewise, NMDA antagonist can protect the GEPR from seizure induced under hyperbaric condition (Millan et al., 1991). These findings suggest that EAA receptors might play an important role in predisposing the GEPR to seizure generation.

In line with this idea, the basal levels of EAAs are found to be significantly elevated in the inferior colliculus, hippocampus, neocortex, thalamus and cerebellum of the GEPR (Ribak et al., 1988; Lehmann, 1989; Lasley, 1991). Moreover, the levels of EAAs in these cortical areas are further augmented by the occurrence of audiogenic seizure (Chapman et al., 1986; Lasley, 1991). These observations are consistent with the fact that the release of endogenous EAAs is up-regulated in the GEPR brain (Lehmann et al., 1986; Lehmann, 1989).

5.2. *WAG/Rij rat*

The WAG/Rij rat represents an animal model for human non-convulsive absence epilepsy. According to Peeters and co-workers, EAA receptors are involved in the electroencephalogram spike-wave discharges of this inbred strain. They reported that the injection of NMDA receptor antagonists can reduce the frequency and duration of spike-wave discharges (Peeters et al., 1989,1990). More recently, these authors have also found that the non-NMDA receptor antagonist CNQX can produce similar

anti-convulsant effects in the WAG/Rij rat (Ramakers et al., 1991).

5.3. *El mouse*

The El mouse is considered to be a model for human temporal lobe epilepsy. According to pharmacological experiments, the occurrence of seizures in El mice is dependent on the activation of NMDA receptors since NMDA antagonists can potently suppress tonic-clonic convulsions (Shimada et al., 1987; Sato et al., 1989). In contrast, autoradiographic studies have reported that the K_d (affinity) and B_{max} (density) of NMDA receptors in the El brain are similar to or lower than the ones found in its wildtype counterpart (Kawasaki et al., 1991; Oguro et al., 1990,1991). One possible explanation for this discrepancy is the release of endogenous EAAs which is significantly elevated in the El brain (Flavin et al., 1991). A reduction in the affinity and density of NMDA receptors might act as a compensatory mechanism protecting the neurons from the neurotoxic consequence of elevated EAA concentrations.

5.4. *DBA/2 mouse*

Similar to the GEPR (see section 5.1), the DBA/2 mouse is also hypersensitive to acoustic stimulation. When exposed to loud sound, DBA/2 mice often display tonic-clonic motor seizures. Using this model, Croucher et al. (1982) first demonstrated that NMDA antagonists can potently suppress audiogenic seizure in DBA/2 mice and proposed that NMDA antagonists might represent a new class of

anticonvulsants. Since then, similar findings have been described by others (Meldrum et al., 1983b; Jones et al., 1984; Patel et al., 1990; Chapman et al., 1991b). With the aid of 7-chlorokynurenic acid, Singh et al. (1990) further reported that the involvement of NMDA receptors in DBA/2 mice seizure is modulated by its strychnine-insensitive glycine site.

Audiogenic seizure in DBA/2 mice is also dependent on the activation of non-NMDA receptors. Earlier studies had shown that this type of seizure is sensitive to broad spectrum EAA antagonists (Croucher et al., 1982,1984; Chapman et al., 1985). Recently, Chapman et al. (1991a) have used more selective non-NMDA receptor antagonists to directly demonstrate that the kainate and quisqualate receptors can also play an important role in audiogenic seizure of DBA/2 mice.

5.5. *Papio Papio baboon*

In agreement with all of the genetic models described above, the appearance of seizure in *Papio Papio* baboons is also mediated by the NMDA receptor. For example, NMDA antagonists can exhibit potent anticonvulsant action against seizure induced by light or stroboscopic stimulation of the animal (Meldrum et al., 1983a; Patel et al., 1990; Chapman et al., 1991a). Interestingly, the degree of photosensitivity in *Papio Papio* correlates directly with the levels of asparagine (an aspartate metabolite) in the cerebrospinal fluid (Lloyd et al., 1986).

6. OBJECTIVES OF THIS THESIS

The findings discussed in the preceding sections indicate that blockers of EAA receptors can act as potent anticonvulsants against the various experimental models of epilepsy (see Table 2). Moreover, knowledge on the functional properties of EAA receptors has provided greater insight into the mechanisms underlying neuronal hyperexcitability. However, what relevance does the above have to human epilepsy?

One approach to this question is to perform pharmacological studies on the cellular activity recorded from epileptogenic human brain slices maintained in vitro. In this project, the EAA antagonists APV, CNQX, CPP and MK-801 (see Table 1) were used to examine the role of NMDA and non-NMDA receptors in excitatory synaptic transmission in human neocortical slices resected from the epileptic focus. The slices were either maintained in normal artificial cerebrospinal fluid (ACSF; see chapter 5) or maintained in an ACSF medium containing bicuculline, an antagonist of the $GABA_A$ receptor (see chapter 6). The rationale behind these latter experiments is that extreme experimental conditions, such as disinhibition, might help to reveal some cellular anomalies that are not discernible in normal ACSF.

Ideally, a similar line of studies should be performed in neocortical slices of non-epileptic patients. The problem, however, is that the availability of this type of control specimen is very limited. As an alternative, neocortical slices of normal rats were used for comparison in this project. Like the human slices, the rat slices were subjected to the perfusion with normal ACSF (see chapter 2) or with an ACSF medium containing bicuculline (see chapters 2 to 4). Although imperfect, this type of parallel study between the rat and the human represents the most direct approach

to understanding the involvement of EAAs in human epilepsy.

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Table 1. Classification of EAA receptors and their pharmacology. See section 1.2 in text for further explanation. Abbreviations: ***trans*-ACPD**, *trans*-1-amino-cyclopentyl-1,3-dicarboxylate; **AMPA**, α-amino-3-hydroxyl-5-methyl-4-isoxazolepropionate; **APV**, 2-amino-5-phosphonovalerate; **L-AP4**, L-2-amino-4-phosphonobutyrate; **L-ASP**, L-aspartate; **7-CL-KYN**, 7-chlorokynurenic acid; **CNQX**,6-cyano-7-nitroquinoxaline-2,3-dione;**CPP**,3-((±)-2-carboxypiperazin-4-yl)-propyl-1-phosphonate; **DNQX**, 6,7-dinitro-quinoxaline-2,3-dione; **L-GLU**, L-glutamate; **GLY**, glycine; **MK-801**, (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine; **NMDA**, N-methyl-D-aspartate; **PCP**, phencyclidine; **QUIS**, quisqualate; **D-SER**, D-serine.

Receptor	Agonist	Antagonist
Ionotropic		
1) NMDA		
agonist site	NMDA, L–GLU, L–ASP	CPP, APV
ionophore		Mg^{2+}, PCP, MK–801, Ketamine
glycine site	GLY, D–SER	7–CL–KYN
2) Kainate	Kainate, L–GLU	CNQX, DNQX
3) AMPA	AMPA, QUIS, L–GLU	CNQX, DNQX
4) L–AP4	L–GLU	L–AP4
Metabotropic		
5) *trans*–ACPD	*trans*–ACPD, L–GLU, QUIS	

Table 1

Table 2. Effects of EAA antagonists in different models of epilepsy. This table is designed as an organizational aide to the text. References and detailed explanation can be found in sections 2 to 5.

Model		NMDA antagonist	Non–NMDA antagonist
Low $[Mg^{2+}]_o$	Interictal	++	++
	Ictal	++	++
High $[K^+]_o$	Interictal	–	++
	Ictal	++	N/T
Kindled seizure	Induction	++	N/T
	Maintenance	+	N/T
GEP rat	Seizure	++	N/T
WAG/Rij rat	Seizure	++	++
EI mouse	Seizure	++	N/T
DBA/2 mouse	Seizure	++	++
Papio Papio baboon	Seizure	++	N/T

++	very effective
+	partially effective
–	ineffective
N/T	not tested

Table 2

CHAPTER 2

EXCITATORY POSTSYNAPTIC POTENTIALS RECORDED FROM REGULAR-SPIKING CELLS IN LAYERS II/III OF RAT SENSORIMOTOR CORTEX

SUMMARY AND CONCLUSIONS

1. Intracellular recording techniques were used to investigate the physiological and pharmacological properties of stimulus-induced excitatory postsynaptic potentials (EPSPs) recorded in regular-spiking cells located in layers II/III of rat sensorimotor cortical slices maintained in vitro.

2. Depending on the strength of the extracellular stimuli, a pure EPSP or an EPSP-inhibitory postsynaptic potential sequence was observed under the perfusion with normal medium. The EPSPs displayed short latency of onset (2.4 $\pm$ 0.7 ms) and were able to follow repetitive stimulation (tested up to 5 Hz). Variation of the membrane potential (Vm) revealed 2 types of voltage behavior for the short-latency EPSP. The first type decreased in amplitude with depolarization and increased in amplitude with hyperpolarization. In contrast, the second type behaved anomalously by increasing and decreasing in size after depolarization and hyperpolarization, respectively.

3. Several experimental procedures were carried out to investigate the mechanism underlying the anomalous voltage behavior of the EPSP. Results indicated that this type of Vm dependency could be mimicked by an intrinsic response evoked by a brief pulse of depolarizing current and abolished by N-(2,6-dimethylphenylcarbamoyl-methyl)triethylammonium bromide (QX-314, 50 mM). Furthermore, the EPSP was not sensitive to the N-methyl-D-aspartate (NMDA) receptor antagonist 3-((±)-2-carboxypiperazin-4-yl)-propyl-1-phosphonate (CPP, 10 μM). Thus, the anomalous voltage behavior was attributable to the nonlinear current-voltage relationship of the

neuronal membrane.

4. The involvement of non-NMDA receptors in excitatory synaptic transmission was investigated with their selective antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 1-10 μM). This drug greatly reduced or completely blocked the EPSP in a dose-dependent manner (1-10 μM). The IC_{50} for the CNQX effect was about 2 μM. In the presence of CNQX (10 μM) and glycine (10 μM), synaptic stimulation failed to elicit firing of action potential. However, a CPP-sensitive EPSP was observed.

5. When synaptic inhibition was reduced by low concentration of bicuculline methiodide (BMI, 1-2 μM), extracellular stimulation revealed late EPSPs (latency to onset: 10-30 ms) that were not discernible in normal medium. Similar to the short-latency EPSP, the Vm dependency displayed by this late EPSP could be modified by inward membrane rectifications. The late EPSP appeared to be polysynaptic in origin because 1) its latency to onset was long and variable and 2) it failed to follow repetitive stimuli delivered at a frequency that did not depress the short-latency EPSP.

6. The role of NMDA receptors in mediation of the short-latency and late EPSPs evoked in the presence of BMI was examined. Bath perfusion of CPP (3-10 μM) progressively increased the latency to onset of the late EPSP while reducing its amplitude until the response was completely abolished. In contrast, CPP attenuated the decay phase of the short-latency EPSP, whereas its peak amplitude remained insensitive to the drug.

7. Our data indicate that excitatory synaptic transmission in the sensorimotor cortex

is predominately mediated by the CNQX-sensitive non-NMDA receptors. Although a CPP-sensitive EPSP was observed in the presence of CNQX and glycine, the appearance of this NMDA-mediated potential might be explained by the effects of CNQX which reduces the IPSP and glycine that augment NMDA-mediated responses. In addition, our results demonstrate that a slight reduction of inhibition by BMI is sufficient to reveal latent polysynaptic pathways that are mediated by NMDA receptors. Under such condition, NMDA receptors can play a crucial role in neuronal synchronization.

INTRODUCTION

The amino acids L-glutamate and L-aspartate are putative excitatory transmitters within the mammalian central nervous system (for review, see Streit, 1984). The receptors activated by these excitatory amino acids are generally classified into the N-methyl-D-aspartate (NMDA) type and the non-NMDA type (for review, see Monaghan et al., 1989). In the neocortex, these receptors have been shown to participate in important functions. For example, NMDA receptors are involved in behavioral plasticity of the visual cortex during postnatal development (Kleinschmidt et al., 1987; Rauschecker and Hahn, 1987; Bear et al., 1990), as well as in reorganization of the somatosensory map after hindlimb deafferentation (Kano et al., 1991). In addition, it has also been shown that visual responses in the adult animal can be blocked by broad-spectrum antagonists of excitatory amino-acid receptors (Tsumoto et al., 1986,1987).

The role of excitatory amino-acid receptors in the neocortex has also been examined at the cellular level by analysis of the excitatory postsynaptic potentials (EPSPs). However, it appears from the data reported to date that the contribution of NMDA and non-NMDA receptors to synaptic transmission is not homogenous in the various neocortical regions. In the sensorimotor cortex (Thomson and Lodge, 1985; Thomson et al., 1985; Thomson, 1986), synaptic activation of regular-spiking pyramidal cells can evoke an EPSP that displays voltage behavior that is in accordance with the $[Mg^{2+}]_o$ gating effect of the NMDA receptor-mediated ionophore (Mayer et al., 1984; Nowak et al., 1984) and is readily blocked by selective

antagonists of NMDA receptors. In the visual cortex, NMDA-like voltage dependency of the EPSP is only observed in the intrinsically bursting cells while the regular-spiking cells displayed an EPSP with conventional voltage behavior (Artola and Singer, 1987). Pharmacological experiments indicate that both NMDA and non-NMDA receptors are involved in the regular-spiking cell's EPSP, with the non-NMDA receptors playing the dominant role (Artola and Singer, 1987,1990; Jones and Baughman, 1988; Shirokawa et al., 1989). In the frontal cortex, focal extracellular stimuli can evoke a biphasic (early/late) EPSP in regular-spiking cells (Howe et al., 1987; Sutor and Hablitz, 1989a,b,c; Hablitz and Sutor, 1990). Although the early EPSP displays NMDA-like voltage dependency (Sutor and Hablitz, 1989b,c), it remains insensitive to NMDA receptor antagonists (Sutor and Hablitz, 1989a,c; Hablitz and Sutor, 1990). In contrast, the late EPSP, which displays conventional voltage behavior (Sutor and Hablitz, 1989b), is blocked by NMDA receptor antagonists (Sutor and Hablitz, 1989a,c).

The present study was undertaken to examine the physiological and pharmacological features of the EPSP evoked in the rat sensorimotor cortical slices maintained in vitro. We chose to study the sensorimotor area for several reasons. First, there is no information pertaining to the contribution of non-NMDA receptors to excitatory synaptic transmission in this area. Second, the role of NMDA receptors in the sensorimotor cortex remains controversial. It has been reported that the EPSP waveform evoked at the various membrane potentials is governed by NMDA receptor activation (Thomson and Lodge, 1985; Thomson et al., 1985; Thomson, 1986).

However, the NMDA-like voltage dependency of EPSPs might also be explained by the nonlinear current-voltage relationship of the neuronal membrane (Stafstrom et al., 1985; Thomson et al., 1988). This second idea is further corroborated by a recent demonstration in the frontal cortex where the NMDA-like voltage behavior of the early EPSP is sensitive to blockers of voltage-dependent intrinsic conductances (Sutor and Hablitz, 1989c). Third, most of our knowledge on the mechanisms underlying neocortical epileptogenesis is based on studies carried out in tissues obtained from the sensorimotor region (for review, see Connors and Gutnick, 1984). Therefore, an improved understanding of the roles played by excitatory amino-acid receptors within this area might lead to greater insight into the mechanism of focal epilepsy. Part of these results have been communicated in abstract form (Hwa and Avoli, 1990).

METHODS

Preparation and incubation of brain slices

Male Sprague-Dawley rats weighing 225-300 g were decapitated under ether anesthesia. Their brains were removed rapidly and immersed in cold (1-3 °C) artificial cerebrospinal fluid (ACSF). A block of the neocortex was dissected from the sensorimotor region (Zilles and Wree, 1985) and cut transversely into 450-550 μm thick slices using a McIlwain tissue chopper. Slices were then transferred to a fluid-gas interface chamber where they were perfused with warmed (34$\pm$1 °C), oxygenated (95% O_2, 5% CO_2) ACSF and exposed to humidified atmosphere saturated with 95% O_2/5% CO_2. The perfusing ACSF (pH 7.4), which was kept at

a constant rate during each experiment, had the following composition (in mM): NaCl 124, KCl 2, KH_2PO_4 1.25, $MgSO_4$ 2, $CaCl_2$ 2, $NaHCO_3$ 26, glucose 10. The following drugs were applied by bath perfusion. Bicuculline methiodide (BMI, 1-5 μM) and glycine (10 μM) were obtained from Sigma. 3-((±)-2-carboxypiperazin-4-yl)-propyl-1-phosphonate (CPP, 3-10 μM) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 1-10 μM) were supplied by Tocris Neuramin.

Recording and stimulation

Conventional intracellular recordings were made from neurons located in the superficial layers (II/III). Sharp electrodes (tip resistance: 40-80 MΩ) were pulled from 1.5 mm OD glass micropipettes on a horizontal puller (Narishige) and were usually backfilled with 4 M K-acetate (pH 7.2). In some cases, the electrodes contained 50 mM of N-(2,6-dimethylphenylcarbamoyl-methyl)triethylammonium bromide (QX-314, supplied by Astra) dissolved in 4M K-acetate for blocking intrinsic voltage-dependent conductances. The ejection of QX-314 was facilitated by passing pulses (100-400 ms) of depolarizing current (1.0-3.0 nA) through the recording electrode. We considered that QX-314 had taken its effects when the Na^+-dependent fast action potential and depolarizing inward rectification were largely reduced or abolished (Connors and Prince, 1982).

The intracellular signal was fed to a high-impedance negative-capacitance amplifier (Axoclamp-2A) with a standard bridge circuit for passing current through the recording electrode. The bridge balance was monitored routinely throughout the experiments and adjusted when necessary. The recorded signals were displayed on

a storage oscilloscope and/or a GOULD pen writer. In the latter case, a 20 KHz waveform digitizer was often used to avoid distortion of the signals due to slow time response of the pen. In some cases, data were also recorded on FM tape for latter analysis. The intracellular results shown in this study were taken from a total of 72 neurons that displayed resting membrane potential (Vm) of -79±9 mV (mean±SD, n=55), input resistance of 27±8 MΩ (n=47) and action potential amplitude of 100±14 mV (n=46). These neurons were identified as regular-spiking cells with the use of the electrophysiological criteria reported previously in rodents (Connors et al., 1982; McCormick et al., 1985; Chagnac-Amitai and Connors, 1989b; Connors and Gutnick, 1990).

A bipolar stainless steel electrode was used to deliver pulses of extracellular stimuli (90 μs, 10-2000 μA) to the white matter or deep layers (V/VI). The stimulation and recording electrodes were aligned in a straight line that was perpendicular to the pia surface.

RESULTS

Characteristics of PSPs evoked in normal ACSF

Responses to focal extracellular stimulation were examined in 28 neurons bathed in normal ACSF. At resting Vm, all of the neurons responded to extracellular stimulation with a monophasic depolarizing postsynaptic potential (PSP). Stepwise increment of stimulus intensity increased the amplitude and duration of the depolarizing PSP until it was capable of triggering action potential. Within the Vm

range tested (-50 to -120 mV), the PSPs displayed two distinctive types of voltage behavior.

The first type is illustrated in figures 1A and 1B. The PSP consisted of an initial EPSP that was followed by an inhibitory postsynaptic potential (IPSP). The amplitude of the EPSP increased with hyperpolarization and decreased with depolarization (Fig. 1B, early). The IPSP, which was usually inverted at rest, reversed in polarity when the Vm was more positive than -70 mV (Fig. 1B, late). This suggests that it was mediated by gamma-aminobutyric acid $(GABA)_A$ receptors (Avoli, 1986; Howe et al., 1987; Connors et al., 1988; Deisz and Prince, 1989). In some instances, the increase in the size of the EPSP and the inverted IPSP during membrane hyperpolarization could reach a plateau when the Vm was more negative than -85 to -90 mV (Fig. 1B).

The second type of voltage behavior is shown in figures 1C and 1D. The PSP was considered as a pure EPSP because depolarization of the Vm to more positive than -70 mV did not reveal any IPSP component (Fig. 1D). Although variation of the Vm had no apparent influence on the peak amplitude of the EPSP (Fig. 1D, early), its decay phase increased in amplitude with membrane depolarization and decreased in amplitude with membrane hyperpolarization (Fig. 1D, late). Because of a shunting effect that the IPSP can impose on the EPSP (Fig. 1E), this type of anomalous voltage behavior was only observed in EPSP evoked by stimulus intensities that were below the threshold for IPSP generation. When the neocortical slice was perfused with $GABA_A$ receptor antagonist BMI (2-5 μM, n=19), the EPSP always displayed anomalous voltage behavior regardless of the stimulus intensity (Fig. 1F).

All of the EPSPs could follow repetitive stimulation (tested up to 5 Hz). Their latency of onset, which ranged between 1.6 and 4 ms (2.4 ± 0.71 ms, n=20), was similar to the value (2.13 ± 0.59 ms) derived from single-axon stimulation (cf. Thomson et al., 1988). Therefore, we presumed that these EPSPs were monosynaptically activated.

Possible mechanisms underlying the anomalous voltage behaviour

The anomalous voltage behavior of the EPSP described above is similar to that reported previously in the sensorimotor cortex. However, it remained unclear whether NMDA receptor-mediated conductance (Thomson and Lodge, 1985; Thomson et al., 1985; Thomson, 1986) or voltage-dependent intrinsic conductances (Stafstrom et al., 1985; Thomson et al., 1988) were responsible for such changes in the EPSP's waveform during Vm variation.

We used two criteria to discriminate between these possibilities. First, the anomalous Vm dependency of the EPSP was tested with 10 μM of the NMDA receptor antagonist CPP (Harris et al., 1986). In five of five neurons examined, neither the amplitude nor the Vm dependency of the EPSP was influenced by CPP (Fig. 2A). Second, if voltage-dependent intrinsic conductances were involved in shaping the EPSP, then one would expect the Vm dependency of a depolarizing response induced by either current pulse injection or synaptic stimulation to behave in a similar manner. The following protocol was used to address this question. EPSPs that displayed anomalous Vm dependency were obtained in the presence or absence

of BMI (Fig. 2B, synaptic). Then brief pulses of positive current were injected into the same cell and adjusted to elicit a voltage response that resembled the EPSP (Fig. 2B, intrinsic). In all of the neurons tested (n=12), both the EPSP and the intrinsic response were influenced by Vm changes in a similar fashion (Fig. 2B). Therefore, these results indicate that the unconventional voltage characteristic of the EPSP is not due to the Mg^{2+} gating of the NMDA receptor-ionophore, but is governed by voltage-dependent intrinsic conductances.

Effects of QX-314 on intrinsically and synaptically evoked responses

Stafstrom et al. (1985) have suggested that a persistent Na^+ current, which underlies the depolarizing inward rectification, can influence the time course of the EPSP in the cat sensorimotor cortex. To test this hypothesis in the rat, we injected the cells with QX-314 (50 mM).

In the first series of experiments, the effects of QX-314 on the Vm dependency of the intrinsic response were analyzed (n=5). This procedure could help to determine the effectiveness of QX-314 in blocking the intrinsic conductances that may contribute to the shape of the EPSP. As illustrated in figure 3A, the intrinsic response displayed anomalous voltage behavior very similar to that of the EPSP (control). After the intracellular injection of QX-314, this type of Vm dependency disappeared completely (Fig. 3Aa, QX-314). Consequently, the amplitude of the intrinsic response was no longer dependent on the changes in Vm (Fig. 3Ab, QX-314).

Since QX-314 is known to block the depolarizing inward rectification in

neocortical cells (Connors et al., 1982; Stafstrom et al., 1985; Sutor and Hablitz, 1989c), the above results might suggest that this type of Na^+ conductance is responsible for the anomalous voltage behavior. If this i,s the case, then the response should not vary in size within the Vm region where the Na^+ channel is not activated (more negative than resting level; cf. Connors et al., 1982; Stafstrom et al., 1985). However, our data showed that the response continued to reduce in size with Vm hyperpolarization from -85 mV (Fig. 3Ab, control). This suggests that the anomalous voltage behavior might also be contributed by the hyperpolarizing inward rectification that is sensitive to QX-314 (Hwa and Avoli, 1991a). In agreement, at a Vm level where the hyperpolarizing inward rectifier showed steep activation (as characterized by a "sag" of the membrane potential towards the resting level), the response was almost completely shunted (see -1.0nA in Fig. 3Aa, control). When the hyperpolarizing inward rectification was blocked by QX-314, the response amplitude was enhanced by about 100% (see -0.4nA in Fig. 3Aa, QX-314). Therefore, at Vm levels more positive than rest, the increase in response amplitude was due to the activation of the depolarizing inward rectification. At Vm levels more negative than rest, the decrease in response amplitude was due to a shunting effect imposed by the hyperpolarizing inward rectification.

In the second part of the QX-314 experiments, we investigated the effects of this lidocaine derivative on the EPSP directly. In all of the experiments (n=5), the slices were bathed in BMI (2-5 μM) to avoid contamination from the IPSP. Under such condition, extracellular stimulation always evoked a pure EPSP that displayed

anomalous voltage behavior (Fig. 3B, control). After the intracellular injection of QX-314, the Vm dependency of the EPSP evoked at the same stimulus intensity was completely altered (Fig. 3B, QX-314). Note in the same figure (3B) that the EPSP evoked at Vm level of about -66 mV displayed a much faster rate of decay than in control, presumably because of the blockade of the depolarizing inward rectifier. In contrast, the removal of the hyperpolarizing inward rectifier appeared to potentiate the EPSP evoked at Vm level of -116 mV. Plots of the voltage-response relationship showed that both the early (Fig. 3Ca, QX-314) and late (Fig. 3Cb, QX-314) components of the EPSP increased with hyperpolarization and decreased with depolarization.

Effects of CNQX on the EPSP

The participation of non-NMDA receptors in the EPSP was investigated in 13 cells by the use of the selective antagonist CNQX (Honoré et al., 1988). In most of the experiments, 10 μM of glycine was also included in the control medium. This could help to minimize the undesired interaction of high doses of CNQX with the NMDA receptor (Hablitz and Sutor, 1990; Thomson, 1990).

The experiment depicted in figure 4 was carried out in the presence of glycine. In control, stepwise increment of the stimulus intensity progressively increased the peak amplitude of the EPSP until it reached a plateau (Fig. 4B, control). The bath application of CNQX (1-10 μM) reduced the amplitude and rate of rise of the EPSPs in a dose-dependent manner (Fig. 4A). Meanwhile, the latency of onset of the EPSPs

remained unchanged (Fig. 4A). This suggests that these EPSPs were monosynaptically activated through the non-NMDA receptors. The dose-dependent effect of CNQX was also illustrated in the stimulus-response relationship where CNQX shifted the input-output curve downward to the right (Fig. 4B). Interestingly, the IC_{50} of CNQX on the EPSP (about 2 μM, Fig. 4C) evoked in media containing glycine was similar to the value obtained from media that lack exogenous glycine.

The action of CNQX was always more potent against the smaller EPSPs evoked by near-threshold stimuli than against the larger EPSPs evoked by suprathreshold stimuli. In the example shown in figure 4, 1 μM of CNQX induced a 40% reduction of the smaller EPSP (Fig. 4Aa, 1μM) as compared with an 18% reduction observed in the larger EPSP (Fig. 4Ab, 1μM). Raising the CNQX concentration to 3 μM virtually abolished the smaller EPSP (Fig. 4Aa, 3μM) but only depressed the larger EPSP by 65% (Fig. 4Ab, 3μM). At 5-10 μM of CNQX, the stimulus intensity used for evoking the smaller EPSP could no longer evoke any synaptic response (Fig. 4Aa, 5 and 10μM). However, the stimulus intensity used for evoking the larger EPSP could still elicit a 2- to 4-mV depolarizing response (Fig. 4Ab, 5 and 10μM).

CNQX-resistant component of the PSP

The PSPs evoked in the presence of CNQX (5-10 μM) and glycine (10 μM) were examined in 25 cells. At resting membrane level, the PSP was always depolarizing in shape (Fig. 5Aa). The PSPs displayed a latency to onset of about 2.4 $\pm$ 0.63 ms (n=23) and could follow repetitive stimuli that were tested up to 5 Hz. Plots of the

PSP's amplitude as a function of the stimulus strength indicated that the PSP was graded in nature. As shown in figure 5Ab, the peak amplitude of the PSP (open circle) increased with stepwise increment in stimulus intensity until the response reached a plateau. The decay phase of the PSP (Fig. 5Ab, solid circle), on the other hand, displayed an initial increase that was followed by a decrease. This decline in input-output relationship of the decay phase appeared to coincide with the activation of the IPSP (Fig. 5B). In all of the neurons tested at resting Vm (n=22), synaptic stimulation (even at suprathreshold intensity) was not able to trigger firing of action potential. However, action potentials could be generated by injecting a depolarizing pulse of current into the cell. The maximum amplitude attained by the CNQX-resistant PSP was 9 $\pm$ 2 mV (n=22).

The participation of NMDA receptors in the CNQX-resistant PSP was tested with CPP (n=6). As illustrated in figure 5C, the PSP evoked by a low-intensity stimulus was completely abolished by CPP (Fig. 5C, 30 μA), thereby suggesting that it was a NMDA receptor-mediated EPSP. In contrast, the PSP evoked at suprathreshold intensity was only partially reduced by CPP (Fig. 5C, 90 μA). In the presence of CNQX and CPP, focal stimulation could still evoke a depolarizing potential (90 μA in Fig. 5C, CPP) that would reverse in polarity at about -70 mV. This suggests that the residual depolarization was a Cl^--mediated IPSP.

Reduction in GABAergic inhibition revealed NMDA receptor-mediated late EPSPs

A reduction in the GABAergic inhibitory mechanism can help to alleviate the

voltage-dependent blockade of the NMDA receptor-ionophore by $[Mg^{2+}]_o$ (Herron et al., 1985; Dingledine et al., 1986). Therefore, we examined the EPSPs evoked in slices that were perfused with low concentrations of BMI (1-2μM, n=12).

In the presence of BMI, near-threshold synaptic stimuli could evoke a short-latency, monophasic EPSP (Fig. 6A, 20 and 30μA). When the stimulus strength was raised to higher levels, late EPSPs that were biphasic or multiphasic in shape were observed (Fig. 6A, > 30μA). The latency to onset of these late EPSPs was variable and ranged between 10 and 30 ms. At suprathreshold stimulus intensities, the short-latency and late EPSPs became hardly distinguishable (Fig. 6A, 200 and 500μA). The voltage behavior of the late EPSP was variable. Figure 6B was obtained from a neuron in which the Vm dependency of the EPSP was presumably modified by the evident presence of inward rectification in the hyperpolarizing direction (traces 2-5). On the other hand, in neurons in which membrane rectification was less apparent, the late EPSP behaved like a conventional EPSP by increasing and decreasing in amplitude with Vm hyperpolarization and depolarization respectively.

The long latency to onset and the variability in shape of the late EPSPs suggested that they were polysynaptic in origin. To test this possibility, we examined the ability of the late EPSPs to follow repetitive stimulation. As illustrated in figure 6C, a stimulus frequency of 0.1 Hz had no effect on the appearance of the late EPSP. On the other hand, higher frequency repetitive stimuli delivered at 2 Hz progressively reduced the amplitude and latency to onset of the late EPSP (stimulus #12 in Fig. 6Cb, 2Hz) until it had completely disappeared (stimulus #30 in Fig. 6Cb, 2Hz). This

frequency-dependent depression of the late EPSP was fully reversible after a pause of stimulation (Rest in Fig. 6Cb, 2Hz). This suggests that the late EPSPs were activated through polysynaptic pathways (cf. Berry and Pentreath, 1976).

The involvement of NMDA receptor in the short-latency and late EPSPs was investigated with CPP (3-10 μM) in six cells. A typical experiment is illustrated in figure 7. In control, focal stimulation evoked a short-latency EPSP that was followed by a late EPSP (Fig. 7A). After the bath perfusion of CPP, the peak amplitude of the short-latency EPSP remained basically unchanged (Fig. 7A and B) whereas its decay phase was attenuated by CPP (Fig. 7A, 30 min). This demonstrated the existence of a NMDA receptor-mediated component within the short-latency EPSP when GABAergic inhibition is reduced. The effect of CPP on the late EPSP was more potent. The drug caused a progressive increase in the latency to onset of the late EPSP and gradual reduction in its amplitude (Fig. 7A, CPP 20 and 25 min), until the entire response was completely abolished (Fig. 7A, CPP 30 min). A plot of the changes caused by CPP over time indicates that the increase in latency occurred about 10 min before the reduction in amplitude (Fig. 7B). The CPP-induced blockade of the late EPSPs was reversible upon washout of the drug (Fig. 7A, wash).

DISCUSSION

The present experiments carried out in regular-spiking cells of the rat sensorimotor cortex demonstrate that 1) the anomalous voltage behavior of the EPSP was caused by intrinsic membrane rectifications rather than the voltage-dependent

gating effect of $[Mg^{2+}]_o$ on the NMDA receptor-ionophore; 2) excitatory synaptic transmission is primarily mediated by non-NMDA receptors; and 3) a slight reduction in synaptic inhibition can disclose NMDA receptor-mediated EPSPs that were polysynaptic in origin.

Anomalous voltage-dependency of the EPSP

On the basis of three lines of evidence, we conclude that the activation and inactivation of intrinsic conductances were responsible for the anomalous voltage behavior of the EPSP described here. First, the blockade of NMDA receptors with CPP had no apparent influence on the anomalous voltage behavior. Second, analysis of the Vm variation on the response evoked by positive current pulse indicated that intrinsic conductances can contribute to the anomalous voltage behavior. Third, the blockade of inward membrane rectifications with QX-314 readily abolished the anomalous voltage behavior. This conclusion supports the hypothesis of Stafstrom et al. (1985).

It should be pointed out that the pharmacological feature of the EPSP described in our study (lack of sensitivity towards NMDA antagonist) is different from the one reported previously in the sensorimotor cortex (Thomson and Lodge, 1985; Thomson et al., 1985; Thomson, 1986), where these authors concluded that the EPSP's anomalous voltage behavior was due to the extracellular Mg^{2+} gating of the NMDA ionophore. This difference, however, does not allow one to exclude the possibility that intrinsic membrane current can also influence the voltage dependency of an EPSP

that is mediated by NMDA receptors. For example, we found that the late EPSP, which was readily blocked by CPP, only displayed NMDA-like voltage dependency when inward membrane rectification was apparent. Similarly, Lambert and Jones (1990) have observed in dentate granule cells a NMDA receptor-mediated EPSP that still displayed anomalous voltage dependency even when Mg^{2+} was completely removed from the extracellular medium (Lambert and Jones, 1990). Sutor et al. (1987) have also found in frontal cortical neurons a NMDA-induced response that still contained a NMDA-like, voltage-dependent component in the absence of $[Mg^{2+}]_o$.

Any interpretation on the voltage behavior of an EPSP will also depend on the locations of the postsynaptic receptors and the electrotonic compactness of the cell. If the NMDA receptors were located in the dendrites of neocortical neurons (Sutor and Hablitz, 1989c), then variation of the Vm at the cell body (i.e., the recording site) might not be sufficient to influence the Mg^{2+}-gating effect on the dendritic NMDA channels. On the other hand, the somatic ion channels that gate intrinsic conductances can come under the influence of this Vm variation and subsequently modify the shape of the EPSP.

Non-NMDA receptors and the EPSP

Although CNQX remains one of the most selective non-NMDA-receptor blockers available to date (Honoré et al., 1988), some studies have shown that this quinoxalinedione can also antagonize the NMDA receptor by interacting with its

glycine site (Hablitz and Sutor, 1990; Thomson, 1990). Interestingly, we found that the IC_{50} of CNQX on the EPSP was not affected by glycine. This might be explained by the fact that the IC_{50} range of CNQX observed in our experiments (about 2 μM) was well below the concentrations ($>$ 5 μM) at which the drug is known to reduce NMDA-evoked depolarization in neocortical cells (Hablitz and Sutor, 1990).

The EPSPs described in the present study were evoked by stimulation of the deep layers (V/VI) or the white matter. Our results showed that the EPSPs were greatly reduced or completely blocked by CNQX in a concentration-dependent manner. Moreover, in the presence of CNQX, extracellular stimuli delivered at suprathreshold intensity could no longer elicit firing of action potentials. Thus, it appears that excitatory synaptic transmission in the sensorimotor cortex is predominately mediated by the non-NMDA receptors. Non-NMDA receptors have also been shown to mediate layers II/III to V and the geniculocortical pathways in the visual cortex (Jones and Baughman, 1988; Shirokawa et al., 1989), as well as the thalamocortical input into the frontal cortex (Hablitz and Sutor, 1990).

The stimulus-response relat' nship showed that the action of CNQX was not uniform against all sizes of EPSP elicited in the same cell. The drug appeared to be more effective against the EPSPs evoked by lower stimulus strength than the ones evoked by higher stimulus strength. Because the size of an EPSP should theoretically reflect the degree of transmitter release across the synapse, our results imply that the amount of CNQX necessary to block a given EPSP is proportional to the amount of excitatory amino acids used to elicit this EPSP. This is in line with the competitive

nature of CNQX (Birch et al., 1988; Honoré et al., 1988).

Role of NMDA receptor

According to our findings, the blockade of non-NMDA receptors with CNQX consistently disclosed an EPSP that was sensitive to CPP. The latency to onset of this EPSP and its ability to follow high-frequency, repetitive stimuli suggest that it was monosynaptically driven. Although this indicates that excitatory synaptic transmission in the sensorimotor cortex contains a component that is mediated by the NMDA receptor, we failed to observe such a component under the perfusion of normal ACSF.

This discrepancy might be related to the experimental conditions (i.e., normal ACSF vs. ACSF containing CNQX and glycine). It has been demonstrated that CNQX can reduce the IPSP, possibly through a blockade of the non-NMDA excitatory pathways that synapse with the inhibitory interneurons (Andreasen et al., 1989; Davies and Collingridge, 1989; Hablitz and Sutor, 1990; Higashi et al., 1991). This disinhibitory effect could provide a means for activating NMDA receptors that were normally silenced by the IPSP (Herron et al., 1985; Dingledine et al., 1986). Indeed, our BMI experiments showed that the NMDA receptor was involved in the decay phase of the short-latency EPSP when inhibition was reduced. The NMDA receptor-mediated conductance can also be augmented by glycine (Minota et al., 1989; Thomson et al., 1989). Therefore, it is plausible that the glycine concentration (10 μM) employed in our experiments, which was at least 10-fold higher than the

level present in the CNS perfused with normal ACSF (cf. Gaiarsa et al., 1990), could enhance the activation of NMDA receptors.

The blockade of $GABA_A$ receptor-mediated inhibition with high concentrations of bicuculline (50 μM) is known to induce epileptiform discharge in neocortical slices (Gutnick et al., 1982; Connors, 1984; Hwa and Avoli, 1989,1991b). This type of epileptiform activity is believed to be initiated by a subset of intrinsically bursting cells located in the middle cortical layers (Gutnick et al., 1982; Connors, 1984; Chagnac-Amitai and Connors, 1989b). In the present study, slices were bathed in low concentration of BMI (1-2 μM) which should only reduce GABAergic inhibition by about 20% (cf. Chagnac-Amitai and Connors, 1989a). This procedure provided an experimental condition in which one can analyze subthreshold synaptic response evoked in a synchronized network of neurons. According to our data, BMI induced the appearance of late EPSPs that were not discernible in normal ACSF. These late EPSPs appeared to be activated by NMDA-mediated polysynaptic pathways because 1) their latency to onset (10-30 ms) was variable and was much longer than the ones displayed by synaptic responses evoked by single-axon stimulation (cf. Thomson et al., 1988); 2) they failed to follow repetitive stimuli delivered at a frequency that did not interfere with the short-latency, monosynaptic EPSP; 3) they were readily abolished by the NMDA receptor antagonist CPP; and 4) CPP increased their latency to onset before a reduction in their amplitude was observed.

What is the implication of the NMDA receptor's involvement in these polysynaptic EPSPs? Autoradiographic studies have shown that the superficial layers

of the rat neocortex have abundant NMDA-binding sites (Monaghan and Cotman, 1985). Therefore, although NMDA receptors do not usually participate in excitatory synaptic transmission in these layers, the existence of NMDA-binding sites might provide the cells with additional anatomical substrates to interact with each others under certain conditions, such as epileptogenesis. Using a dual recording technique, Miles and Wong (1987) have shown that tetanic stimulation, which depresses inhibition, can reveal latent excitatory interactions between CA3 pyramidal cells. Based on this scheme, we propose that a slight reduction in inhibition by BMI in the sensorimotor cortex is sufficient to uncover latent polysynaptic connections that are mediated by NMDA receptors. Once activated, these NMDA-mediated pathways can amplify the excitatory input from the intrinsically bursting cells, thereby entraining the whole neocortical population into widespread synchronization.

The location of latent NMDA synapses remains a matter of speculation. According to our findings, the NMDA-mediated EPSP evoked in the presence of CNQX was monosynaptically activated since it displayed short latency of onset and was able to follow repetitive stimulation. This indicates that NMDA receptors are present on layer II/III regular-spiking cells. It is also conceivable that NMDA receptors are present on excitatory interneurons within the latent polysynaptic circuit.

In conclusion, the above idea on the role of NMDA receptors is in line with findings obtained from epileptogenic neocortical slices resected from patients for treatment of intractable seizures. Although intracellular recordings performed in these tissues have demonstrated the presence of synaptic inhibition (Schwartzkroin and

Haglund, 1986; Avoli and Olivier, 1989; McCormick, 1989; Hwa et al., 1991c), we found that NMDA receptors participate in the late EPSP and bursting discharge evoked by above-threshold synaptic stimuli (Avoli and Olivier, 1987,1989). Interestingly, our preliminary findings indicate that this type of NMDA receptor-mediated hyperexcitability is not reproducible in "control" human tissues obtained from non-epileptic patients.

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Figure 1. Electrophysiological features of the postsynaptic potential (PSP) evoked in sensorimotor cortical neurons. We found two types of PSP on the basis of voltage dependence. A and B: The first type consists of an excitatory postsynaptic potential (EPSP)-inhibitory postsynaptic potential (IPSP) sequence. The EPSP increases and decreases in amplitude with membrane hyperpolarization and depolarization respectively. The IPSP is positive-going at rest (Vm = -78 mV) and reverses in polarity at about -70 mV. C and D: The second type of PSP consists of a pure EPSP since depolarization of the Vm to more positive than -70 mV does not reveal any Cl-mediated IPSP. The peak amplitude of the EPSP (open circle) is not affected by variation of the Vm. In contrast, the decay phase of the EPSP (solid circle) increases and decreases in amplitude with membrane depolarization and hyperpolarization respectively. The resting Vm was -69 mV. E. The stimulus-response relationship shows that the decay phase of the EPSP which was evident at low intensity stimuli (20,25 μA) is shunted by the activation of the IPSP at higher stimulus strength (> 30μA). The Vm of this neuron was maintained at -55 mV by passing depolarizing direct current through the recording electrode. F: When synaptic inhibition is reduced by bicuculline methiodide (BMI, 3 μM), the EPSP always displays anomalous voltage dependency. The resting Vm was -86 mV.

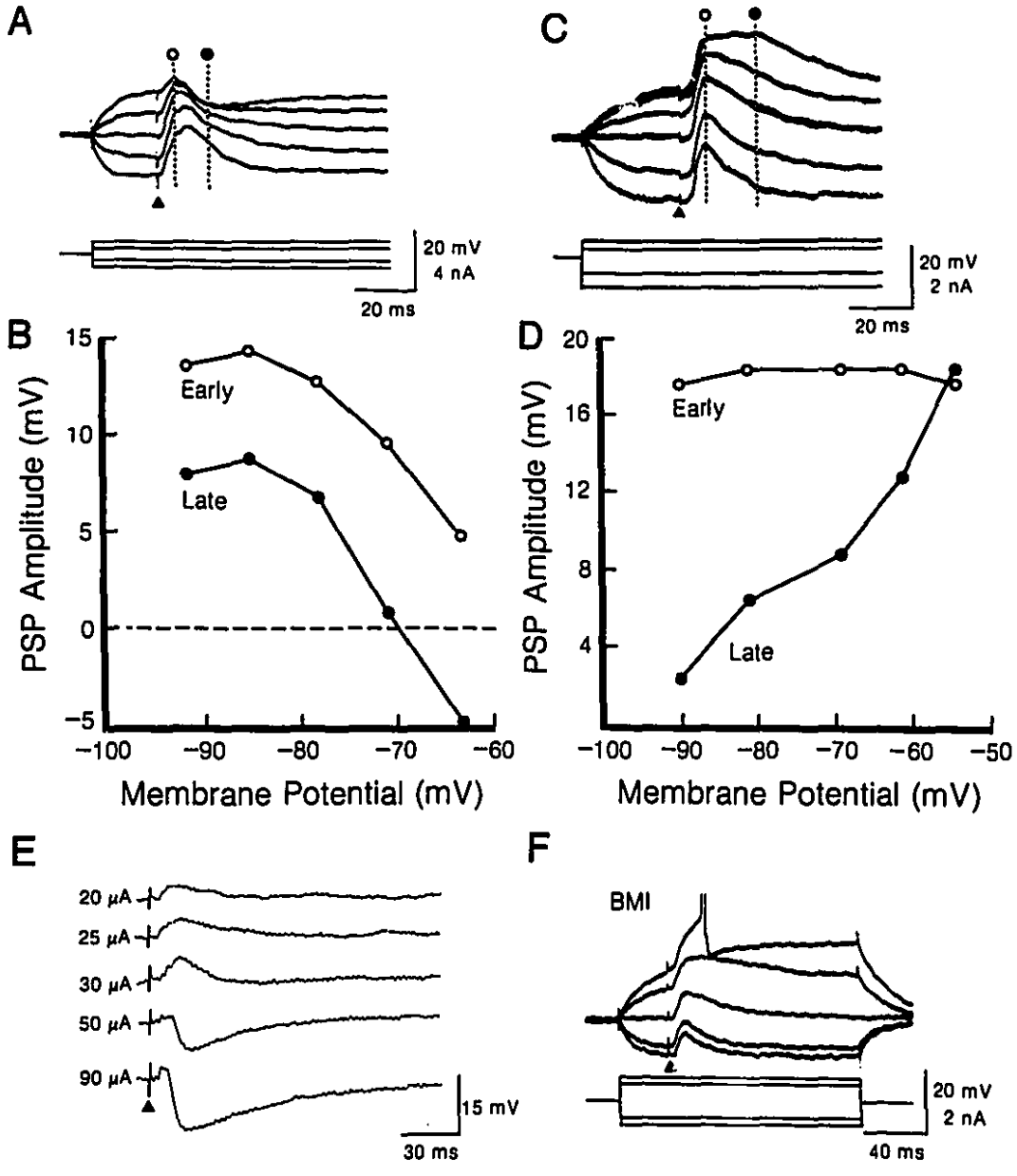
A
20 mV
4 nA
20 ms
C
20 mV
2 nA
20 ms
B
PSP Amplitude (mV)
15
10
5
0
−5
Early
Late
−100
−90
−80
−70
−60
Membrane Potential (mV)
D
PSP Amplitude (mV)
20
16
12
8
4
Early
Late
−100
−90
−80
−70
−60
−50
Membrane Potential (mV)
E
20 μA
25 μA
30 μA
50 μA
90 μA
15 mV
30 ms
F
BMI
20 mV
2 nA
40 ms

Figure 1

Figure 2. Possible mechanisms underlying the anomalous voltage-dependency of the EPSP. A: NMDA receptor antagonist CPP (10 μM) fails to modify the shape of the EPSP evoked at the various membrane levels (rest Vm = -67 mV). Note that the anomalous voltage dependency remains unchanged in the presence of CPP. This demonstrates that the EPSP is not mediated by NMDA receptor-associated conductance. B: The anomalous Vm-dependency of the EPSP (synaptic) can be mimicked by an intrinsic response evoked by a brief pulse of depolarizing current (intrinsic). This suggests that voltage-dependent intrinsic conductances are involved in the voltage behavior of the EPSP. The resting Vm = -69 mV.

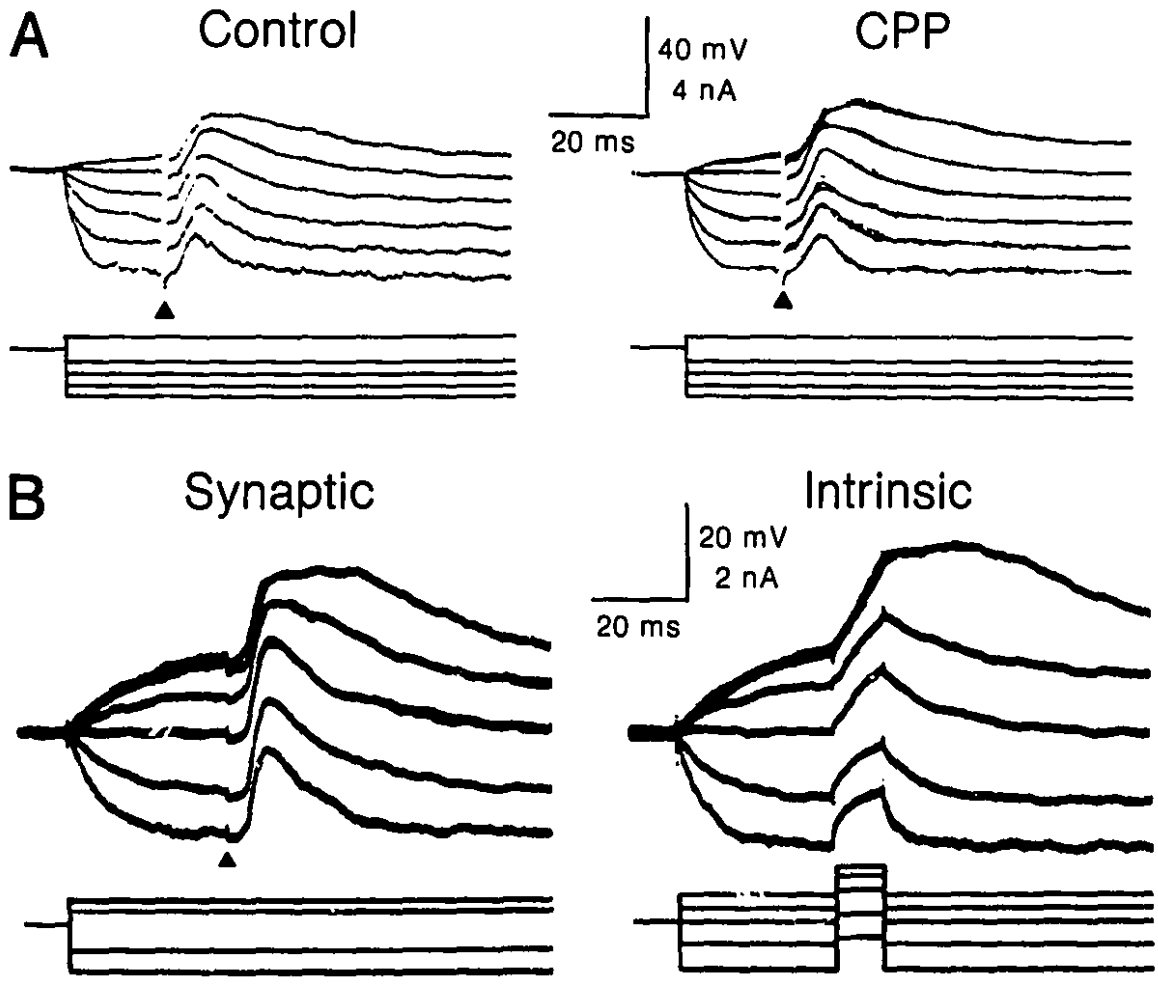
A
Control
CPP
40 mV
4 nA
20 ms
B
Synaptic
Intrinsic
20 mV
2 nA
20 ms

Figure 2

Figure 3. The intracellular injection of QX-314 modifies the anomalous voltage behavior. A: An intrinsic response is evoked at the various membrane levels (RL = -70 mV). The onset and offset of the positive current pulse is represented by up and down arrows respectively. Following the injection of QX-314, the intrinsic response which behaves anomalously in control is no longer dependent on the changes in Vm. B and C: The EPSP evoked in the presence of BMI (control) is also sensitive to the injection of QX-314 (QX-314). According to the voltage-response plots, the decay of the EPSP which behaves anomalously in control (Cb, control) is modified by QX-314 into an EPSP that increases in amplitude with Vm hyperpolarization and decreases in amplitude with Vm depolarization (Cb, QX-314). This type of voltage relationship is also observed in the early phase of the EPSP following QX-314 injection (Ca, QX-314). The resting Vm = -82 mV.

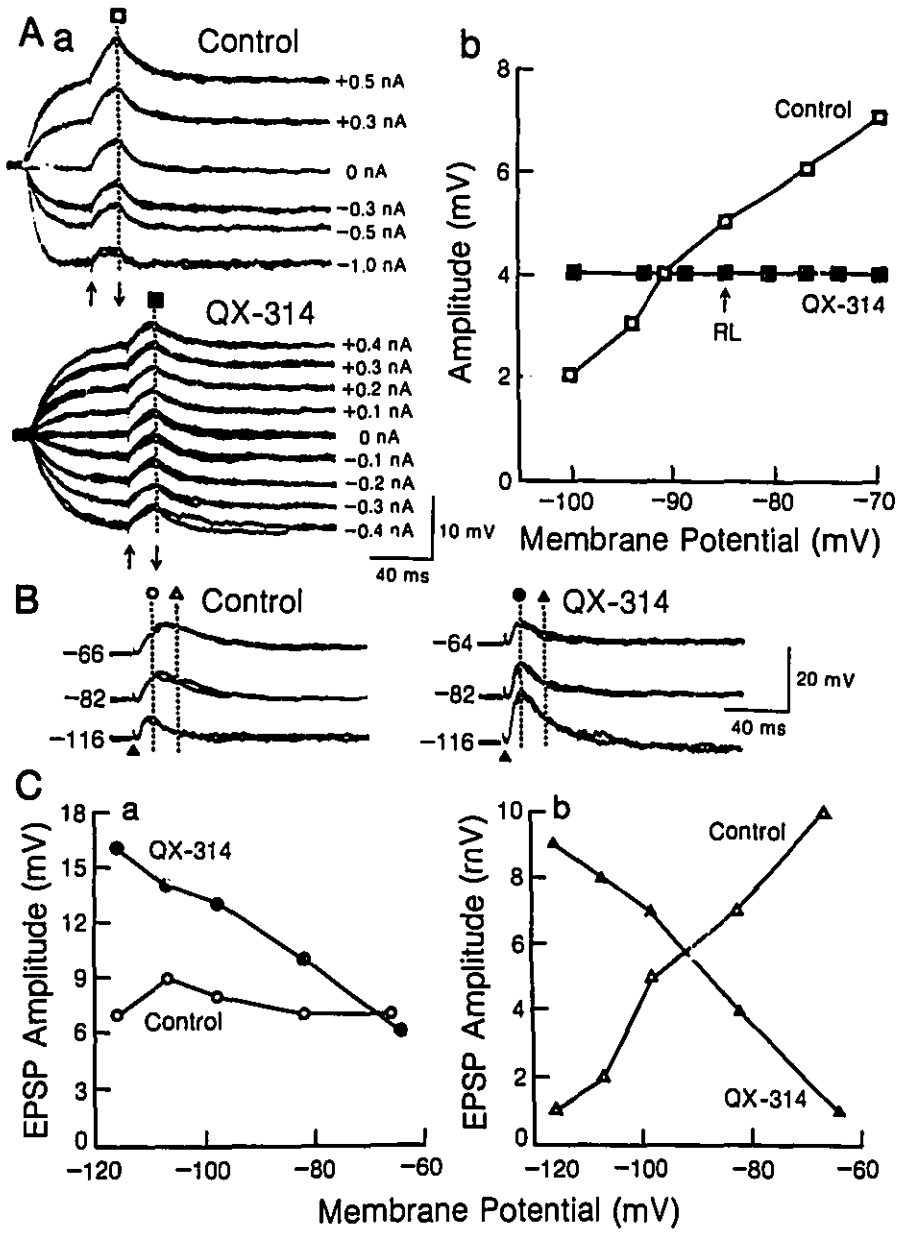
A a
Control
+0.5 nA
+0.3 nA
0 nA
−0.3 nA
−0.5 nA
−1.0 nA
QX-314
+0.4 nA
+0.3 nA
+0.2 nA
+0.1 nA
0 nA
−0.1 nA
−0.2 nA
−0.3 nA
−0.4 nA
10 mV
40 ms
b
Control
QX-314
RL
Amplitude (mV)
Membrane Potential (mV)
B
Control
QX-314
−66
−82
−116
−64
−82
−116
20 mV
40 ms
C
a
QX-314
Control
EPSP Amplitude (mV)
b
Control
QX-314
EPSP Amplitude (mV)
Membrane Potential (mV)

Figure 3

Figure 4. Response of the EPSP to the non-NMDA receptor antagonist CNQX. Glycine (10 μM) was also included in the control medium to avoid interactions between CNQX and NMDA receptors. A: CNQX reduces or blocks the EPSPs in a concentration-dependent manner (1-10 μM). At CNQX concentration of 3-5 μM, the EPSP evoked by low-intensity stimulus (50 μA) is completely blocked. In contrast, suprathreshold intensity (200 μA) can still evoke a small EPSP (Ab, 10 μM) in the presence of 10 μM of CNQX. B: The effect of CNQX on the stimulus-response relationship is illustrated graphically in an input-output plot where the drug shifted the curve downward to the right. C: The dosage-response curve indicates that the IC_{50} of CNQX on the EPSP was about 2 μM. Each point represents the mean $\pm$ SD of 7 EPSPs.

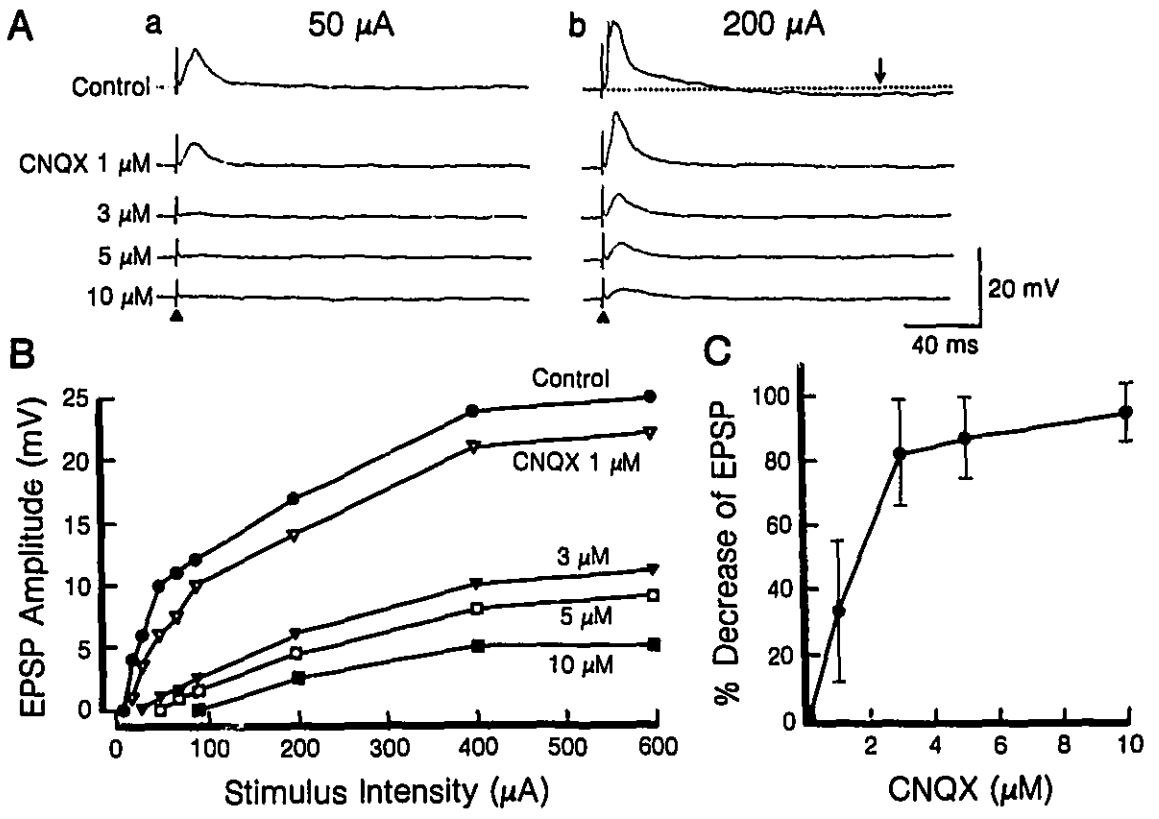
A
a
50 μA
b
200 μA
Control
CNQX 1 μM
3 μM
5 μM
10 μM
20 mV
40 ms
B
EPSP Amplitude (mV)
Control
CNQX 1 μM
3 μM
5 μM
10 μM
Stimulus Intensity (μA)
C
% Decrease of EPSP
CNQX (μM)

Figure 4

Figure 5. Characteristics of the PSP evoked in the presence of CNQX (10 μM) and glycine (10 μM). A: The CNQX-resistant PSP is a graded potential and its shape varies in accordance with the strength of the extracellular stimulus. The early component of the PSP (open circle) increases in amplitude with increasing stimulus strength (from 0-200 μA) until it reaches a plateau at about 200 μA. In contrast, the decay phase of the PSP (solid circle) shows an initial increase in amplitude at stimulus intensity between 0 and 80 μA, but begins to decrease when the stimulus strength is greater than 80 μA. The resting Vm = -82 mV. B: The PSP evoked by suprathreshold stimuli consists of an EPSP-IPSP sequence. The inverted IPSP observed at resting Vm (-82 mV) reverses in polarity with membrane depolarization (-62 mV). C: The NMDA receptor antagonist CPP (10 μM) readily blocks the EPSP evoked by near-threshold stimulus (30 μA) but only attenuates the EPSP evoked by suprathreshold stimulus (90 μA). The resting Vm = -91 mV.

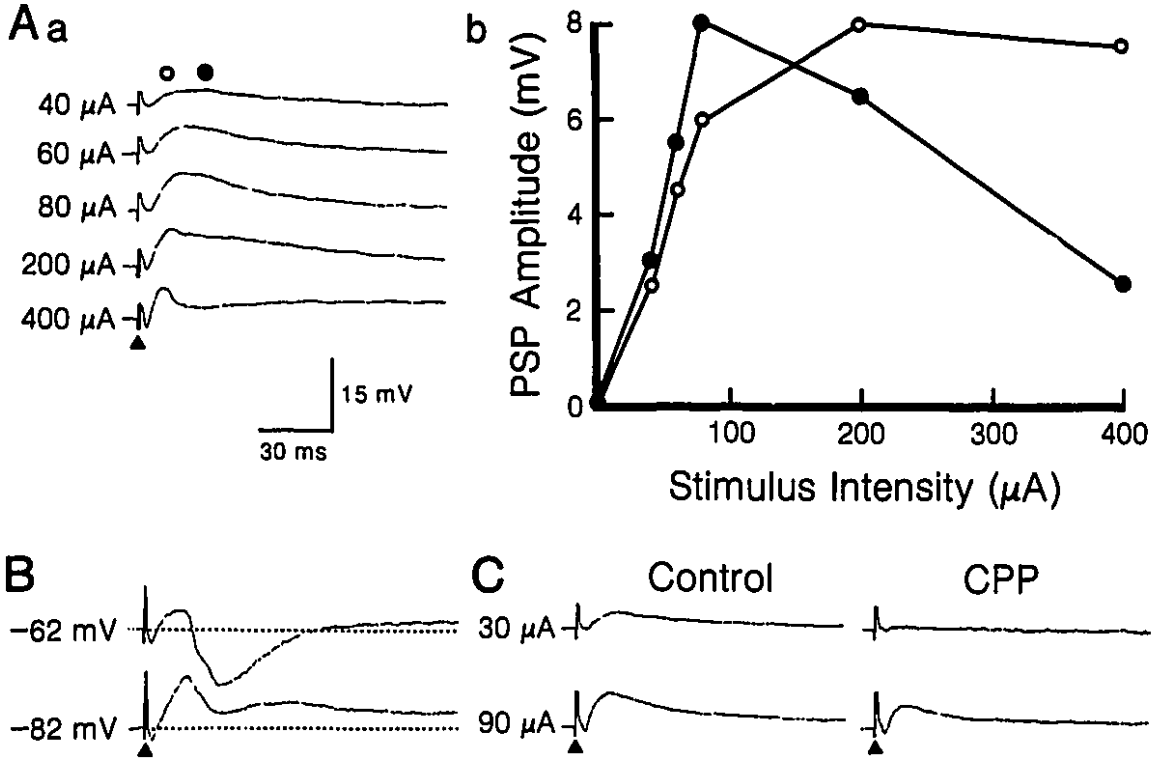
A a
40 μA
60 μA
80 μA
200 μA
400 μA
15 mV
30 ms
b
PSP Amplitude (mV)
0
2
4
6
8
100
200
300
400
Stimulus Intensity (μA)
B
−62 mV
−82 mV
C
Control
CPP
30 μA
90 μA

Figure 5

Figure 6. Electrophysiological features of the EPSPs evoked in the presence of $GABA_A$ receptor antagonist BMI (1-2 μM). A: The intensity of the extracellular stimulus is increased progressively from threshold level (20,30 μA) to suprathreshold level ($\geq$70 μA). Note that suprathreshold stimuli lead to the recruitment of biphasic or multiphasic late EPSPs. The resting Vm = -87 mV. B: Voltage dependency displayed by some of the late EPSPs. The reduction in late EPSPs' amplitude with stepwise membrane hyperpolarization coincides with the progressive activation of inward rectification (traces 2-5). Note the sag of membrane potential towards the resting level (trace 5) which reflects the steep activation of the hyperpolarizing inward rectifier. Depolarization of the Vm, however, fails to significantly modify the shape of the late EPSPs (compare trace 1 with 2). Resting Vm = -85 mV. C: The appearance of the late EPSP is dependent on the stimulation frequency. Following repetitive stimuli delivered at 2 Hz, the late EPSP decreases in amplitude and increases in latency to onset progressively (stimulus #12, L) until the response completely fails (stimulus #30, L). The use-dependent blockade of the late EPSP is fully reversible after a pause of stimulation for about 1 min (Rest, L). The resting Vm = -81 mV.

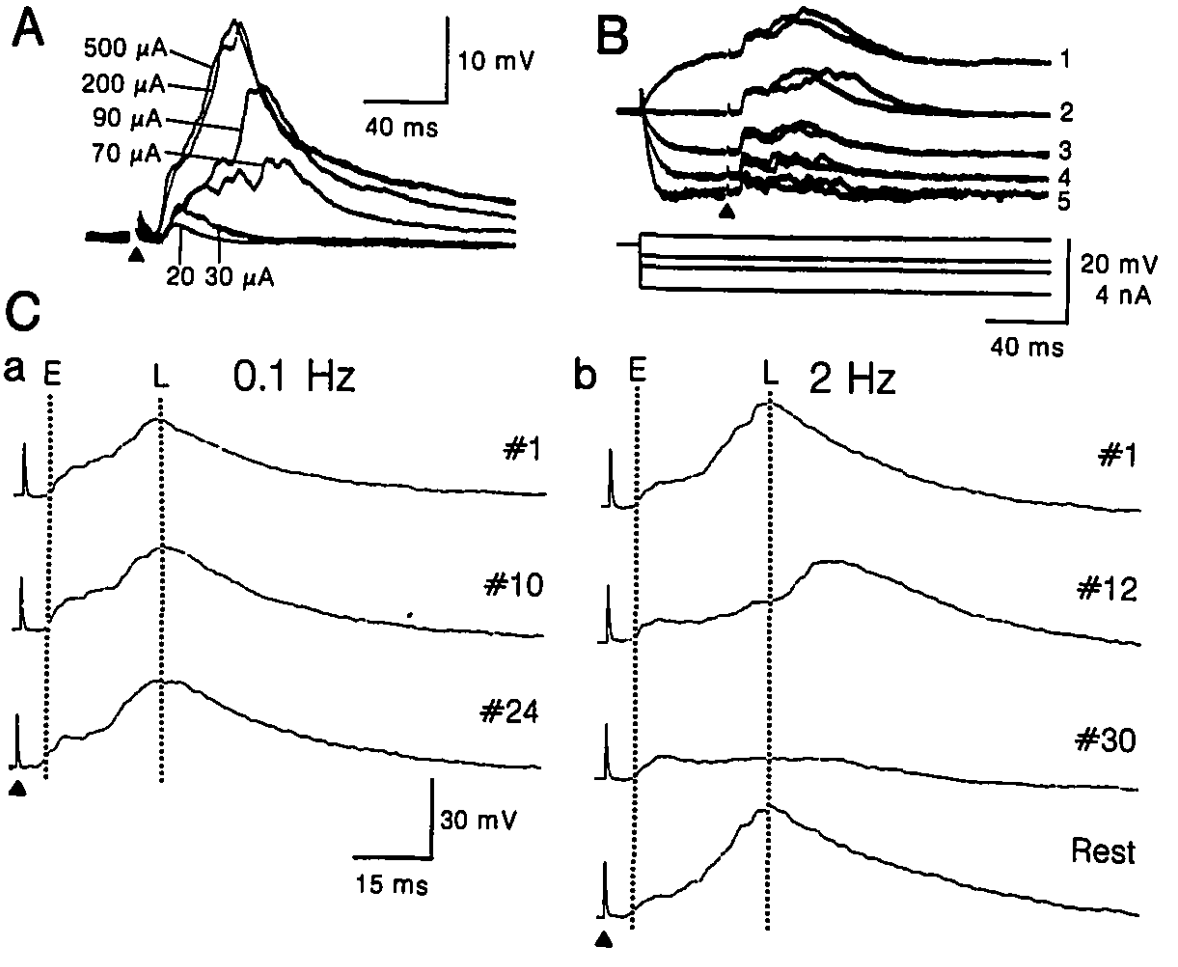
A
500 μA
200 μA
90 μA
70 μA
10 mV
40 ms
20 30 μA
B
1
2
3
4
5
20 mV
4 nA
40 ms
C
a E L 0.1 Hz
#1
#10
#24
30 mV
15 ms
b E L 2 Hz
#1
#12
#30
Rest

Figure 6

Figure 7. Pharmacology of the late EPSP induced by BMI (1 μM). A: Bath perfusion of CPP (3 μM) progressively reduces the amplitude of the late EPSP while increasing its latency to onset (20,25 min) until the response is completely abolished (30 min). The effect of CPP is fully reversible following washout of the drug (Wash). This demonstrates that the late EPSP is mediated by NMDA receptors. Although CPP has no apparent influence on the peak amplitude of the short-latency EPSP (also see B), its decay phase is attenuated by the drug (30 min). This demonstrates a NMDA receptor-mediated component in the short-latency EPSP when GABAergic inhibition is reduced. B: A plot of the drug's effect over time indicates that the increase in latency to onset occurs prior to the reduction in amplitude. Each point represents the mean $\pm$ SD of 5-8 responses. Both A and B were obtained from the same cell with resting Vm = -92 mV.

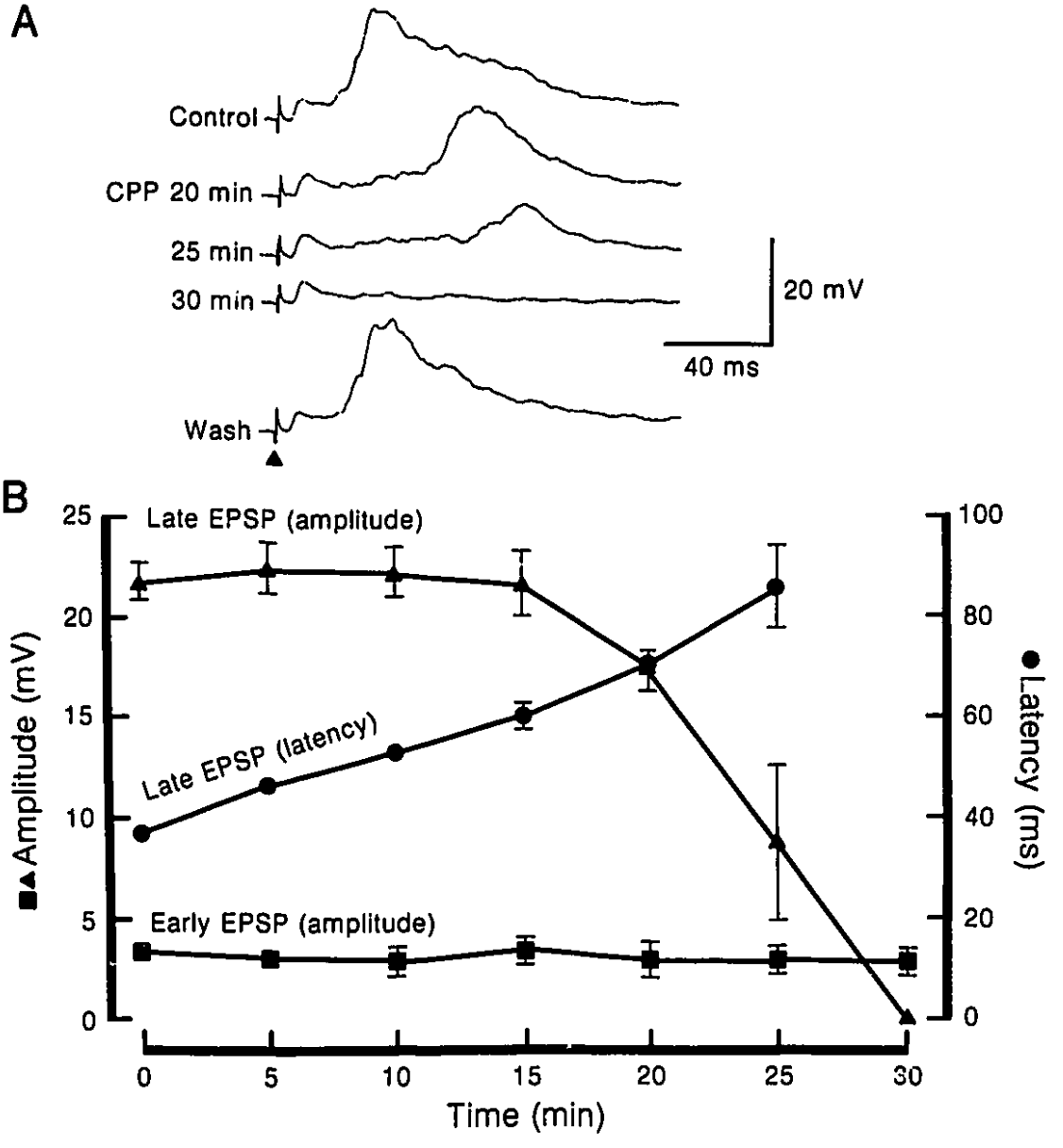
A
Control
CPP 20 min
25 min
30 min
Wash
20 mV
40 ms
B
Late EPSP (amplitude)
Late EPSP (latency)
Early EPSP (amplitude)
▲■ Amplitude (mV)
● Latency (ms)
Time (min)
0
5
10
15
20
25
30
0
5
10
15
20
25
0
20
40
60
80
100

Figure 7

CHAPTER 3

THE INVOLVEMENT OF EXCITATORY AMINO ACIDS IN NEOCORTICAL EPILEPTOGENESIS: NMDA AND NON-NMDA RECEPTORS

SUMMARY

Conventional intracellular recording techniques were used to investigate the N-methyl-D-aspartate (NMDA) and non-NMDA mediated synaptic mechanisms underlying the stimulus-induced paroxysmal depolarization shift (PDS) generated by cells in rat neocortical slices treated with bicuculline methiodide (BMI). The NMDA receptor antagonists CPP or MK-801 were ineffective in abolishing the PDS. However, both drugs were able to attenuate the late phase of the PDS and delay its time of onset. In contrast, the non-NMDA receptor blocker CNQX demonstrated potent anticonvulsant property by reducing the PDS into a depolarizing potential that was graded in nature. This CNQX-resistant depolarizing potential was readily blocked by CPP. Voltage-response analysis of the PDS indicated that the entire response (including its NMDA-mediated phase) displayed conventional voltage characteristics reminiscent of an excitatory postsynaptic potential that is mediated by non-NMDA receptors. We conclude that the activation of non-NMDA receptors is necessary and sufficient to induce epileptiform activity in the neocortex when the GABAergic inhibitory mechanism is compromised. The NMDA receptors contribute to the process of PDS amplification by prolonging the duration and reducing the latency to onset of each epileptiform discharge. However, the participation of NMDA receptors is not essential for BMI-induced epileptogenesis, and their partial involvement in the PDS is dependent upon the integrity of the non-NMDA mediated input. The lack of NMDA-like voltage dependency observed in the PDS's late phase might reflect an uneven distribution of

NMDA receptors along the cell and/or an association of this excitatory amino acid receptor subtype in the polysynaptic pathways within the neocortex.

INTRODUCTION

It is well documented that epileptiform activities can be evoked by reducing synaptic inhibition mediated by gamma-aminobutyric acid A ($GABA_A$) receptor with chemical convulsants like bicuculline or picrotoxin (for review, see Avoli, 1988). Based on this experimental approach, previous investigations have provided some insights into the basic mechanisms underlying epileptogenesis. It is generally believed that the initiation of focal epileptiform activity in the neocortex is highly dependent upon a subpopulation of endogenously bursting cells located in the middle cortical layers (Gutnick et al., 1982; Connors, 1984, Chagnac-Amitai and Connors, 1989). However, the mechanism responsible for the propagation of epileptiform activities in the neocortex is less clear. While nonsynaptic transmission such as electronic coupling has been implicated in this process (Gutnick and Prince, 1981), it appears more likely that widespread epileptiform synchronization is achieved through the excitatory synaptic interactions between neurons (Johnston and Brown, 1986).

N-methyl-D-aspartate (NMDA) and non-NMDA receptors have been widely recognized as two major subclasses of excitatory amino-acid receptors responsible for synaptic transmission in the mammalian central nervous system (for review, see Cotman et al., 1987). In recent years, the role played by these excitatory receptors in the physiopathogenesis of epilepsy has attracted much attention. Following the *in vivo* demonstrations that NMDA receptor antagonists can exhibit potent anticonvulsant actions (Croucher et al., 1982; Meldrum et al., 1983; Peterson et al., 1983), intracellular studies carried out in the hippocampal or entorhinal cortical slices

indicated that the epileptiform discharge resulting from a reduction in GABAergic inhibition is sensitive to NMDA receptor antagonists (Herron et al., 1985; Dingledine et al., 1986; Hablitz and Langmoen, 1986; Jones, 1988; Lee and Hablitz, 1990; Watson and Lanthorn, 1990). However, the involvement of excitatory amino acid receptors within the disinhibited neocortex remains unclear. In fact, it has been reported that NMDA receptors do not play a role in the picrotoxin-induced epileptiform discharge evoked in rat neocortical slices (Thomson and West, 1985,1986).

The goal of this study was to examine the participation of NMDA and non-NMDA receptors in the bicuculline methiodide (BMI)-induced epileptiform discharge evoked in rat neocortical slices maintained *in vitro*. The role of NMDA receptor was tested with its potent antagonists 3-((±)-2-carboxypiperazin-4-yl)-propyl-1-phosphonate (CPP) (Davies et al., 1986) or (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate (MK-801) (Wong et al., 1986). The role of non-NMDA receptors was tested with 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) which represents the most specific non-NMDA receptor blocker available to date (Honoré et al., 1988). Some of these results have appeared in preliminary forms (Hwa and Avoli, 1988,1989).

MATERIALS AND METHODS

Male Sprague-Dawley rats (200-300 g) were anesthetized with ether. Upon decapitation, their brains were rapidly removed and placed in cold (1-3 °C) artificial cerebrospinal fluid (ACSF). A block of the neocortex was dissected from the region

corresponding to the sensorimotor cortex (Zilles and Wree, 1985) and cut coronally at a thickness of 450-550 μm using a McIlwain tissue chopper. Slices were then transferred to a tissue chamber where they were laid at an interface between humidified gas (saturated with 95% O_2/5% CO_2) and warmed (34$\pm$1 °C), oxygenated (95% O_2, 5% CO_2) ACSF. The ACSF was perfused at a constant rate (0.5-1.5 ml/min) during each individual experiment. The composition of the ACSF (pH 7.4) was (in mM): NaCl 124, KCl 2, KH_2PO_4 1.25, $MgSO_4$ 2, $CaCl_2$ 2, $NaHCO_3$ 26, glucose 10. Following each slicing procedure, the neocortical slices were always incubated in control ACSF for at least 1 h prior to recording. BMI (Sigma), MK-801 (Merck Sharp & Dohme), CPP and CNQX (Tocris Neuramin) were all applied by bath perfusion.

Conventional intracellular recordings were obtained from the superficial layers (II-III) with glass microelectrodes (diameter: 1.5-mm, tip resistance: 40-80 MΩ) backfilled with either 4 M K-acetate or 50 mM QX-314 (Astra) dissolved in 4 M K-acetate. Injection of QX-314 was achieved by passing pulses (100-400 ms) of depolarizing current (1.0-3.0 nA) through the recording electrode. Signals were fed to a high-impedance amplifier (Axoclamp-2A) with an internal bridge circuit for passing intracellular current. The bridge balance was monitored routinely and adjusted when necessary. The recorded responses were displayed on a storage oscilloscope and/or a GOULD pen writer. In the latter case, a 20 KHz waveform digitizer was often used to avoid distortion of the signals due to slow time response of the pen. In some experiments, the data were also recorded on FM magnetic tape for subsequent analysis.

Constant current stimuli (0.01-10 mA; 10-180 μs) were delivered through a sharpened bipolar tungsten stainless steel electrode which was positioned either in the underlying white matter or within the grey matter. The alignment of the stimulation electrode with respect to the recording site formed an imaginary line which was perpendicular to the pia surface. Comparison of the responses evoked by either white or grey matter stimulation showed that they were very similar in shape and characteristics.

All statistical analyses were performed with the paired-sample Student *t*-test. The quantitative results were expressed as the mean $\pm$ SD. The duration of the epileptiform burst was measured at the halfway point between the baseline and the response's peak amplitude. The latency to onset of the response was represented by the distance between the stimulus artifact and the peak of the first overriding action potential.

RESULTS

Results obtained from 33 neocortical cells were included in the present study. Although no attempt was made to identify them morphologically, the electrophysiological properties of these neurons were similar to the regular-spiking pyramidal cells described in the guinea pig (Connors et al., 1982; McCormick et al., 1985) and the rat neocortex (Sutor and Zieglgänsberger, 1987; Chagnac-Amitai and Connors, 1989). In the presence of BMI, a sample of the neurons displayed resting membrane potential (Vm) of -80$\pm$13 mV (mean$\pm$SD, n=25), input resistance of 28$\pm$10 MΩ (n=22), and action potential amplitude of 100$\pm$14 mV (n=26).

Characteristics of the BMI-evoked response

Neocortical slices were first perfused with 50 μM BMI for at least 30-60 min prior to each experiment. Under such condition, spontaneously occurring epileptiform discharges were rarely observed. However, electrical stimulation of the underlying white matter or within the grey matter evoked an all-or-none epileptiform burst that closely resembled the paroxysmal depolarization shift (PDS) (for review, see Prince and Connors 1986). Briefly, the stimulus-induced PDS was characterized by a depolarizing envelope (20-40 mV in amplitude, > 50 ms in duration) which triggered a discharge of action potentials (Fig. 1A).

The appearance of the PDS was dependent upon the strength and the frequency of stimulation. At near-threshold intensity stimulation (Fig. 1A, 50μS), the PDS was sometimes preceded by a short-latency excitatory postsynaptic potential (EPSP). However, because the PDS latency was inversely related to the stimulus strength, the short-latency EPSP was no longer discernible in a response evoked by above-threshold stimuli (Fig. 1A, 90μS). When the stimulus was delivered repetitively at a frequency of 0.10 Hz or higher, failure of triggering the PDS often occurred. This refractory period was characteristic of all the neocortical slices subjected to this protocol.

The voltage behavior of the PDS was investigated by injecting steady DC current of negative or positive polarities through the K-acetate-filled recording microelectrode. During membrane depolarization, the PDS's peak amplitude decreased, while the duration measured at the depolarization plateau and the firings of action potentials increased (Fig. 1B, +15, +20mV). A post-burst hyperpolarization, which lasted over

1 sec, was also disclosed at these voltage levels (Fig. 1B, +15,+20mV). In contrast, hyperpolarization of the Vm enhanced the PDS's peak amplitude, and reduced the plateau duration and action potential discharge (Fig. 1B, -10mV). In addition, the post-burst hyperpolarization became inverted in this voltage range (Fig. 1B, -10mV).

Effects of CNQX

Using low concentrations of CNQX (0.5-5 μM), we examined how the blockade of non-NMDA receptors would affect the BMI-induced PDS (n=9 cells). A typical experiment is illustrated in figure 2A. In control condition, the PDS was elicited by synaptic stimulation of the underlying white matter (Fig. 2Aa, control). The addition of CNQX progressively depressed the epileptiform response by reducing its duration and consequently modifying the firing pattern of its action potential discharge (Fig. 2Aa, 20 min). Moreover, the PDS's latency to onset was also increased (Fig. 2Ab, 20 min). After about 30-40 min following the onset of CNQX application, the stimulus-induced PDS was completely abolished (Figs. 2Aa,b 40 min). In place of it there was a residual depolarizing potential (Figs. 2Aa,b 40 min; also see below).

The effects induced by CNQX were very persistent. In three experiments, attempts were made to reverse the blockade of CNQX with control ACSF containing BMI. We observed that the washout of CNQX for over 100 min did not bring about any noticeable change. This long-lasting effect of CNQX was supported by previous reports that the recovery from CNQX is an extremely slow process (Blake et al., 1988; Neuman et al., 1988a; Andreasen et al., 1989; Davies and Collingridge, 1989). It is

unlikely that the changes observed following the application of CNQX were due to general depressant effects of the drug since the neuron's resting Vm, input resistance and intrinsic firing evoked by an intracellular pulse of depolarizing current were not altered by CNQX (Fig. 2Ac).

The CNQX-resistant component

The stimulus-induced depolarizing potential observed in the presence of CNQX was analyzed in detail in 5 cells. Further enhancement in the intensity of the extracellular stimulus gradually augmented the rate of rise and the peak amplitude of the depolarizing potential (Fig. 2Ba). Moreover, higher stimulus strength could bring the depolarizing potential within the Vm threshold for the triggering of action potentials (Fig. 2Ba). However, because this type of bursting discharge was graded in nature, it was not considered as an epileptiform event which is defined as an all-or-none phenomenon (Prince and Connors, 1986).

Variation of the Vm indicated that the depolarizing potential increased in size during membrane depolarization and decreased in size during membrane hyperpolarization (Fig. 2Bb). Following the bath perfusion of CPP, the CNQX-resistant potential was completely abolished (Fig. 2Bc). This suggests that the residual depolarizing potential is mediated by NMDA receptor-associated conductance. In the presence of both CNQX and CPP, focal stimulation at the highest possible intensity (10 mA, 180 μs) could no longer elicit any synaptic response.

Effects of NMDA antagonists

The results reported in the previous section indicate that the activation of NMDA receptors alone is not sufficient to evoke BMI-induced epileptiform discharge. However, the extent to which NMDA receptors participate in the BMI-induced PDS when the non-NMDA mechanisms are operant in the rat neocortex was not clear. Therefore, the competitive NMDA antagonist CPP and the non-competitive NMDA antagonist MK-801 were used to establish the role of NMDA receptor in this type of interictal-like discharge.

In all of the neurons tested (n=6), application of CPP (3-5 μM) reduced the PDS's duration by 51$\pm$22% (paired *t*-test, $P < 0.025$). The diminution in duration was caused by an attenuation of the PDS's falling phase (Fig. 3A, CPP), and it was fully reversible upon the washout of the drug (Fig. 3A, Wash). Moreover, the effects induced by CPP were not reflected by any apparent changes in the membrane excitability of the neuron (e.g. resting Vm, input resistance, and repetitive firing; not illustrated).

CPP also retarded the time of onset of the stimulus-induced epileptiform response (Fig. 3B). Comparison of the response's latency to onset between the control and CPP application in 5 experiments showed that CPP caused an increase of 46$\pm$5 % (Fig. 3C). This prolongation in the response's time of onset was significant (paired *t*-test, $P < 0.005$) and was partially reversible after washing (Figs. 3B,C). The changes induced by CPP always occurred progressively within the first 15 min following its introduction and reached a steady state in about 30 min. Our results also showed that the

short-latency EPSP which precedes the PDS was a potential that was resistant to CPP. During the perfusion of the NMDA antagonist, the EPSP's peak amplitude, its latency to onset and its rate of rise remained unchanged at a time when both the duration and latency to onset of the PDS were modified (Fig. 3B). This short-latency EPSP was primarily mediated by CNQX-sensitive non-NMDA receptor subtypes (Hwa and Avoli, 1990, 1992).

Changes analogous to those seen with CPP in the PDS's shape and latency to onset were also observed in 4 neurons treated with MK-801 (3-5 μM). As illustrated in figure 4A, MK-801 attenuated the late phase of the PDS and lessened the firings of its associated action potentials. In this example, we have also monitored the amplitude of the underlying depolarizing envelope measured at two different latencies from the stimulus artifact (Fig. 4B). After the perfusion of MK-801 for 10 min, a decrease in the late phase of the envelope was observed (Fig. 4B, 150ms). While the reduction in the late phase was a progressive event and reached a steady state after 35 min, the early phase of the envelope measured at its peak amplitude showed no significant changes (Fig. 4B, 50ms). Although these effects were not associated with any changes in the cell's resting Vm, input resistance and repetitive firing (not shown), the action of MK-801 was very persistent and remained irreversible even after 2 h of rinsing with control ACSF (Fig. 4Ac,Bc). This long-lasting effect of MK-801 has been described previously (Coan et al., 1987; Drapeau et al., 1988).

The PDS recorded with QX-314 electrode

Studies in the mammalian neocortex have shown that NMDA-activated response displayed unconventional voltage characteristics by decreasing and increasing in amplitude during membrane hyperpolarization and depolarization respectively (Flatman et al., 1986; Thomson, 1986a,b). This prompted us to see whether this type of anomalous voltage behavior was also observable in the NMDA-sensitive (late) phase of the PDS. Moreover, we were also interested in seeing if the antagonistic action of CPP was a phenomenon that was dependent upon the Vm of the cell. Therefore, in 4 additional experiments, the neurons were injected with QX-314 (50 mM) which can provide a more accurate analysis of the synaptic potential that underlies the PDS. The intracellular injection of this lidocaine compound blocks the inward, Na^+-dependent conductance (Connor and Prince, 1982; Connors et al., 1982; Stafstrom et al., 1985; Sutor and Hablitz, 1989) and subsequently reduces the activation of outward rectifying conductances that follow the burst of fast action potentials. In addition, QX-314 also blocks the hyperpolarizing inward rectification (Hwa and Avoli, 1991a) that can impose a shunting effect on synaptic conductances (Sutor and Hablitz, 1989).

Figure 5 shows sample recordings of the epileptiform response evoked in a neuron injected with QX-314. The Vm of the cell was altered by passing steady DC hyperpolarizing or depolarizing current through the recording microelectrode. At resting level (Fig. 5A, control -70mV), the stimulus-induced response consisted of a depolarizing envelope which lacked the superimposed action potential firing normally observed in recordings with K-acetate electrodes (compare with Fig. 1). This absence of action potential discharge was observed at the various Vm (Fig. 5A, control) and

was presumably due to the blockade exerted by QX-314. The voltage behavior of the epileptiform response was analyzed by measuring the amplitude of the depolarizing envelope at 2 different latencies from the stimulus artifact (Fig. 5A,control: circle, triangle). According to the voltage-response curve, the entire envelope behaved as a monotonic function of the Vm by increasing and decreasing in amplitude during membrane hyperpolarization and depolarization respectively (Figs. 5Ba,b, control). This type of voltage characteristic was reminiscent of a conventional EPSP.

At the various membrane levels tested, CPP did not cause any apparent change in the response's peak (early) phase (Figs. 5A,Ba) while a clear reduction could be seen in the response's falling (late) phase (Figs. 5A,Bb). This was in line with the findings obtained with K-acetate-filled microelectrodes (see Fig. 3). The relative change in synaptic conductance was derived from the slopes of the voltage-response plots. In the example shown in figure 5, the NMDA receptor antagonist only produced a less than 4 % decrease in the response's early phase (Fig. 5Ba), while a decrease of over 21 % was observed in the response's late phase (Fig. 5Bb). These results indicate that the synaptic conductance underlying the PDS's late phase is much more susceptible to the antagonistic action of CPP than the one underlying the early phase. Interestingly, it should be pointed out that the late phase of the epileptiform response, which showed CPP-sensitivity, did not display any NMDA-like voltage dependency.

DISCUSSION

Roles of excitatory amino-acid receptors in BMI-induced epileptogenesis

The present findings demonstrate that the quinoxaline derivative CNQX is an effective agent for abolishing the PDS evoked by the $GABA_A$ receptor blocker BMI in the rat neocortical slice. Since the introduction of CNQX as a potent non-NMDA receptor antagonist (Honoré et al., 1988), several studies have indicated that CNQX can also affect the NMDA receptor by interacting with its glycine modulatory site (Birch et al., 1988; Harris and Miller, 1989; Lester et al., 1989). However, this action of CNQX appears not to be pertinent to our findings since the concentration range of CNQX described in these studies was much higher (up to 100 μM) than the ones chosen in our experiments. Moreover, ionophoretic study in neocortical slices have shown that the doses of CNQX employed by us are not high enough to interact with NMDA receptors (Hablitz and Sutor, 1990). Therefore, it appears that the BMI-induced PDS evoked in the neocortex is predominantly mediated by the non-NMDA receptors.

Our results also show that CPP or MK-801 (3-5 μM) depressed the PDS by shortening its duration and delaying its time of onset. At this concentration of CPP or MK-801, the NMDA mechanisms in the neocortex should be inactivated completely since similar dosages of CPP or MK-801 (1-5 μM) can readily abolish the Mg^{2+}-free neocortical epileptiform discharge (Drapeau et al., 1988) which is mainly dependent on NMDA receptor-associated conductance (Thomson and West, 1986; Avoli et al., 1987; Drapeau et al., 1988; Sutor and Hablitz, 1989). This demonstrates that BMI-induced epileptiform activity in the rat neocortex is only partially mediated by NMDA receptors. We have also shown that the partial role played by NMDA receptors was dependent upon the integrity of the non-NMDA mediated mechanisms, since the PDS

could not be triggered when CNQX was present in the perfusing medium. However, this unilateral dependency only applied to the genesis of epileptiform bursting because a residual depolarizing potential that displayed CPP-sensitivity was observed in the presence of CNQX.

Although the degree of antagonism of the PDS achieved by CPP/MK-801 or CNQX was different in our experiments, the manners by which they suppressed the PDS were very similar - shortening the duration of the PDS and prolonging its latency to onset. These similarities suggest that a common underlying mechanism might be shared between the NMDA and non-NMDA receptors in generating the BMI-induced epileptiform discharge in the neocortex.

A previous study in the neocortical slice had shown that NMDA receptor antagonists have no effect on the PDS induced by picrotoxin (Thomson and West, 1985,1986). This disagreement with our data might be attributable to a difference in experimental conditions employed between the two laboratories. The PDS described in the previous report was evoked in the presence of low dosages of picrotoxin (1.65-5 μM) (Thomson and West, 1986). Thus, it is possible that the degree of GABAergic blockade achieved by low concentrations of picrotoxin (1.65-5 μM) was not sufficient to recruit enough NMDA receptors to participate in epileptiform bursting. Interestingly, a preliminary report has indicated that epileptiform discharges induced by much higher concentrations of picrotoxin (50-100 μM) are reduced by NMDA receptor antagonists in the neocortical slice (Aram and Lodge, 1987).

The absence of NMDA-like voltage dependency

According to findings obtained from the CA1 region of the hippocampus, it was proposed that the bicuculline- or picrotoxin-induced PDS consists of an initial non-NMDA phase and a late NMDA phase which is governed by the $[Mg^{2+}]_o$ gating of the NMDA channel (Herron et al., 1985; Dingledine, 1986; Dingledine et al., 1986). Here in the neocortex, although the pharmacological feature of the PDS is in keeping with this scheme, analysis of the voltage behavior of the PDS showed that its CPP-sensitive phase did not display the non-linear voltage relationship that is typical of NMDA receptor-associated conductance.

Any explanation for this discrepancy may depend on how NMDA receptors are distributed along the neocortical cell. The electrophysiological features (e.g. repetitive firing, action potential's width) of the neuron suggested that we were recording at or close to the cell body. Therefore, if NMDA receptors were located predominately in the dendrites, Vm manipulation through the recording electrode would have little influence on these more distally situated receptors. On the other hand, if NMDA receptors were populated around the neuronal soma, then the late (NMDA) phase of the PDS should be influenced by Vm manipulation. However, if the somatic density of the non-NMDA sites was much richer than the NMDA sites, then the conductances activated by non-NMDA receptors would simply mask the NMDA negative-slope conductance. Based on ionophoretic studies, both somatic (Thomson, 1986b) and dendritic (Sutor and Hablitz, 1989) locations of NMDA receptors have been proposed. Perhaps, it would be of interest to use autoradiographic techniques to establish the

position and density of this excitatory amino-acid receptor in fully developed and identifiable neocortical neurons.

Alternatively, one might ask whether the above hippocampal scheme is applicable to the neocortex. In the hippocampus, the PDS was evoked by afferent stimulation of the Schaffer collaterals which synapse directly with the CA1 pyramidal cell (Herron et al., 1985, Dingledine et al., 1986). Since there exists little evidence for recurrent excitatory interactions within the CA1 subfield (Knowles and Schwartzkroin, 1981), the orthodromically evoked PDS in the CA1 was viewed as a monosynaptic response with its initial non-NMDA component that functions to alleviate the $[Mg^{2+}]_o$ blockade of its late (NMDA) component (Herron et al., 1985; Dingledine, 1986; Dingledine et al., 1986). However, because of the complex nature of the intrinsic neocortical pathways, it is likely that the BMI-induced PDS described in our experiments was generated through a series of synaptic cascades involving reverberating activities within a neuronal aggregate. Therefore, any attempt to temporally dissect the neocortical PDS as a monosynaptic response might be an oversimplification of events. In light of this, perhaps it is not surprising that the late phase of the PDS did not demonstrate NMDA-like voltage dependency. The attenuation of the PDS's late phase might simply reflect a collective blockade of the NMDA receptors within the polysynaptic pathways that underlie this type of epileptiform activity. Our observation that the PDS's latency to onset was increased by CPP or MK-801 appears to support this view.

Conclusion

The primary goal of this study was to examine the excitatory synaptic mechanisms underlying the BMI-induced interictal-like discharge evoked in the neocortical slice. We have shown that the CNQX-sensitive non-NMDA receptors play a paramount role since their activation is a necessary and sufficient condition for evoking BMI-induced epileptiform discharge. The association of non-NMDA receptors with epilepsy has been documented in other *in vitro* models (Savage et al., 1984; Turski et al., 1987; Neuman et al., 1988b, Hwa and Avoli, 1991b; Perreault and Avoli, 1991). However, the clinical relevance of CNQX as a possible anticonvulsant drug remains to be determined since CNQX also blocks normal synaptic transmission in the neocortex (Hwa and Avoli, 1990).

Our results also indicate that the NMDA receptor only contributes to the process of PDS amplification and not its generation in the neocortical slice. Because the spread of BMI-induced epileptiform activity in the neocortex is highly dependent upon its intrinsic excitatory connections (Chervin et al., 1988), the decrease in PDS's duration and the increase in its latency to onset following the blockade of NMDA receptors probably reflect the participation of this excitatory amino-acid receptor in the positive feedback mechanism within the neocortical slice. Moreover, it is also possible that the NMDA receptor may participate in this type of epileptogenesis at a more subtle level. It has been documented that each picrotoxin-induced epileptiform event is associated with massive entry of extracellular Ca^{2+} into the cell through the NMDA receptor-ionophore (Köhr and Heinemann, 1989). Consequently, this massive build-up of

intracellular Ca^{2+} can trigger second messenger systems which may lead to the more long-lasting damage observed in some seizure disorders (Gloor, 1989).

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Figure 1. Characteristics of the stimulus-induced response evoked in the presence of bicuculline methiodide (BMI). Responses were evoked by focal stimulation of the underlying white matter (solid triangle). Calibration in A also applies to B. The resting membrane potential (Vm) of the cell was about -70 mV. A: Stimulus delivered at near-threshold (50 μS) or above-threshold (90 μS) intensities evoke an all-or-none epileptiform burst which resembles the paroxysmal depolarization shift (PDS). The time of onset of the PDS is dependent upon the stimulus strength. As the stimulus intensity is increased from 50 μS to 90 μS, the response's latency to onset decreases accordingly. Note that the response evoked at near-threshold strength is sometimes preceded by a short-latency excitatory postsynaptic potential. B: Alteration of the Vm shows that the PDS behaves in a voltage-dependent manner. Note that a post-burst hyperpolarization is disclosed by membrane depolarization from rest (+15 & 20 mV).

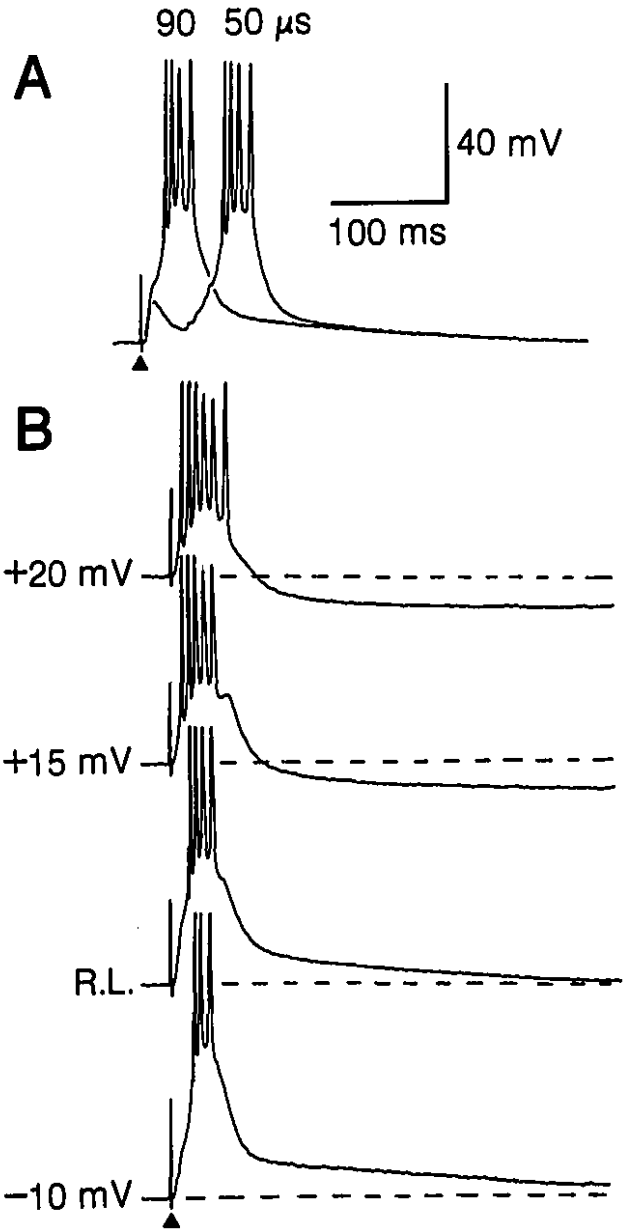

Figure 1

Figure 2. The CNQX-sensitive (A) and CNQX-resistant (B) synaptic potentials evoked in the presence of BMI (50 μM). A: Bath perfusion of the non-NMDA receptor antagonist CNQX (2 μM) initially attenuates the late phase of the BMI-induced PDS (Aa, 20 min) and increases its time of onset (Ab, 20 min). When the action of CNQX reaches equilibrium, the stimulus-induced PDS is no longer discernible. In place of it there is a small depolarizing potential (Aa,b 40 min). Note that the intrinsic firing of the cell is not influenced by CNQX (Ac). B: Characteristics of the depolarizing potential evoked in the presence of CNQX (5 μM) and BMI. Stepwise increment in stimulus intensity progressively increases the size of the CNQX-resistant potential until it triggers burst firing (Ba). Variation of the membrane potential by injecting pulses of positive or negative current indicates that the CNQX-resistant potential increases and decreases in size during membrane depolarization and hyperpolarization respectively (Bb). This CNQX-insensitive potential is readily blocked by the NMDA receptor antagonist CPP (5 μM). The resting level of the cell was -76 mV in A and -77 mV in B.

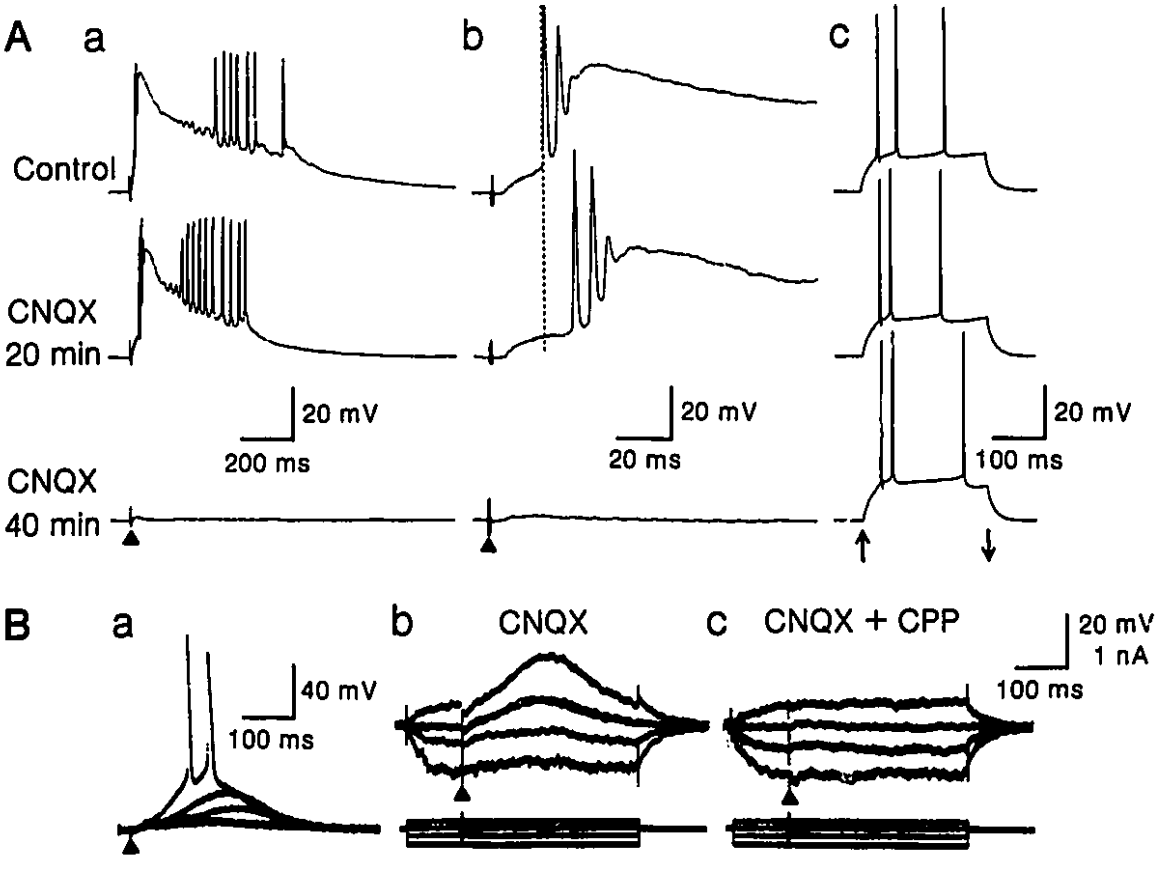
A
a
b
c
Control
CNQX
20 min
CNQX
40 min
20 mV
200 ms
20 mV
20 ms
20 mV
100 ms
B
a
b
CNQX
c
CNQX + CPP
40 mV
100 ms
20 mV
1 nA
100 ms

Figure 2

Figure 3. Effects of the NMDA receptor antagonist CPP on the stimulus-induced PDS. A: Bath application of CPP (3 μM) reduces the duration of the PDS by mainly attenuating its falling phase. This change is fully reversible upon the washout of the drug. B: The latency to onset of the PDS is also increased by CPP in a reversible manner. The traces shown in A and B were obtained from the same cell with a resting Vm of -71 mV. C: Quantitative analysis of the time of onset in 5 cells shows that CPP causes an average increase of 46 % in the PDS's latency to onset. Each vertical bar represents the mean $\pm$ SD of the responses' latencies measured in control, CPP perfusion and its washout. Paired Student *t*-test shows that the increase is significant ($P < 0.005$).

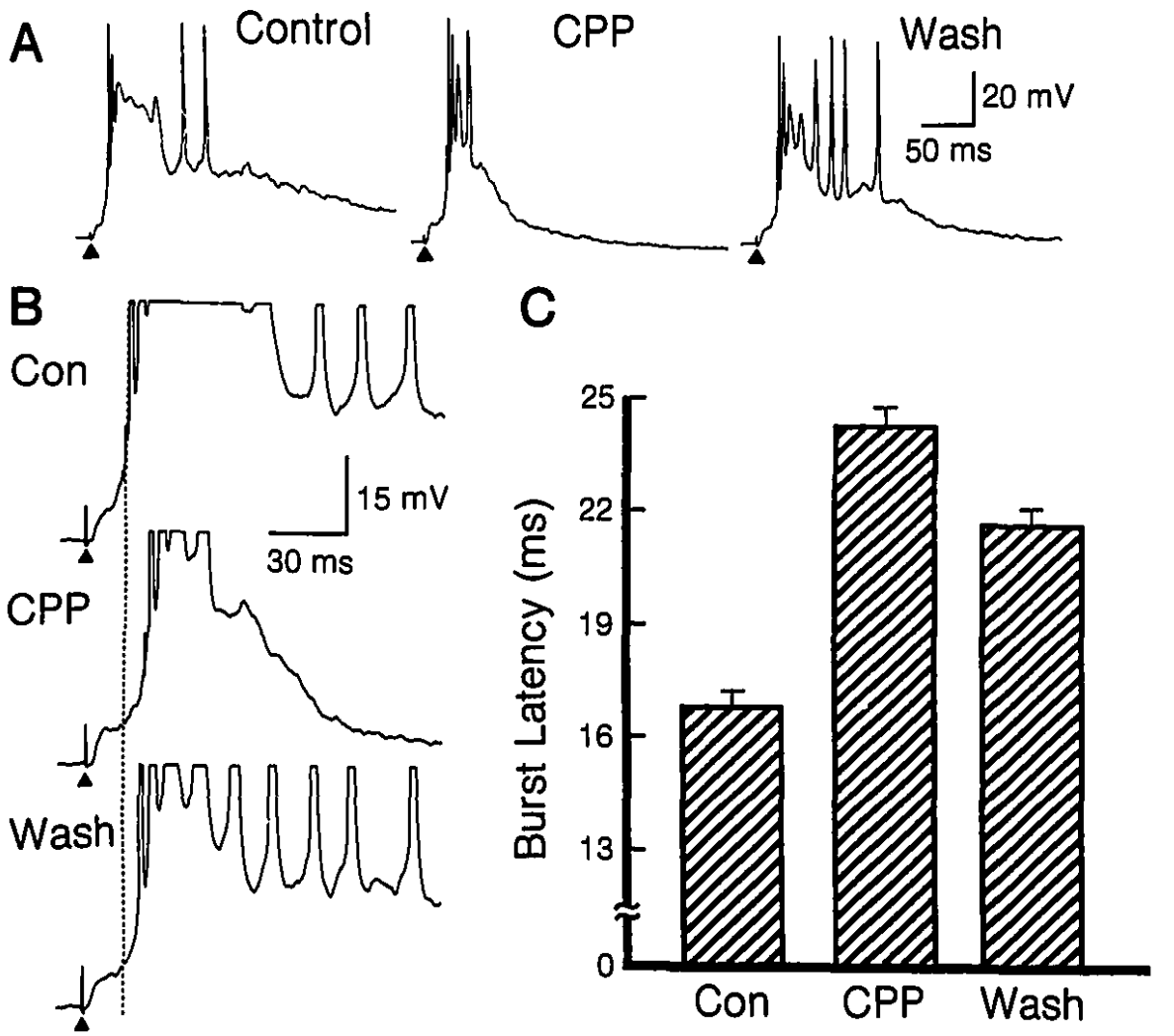
A
Control
CPP
Wash
20 mV
50 ms
B
Con
15 mV
30 ms
CPP
Wash
C
Burst Latency (ms)
25
22
19
16
13
0
Con
CPP
Wash

Figure 3

Figure 4. Changes invoked by the NMDA receptor antagonist MK-801 on the stimulus-induced PDS recorded in a neocortical cell. The a,b,c indicated in A and B correspond with one another respectively. The resting Vm of the cell was -83 mV. A: The solid triangle points at the stimulation artifact. Bath application of MK-801 (3 μM) attenuates the falling phase of the PDS but has no apparent effect on its early phase (Ab). These changes are not reversible upon washout of MK-801 (Ac). B: The sensitivity of the underlying depolarizing envelope towards MK-801 is examined at the response's peak amplitude (early phase, 50ms) and the response's falling phase (late phase, 150ms). Each point represents the mean $\pm$ SD of 6 trials. The horizontal crossed bar designates the period in which the neocortical slice was perfused with the NMDA antagonist. In the presence of MK-801, the early phase of the depolarizing envelope shows no significant change. In contrast, the late phase of the response decreases in amplitude progressively and reaches a steady state 35 min following the introduction of the drug. In this particular experiment, the washout duration was in excess of 2 h.

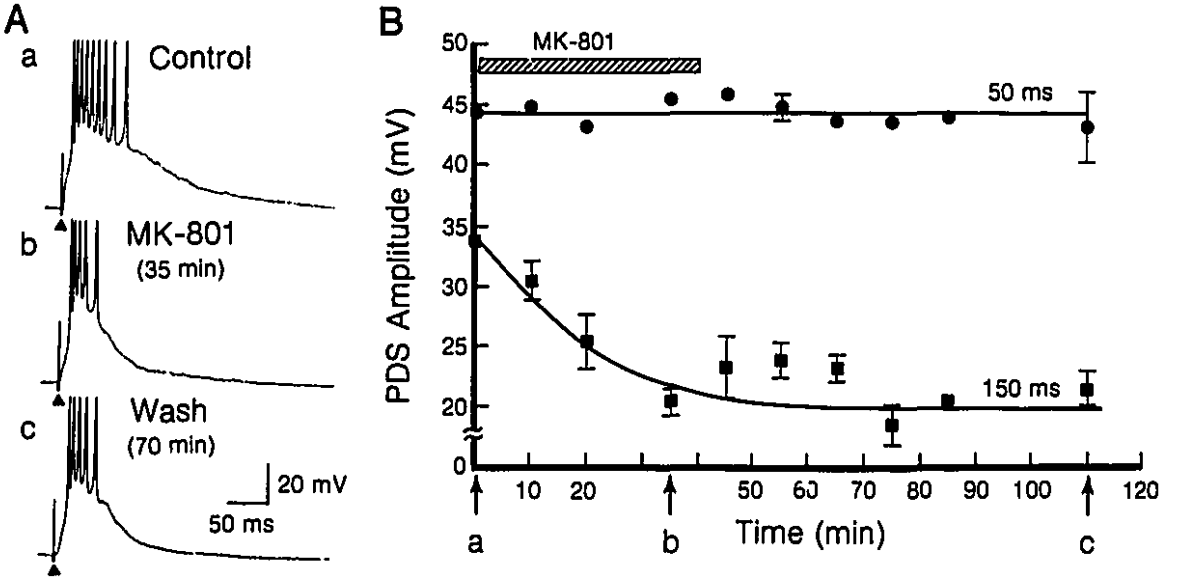
A
a
Control
b
MK-801
(35 min)
c
Wash
(70 min)
20 mV
50 ms
B
MK-801
50 ms
150 ms
PDS Amplitude (mV)
50
45
40
35
30
25
20
0
10
20
50
60
70
80
90
100
120
a
b
Time (min)
c

Figure 4

Figure 5. The voltage behavior and the CPP sensitivity of the BMI-induced PDS evoked in a neuron injected with QX-314. Alteration of the membrane potential (Vm) was achieved by intracellular injection of steady, DC current of positive or negative polarities. Both A and B are from the same neuron with a resting Vm of -70 mV. The circles and triangles in A and B correspond with each other. A: The stimulus-induced response consists of a depolarization shift which lacks the superimposed firings of action potentials. Application of CPP (5 μM) only attenuates the late phase of the response (compare open and solid triangles). This reduction is independent of the neuronal Vm. B: The voltage-response plots show that CPP produces a drop in synaptic potential in the response's falling phase (Bb) but has no apparent effect on the response's early phase (Ba). This modification of synaptic potential was also quantified as changes in synaptic conductance by calculating the slopes of the voltage-response plots. CPP induces a $< 4\%$ and $> 21\%$ decrease in slopes as measured at the early phase (Ba) and the late phase (Bb) respectively. Note that both the CPP-sensitive (Bb, control) and CPP-resistant (Ba, control) components of the depolarizing envelope behave like a conventional excitatory postsynaptic potential by increasing in size during membrane hyperpolarization and decreasing in size during membrane depolarization.

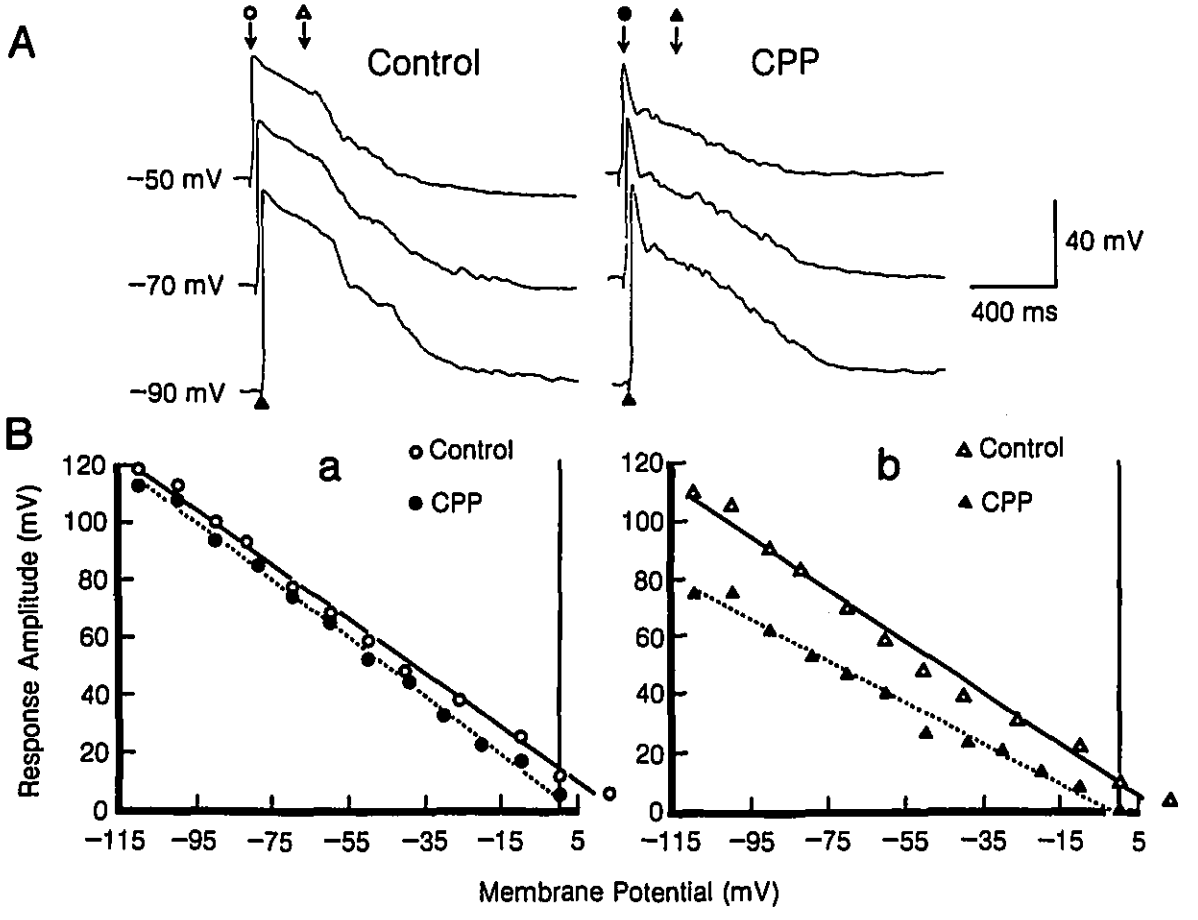
A
Control
CPP
−50 mV
−70 mV
−90 mV
40 mV
400 ms
B
a
b
Control
CPP
Response Amplitude (mV)
Membrane Potential (mV)
120
100
80
60
40
20
0
−115
−95
−75
−55
−35
−15
5

Figure 5

CHAPTER 4

CESIUM POTENTIATES EPILEPTIFORM ACTIVITIES INDUCED BY BICUCULLINE METHIODIDE IN RAT NEOCORTEX MAINTAINED IN VITRO

SUMMARY

In the present study, we report that extracellular application of Cs^+ (3 mM) potentiated the epileptiform discharge evoked by $GABA_A$ receptor antagonist BMI (50 μM) in rat neocortical slices maintained *in vitro*. Cs^+ changed the few hundred ms long BMI-induced epileptiform burst evoked by extracellular focal stimuli into a few sec long (1.8-7.0 sec) epileptiform discharge. Moreover, Cs^+ induced the appearance of spontaneously occurring epileptiform activities (0.038-0.15 Hz). Simultaneous intracellular/extracellular recordings indicated that each intracellular epileptiform burst was correlated with a field discharge. Variation of the membrane potential modified only the amplitude of the epileptiform burst and did not alter its frequency of occurrence. This indicated that each discharge was a synchronous population event. The epileptiform discharges were not blocked by the NMDA receptor antagonist CPP (5-10 μM). In contrast, the non-NMDA receptor antagonist CNQX (0.5-5 μM) greatly reduced the duration of each epileptiform discharge by abolishing its afterdischarges in a concentration-dependent manner. This reduction in duration was, however, accompanied by an increase in frequency of occurrence. Following the blockade of non-NMDA receptors with CNQX, a CPP-sensitive spontaneous discharge could be observed. These findings indicate that the inorganic cation Cs^+, when applied extracellularly, can induce spontaneously occurring epileptiform activities in BMI-treated neocortical slices. In addition, receptors of excitatory amino acids play a major role in synchronizing this type of Cs^+/BMI-induced spontaneous epileptiform activities.

INTRODUCTION

The study of experimental focal epilepsy during the past decade has been based primarily on experiments carried out in the *in vitro* brain slice preparation (for review, see Schwartzkroin, 1986; Taylor, 1988). Many of these studies have employed acute convulsants such as penicillin, bicuculline or picrotoxin that block synaptic inhibition mediated by gamma-aminobutyric acid A ($GABA_A$) receptors. One feature that has been consistently observed in these acute models, when applied to the neocortical slice, is the lack of spontaneous synchronous epileptiform activities (Courtney and Prince, 1977; Gutnick et al., 1982; Connors, 1984; Thomson and West, 1986; Hwa and Avoli, 1989a,1991a; Hwa et al., 1991c). Consequently, the epileptiform discharges described in these studies occurred only in response to focal electrical stimuli.

In the course of experiments performed in neocortical slices perfused with bicuculline methiodide (BMI), we observed that extracellular application of cesium (Cs^+) induced the appearance of spontaneously occurring epileptiform activities. We originally applied Cs^+ to block the hyperpolarizing inward rectification (Halliwell and Adams, 1982; Spain et al., 1987; Avoli and Olivier, 1989) while analyzing the membrane potential dependency of the stimulus-induced epileptiform discharge. Although the proconvulsant effect of Cs^+ was a serendipitous discovery, it appeared important to further characterize this type of epileptiform activity since epileptic discharges recorded in the electroencephalogram of patients occur spontaneously. In the present paper, we describe some electrophysiological features of this novel type

of Cs^+-activated epileptiform potentials. In addition, we have also examined the involvement of the N-methyl-D-aspartate (NMDA) and non-NMDA receptors in this kind of epileptiform discharge. Some of these findings have been communicated in abstract form (Avoli and Hwa, 1989; Hwa and Avoli, 1989b).

MATERIALS AND METHODS

Preparation and maintenance of brain slices

The experiments were performed on neocortical slices prepared from adult male Sprague-Dawley rats weighing 200-300 g. The animals were decapitated under ether anesthesia. Their brains were rapidly removed from the skull and immersed in artificial cerebrospinal fluid (ACSF) at 1-3 °C. Then a block of neocortex corresponding to the sensorimotor region (Zilles and Wree, 1985) was dissected free and cut coronally at a thickness of 450-550 μm with a McIlwain tissue chopper. The slices were transferred to an interface tissue chamber where they were continuously perfused with warmed (33-35 °C), oxygenated (95% O_2, 5% CO_2) ACSF. The composition of the ACSF (pH 7.4) was (in mM): NaCl 124, KCl 2, KH_2PO_4 1.25, $MgSO_4$ 2, $CaCl_2$ 2, $NaHCO_3$ 26, glucose 20. The perfusion rate (0.3 to 1 ml/min) was kept constant during each experiment.

Recording and stimulation

Conventional intracellular and extracellular recordings were carried out with glass microelectrodes. The intracellular electrodes (resistance: 40-80 MΩ) and the extracellular electrodes (2-5 MΩ) were backfilled with 4 M K-acetate and 2 M NaCl

respectively. Both intracellular and extracellular potentials were obtained from layers II/III. Simultaneous intracellular and extracellular recordings were made where the two microelectrodes were placed in the same layer no more than 400 μm apart. The recorded signals were fed to a high-impedance amplifier (Axoclamp-2A and/or WPI 707) which was equipped with an internal bridge circuit for passing intracellular current. The bridge balance was monitored routinely and adjusted when necessary. The intracellular data reported here were obtained from 14 regular-spiking cells (Connors et al., 1982; McCormick et al., 1985) that demonstrated the following characteristics: resting membrane potential of -71 $\pm$ 6 mV (mean$\pm$SD), input resistance of 34 $\pm$ 5 MΩ, and action potential amplitude of 97 $\pm$ 14 mV. All recorded responses were displayed on a storage oscilloscope and/or GOULD pen recorder. In the latter case, a 20 KHz waveform digitizer was often used to avoid distortion of the signals due to the slow response time of the pen. In some experiments, data were also recorded on FM magnetic tape for subsequent analysis.

Constant current stimuli (10-500 μA; 10-90 μs) were delivered through a sharpened bipolar tungsten-stainless steel electrode placed either in the underlying white matter or within the deep layers.

Drugs

All chemicals were applied to the slice by bath perfusion. The $GABA_A$ receptor antagonist BMI was obtained from Sigma. The excitatory amino acid receptor antagonists 3-((±)-2-carboxypiperazin-4-yl-propyl-1-phosphonic acid (CPP) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) were supplied by Tocris Neuramin. The

cation Cs^+, which was introduced into the extracellular medium as CsCl, was obtained from Sigma.

RESULTS

Cs^+ potentiates BMI-induced epileptiform discharges

The effects induced by Cs^+ (3 mM) are illustrated in figure 1. In control ACSF medium which contained BMI (50 μM), focal stimulation of the slice could evoke an all-or-none epileptiform discharge that lasted a few hundred milliseconds (Fig. 1A, control). Previous studies in the rodent neocortex (Gutnick et al., 1982; Connors, 1984; Thomson and West, 1986; Hwa and Avoli, 1989a,1991a) had shown that this BMI-induced epileptiform discharge displayed electrophysiological characteristics very similar to those of the paroxysmal depolarization shift (Matsumoto and Ajmone Marsan, 1964). Following the application of Cs^+, the stimulus-induced discharge was transformed into a long-lasting (> 1 sec) epileptiform event (Fig. 1A, Cs^+). This was also accompanied by the concurrent appearance of spontaneous epileptiform activities (Fig. 1B, Cs^+) that were not evident in the presence of BMI alone (Fig. 1B, control). These changes induced by Cs^+ were reversible when the slice was rinsed with an ACSF medium containing BMI (not shown).

Electrophysiological features of epileptiform discharges

In the presence of both BMI and Cs^+, epileptiform discharge occurred spontaneously and could be evoked by extracellular stimuli (n=14 cells). The

spontaneous discharge and stimulus-induced discharge evoked in the same cell showed similar duration and shape (compare Figs. 1A Cs^+ with 1B Cs^+).

The time course of the epileptiform discharge could last from about 1 sec (Fig. 1, Cs^+) to over 5 sec (Fig. 3Ab, control). It usually consisted of an initial depolarization shift of 200-400 ms, that was followed by a lower amplitude sustained depolarization of variable duration (2-5 sec). This sustained depolarization in turn would trigger a series of overriding afterdischarges that showed variable firing patterns (e.g. Fig. 1 Cs^+; Fig. 3Ab control).

We investigated the membrane potential (Vm)-dependency of the epileptiform discharge by injecting steady direct current through the recording microelectrode (n=7 cells). Figure 2A shows sample recordings of the discharge that occurred spontaneously. Its stimulus-induced counterpart also behaved in a similar voltage-dependent manner (not illustrated). At resting Vm, the discharge was characterized by an initial 300 ms long depolarization shift followed by an afterdischarge of rhythmic depolarizations that triggered single or multiple action potential firings (Fig. 2A, R.L.). When the membrane of the cell was hyperpolarized, the entire initial depolarization shift increased in size while the number of superimposed action potential firings decreased (Figs. 2A, -90mV,-110mV). Furthermore, hyperpolarization of the Vm also increased the amplitudes of the late, rhythmic depolarizations. Consequently, the action potentials associated with each rhythmic depolarization were either reduced or blocked (Fig. 2A, -90,-110mV). The voltage characteristic displayed by the rhythmic depolarizations suggests that each event was

mediated by an excitatory postsynaptic potential. Variation of the Vm within a wide voltage range did not affect the frequency of occurrence of the spontaneous epileptiform discharges (Fig. 2B).

In 8 experiments, we performed simultaneous intracellular and extracellular recordings. As shown in figure 2C, each intracellularly recorded epileptiform discharge (upper trace) was correlated with a negative-going field potential (lower trace) that reflected synchronized population discharge. The shape of the extracellular field discharge was characterized by an initial, fast biphasic transient (Fig. 3Aa control, insert) followed by a negative-going shift that could last several seconds. This negative shift was always superimposed by a series of afterdischarges (Fig. 3Aa, control). The epileptiform discharges recorded extracellularly displayed a frequency of occurrence of 0.075 $\pm$ 0.03 Hz (n=25; range: 0.038-0.15 Hz) and a mean duration of 3.5 $\pm$ 1.8 sec (n=25; range: 1.8-7.0 sec).

Effects induced by excitatory amino acid receptor antagonists

We used the NMDA receptor antagonist CPP (Harris et al., 1986) and the non-NMDA receptor antagonist CNQX (Honoré et al., 1988) to examine the roles played by these excitatory amino acid receptors in the epileptiform discharge that occurred spontaneously. In all of the slices examined (n=6), bath application of CPP (5-10 μM) modified the firing pattern of each spontaneous epileptiform discharge. This was reflected by a decrease in firing frequency of the afterdischarges (Fig. 3Aa, CPP). However, the amplitude of each afterdischarge (Fig. 3Aa, CPP) and the size of the

initial biphasic transient (Fig. 3Aa CPP, insert) were enhanced.

While the increase in amplitude of the afterdischarges and the initial transient might indicate a proconvulsant effect of CPP, the decrease in firing frequency of the afterdischarges suggests some anticonvulsant action. Therefore, we carried out intracellular recordings to analyze further the effect induced by CPP (5 μM) on the epileptiform burst (n=2 cells). Under control condition, the epileptiform burst consisted of an initial depolarization shift that was followed by high frequency afterdischarges of action potentials (Fig. 3Ab, control). Following the perfusion of CPP, the high-frequency afterdischarges were transformed into more isolated bursting events that were individually clustered with more action potential firings (Fig. 3Ab, CPP). CPP also increased the frequency of action potential firings associated with the initial depolarization shift (Fig. 3Ab, CPP). Therefore, these intracellular results suggested that the increase in amplitude of the afterdischarges and of the initial biphasic transient observed extracellularly could be associated with the increase in synchronized action potential firings. One consequence of this increase in synchronized action potential firings would be an enhancement of the repolarizing trend that follows it. This secondary effect might account for the reduction in firing frequency of the afterdischarges.

The effects of CPP on the frequency of occurrence and the overall duration of each epileptiform discharge were variable. In 4/6 slices, CPP increased the occurrence rate by an average of 13% while in the remaining 2 slices, a decrease of 18% was observed. Similarly, the duration of the epileptiform discharge was either

increased (n=2/6) or decreased (n=3/6) by an average of 5%, or remained unaltered (n=1/6). In no case was there any discernible correlation between the changes in frequency and the changes in duration. When the results obtained from all 6 experiments were pooled, the changes in frequency and duration of the spontaneous epileptiform discharges were not significant (Fig. 3B; $P>0.1$, Student *t*-test).

The fact that the blockade of NMDA receptors had limited effects on the epileptiform discharge prompted us to test it with the non-NMDA receptor antagonist CNQX. In 6/6 experiments, we observed that micromolar doses of CNQX (0.5-5 μM) depressed the spontaneously occurring epileptiform discharges in a concentration-dependent manner. As illustrated in figure 4, stepwise increments of CNQX dosage progressively shortened the duration of the epileptiform discharge by attenuating its afterdischarges. This decrease in duration was accompanied by an increase in frequency of occurrence (Fig. 4B). Once the afterdischarges were abolished by CNQX, a synchronous spontaneous discharge became discernible (Fig. 4A, 3 μM). The spontaneous discharge was typically characterized by a fast biphasic transient (about 20 ms in duration) that was followed by a late negative wave (Fig. 4A, 3 μM). Interestingly, the shape and time course of the spontaneous discharge resembled the stimulus-induced epileptiform field discharge evoked in the presence of BMI alone (as compared with Fig. 1 in Connors, 1984). However, unlike this BMI-induced epileptiform discharge which was only partially sensitive to CPP (Hwa and Avoli, 1989a), this CNQX-resistant spontaneous discharge was reversibly blocked by CPP (Fig 4A, CNQX+CPP).

DISCUSSION

The present findings demonstrate that the extracellular application of Cs^+ induces proconvulsant effects on rat neocortical slices in which GABAergic inhibition had been reduced by BMI. When Cs^+ was introduced into the BMI-ACSF perfusate, the few hundred ms long stimulus-induced paroxysmal depolarization shift was transformed into an epileptiform discharge that lasted for a few seconds. In addition, Cs^+ also induced the simultaneous appearance of spontaneously occurring epileptiform activities. Intracellular analysis of the epileptiform discharges indicated that only their amplitudes and not their probability of occurrence were voltage-dependent. This suggests that this type of epileptiform activity reflects the synchronous activities of a neuronal population (Johnston and Brown, 1981,1984). This conclusion is also supported by simultaneous intracellular and extracellular recordings which showed that the duration and frequency of occurrence of each intracellular epileptiform burst was closely correlated with a synchronous field discharge.

Studies of focal epilepsy in the neocortical slice with models such as BMI (Gutnick et al., 1982; Connors, 1984; Thomson and West, 1986; Hwa and Avoli, 1989a,1991a; Hwa et al., 1991c), high $[K^+]_o$ (Avoli, 1986; Thompson et al., 1988) or low $[Cl^-]_o$ (Thompson et al., 1988) did not reveal any spontaneously occurring epileptiform activities. Similarly, neocortical slices obtained from chronic alumina foci (Schwartzkroin et al., 1983) or freeze lesions (Lighthall and Prince, 1983) showed only stimulus-induced discharges. To our knowledge, the occurrence of

spontaneous epileptiform activities in the neocortical slice can only be reproduced consistently in low Mg^{2+} ACSF (Thomson and West, 1986; Aram and Lodge, 1987; Avoli et al., 1987; Fletcher et al., 1988; Sutor and Hablitz, 1989). These studies have demonstrated that this type of epileptiform discharge is readily abolished by NMDA receptor antagonists. Therefore, one might suggest that the activation of NMDA receptor is important for the generation of spontaneous epileptiform activities in the neocortical slice. Obviously, any interpretation of the low $[Mg^{2+}]_o$ model is complicated by the fact that this type of epileptiform discharge is associated with the activation of NMDA receptor-mediated conductances. In contrast, the Cs^+-induced epileptiform discharges described here can provide an alternative for understanding some of the mechanisms underlying spontaneously occurring epileptogenesis in the neocortical slice.

Roles of NMDA and non-NMDA receptors

With the aids of NMDA receptor antagonist CPP (5-10 μM) and non-NMDA receptor antagonist CNQX (0.5-5 μM), we investigated the relative contribution of each excitatory amino acid receptor subtype to the spontaneous epileptiform activities recorded in the presence of Cs^+ and BMI. The concentrations of CPP and CNQX were chosen because of the established efficacy of each drug in blocking either NMDA (Harris et al., 1986) or non-NMDA (Hablitz and Sutor, 1990) receptors selectively. Our results showed that CPP had no significant influence on the overall duration and frequency of occurrence of the spontaneous epileptiform discharges.

CNQX, however, was able to abolish completely the afterdischarges associated with each epileptiform event. In this respect, the participation of non-NMDA receptors appears to be more important than that of NMDA receptors in this type of epileptiform synchronization. This also demonstrates that the activation of NMDA receptors is not a necessary condition for inducing all types of spontaneous epileptiform activities in the neocortical slice.

Although the frequency and duration of the epileptiform discharges were not altered by CPP, the drug was able to modify the firing pattern of each discharge and could independently modulate spontaneous synchronization. This indicates that NMDA receptors do play a role in generating this type of epileptiform activity. Radioligand binding studies have shown that NMDA receptors are distributed preferentially in the superficial layers (Monaghan and Cotman, 1985; Geddes et al., 1989) where neurons are known to send collaterals onto their neighbouring cells. Thus, it is possible that the function of NMDA receptors is to subserve these polysynaptic pathways. The CPP-induced modification of the firing pattern of each epileptiform discharge might simply reflect a disruption of the polysynaptic pathways mediated by NMDA receptors. When the non-NMDA receptors were blocked, the activity mediated through the NMDA-polysynaptic pathways could be isolated and seen collectively as a synchronous spontaneous discharge.

Our results also show that the rate of occurrence of the spontaneous discharges was enhanced by CNQX. It is unlikely that this increase in frequency is due to non-specific actions of CNQX, since this type of inverse relationship between the

epileptiform discharge duration and its frequency of occurrence had been illustrated in other seizure models (Agopyan and Avoli; 1988; Neuman et al., 1988; Tancredi and Avoli, 1987; Chamberlin and Dingledine, 1989; Tancredi et al., 1990). A more conceivable explanation is that the increase in frequency is a secondary effect due to the decrease in duration, i.e. a decrease in the duration of each discharge would reduce its postburst afterhyperpolarization, which determines the interburst interval.

It is interesting to point out that the non-NMDA receptors do not participate in reduced $[Mg^{2+}]_o$ epileptogenesis in the neocortical slice (Fletcher et al., 1988). This is in contrast with our model where the non-NMDA receptors appear to play a dominant role as compared to a limited role played by the NMDA receptor. Thus, it appears that the mechanisms of neuronal synchronization involving excitatory amino acid receptors within the neocortex are very flexible. Although both NMDA and non-NMDA pathways have the capacity of participating in epileptiform synchronization, the involvement of each receptor subtype is ultimately dependent on the model of epilepsy.

Possible mechanisms for increase in hyperexcitability

Several explanations could account for the proconvulsant effects of Cs^+. First, Cs^+ can increase the input resistance of neurons (Halliwell and Adams, 1982; Avoli and Olivier, 1989; Hwa and Avoli, 1989c, 1991b), thereby strengthening the synaptic transfer. Given that the inhibitory mechanism mediated by $GABA_A$ receptors has been reduced by BMI, each strengthened synaptic input might be greatly potentiated within

the disinhibited neocortical network.

Second, preliminary data have shown that action potential duration is prolonged by the extracellular application of Cs^+ (Hwa and Avoli, 1989c), presumably caused by the blockade of some K^+ conductances underlying spike repolarization. This broadening of action potential could enhance the evoked and/or spontaneous release of excitatory transmitters. Similar concentration of Cs^+ has been shown to increase the quantal release of excitatory transmitters in bullfrog neurons (Kumamoto and Kuba, 1985).

Third, it has been shown that Cs^+ (3-5 mM) can reduce the $GABA_B$ receptor-mediated late inhibitory postsynaptic potential (IPSP) in the hippocampus and neocortex (Alger, 1984; Hwa and Avoli, 1989c), and interact with baclofen-sensitive K^+ channels in the neocortex (Ong et al., 1990). These findings indicate that the concentration of Cs^+ employed by us can antagonize $GABA_B$ receptors. Consequently, the potentiation of the BMI-induced epileptiform discharge by Cs^+ might be attributable to the blockade of the late IPSP. In line with this idea are the observations that the $GABA_B$ receptor antagonist phaclofen can potentiate the bicuculline-induced epileptiform discharge evoked in the human neocortex (McCormick, 1989), and in the hippocampal slice culture (Malouf et al., 1990).

Finally, Cs^+ is known to be an extracellular blocker of the hyperpolarizing inward rectification observed in hippocampal and neocortical neurons (Halliwell and Adams, 1982; Spain et al., 1987). This inward rectifier can contribute to the medium afterhyperpolarization that follows repetitive firing evoked in these neurons (Schwindt

et al., 1988; Williamson and Alger, 1990). Therefore, it might be suggested that the Cs^+-induced changes we described may result in part from a reduction in the afterhyperpolarization that follows each BMI-induced PDS.

Electrophysiological studies have revealed a subpopulation of endogenously bursting cells in the middle cortical layers (Connors et al., 1982; McCormick et al., 1985). Although these cells might serve as pacemakers in the initiation of epileptiform activities when synaptic inhibition is reduced (Connors, 1984; Chagnac-Amitai and Connors, 1989), widespread spontaneous synchronization appears to require additional mechanisms since spontaneous epileptiform activities were not observed in the presence of BMI. Here, we propose that the application of Cs^+ can produce a general increase in excitability within the neocortical network which then can amplify the activities initiated by the pacemaker cells through the NMDA and non-NMDA receptor-mediated pathways, thereby leading to the formation of spontaneous epileptiform activities.

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Figure 1. The effects of Cs^+ on the epileptiform burst induced by bicuculline methiodide (BMI). In control, the neocortical slice has been perfused with BMI (50 μM) for over 1 hr. A. Cs^+ (3 mM) prolongs the few hundred milliseconds long stimulus-induced epileptiform burst into a long-duration epileptiform event that lasts over 1 sec. Note that the long-duration epileptiform burst consists of a tonic-phasic firing of afterdischarges. B. Cs^+ also induces the appearance of spontaneously occurring epileptiform activities that are not observed in the presence of BMI alone. The resting Vm of the cell was -73 mV.

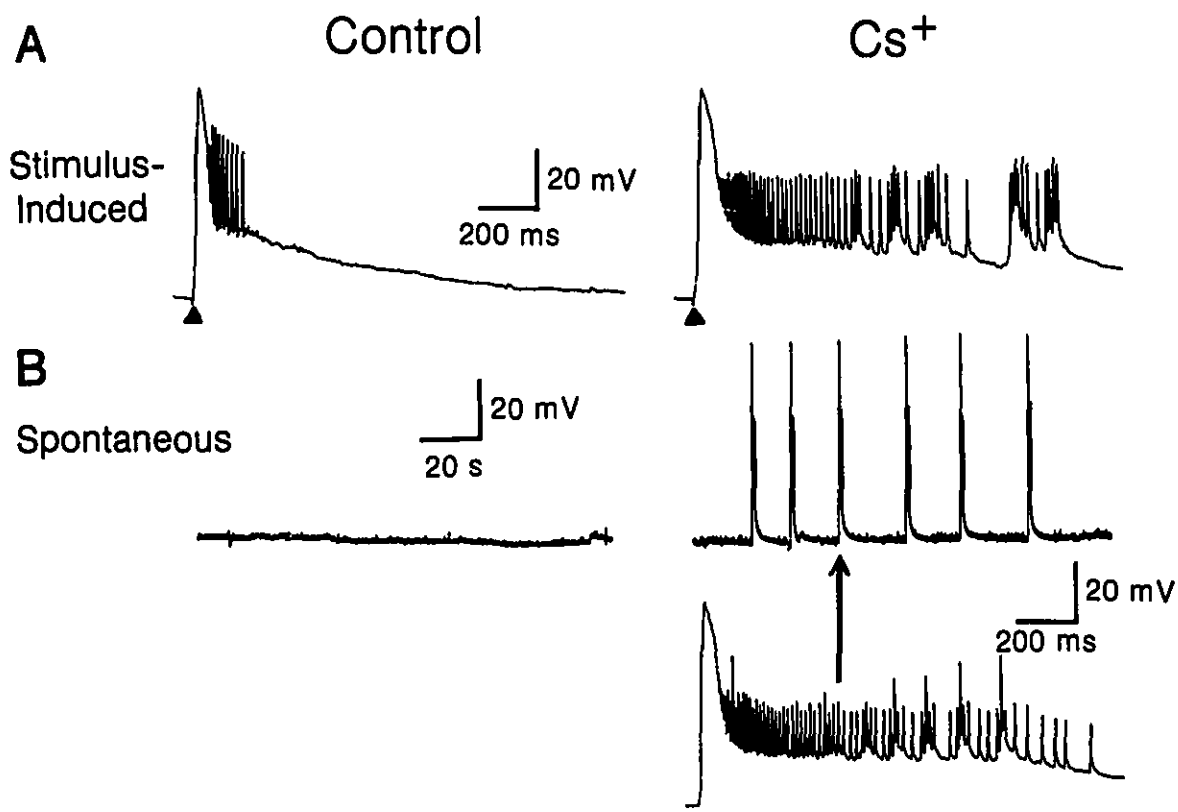
A
Control
Cs+
Stimulus-
Induced
20 mV
200 ms
B
Spontaneous
20 mV
20 s
20 mV
200 ms

Figure 1

Figure 2. Electrophysiological features of the Cs^+-induced epileptiform discharge evoked in BMI-treated neocortical slices. A. The epileptiform burst behaved in a voltage-dependent manner. The initial depolarization shift and its afterdischarge of rhythmic depolarizations increase in size during membrane hyperpolarization. Consequently, the action potential firings associated with the initial depolarization shift and the rhythmic depolarizations are reduced or blocked. B. The frequency of occurrence of the epileptiform burst is not dependent on the membrane potential of the cell. Each point represents the mean $\pm$ SD of at least 5 interburst intervals. A and B were obtained from two neurons recorded from 2 different slices. The resting level (R.L.) of the cell was -70 mV in A and -53 mV in B. C. Simultaneous intracellular/extracellular recordings from another neocortical slice. Note that each intracellular epileptiform burst (upper trace) is correlated with an epileptiform field discharge (lower trace) of similar duration. The resting Vm of this cell was -70 mV.

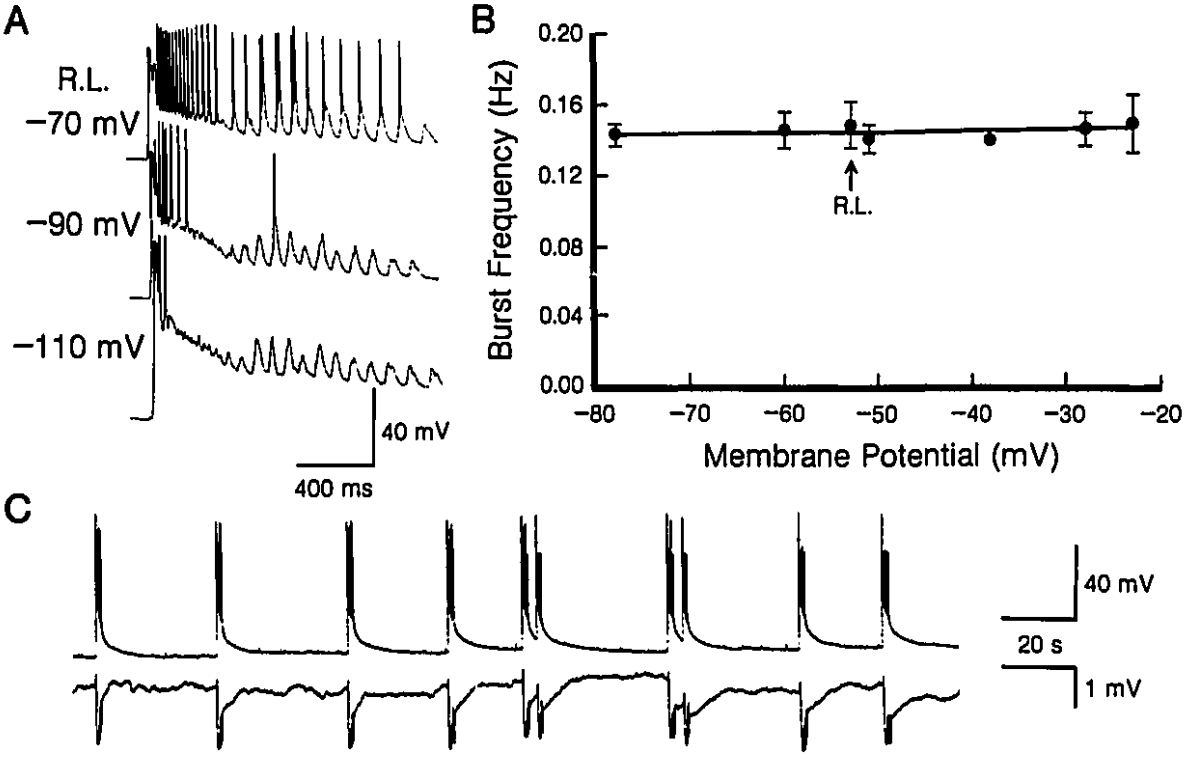
A
R.L.
−70 mV
−90 mV
−110 mV
40 mV
400 ms
B
Burst Frequency (Hz)
0.20
0.16
0.12
0.08
0.04
0.00
R.L.
−80
−70
−60
−50
−40
−30
−20
Membrane Potential (mV)
C
40 mV
20 s
1 mV

Figure 2

Figure 3. Effects of NMDA receptor antagonist CPP on Cs^+-induced epileptiform discharges that occur spontaneously in BMI-treated neocortical slices. A. The field epileptiform discharge consists of an initial biphasic transient (insert) followed a negative-going shift that is superimposed with an afterdischarge of fast spikes. CPP (10 μM) reduces the firing frequency of the superimposed fast spikes, but increases the amplitude of the initial transient and of each remaining fast spike. B. CPP (5 μM) modifies the intracellular epileptiform burst in a similar fashion. Note that the firing frequency of the afterdischarges that follow the initial depolarization shift is reduced. However, the action potential firings associated with the initial depolarization shift and each remaining afterdischarges are also enhanced. The resting Vm of the cell was -77 mV. A and B were obtained from 2 different slices. C. CPP (5-10 μM) has no significant effect on the duration and frequency of occurrence of the epileptiform discharges recorded extracellularly from 6 slices ($P > 0.1$, Student *t*-test). Each bar represents the mean $\pm$ SD.

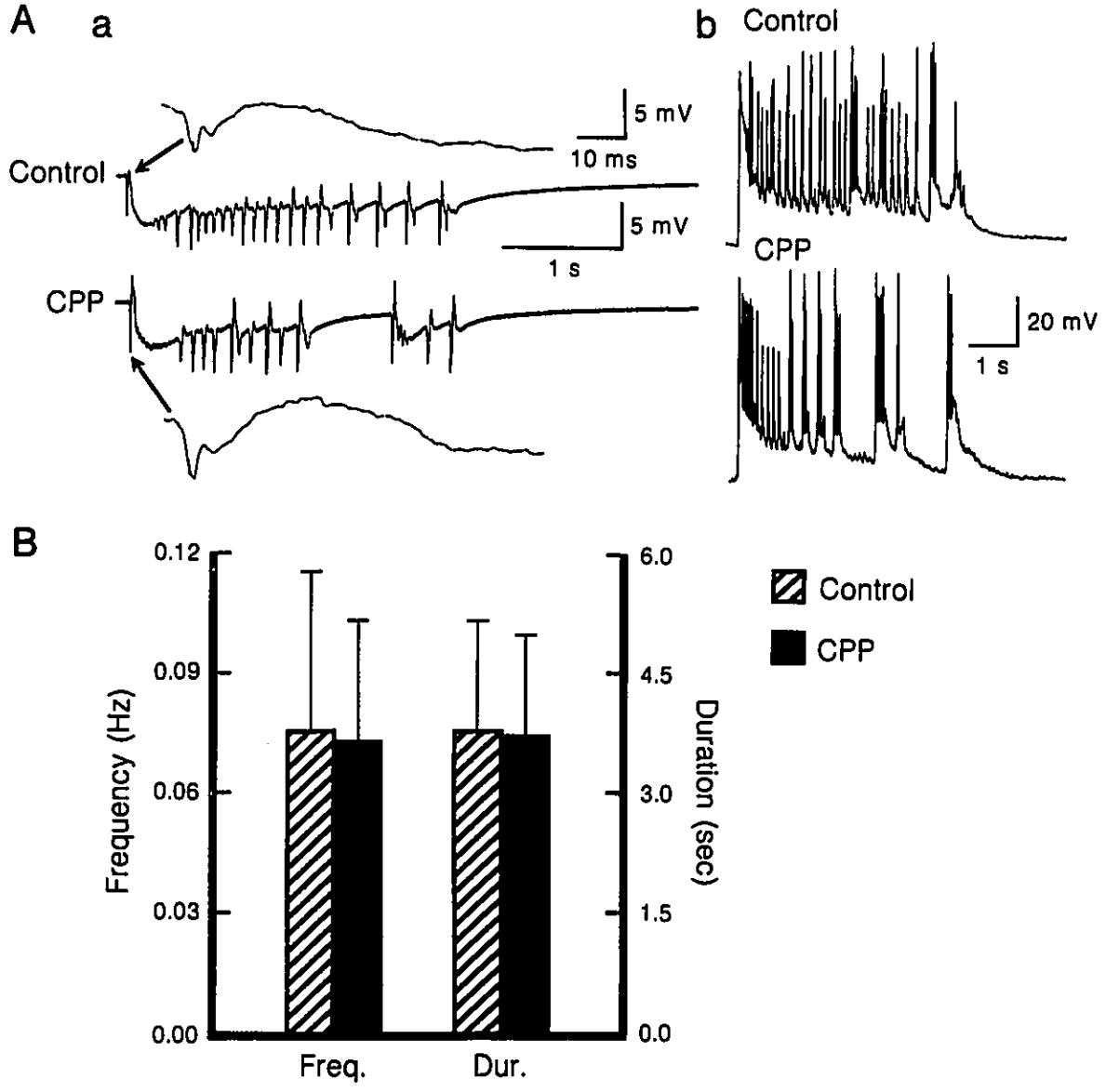
A
a
Control
5 mV
10 ms
5 mV
1 s
CPP
b
Control
CPP
20 mV
1 s
B
0.12
0.09
0.06
0.03
0.00
Frequency (Hz)
6.0
4.5
3.0
1.5
0.0
Duration (sec)
Freq.
Dur.
Control
CPP

Figure 3

Figure 4. Effects of the non-NMDA receptor antagonist CNQX on the Cs^+-induced epileptiform discharges that occur spontaneously in BMI-treated neocortical slices. A. Stepped increments of CNQX doses (0.5-3.0 μM) progressively reduce the duration of each epileptiform field potential by attenuating its associated afterdischarges. In the presence of 3 μM of CNQX, there remains a brief spontaneous discharge that is reversibly abolished by CPP (5 μM). B. The concentration-dependency of the epileptiform discharges on CNQX is displayed in a graphical manner. Note the inverse relationship between the decrease in duration of the epileptiform discharge and its increase in frequency of occurrence. Each point represents the mean $\pm$ SD of 6 responses. A and B were obtained from the same slice.

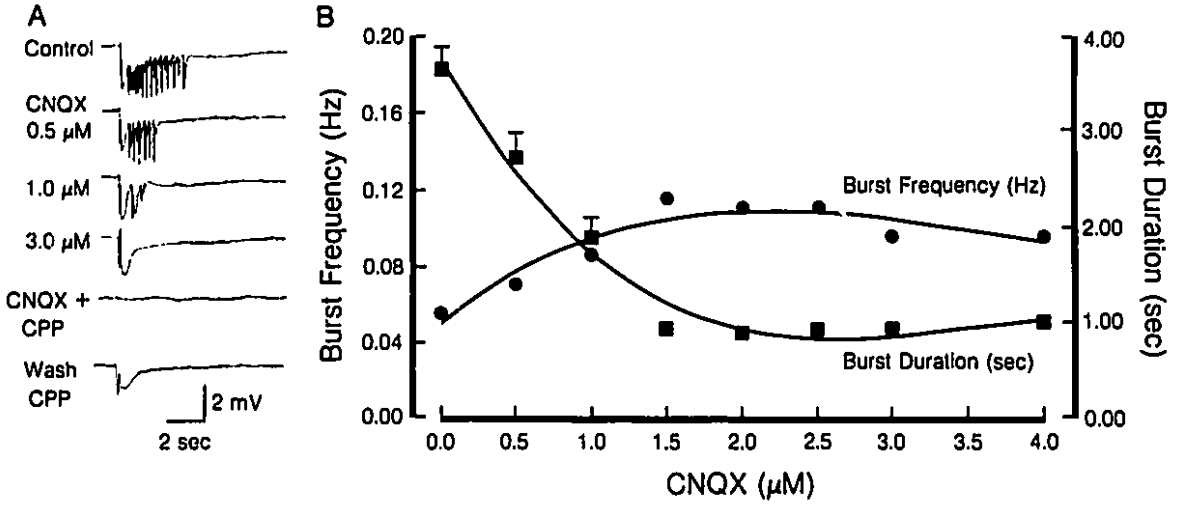
A
Control
CNQX
0.5 μM
1.0 μM
3.0 μM
CNQX +
CPP
Wash
CPP
2 mV
2 sec
B
Burst Frequency (Hz)
Burst Duration (sec)
0.20
0.16
0.12
0.08
0.04
0.00
4.00
3.00
2.00
1.00
0.00
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
CNQX (μM)

Figure 4

CHAPTER 5

EXCITATORY SYNAPTIC TRANSMISSION MEDIATED BY NMDA AND NON-NMDA RECEPTORS IN THE SUPERFICIAL/MIDDLE LAYERS OF THE EPILEPTOGENIC HUMAN NEOCORTEX MAINTAINED IN VITRO

SUMMARY AND CONCLUSIONS

1. Conventional intracellular recording techniques were used to examine the involvement of excitatory amino-acid receptors in synaptic transmission in epileptogenic human neocortical slices maintained in vitro. Extracellular stimuli were delivered to the deep layers. Recordings were made from regular-spiking cells located in layers II-IV.

2. Stimuli, that were below the threshold for generating action potentials, evoked a short-latency excitatory postsynaptic potential (EPSP) followed by an early and a late inhibitory postsynaptic potential. When suprathreshold stimuli were delivered, 95% of the neurons fired a single action potential. In 5% of the population, however, suprathreshold stimuli could elicit an all-or-nothing bursting discharge.

3. The EPSP and the bursting discharge were tested with the N-methyl-D-aspartate (NMDA) receptor antagonist 3-((±)-2-carboxypiperazin-4-yl)propyl-1-phosphonate (CPP, 5 μM) or the non-NMDA receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 4 μM). In the presence of CNQX, the peak amplitude of the EPSP was reduced by 85% and the bursting discharge was abolished completely. By contrast, CPP reduced the peak amplitude of the EPSP by 52%, attenuated the late phase of the bursting discharge and increased its threshold.

4. These results indicate that excitatory amino acids function as excitatory transmitters in the human brain. While the involvement of non-NMDA receptors in the EPSP is in line with data from normal neocortical slices of other mammals, the participation of NMDA-mediated conductances to the EPSP appears peculiar to the

epileptogenic human neocortex. This evidence, together with the contribution of NMDA and non-NMDA receptors to the all-or-nothing bursting discharge suggests that excitatory amino acid-mediated transmission might be modified in the epileptogenic human neocortex.

INTRODUCTION

Animal studies have provided valuable information on the basic mechanisms underlying neuronal hyperexcitability (for review, see Jefferys, 1990). With this information as background, some neurophysiologists have searched for evidence of cellular anomalies in human neocortical slices resected from patients for the treatment of intractable epilepsy. To date, intracellular recordings made from this type of "epileptic" biopsy did not reveal any abnormalities in the intrinsic electrical properties of neurons (Avoli and Olivier, 1989; Foehring et al., 1991; Lorenzon and Foehring, 1992). However, some studies on synaptic responses had reported the appearance of bursting discharges that are graded (Schwartzkroin and Prince, 1976; Schwartzkroin et al., 1983; Schwartzkroin and Haglund, 1986; Avoli and Olivier, 1987,1989) or all-or-nothing (Prince and Wong, 1981; Schwartzkroin and Haglund, 1986) in origin. Furthermore, these bursting discharges were not seen in neocortical slices excised from "non-epileptic" patients (Prince and Wong, 1981; M. Avoli and G. Hwa, unpublished results). This suggests that the cellular anomalies in the human epileptic brain tissue might be related to the network properties of neurons (cf. Johnston and Brown, 1986).

In light of these findings, we have begun to investigate the role of excitatory amino acid-mediated synaptic transmission in the human epileptic neocortex maintained in vitro. We found that the N-methyl-D-aspartate (NMDA) receptor antagonist DL-2-amino-5-phosphonovalerate (APV) can selectively reduce or block the graded burst evoked in the deep layers (Avoli and Olivier, 1987, 1989). By

contrast, the excitatory postsynaptic potential (EPSP) evoked in these layers is not sensitive to APV (Avoli and Olivier, 1987,1989). As a continuation of this work, the present study was aimed at analyzing neurons located in the superficial/middle neocortical layers. The experiments were designed to address the following questions. 1) Can synaptic stimulation evoke the appearance of bursting discharge (graded or all-or-nothing) in the superficial/middle layers? 2) If so, which are the respective roles of NMDA and non-NMDA receptors? 3) How do the NMDA and non-NMDA receptors contribute to the EPSP recorded from superficial/middle layer cells?

METHODS

Human neocortical tissue examined in the present study was removed for the treatment of intractable epilepsy. The patients, who were mostly adult (mean age: 30 $\pm$ 12 yr; n=22), had been maintained on a variety of antiepileptic drugs that were progressively discontinued or markedly reduced during the week preceding surgery. During each surgical procedure, an electrocorticogram (ECoG) was performed to define the cortical region(s) that was capable of generating interictal spikes. In 15 cases, the brain specimens were excised from regions that showed active ECoG spiking. In the remaining 7 cases, the specimens were obtained from regions that did not display any clear ECoG spiking. However, this cannot be considered as valid evidence against the epileptogenicity of these specimens (cf. Avoli and Olivier, 1989). Among the 22 cases, 18 were from the temporal lobe, 3 were from the frontal lobe and 1 was from the occipital lobe. Neuropathological examination of the

removed brain tissue revealed a moderate degree of gliosis and neuronal loss.

The procedures for slicing and incubating human neocortical slices were similar to those described previously (Avoli and Olivier, 1989). Briefly, the biopsy specimen was cut with a McIlwain tissue chopper into slices that were 500-700 μm thick. The plane for cutting was usually normal to the longitudinal axis of the gyrus. Slices were then transferred to an interface tissue chamber where they were perfused at 34 $\pm$ 1 °C with oxygenated (95% O_2-5% CO_2) artificial cerebrospinal fluid (ACSF) and exposed to a humidified atmosphere saturated with 95% O_2-5% CO_2. The composition of the ACSF (pH 7.2) was (in mM): NaCl 124, KCl 2, KH_2PO_4 1.25, $MgSO_4$ 2, $CaCl_2$ 2, $NaHCO_3$ 26, glucose 10. In some of the experiments, the excitatory amino-acid antagonists 3-((±)-2-carboxypiperazin-4-yl)-propyl-1-phosphonate (CPP, 5μM) or 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 4μM) were added to the ACSF perfusate. Both drugs were obtained from Tocris Neuramin.

Conventional intracellular recordings were made from neurons located in the superficial/middle neocortical layers (II-IV). The recording electrodes (resistance: 40-80 MΩ) were backfilled with 4 M K-acetate. The intracellular signal was fed to a high-input resistance amplifier (Axoclamp-2A) equipped with an internal bridge circuit for passing current through the recording electrode. The bridge balance was monitored routinely throughout the experiments and adjusted when necessary. The recorded signals were displayed on a storage oscilloscope and/or GOULD pen writer equipped with a 20 KHz waveform digitizer. In some cases, the signals were also recorded on FM tape for subsequent anal[illegible]. A bipolar stainless steel electrode was

used to deliver pulses of extracellular stimuli (90 μs, 10-5000 μA) to the deep layers.

RESULTS

Intrinsic membrane properties

The data described in the present study were obtained from 64 neurons with an average resting membrane potential (Vm) of -74 $\pm$ 10 mV (mean$\pm$SD, n=50), input resistance of 30 $\pm$ 15 MΩ (n=48) and action potential amplitude of 92 $\pm$ 12 mV (n=44). On the basis of their repetitive firing properties (Fig. 1A), these neurons could be classified as the regular-spiking type (Connors and Gutnick, 1990).

When the subthreshold membrane properties of the neurons were examined by the intracellular injection of positive and negative current pulses (Fig. 1B), most of the cells displayed non-linear current-voltage relationship especially at hyperpolarized membrane levels (Fig. 1C). This kind of inward rectification is similar to that described previously in human neocortical cells (Avoli and Olivier, 1989; Wuarin et al., 1990; Foehring et al., 1991).

Characteristics of synaptic responses

Under the perfusion of normal ACSF, postsynaptic potentials (PSPs) were evoked by focal stimulation of the deep layers. The stimulus intensity required to elicit a PSP varied between slices. In spite of this, the PSP characteristics observed in most of the neurons were very similar (see below).

Among the 64 cells, 95% (n=61) showed the following input-output relationship.

At low intensity stimulation (50 μA in Fig. 2Aa), a small depolarizing PSP was elicited. These PSPs had short latency to onset (0.8-4 ms) and could follow repetitive stimulation. Occasionally, late EPSPs were also encountered (see arrow in Fig. 2Ab, 50 μA). However, their appearance was unstable. Raising the stimulus strength to higher intensities (70-300 μA in Fig. 2A) progressively increased the amplitude and duration of the PSP until it reached the membrane threshold for triggering an action potential. When the stimulus intensity was further augmented to suprathreshold level (5000 μA in Fig. 2A), these neurons still fired a single action potential.

Within this group of 61 neurons, spontaneously occurring PSPs similar to those described previously (Schwartzkroin and Knowles, 1984; Schwartzkroin and Haglund, 1986; Avoli and Olivier, 1989; McCormick, 1989) were encountered in 6 cases. At resting Vm, the spontaneous PSPs were mainly depolarizing in shape and their size was variable (Figs. 2Ba,b). At times, they could also elicit firing of action potentials (Fig. 2Bb3). Because of their high frequency of occurrence (about 2Hz), the spontaneous PSPs could contribute to the stimulus-induced response (Fig. 2Bc). Therefore, these neurons were not included in the pharmacological experiments described below.

In the remaining 5% of the population (n=3/64 cells), synaptic stimuli delivered at suprathreshold intensities did not trigger the firing of a single action potential. Instead, an all-or-nothing bursting discharge was elicited (150 μA in Fig. 3A). Hyperpolarization of the Vm to a level that blocked most of the action potentials indicates that the discharge was underlain by a large amplitude depolarization (Fig.

3B). Upon the intracellular injection of a depolarizing current pulse, these neurons fired in a regular fashion and did not show any intrinsic bursting capability (Fig. 6C).

Voltage behavior of the PSP

Because of the unusual voltage-dependent gating effect of extracellular Mg^{2+} on the NMDA ionophore (Mayer et al., 1984; Nowak et al., 1984), Thomson et al. (1985) proposed that the voltage behavior of an EPSP might be a useful criteria for determining the involvement of NMDA receptors in excitatory synaptic transmission. Therefore, the Vm sensitivity of the subthreshold PSPs was investigated.

Variation of the Vm (between -60 and -110 mV) showed that the PSP evoked by high-intensity stimulation consists typically of both excitatory and inhibitory components. As illustrated in figure 4Aa, the temporal order was a short-latency EPSP (open circle) followed by an early (solid circle) and a late (open triangle) inhibitory postsynaptic potential (IPSP). The latency to peak (see Fig. 4Aa) and the reversal potential (see Fig. 4Ab) of the early and the late IPSPs suggest that they were mediated by Cl^- and K^+ conductance respectively (McCormick, 1989).

The EPSP component of these PSPs displayed conventional voltage behavior by increasing in amplitude following Vm hyperpolarization and decreasing in amplitude following Vm depolarization (Fig. 4B, 250 μA). However, when the stimulus strength was reduced to lower intensities, the smaller EPSP showed an opposite type of Vm dependency. The response increased in size with membrane depolarization and decreased in size with membrane hyperpolarization (Fig. 4B, 150 μA). Although this

type of Vm sensitivity resembles the one displayed by NMDA receptor-mediated conductance, it can also be caused by inward rectification of the neuronal membrane (Sutor and Hablitz, 1989; Hwa and Avoli, 1992).

In order to rule out this latter possibility, the voltage behavior of an intrinsic response evoked by a brief pulse of positive current was compared to that of the smaller EPSP in the same cell (see Hwa and Avoli, 1992). Interestingly, the intrinsic response also behaved anomalously by increasing in size with Vm depolarization and decreasing in size with Vm hyperpolarization (Fig. 4B, intrinsic). This implies that the activation and inactivation of voltage-dependent intrinsic conductances can contribute to the shape of the EPSP in these human neocortical cells. Therefore, their EPSP's voltage behavior might not be an accurate reflection of the Vm sensitivity of its underlying synaptic conductances. To avoid bias, pharmacological agents were used to identify the involvement of NMDA receptors in excitatory synaptic transmission.

Effects of excitatory amino-acid antagonists

The EPSP was assessed with the NMDA receptor antagonist CPP (Harris et al., 1986) and the non-NMDA receptor antagonist CNQX (Honoré et al., 1988). Depending on the stimulus strength used to elicit an EPSP, the antagonists could either reduce or abolish the response. For the representative neuron shown in figure 5, perfusion of CPP could almost abolish the EPSP evoked by low intensity stimulation (10 μA, CPP) and reduced by 55% the peak amplitude of the EPSP

evoked by high intensity stimulation (90 μA, CPP). By contrast, CNQX completely blocked the smaller EPSP (Fig. 5A, CNQX) and reduced the peak amplitude of the larger EPSP by 82% (Fig. 5B, CNQX). In 9 additional experiment where EPSPs evoked by high intensity stimulation were tested, CNQX was found to reduce their peak amplitudes by an average of 85% (n=4) as compared with a mean decrease of 52% produced by CPP (n=5).

The involvement of NMDA and non-NMDA receptors in the stimulus-induced bursting discharge was also investigated. As illustrated in figure 6, application of CPP abolished the bursting discharge evoked by threshold intensity (150 μA, CPP) and attenuated its late phase when evoked by suprathreshold intensity (300 μA, CPP). Meanwhile, the repetitive firing property of the neuron remained unchanged (Fig. 6C). After washout of the drug (Fig. 6A and B, wash), CNQX was introduced into the ACSF. Unlike CPP, CNQX could also abolish the discharge induced by suprathreshold intensity (300 μA, CNQX). Moreover, the CNQX-insensitive EPSP observed at threshold intensity (150 μA, CNQX) was much smaller than the CPP-insensitive EPSP elicited by the same stimulus strength (150 μA, CPP).

DISCUSSION

The present study examined excitatory synaptic transmission in the superficial/middle layers of epileptogenic human neocortical slices maintained in vitro. In response to focal extracellular stimulation, most of the sampled neurons displayed EPSP-IPSP sequence and firing of a single action potential. However in 5%

of the population, synaptic stimuli could elicit an all-or-nothing bursting discharge (also see Prince and Wong, 1981; Schwartzkroin and Haglund, 1986). Pharmacological experiments indicate that both the EPSP and the bursting discharge were mediated by excitatory amino-acid receptors.

According to our previous investigation of human neocortical slices resected from epileptic patients, the stimulus-induced EPSP evoked in layers V-VI is not sensitive to the NMDA receptor antagonist APV (Avoli and Olivier, 1987, 1989). However, the stimulus-induced field potential recorded from these layers is blocked by kynurenic acid which is a broad spectrum antagonist of the NMDA and non-NMDA receptors (Avoli, 1991). Using a similar approach, but on a much younger group of patients, Wuarin et al. (1990) found that kynurenic acid can reduce the stimulus-induced EPSP evoked in layers III-V. Therefore, it seems likely that the non-NMDA receptors are involved in excitatory synaptic transmission in the epileptogenic human neocortex. This hypothesis is now confirmed by our CNQX experiments where the drug could abolish the EPSP elicited by low-intensity stimuli and potently reduced by 85% the EPSP elicited by high-intensity stimuli. The percent reduction of the EPSP by CNQX is greater than the 60-80% decrease reported by Wuarin et al. (1990) using kynurenic acid. This difference probably reflects a higher affinity of CNQX for non-NMDA receptors.

Considering that the activation of non-NMDA receptors had been shown to be crucial for excitatory synaptic transmission in the frontal (Hablitz and Sutor, 1990), sensorimotor (Hwa and Avoli, 1992), cingulate (Higashi et al., 1991; Sah and Nicoll,

1991) and visual (Jones and Baughman, 1988; Artola and Singer, 1990) cortical slices of normal rats, it is perhaps reasonable to find that the EPSP described here was sensitive to the blockade of CNQX. However, the possibility that the function of non-NMDA receptors is altered in the epileptogenic human neocortex cannot be ruled out. Indeed, our results regarding the potent effect of CNQX on the all-or-nothing bursting discharge suggest that the excitatory mechanism mediated by non-NMDA receptors might be enhanced in the human epileptogenic cortex.

The CPP experiments show that the NMDA antagonist reduced the peak amplitude of the stimulus-induced EPSP by 52%, attenuated the late phase of the all-or-nothing bursting discharge and increased its activation threshold. These are interesting observations for two reasons. First, they reveal that the appearance of the NMDA-mediated graded burst in the deep layers, where the EPSPs are APV insensitive (Avoli and Olivier, 1987,1989), probably involves the activation of NMDA receptors in the superficial/middle layers. In agreement, autoradiographic analysis of human neocortical tissues indicate that the laminar distribution of NMDA receptors is highest in these layers (Jansen et al., 1989). Second, they contrast dramatically with data obtained from neocortical slices of normal rats where the short-latency EPSP evoked in normal ACSF is not sensitive or only slightly sensitive to NMDA antagonists (Artola and Singer, 1987,1990; Jones and Baughman, 1988; Hablitz and Sutor, 1990; Deisz et al., 1991; Hwa and Avoli, 1992). Although this discrepancy might be related to a species difference between the rat and the human, it could also reflect an increase in NMDA-mediated excitation in the epileptogenic

human neocortex.

The idea that the actions of excitatory amino-acid receptors are up-regulated in the epileptogenic human neocortex is supported by several lines of evidence. According to biochemical studies, the levels of glutamate, aspartate and glycine in epileptogenic human neocortical tissues are significantly higher than those found in non-epileptic specimens (Van Gelder et al., 1972; Perry and Hansen, 1981; Kish et al., 1988; Sherwin et al., 1988,1991). Presumably, this increase in glutamate and aspartate concentrations can exert a positive influence on the NMDA and non-NMDA receptors, whereas the increase in glycine level can offset the desensitizing effect, associated with the rise in glutamate and aspartate concentrations, on the NMDA receptor (Mayer et al., 1989).

According to autoradiographic studies, NMDA and non-NMDA binding is markedly increased in the epileptogenic human hippocampus (Represa et al., 1989; Hosford et al., 1991; McDonald et al., 1991). Although this aspect has not been examined adequately in the epileptogenic human neocortex, it is conceivable that similar changes could take place. If this is the case, then these increases could complement the increase in agonist concentrations and further augment the excitatory interaction between neurons.

A slight impairment in synaptic inhibition can also augment the actions of excitatory amino-acid receptors. In rat neocortical slices, a 10-20% blockade of gamma-aminobutyric acid $(GABA)_A$-mediated inhibition is sufficient to activate the NMDA receptor (Hwa and Avoli, 1992) and produce epileptiform synchronization

at a time when intracellular recordings could still reveal the presence of robust IPSPs (Chagnac-Amitai and Connors, 1989). Therefore, although IPSPs are discernible in the epileptogenic human neocortex (Schwartzkroin and Haglund, 1986; Avoli and Oliver, 1989; McCormick, 1989; Hwa et al., 1991; also present study), its inhibitory mechanism could still be impaired. In agreement with this idea, the effects of CPP and CNQX on the all-or-nothing bursting discharge reported here appear remarkably similar to those described in rat neocortical slices treated with $GABA_A$ antagonists (Hwa and Avoli, 1989,1991). Furthermore, Schwartzkroin et al. (1983) found that synaptic inhibition is less effective in human neocortical slices excised from regions that showed maximal ECoG spiking. Abnormal GABAergic inhibition had also been described in the epileptogenic human hippocampus by Masukawa and co-workers. According to their studies, NMDA-mediated activities are increased in the dentate region (Masukawa et al., 1991) where $GABA_A$ inhibition is only 80-90% functional (Masukawa et all., 1989).

By performing pharmacological analysis on the EPSP with NMDA and non-NMDA antagonists, our results provide direct evidence that excitatory amino acids are the putative excitatory transmitters in the human cerebral cortex. The finding that the non-NMDA receptors are the primary mediators of excitatory synaptic transmission is in line with their role in normal animal neocortices. However, the involvement NMDA and non-NMDA receptors in the all-or-nothing bursting discharge suggests that their function might be up-regulated in the epileptogenic human neocortex. In this respect, it would be of great interest to investigate the role

of NMDA and non-NMDA receptors in neocortical slices of "non-epileptic" patients.

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FIGURE LEGENDS

Figure 1. Electrical properties of a human neocortical cell in response to the intracellular injection of positive and negative current pulses (170 ms in duration). A. Depolarizing pulses elicits a single action potential at threshold level (+0.9 nA) and repetitive firing at suprathreshold levels (+1.3 and +1.7 nA). B. Following the injection of subthreshold current pulses, a hump is observed in the depolarizing direction (+0.7 nA) and a sag in the hyperpolarizing direction (-2.3 nA). C. A plot of the current-voltage relationship measured at the steady state (solid circle) reveals inward membrane rectification at the hyperpolarized membrane levels. The resting Vm of the cell was -72 mV.

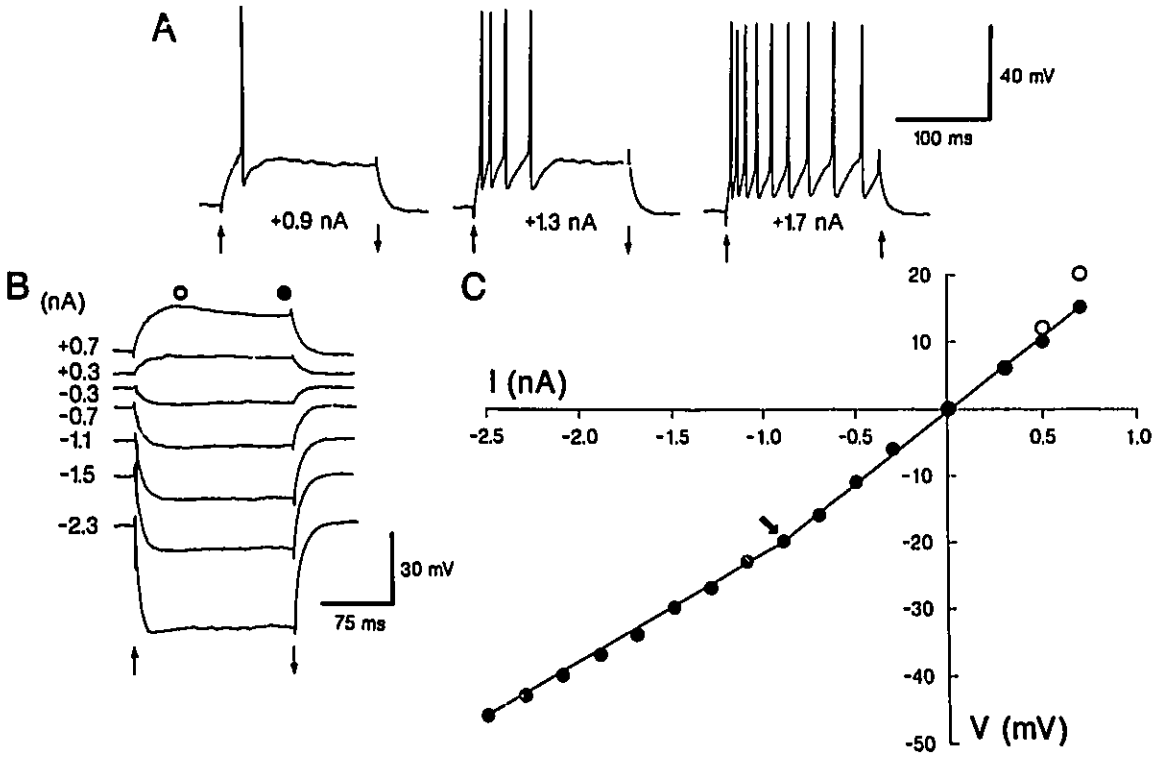
A
+0.9 nA
+1.3 nA
+1.7 nA
40 mV
100 ms
B
(nA)
+0.7
+0.3
-0.3
-0.7
-1.1
-1.5
-2.3
30 mV
75 ms
C
I (nA)
-2.5
-2.0
-1.5
-1.0
-0.5
0.5
1.0
20
10
-10
-20
-30
-40
-50
V (mV)

Figure 1

Figure 2. Stimulus-induced and spontaneous postsynaptic potentials (PSPs). A. Low intensity stimulation (50 μA) evokes a small amplitude PSP with short-latency to onset. Occasionally, late PSP are observed (see arrow in b). When the stimulus strength is progressively augmented (70-300 μA), the amplitude and duration of the PSP increase accordingly until it reaches the membrane threshold for triggering an action potential. At 5000 μA stimulation, the cell still fires a single action potential. The resting Vm of this cell was -87 mV. B. Unlike the neuron illustrated in A, spontaneous PSPs are apparent in this cell at resting Vm (-85 mV). The PSPs occur at a frequency of about 2 Hz (a) and their shapes were variable (b). These spontaneous PSPs can contribute to the shape of the stimulus-induced response (c).

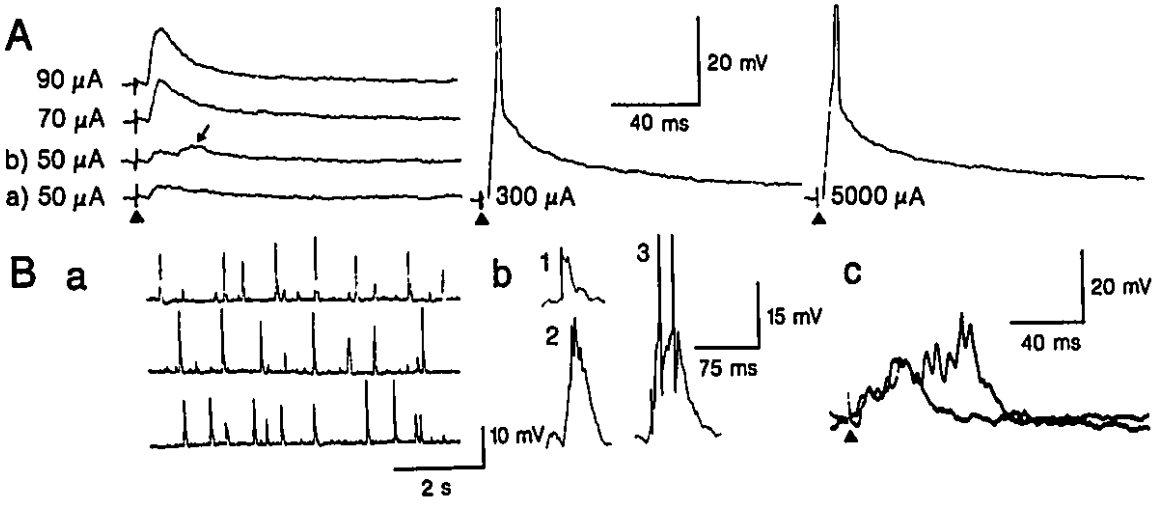
A
90 μA
70 μA
b) 50 μA
a) 50 μA
20 mV
40 ms
300 μA
5000 μA
B a
b
1
2
3
15 mV
75 ms
10 mV
2 s
c
20 mV
40 ms

Figure 2

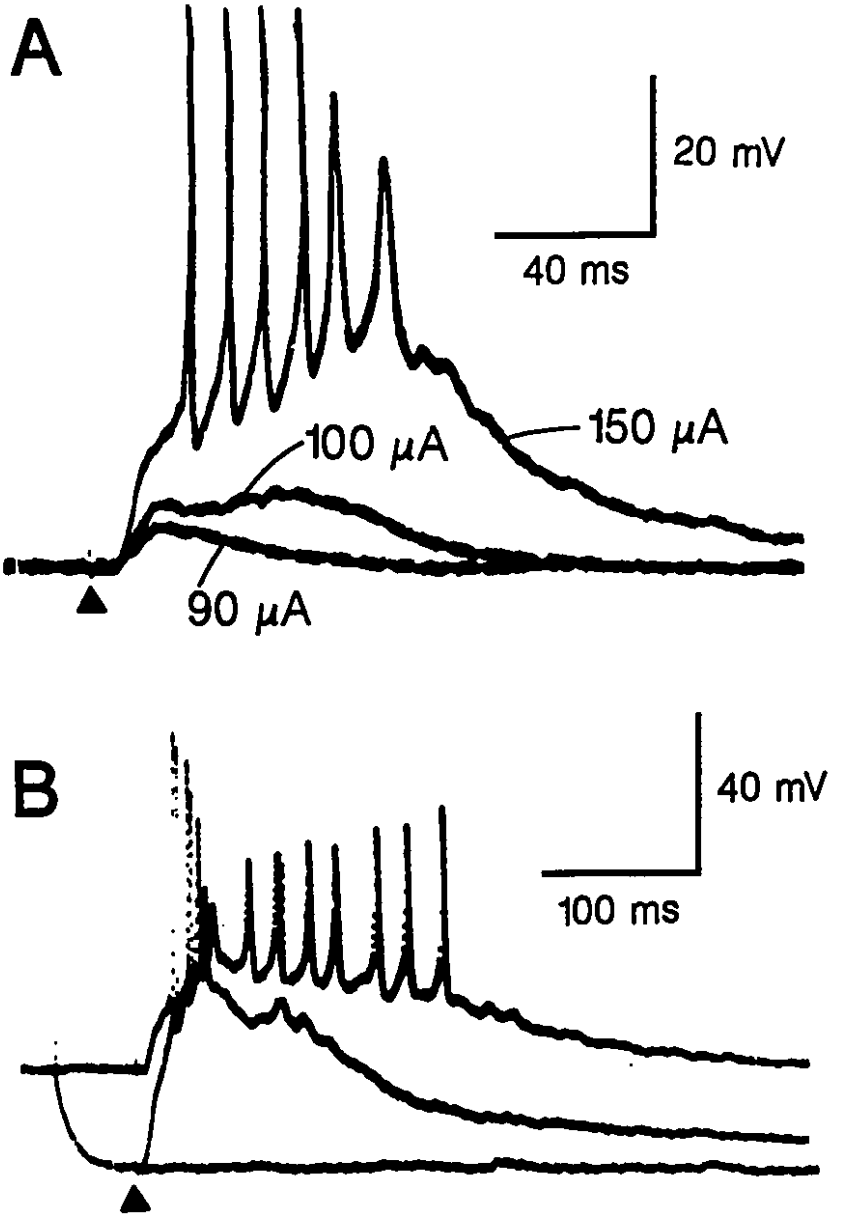

Figure 3

Figure 4. Voltage behavior of the stimulus-induced PSP. A. The PSP evoked by high intensity stimuli consists of a short-latency excitatory postsynaptic potential (EPSP, open circle) followed by an early inhibitory postsynaptic potential (IPSP, solid circle) and a late IPSP (open triangle). The EPSP increases in amplitude with Vm hyperpolarization and decreases in amplitude with Vm depolarization (also see 250μA in B). The early IPSP is inverted at rest (Vm = -82 mV) and can be revealed with membrane depolarization (-62 mV). According to the voltage-response plot (b), the reversal potential for the early and late IPSPs are about -72 mV and -98 mV respectively. B. In contrast, low intensity stimulation (150 μA) evokes a small amplitude EPSP that increases in size with Vm depolarization and decreases in size with Vm hyperpolarization. This type of anomalous voltage behavior can be mimicked by an intrinsic response evoked by a brief pulse of positive current (intrinsic). The resting Vm of this cell was -57 mV.

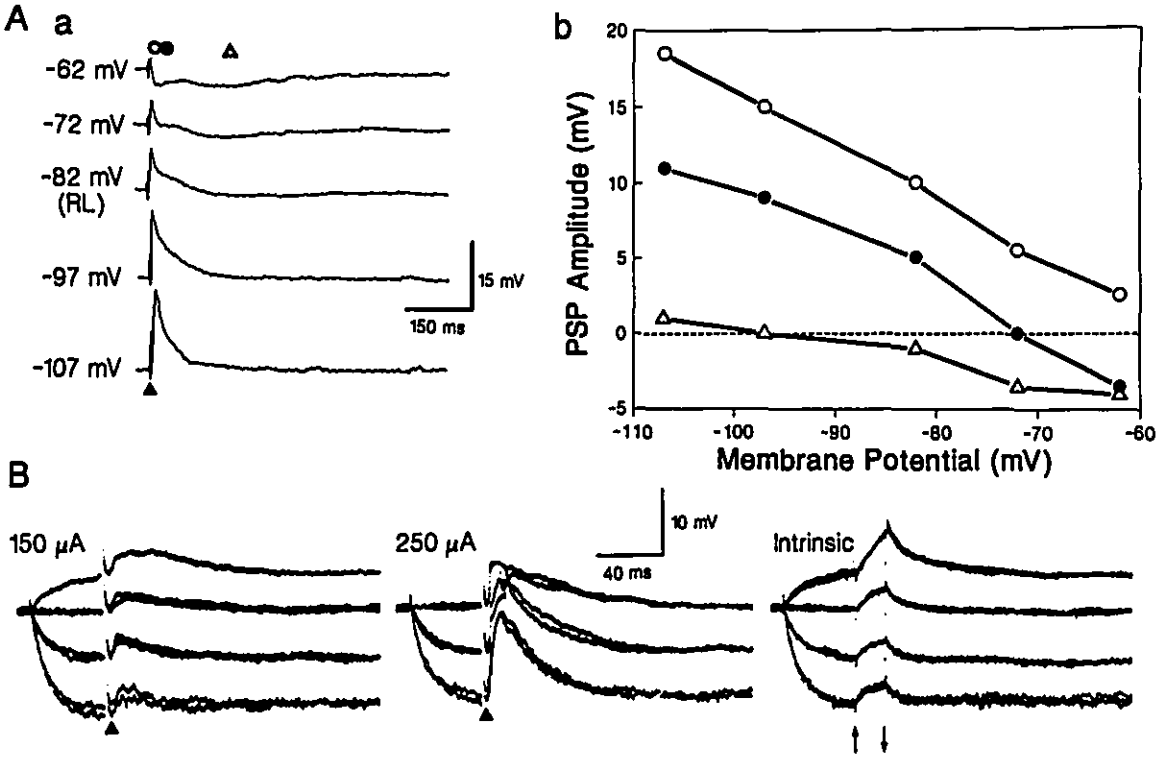
A
a
-62 mV
-72 mV
-82 mV
(RL)
-97 mV
-107 mV
15 mV
150 ms
b
PSP Amplitude (mV)
20
15
10
5
0
-5
-110
-100
-90
-80
-70
-60
Membrane Potential (mV)
B
150 µA
250 µA
10 mV
40 ms
Intrinsic

Figure 4

Figure 5. Effects of the NMDA antagonist CPP and the non-NMDA antagonist CNQX on the stimulus-induced EPSP. Bath application of CPP (5 μM) reduces the peak amplitudes of EPSPs evoked by low (10 μA) and high (90 μA) intensity stimulations by 67% and 55% respectively. Following the washout of CPP (wash), the EPSPs are tested with CNQX (4 μM). In contrast with CPP, the non-NMDA antagonist readily blocks the EPSP evoked by low-intensity stimulation (10 μA) and potently reduces the one (by 82%) evoked by high-intensity stimulation (90 μA). The resting Vm of this neuron was -77 mV.

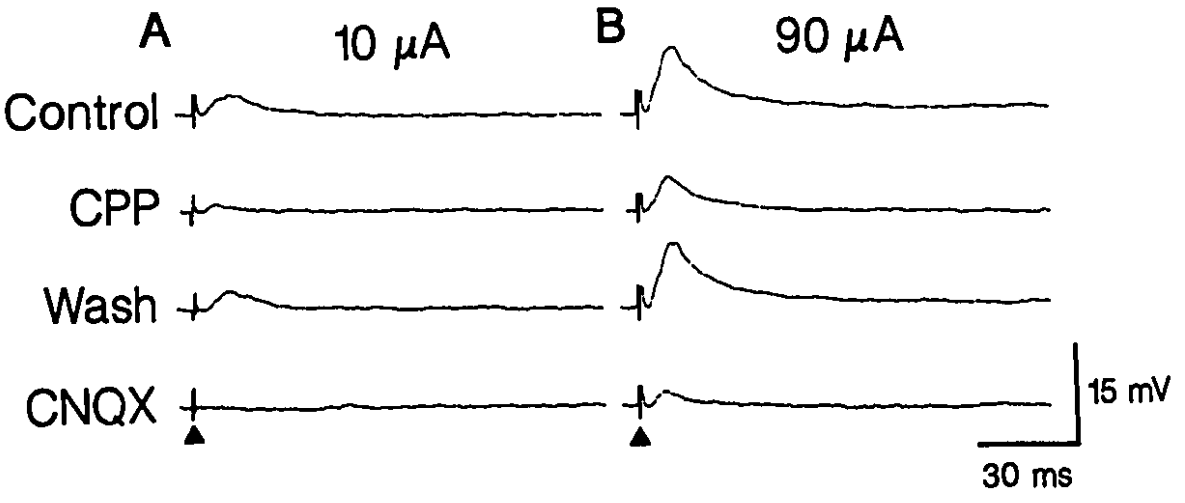

Figure 5

Figure 6. Effects of CPP and CNQX on the all-or-nothing bursting discharge. In control, the discharge is discernible following stimulation by threshold (150 μA) and suprathreshold (300 μA) intensities. The application of CPP (5 μM) reduces the threshold discharge into a depolarizing PSP (150 μA, CPP) and attenuates the late phase of the suprathreshold discharge (300 μA, CPP). Meanwhile, the repetitive firing property of the neuron remains unaffected (C, +0.5 nA). After the effect of CPP is washed out (wash), CNQX (4 μM) can abolish both the threshold (150 μA, CNQX) and suprathreshold (300 μA, CNQX) bursting discharge. Note that the size of the CNQX-insensitive depolarizing PSP (CNQX, 150 μA) is smaller than the CPP-insensitive depolarizing PSP (CPP, 150 μA). The resting level of this cell was -68 mV.

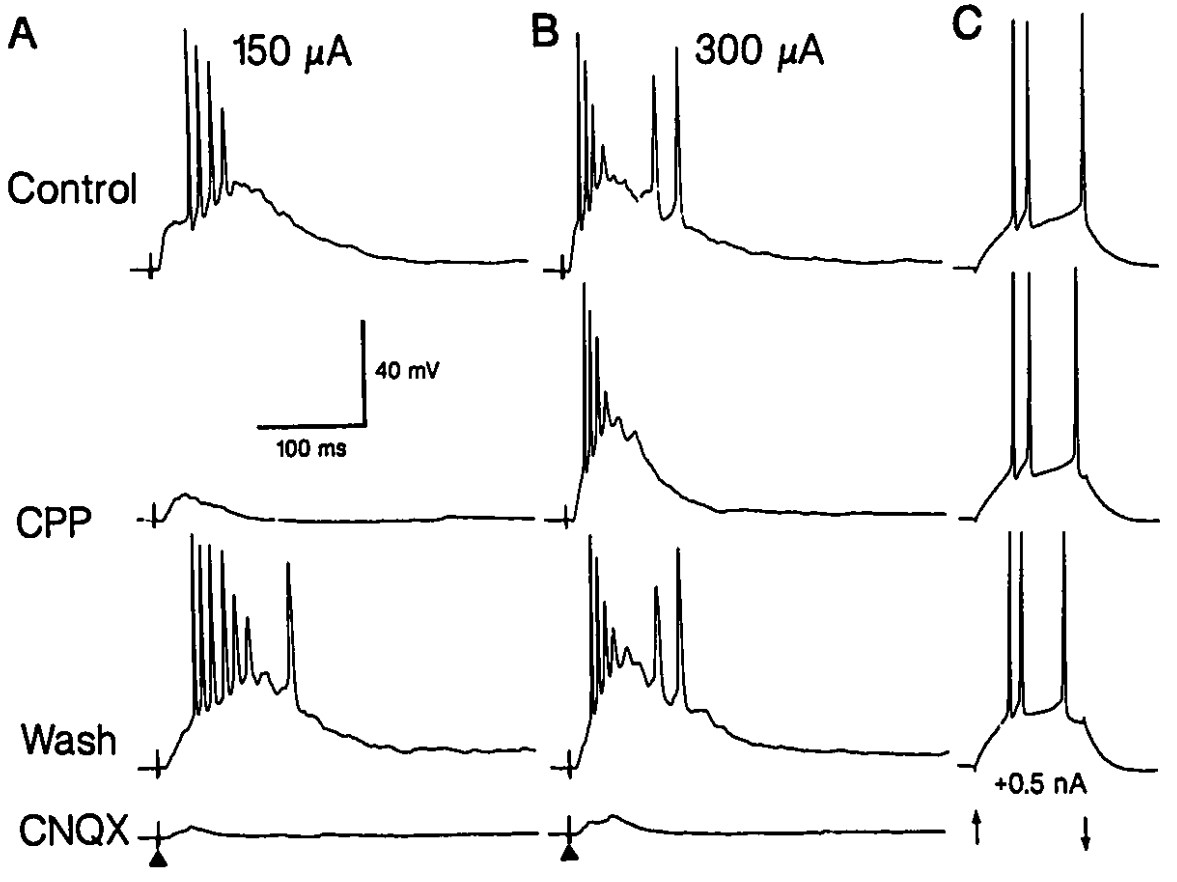

Figure 6

CHAPTER 6

BICUCULLINE-INDUCED EPILEPTOGENESIS IN THE HUMAN NEOCORTEX MAINTAINED IN VITRO

SUMMARY

Intracellular and extracellular recordings were made from human neocortical slices of the temporal lobe maintained in vitro. The slices were treated with bicuculline methiodide to reduce synaptic inhibition mediated by the gamma-aminobutyric acid A ($GABA_A$) receptor. Spontaneously occurring epileptiform activity was never observed in over 60 slices examined. All epileptiform discharges were elicited by single-shock stimuli delivered in the underlying white matter or within the cortical layers. Intracellularly, the stimulus-induced epileptiform discharge resembled the paroxysmal depolarization shift (PDS). This potential was observed in neurons located between 200 and 2200 μm from the pia. It was characterized by a 100-1800 ms long depolarization which triggered burst firing of action potentials, and was at times followed by an afterdischarge. Simultaneous intracellular and extracellular recordings showed that each PDS was reflected by the synchronous discharge of a neuronal aggregate. The voltage behavior of the PDS and its preceding EPSP was analyzed in cells that were injected with the lidocaine derivative N-(2,6-dimethylphenylcarbamoylmethyl)triethyl-ammonium bromide (QX-314). The amplitudes of the PDS depolarizing envelope measured at its peak and during its falling phase both behaved as a monotonic function of the membrane potential by increasing in amplitude during hyperpolarization. In addition, the PDS peak amplitude showed a much greater rate of increase than the early EPSP peak amplitude, thus suggesting that the synaptic conductance underlying the PDS was much greater. Perfusion of the neocortical slices with the N-Methyl-D-aspartate(NMDA)receptorantagonistDL-2-amino-phosphonovalericacid

(APV) reduced both the duration and the amplitude of the paroxysmal field discharge in a dose related fashion. The effects of APV were reflected intracellularly by an attenuation of the PDS's late phase and a blockade of the afterdischarge. Similar findings were also obtained by using the NMDA receptor antagonist 3-((±)-2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid. These data indicate that reduction or blockade of the $GABA_A$ receptor is sufficient to elicit epileptiform discharges in the human neocortex maintained in vitro. Mechanisms dependent upon the NMDA receptor contribute to this type of epileptiform response mainly by prolonging the stimulus-induced depolarizing potential and the associated burst of firing.

INTRODUCTION

The paroxysmal depolarization shift (PDS) generated by cortical neurons in acute and chronic epileptogenic foci represents to date the epileptiform event which has been most extensively studied at the cellular level (Ayala et al. 1973; Prince and Connors 1986). Since it occurs at the same time of the electroencephalogram interictal focal spike, the PDS has been considered the intracellular hallmark of focal, interictal epileptogenesis. The PDS can be reproduced in the in vitro brain slice preparation following pharmacological manipulations. These include the blockade of the gamma-aminobutyric acid A ($GABA_A$) receptor by convulsant drugs such as bicuculline, penicillin and picrotoxin (for review, see Avoli 1988).

Much of our knowledge on the mechanisms underlying the PDS has been obtained from animal studies (Prince and Connors 1986). However, the availability of human cortical biopsies in our laboratory has provided us the valuable opportunity to examine directly this type of epileptiform discharges evoked in the human neocortex. While it has been shown previously that human cortical neurons generate the PDS following the application of bicuculline methiodide (BMI) (Schwartzkroin and Haglund 1986; Avoli and Olivier 1989), detailed information pertaining to its electrophysiological and pharmacological characteristics is still lacking. Therefore, the present study was undertaken to advance our understanding of the electrophysiological attributes of the BMI-induced PDS recorded in the human temporal cortex maintained in vitro. In addition, the role of the N-methyl-D-aspartate (NMDA) receptor in this model of focal

epileptogenesis was also examined. Some of these findings have been presented in a preliminary communication (Avoli and Olivier 1986).

MATERIALS AND METHODS

Human brain tissue was obtained during neurosurgical operations performed for the relief of intractable epilepsy (n=25). All the specimens examined in the present study belonged to blocks of tissue removed strictly for medical reasons. Informed consent was obtained in all cases. While most of the patients who underwent surgery had been maintained on a variety of antiepileptic drugs, the medications were progressively discontinued or markedly reduced during the weeks preceding surgery. Surgical procedures were carried out under local anesthesia in all but 2 cases where general anesthetics with halogenated compounds were used. Patients operated under local anaesthesia received during the operation the analgesic drug sublimaze (0.2 mg/Kg,i.v.) and the short-lasting barbiturate methohexital (0.5 mg/Kg,i.v.). The intrinsic and synaptic properties of the neurons recorded from cortical tissue of patients who received general anesthesia were indistinguishable from those obtained from patients operated under local anesthesia (see also Avoli and Olivier 1989). This suggests that the "acute" effects of drugs present in the brain in situ are readily washed away during the time (1-2 h) allowed for stabilization in the tissue chamber following slicing. The brain samples used in the present experiments were excised from the first (n=6) and second (n=19) temporal gyrus. Neuropathological analysis of the tissue revealed a moderate degree of gliosis and neuronal loss.

The preparation and maintenance of human cortical slices in vitro have been already described in a previous study (Avoli and Olivier 1989). Briefly, neocortical tissue was cut with a McIlwain tissue chopper at thickness ranging from 500-700 μm. The plane for cutting was usually normal to an axis that was longitudinal through the gyrus. The slices were then placed in an interface tissue chamber where they were perfused at 35 ± 1°C with oxygenated (O_2 95%, CO_2 5%) artificial cerebrospinal fluid (ACSF). The composition of the ACSF was in mM: NaCl 124, KCl 2, KH_2PO_4 1.25, $CaCl_2$ 2, $MgSO_4$ 2, $NaHCO_3$ 26 and glucose 10. The perfusion rate (0.5-1.5ml/min) was kept constant in each experiment. The drugs bicuculline methiodide (BMI, Sigma), DL-2-aminophosphono-valeric acid (APV, Sigma) and 3-((±)-2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP, Tocris Neuroamin) were all applied by bath perfusion.

Conventional intracellular recordings were performed with glass micropipettes (diameter: 1.5 mm; resistance: 50-150 MΩ) filled with 4M K-acetate. In some cases, the microelectrodes were filled with the lidocaine derivative N-(2,6-dimethylphenylcarbamoylmethyl)triethyl-ammonium bromide (QX-314) (50 mM dissolved in 2M K-acetate). The signals were fed to a high impedance amplifier (Axoclamp-2A, Axon Instruments) with an internal bridge circuit for passing intracellular current. The bridge balance was monitored routinely and adjusted when necessary. The recorded signals were displayed on an oscilloscope and/or a GOULD pen recorder. In the latter case a 20 KHz waveform digitizer was often used to avoid

distortion of the signals due to slow time response of the pen. In some experiments, the data were also recorded on FM magnetic tape for subsequent analysis.

Extracellular field potentials were measured with microelectrodes (resistance: 2-5 MΩ) filled with 2 M NaCl. In the depth profile experiments, the recording electrode was moved by steps of 100 μm along the neocortical slice following a theoretical line which was normal to the pial surface. Constant current anodal stimuli (0.05-0.5 mA;10-90 μs) were delivered through sharpened and insulated tungsten electrodes which were placed in the underlying white matter or within the cortical layers. Comparison of the responses evoked by either white or grey matter stimulation showed that they were very similar in shape and characteristics.

RESULTS

In the present experiments, neurons were impaled at depths ranging between 200 and 2,200 μm from the pia. This corresponded roughly to cortical layers II to V. The cells displayed in the presence of BMI (50 μM) a resting membrane potential more negative than -55 mV, action potential amplitude greater than 80 mV, and input resistance higher than 20 MΩ. Their electrophysiological characteristics were similar to those reported previously by our laboratory (Lacaille et al. 1988; Avoli and Olivier 1989). Morphological identification with the Lucifer yellow staining technique had shown that these neurons were pyramidal in shape.

Development of the PDS

Neocortical cells perfused with normal ACSF responded to focal extracellular stimuli with a short-latency depolarizing event which lasted 10-40 ms (Fig. 1A, control). This depolarizing response was observed in cells located at any depth within the neocortical slice and was often followed by a 40-200 ms long hyperpolarization (cf. Lacaille et al. 1988, Avoli and Olivier 1989). Manipulation of the membrane potential by injection of steady intracellular current showed that the depolarizing-hyperpolarizing sequence was reminiscent of a conventional excitatory postsynaptic potential (EPSP) and an inhibitory postsynaptic potential (IPSP) respectively.

Perfusion of the slices with ACSF containing BMI markedly altered this biphasic response. As illustrated in figure 1A, the initial hyperpolarizing component (Fig. 1A, control) was progressively replaced by a depolarizing event (Fig. 1A, +15 min) which eventually developed into an epileptiform discharge (Fig. 1A, +25 and +30 min). This example also demonstrates that BMI application only induced pronounced changes in the falling phase of the EPSP, while its rising phase merely showed a slight increase in amplitude. Comparison of the latencies between the EPSP's falling phase and the early IPSP showed that the two events coincided with each other (cf. Avoli 1986; Avoli and Olivier 1989). Therefore the pronounced changes observed in the falling phase of the EPSP during BMI transition were probably a direct result of BMI action on the early IPSP which was partially masked in the control situation by this component of the EPSP. Although these effects were consistently observed in all the cells recorded between layers II to V (n=7), BMI failed to evoke any spontaneous epileptiform

activities in over 60 slices examined. Therefore, all epileptiform discharges in the present study were evoked by extracellular focal stimulation.

Stimulus-induced epileptiform discharge

The epileptiform discharges evoked by focal, extracellular stimuli reached a steady state within 30-60 min of perfusion with BMI. While each epileptiform response was characterized by the classic all-or-none PDS, at least two different discharge patterns could be observed. The first type was recorded in 5 cells and consisted of a 100-350ms long depolarization associated with 4-12 overriding action potentials (Fig.1A, +30 min). The second type, which was seen in 16 cells, comprised of an initial burst of discharge followed by a sustained plateau depolarization during which either a depolarizing block of action potential generation (Fig. 1B) or tonic firing of action potentials could occur (Fig. 1C). Furthermore, this second type of epileptiform activity, which lasted 350-1,800 ms, was sometimes followed by phasic, long-lasting afterdischarges (Fig. 1D). This was recorded in 4 cells.

Despite this variation of the burst duration and morphology among the neurons studied, the PDS recorded from any cell was a stereotyped response autonomous of the stimulus strength (Fig. 2A). At near-threshold intensity, the response was sometimes preceded by an early EPSP and displayed variable time of onset (Fig. 2A, 0.15mA). An inverse relationship between the stimulus intensity and the burst latency to onset became apparent when the stimulus strength was progressively increased. As a result,

the early EPSP was no longer discernible following suprathreshold intensity stimulation (Fig. 2A, 0.40 vs. 0.15 mA).

When the PDS duration between the superficial layer cells (200-1000 μm from the pia) and the deep layer cells (1000-2200 μm from the pia) were compared, no visible distinction between the two neuronal types could be detected (Fig. 2B). This suggests that the heterogeneity in the burst duration was not associated with the location of the recorded neuron. Moreover, this type of variation was only observed when neurons from different slices were compared. In some experiments, up to 6 cells in a given slice were randomly impaled at all the cortical laminae. Under this method of sampling, all the neurons recorded from the same slice displayed PDS with similar features (e.g., duration, discharge pattern). These observations suggest that the PDS is generated primarily by the synchronous interaction among a neuronal population.

The voltage-behaviour

Intracellular injection of steady, DC current of positive or negative polarity modified the amplitude of the depolarizing envelope as well as the pattern of action potential discharge associated with each stimulus-induced epileptiform burst. As illustrated in figure 3, depolarization of the neuronal membrane reduced the amplitude of the underlying depolarization, and concurrently increased the number of overriding action potentials discharge that were associated with its late phase. Conversely, during membrane hyperpolarization, the amplitude of the depolarizing envelope increased, while its plateau duration and action potential firing decreased. In some cells, bringing

the membrane potential into a more depolarized voltage range disclosed or enhanced a post-burst hyperpolarization which could last up to 4 s (Fig. 3B, -52 mV).

The activation and inactivation of the epileptiform burst evoked in the mammalian central neuron is associated with multiple types of voltage dependent currents (Crill and Schwindt 1986). This makes the study of the synaptic potentials underlying the PDS recorded with only potassium-salt filled electrode rather difficult. In order to circumvent this problem, 5 neurons were impaled with microelectrodes filled with QX-314. Intracellular injection of this lidocaine derivative blocked both the inward, Na^+-dependent rectification and the fast Na^+-dependent action potentials (Connors and Prince 1982). Consequently, the activation of the outward rectifying conductances that follow the burst of fast action potentials would also be decreased.

The epileptiform response evoked in all the neurons injected with QX-314 was characterized by a depolarizing envelope which lacked the overriding action potentials (Fig. 4A, insert). When the voltage behaviour of the response's amplitude was measured at the various latencies from the stimulus artifact, it was observed that the entire depolarizing envelope displayed the voltage characteristics of a conventional synaptic potential. As illustrated in figure 4A, both the response's peak amplitude (60 ms) and its falling phase (300, 1000ms) behaved as a monotonic function of the baseline membrane potential by increasing in amplitude during hyperpolarization. In this series of QX-314 experiments, the PDS peak amplitude was also compared with the early EPSP peak amplitude at the various membrane potentials. Both the PDS and EPSP peaks behaved as linear functions of the Vm. However the PDS function

consistently showed a greater rate of increase during membrane hyperpolarization. In the example shown in figure 4B, the slope of the PDS was 5 times greater than the slope of the EPSP. This suggests that the synaptic conductance underlying the PDS was much greater.

PDS as a synchronous event

The extracellular counterpart of the stimulus-induced PDS (Fig. 5A, upper trace) consisted of a synchronous, long-lasting field potential (Fig.5A, lower trace) which displayed different shape and polarity depending on the cortical layer where the recording microelectrode was placed. A typical profile of the epileptiform field potentials is shown in figure 5B. Beginning at the pial surface, the field response consisted of a 100-300 ms long, positive potential which peaked 100-180 ms after the stimulus. This potential became of negative polarity at a depth of 200-400 μm from the pia. It was associated with at least one sharp, biphasic transient which occurred at a latency of 5-10 ms from the stimulus artifact (arrow in 400 μm sample). This early negative component decreased in amplitude as the recording electrode was further advanced to the middle/deep layers (i.e. between 800 and 1,400) while a late, 1,000-2,000 ms long, negative component was also visible at these depths (asterisk in 1,200 μm sample). Finally as the recording electrode approached the white matter (e.g. 2400 μm sample), there was a progressive reduction in the amplitude of the overall response. This type of depth profile was consistently seen in 6 slices following electrical stimuli delivered in either the white matter or the pial surface. In some

instances, 2 to 3 sharp potentials which resembled the population spikes recorded in the hippocampus occurred during the field potential epileptiform response (see Fig. 7A, control).

The appearance of the all-or-none epileptiform field potential was dependent upon the frequency of stimulation. In 18 slices examined, failure of the field response could be observed at stimulus frequency above 0.20 Hz (Fig. 5Cb). Moreover, this refractory period was a function of the stimulus strength. When repetitive stimuli were delivered at a relatively high frequency (Fig. 5Ca, 1Hz), the responses evoked at threshold intensity (Fig. 5Ca, 10μs) showed a much higher failure rate than the responses evoked at suprathreshold strength (Fig. 5Ca, 30μs).

Glial cells recordings

Impalements of glial cells were obtained in all layers. Their electrophysiological characteristics were similar to those described in the animal neocortex in vivo (Kelly et al. 1967; Tzachtenberg and Pollen 1970; Ransom and Goldring 1973a,b) and in vitro (Gutnick et al. 1981). They displayed a resting membrane potential more negative than -80 mV and failed to produce regenerative events during intracellular injection of depolarizing current (as much as 5.0 nA).

In three experiments, the glial membrane potentials were analyzed in detail during the stimulus-induced paroxysmal discharge. As shown in figure 6A, single shock stimuli did not only evoke the expected paroxysmal field potentials (bottom traces), but also elicited simultaneous depolarizations in the intracellularly recorded glial cell (top

traces). Furthermore, the amplitude and the degree of depolarization of the glia varied in unison with the field discharge. The glial response was not associated with any change in conductance as measured by injection of depolarizing or hyperpolarizing current pulses. In keeping with this finding, the stimulus-induced glial depolarization did not change in amplitude or shape during variation of the glial membrane potential obtained by passing steady polarizing current through the recording microelectrode (not shown). During successive glial depolarizations elicited by repetitive stimuli of constant strength, the glial response displayed a tendency to decrementally summate (Fig. 6B, insert). This inverse relationship between the glial response and its serial order in a stimulus train was monotonic in a semi-log plot (Fig. 6B).

Effects evoked by NMDA antagonists

Several animal studies have indicated that conductances activated by NMDA receptor may play a role in epileptogenesis (Croucher et al. 1982; Kemp et al. 1987). In agreement with these findings we have previously demonstrated that neurons recorded in slices of human epileptogenic neocortex perfused with normal ACSF generate a stimulus-induced burst which is blocked by the NMDA receptor antagonist APV (Avoli and Olivier 1987,1989). It appeared therefore of interest to assess the participation of conductances activated through the NMDA receptor in the BMI-induced epileptiform activity recorded in the human neocortex maintained in vitro.

As illustrated in figure 7A, APV reduced both the amplitude and the duration of the epileptiform field potential induced by extracellular focal stimuli. These effects, which were fully reversible upon washout of APV, were dosage dependent (Fig. 7B). By increasing the APV concentration from 50 μM to 200 μM, a significant decrease in the response duration could be observed ($P < 0.005$, Student *t* test). Although this was also accompanied by an overall decrease in the response amplitude, the percent reduction of the amplitude induced by 50 μM and 200 μM of APV were not significantly different ($P > 0.05$, Student *t* test). Interestingly similar concentrations of APV (50-200 μM) did not induce any consistent change in the early part of the epileptiform response as studied by comparing the rate of onset of the initial negative deflection of the field potential before and during perfusion with this NMDA receptor antagonist (Fig. 7A).

The antagonistic actions of APV (25-100 μM) were also reflected intracellularly in 4/4 neurons tested. Figure 8 shows the effects of APV on the burst of longer (>350ms) and shorter (<350ms) durations. In those cells in which a prolonged epileptiform discharge was recorded, APV attenuated the late phase of the initial PDS and blocked the afterdischarges that followed it (Fig. 8A). Again, these effects of APV were fully reversible upon washout of the drug. Similar results were also obtained from neurons that generated the shorter duration burst (Fig. 8B). In the example shown in figure 8B, the effects of APV were more clearly demonstrated in the response evoked at a more hyperpolarized membrane level where the activation of action potentials was prevented (Fig. 8Bb). In agreement with the findings obtained by using extracellular field potential recordings only the falling phase of the depolarization envelope was reduced,

while its early phase (measured at peak amplitude) remained unaffected (Fig. 8C). Similar results were also reproduced in 2/2 neurons examined with the NMDA receptor antagonist CPP (5 μM) (not illustrated). The effects induced by these NMDA receptor antagonists were not accompanied by any changes in the input resistance and the repetitive firing evoked by an intracellular pulse of depolarizing current.

DISCUSSION

The experiments reported in this paper indicate that perfusion of human neocortical slices with ACSF containing the $GABA_A$ receptor antagonist BMI modifies the EPSP-IPSP sequence evoked by extracellular focal stimuli into a PDS. Although the appearance of BMI-induced epileptiform discharges in the human cortex maintained in vitro was originally reported by Schwartzkroin and Haugland (1986) and was later confirmed in our laboratory (Avoli and Olivier 1989), the present experiments reveal some new electrophysiological features that had not previously been described. Furthermore, these data also provide us with information on the participation of NMDA receptor in this type of human interictal activity.

Knowledge on the physiopathogenesis of the epileptiform discharge induced by BMI in the human neocortex is important for determining whether the cellular mechanisms underlying epileptogenesis that have been so far described in the various animal models are in fact applicable to the human brain. Moreover, establishing the cellular bases of the BMI-induced PDS recorded in the human neocortex is relevant for comparing it with the stimulus-induced bursting responses reported to occur in some

human cortical cells recorded from the epileptogenic focus during perfusion with normal ACSF (Schwartzkroin et al. 1983; Avoli and Olivier 1989). In this respect the PDS observed in the present study displays several traits that are different from those of the bursting response seen in the absence of any pharmacological manipulation. Firstly, the BMI-induced discharge is a highly synchronized cellular event, i.e. the PDS can be observed in any cell studied in any given slice. Secondly, the BMI-induced epileptiform discharges are all-or-none in appearance, while the bursting response elicited in cells perfused with normal ACSF is a graded phenomenon. Finally the BMI-induced PDS has a clear refractory period, a phenomenon that is rarely seen in the bursting cells. Thus, these results further indicate that synchronous epileptiform activity in the human neocortex is only observed following pharmacological manipulations (cf. Avoli et al. 1987).

Cellular mechanisms underlying human epileptogenesis

The epileptiform discharges recorded in the human neocortex maintained in vitro during perfusion with ACSF containing BMI are characterized by features which resemble those observed in similar experiments performed in the animal neocortex both in situ and in vitro. Johnston and Brown (1981) had used several criteria to propose that the all-or-none PDS is a "giant EPSP" driven by a neuronal network. The characteristics of the BMI-induced PDS observed in our human neocortical cells appear to satisfy at least two of these criteria. Firstly, the amplitude of the PDS recorded with QX-314 filled microelectrodes shows a linear relationship with the membrane potential

thus suggesting that it depends on the synaptic driving force behind it. Secondly, the increase in conductance underlying the PDS is by several magnitudes greater than the one associated with the preceding, early EPSP's. This difference between the PDS and the EPSP suggests that the former is a giant EPSP. Although the lack of spontaneous activities in our preparation had made it impossible to examine the rate of occurrence of the PDS during changes in the membrane potential, there exists two additional pieces of evidence which suggest that the PDS is indeed a synaptically driven network event. Firstly, the variation in the shape of the PDS is a phenomenon that could only be observed when neurons recorded from different neocortical slices (and not from an individual slice) were compared. Secondly, the NMDA receptor antagonists APV and CPP depress the epileptiform burst at a concentration that did not affect the intrinsic membrane excitability of the neurons.

Depth profile, field potential analysis of the BMI-induced epileptiform activity indicates that the initial component of the stimulus-induced discharge recorded in the human neocortical slice attains its maximal amplitude in the middle layers. This finding suggests that the depolarizing event responsible for the early part of the epileptiform response is presumably located at this neocortical depth. Interestingly, this finding is in line with several studies performed in animal neocortex both in situ and in vitro following treatment with $GABA_A$ receptor antagonists (Gutnick et al. 1982; Connors 1984; Pumain et al. 1983). It has been suggested in these experiments that neurons located in the middle/deep layers might possess membrane properties endowing them with intrinsic bursting capabilities (Gutnick et al. 1982; Connors 1984; Prince and

Connors 1986). Whether this is the case for the human neocortical cells located in the middle layers still awaits cellular, electrophysiological investigation. However the depth profile findings suggest that the human neocortex might display a feature which is shared by the neocortex of lower level mammals such as the rat or the guinea pig.

The roles of NMDA receptors

Much of our understanding of the roles of excitatory amino acids in epileptogenesis was advanced by the availability of several classes of highly specific NMDA receptor antagonists (for review, see Monaghan et al. 1989). In the rodent hippocampus (Herron et al. 1985; Dingledine et al. 1986; Hablitz and Langmoen 1986), entorhinal cortex (Jones 1988) and neocortex (Hwa and Avoli 1989), it had been shown that conductances caused by the activation of the NMDA receptor underlie the late phase of the PDS evoked in the presence of $GABA_A$ receptor antagonists. The findings concerning the effects of APV and CPP on the BMI-induced PDS evoked in the human neocortex are in line with these previous animal studies.

The NMDA receptor-ionophore is gated by extracellular Mg^{2+} in a voltage-dependent manner. Therefore when the neuronal membrane becomes more negative than -30 mV, the NMDA-activated current progressively decreases in amplitude (Mayer et al. 1984; Nowak et al. 1984). In agreement with this mechanisms, a NMDA agonist-induced response decreases in amplitude during membrane hyperpolarization and increases in amplitude during membrane depolarization (Flatman et al. 1983; Thomson 1986a,b). Because of such unique electrophysiological

characteristics, it was proposed that the BMI-induced PDS consisted of two synaptic components. The early phase mediated primarily by non-NMDA receptors would depolarize the membrane into the voltage range where NMDA receptors could be activated. This subsequent activation of NMDA receptors would therefore function to prolong the epileptiform burst (Herron et al. 1985; Dingledine et al. 1986). Based on this hypothesis one might predict that the late phase of the BMI-induced PDS would behave in a NMDA-like voltage dependent manner. However, our QX-314 experiments indicated the contrary. Although the late phase of the PDS did show NMDA antagonist-sensitivity, it did not demonstrate NMDA-like voltage dependency. Instead, the entire response behaved like a conventional synaptic potential and varied linearly within the voltage range in which the NMDA receptors are known to be sensitive to extracellular Mg^{2+}.

Several mechanisms might be considered to explain the discrepancy between the electrophysiological and pharmacological data. First, the lack of a NMDA-like voltage behaviour in the PDS's late phase might be caused by a difference in the distribution of NMDA and non-NMDA receptors along neocortical neuron. While it has been demonstrated that the non-NMDA receptors are spread all over the neocortical cell (Thomson 1986b), the distribution of NMDA receptor remains inconclusive. Since the electrophysiological features of the neuronal responses recorded in our experiments suggest that the intracellular microelectrode was inserted at or close to the neuronal soma, a dendritic location of the NMDA receptors might be sufficient for explaining the lack of voltage sensitivity of the late phase of the PDS. In other words,

manipulation of the membrane potential at the somatic recording site would have no effect on these receptor sites which are more distally situated.

A different explanation could be derived from the findings of Thomson (1986b) which indicate that NMDA-induced responses in the rat neocortex can only be evoked when the ionophoretic pipette is positioned close to the somatic recording site. This would imply that our somatic response is composed of a mixture of NMDA and non-NMDA activated potentials. However, if the non-NMDA receptors are more densely represented than the NMDA receptors at the neuronal soma, then the conductance activated by the non-NMDA receptors may become so overwhelming that it would mask the NMDA-induced negative-slope conductance. Consequently, the NMDA-like voltage dependency of the late phase of the PDS could be hidden within the conventional voltage response of its early phase. In agreement with this notion, an agonist-induced response evoked by glutamate, which is known to activate both NMDA and non-NMDA receptors, displayed a conventional voltage behaviour in 94% of the neurons tested by Thomson (1986b).

Another plausible hypothesis is the involvement of NMDA-mediated polysynaptic excitatory mechanisms within the superficial-middle layers of the neocortex. These polysynaptic pathways may involve excitatory interneurons which play a positive feedback role between neighbouring pyramidal cells during seizure activities (Ayala et al. 1973). Binding studies have shown that cortical laminae I to III in which polysynaptic pathways are abundant, are also rich in NMDA recognition sites (Jansen et al., 1989). Moreover, current source density analysis of the stimulus-induced

response recorded in human neocortical slices excised from the epileptic focus reveal an APV sensitive active sink at 300-700 μm from the pia (J. Louvel and R. Pumain, personal communication). If the excitatory interneurons that relay between the pyramidal cells display NMDA mediated conductances, then intracellular recording performed in pyramidal cells would not reveal any NMDA-like voltage dependency. However, these NMDA-activated conductance of the excitatory interneurons would be sensitive to NMDA antagonists and one consequence of this antagonism would be an attenuation of the PDS. The pharmacological sensitivity of the more prolonged PDS obtained in our present study supports this view. We have observed in these long-lasting paroxysms that only their late phase and the subsequent afterdischarge are sensitive to NMDA antagonists. The long latency (> 1 sec) of these NMDA-sensitive component together with their lack of any NMDA-like voltage dependence, suggest that they might be the expression of activity in polysynaptic excitatory pathways.

Conclusions

Despite the well documented link of inhibition and epileptogenesis, (for review, see Avoli 1988), our results further indicate that inhibitory mechanisms mediated by $GABA_A$ receptors are operant in the human epileptogenic neocortex (Schwartzkroin and Haglund 1986; Avoli and Olivier 1989) and that only following their blockade can epileptiform discharges be elicited. This study also demonstrates that excitatory mechanisms such as the one mediated by the NMDA receptors play role in this pathological state. However, the partial sensitivity of the BMI-induced PDS towards

the NMDA receptor antagonists indicates the possible involvement of other excitatory amino acid receptor subtypes. Obviously, more experiments are required to establish the relative contribution of these different receptor subtypes to the BMI-induced epileptogenesis.

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Figure 1. Effects of bicuculline methiodide (BMI) on the synaptic potentials evoked in human neocortical neurons by single shock stimuli (triangle). Recordings were obtained from 4 different neurons (A,B,C,D). A. Bath application of BMI modified the EPSP-IPSP sequence elicited under pre-BMI condition (control) into a depolarizing event (+15 min) which eventually developed into an all-or-none paroxysmal depolarization shift (+25 and +30 min). At steady state perfusion of BMI, other more prolonged stereotyped discharge patterns were also observed (B,C,D). These prolonged responses are characterized by an initial burst of discharge followed by a sustained plateau depolarization during which either a depolarizing block of action potential generation (B) or tonic firing of action potentials can occur (C). Furthermore, these prolonged paroxysms are sometimes followed by phasic, long-lasting afterdischarges (D).

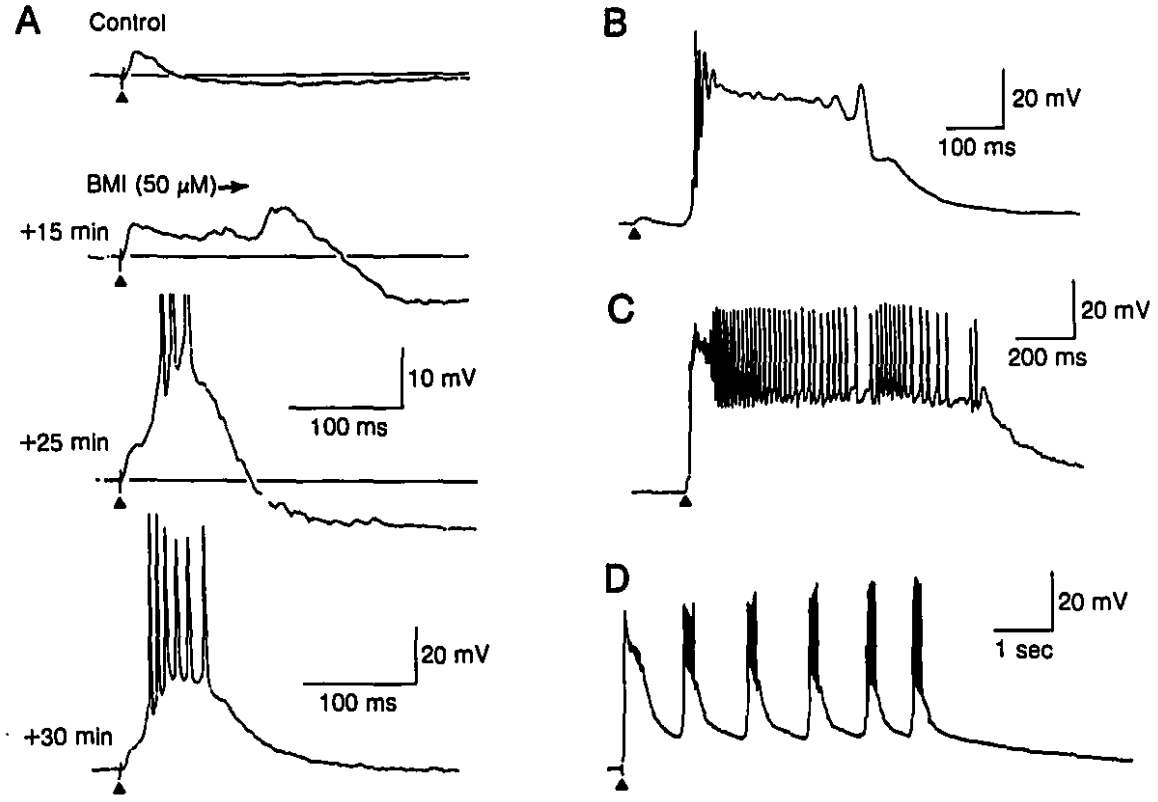
A
Control
BMI (50 μM)
+15 min
10 mV
100 ms
+25 min
20 mV
100 ms
+30 min
B
20 mV
100 ms
C
20 mV
200 ms
D
20 mV
1 sec

Figure 1

Figure 2. Characteristics of the paroxysmal depolarization shift (PDS) in relation to the stimulus intensity (A) and the location of the neuron (B). A. A burst evoked at near threshold intensity stimuli (0.15 mA) displays variable time of onset. As the stimulus strength is progressively increased to suprathreshold levels (0.20, 0.40 mA), a gradual decrease in the PDS latency can be observed. Due to this shift in latency, the early EPSP that precedes the PDS at near threshold intensity is no longer discernible at suprathreshold strength. This type of behaviour is characteristic of all of the neurons examined. B. There is a great degree of variability in the PDS durations (abscissa) recorded from neurons located between cortical layers II-V. Each square represents either a superficial layer cell (cross bar;layers II-III) or a deep layer cell (solid; layers IV-V). Note that the heterogeneity in the PDS duration is not associated with the position of the recording site.

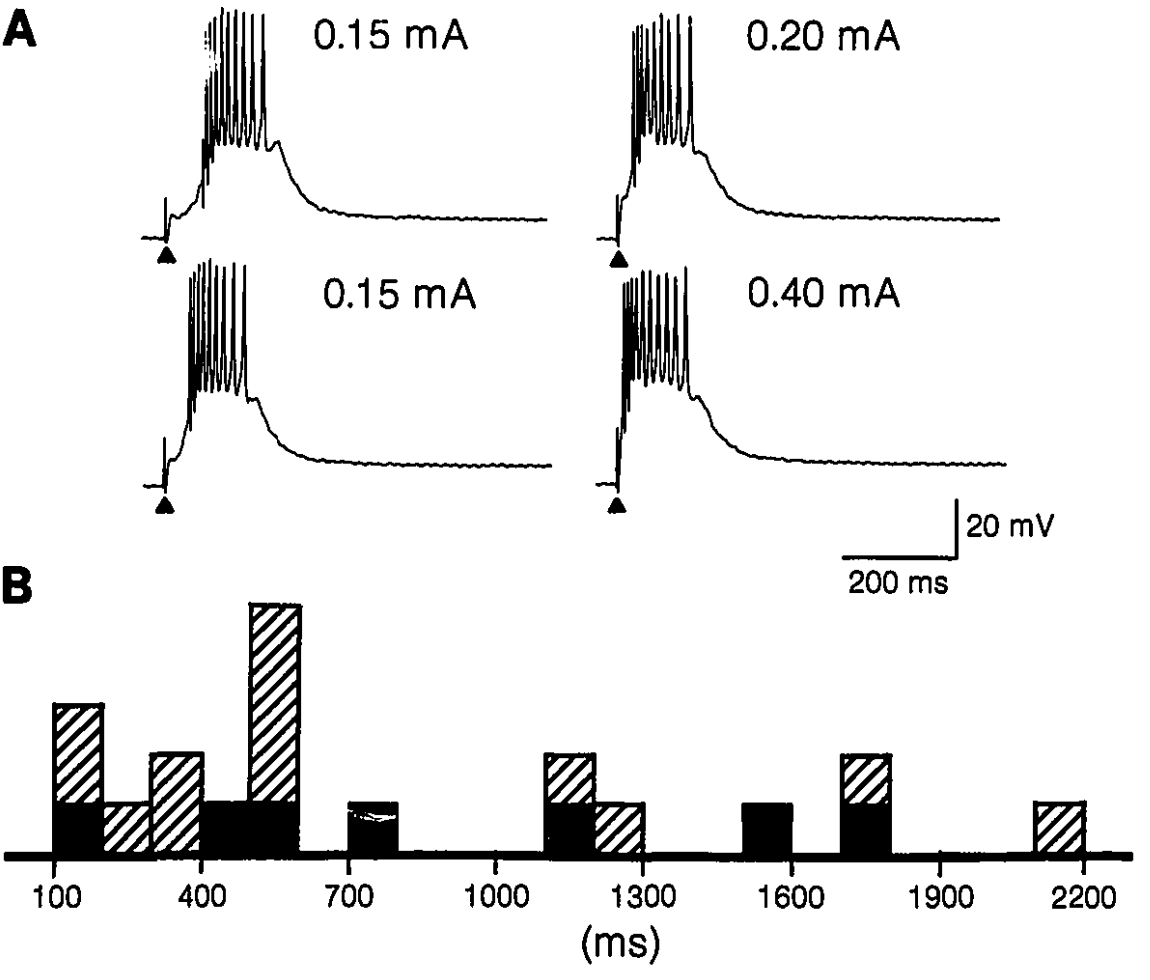
A
0.15 mA
0.20 mA
0.15 mA
0.40 mA
20 mV
200 ms
B
100
400
700
1000
1300
1600
1900
2200
(ms)

Figure 2

Figure 3. Typical voltage behaviour of the paroxysmal discharge recorded with K-acetate filled electrode in two different neocortical cells (A, B). As shown in both examples, hyperpolarization of the membrane with steady, DC current injection increases the peak amplitude of the underlying depolarization envelope. However, the overall duration of the response and the number of action potentials firing associated with its late phase are reduced. B. Depolarization of the membrane to values more positive than -65 mV discloses the post-burst hyperpolarization.

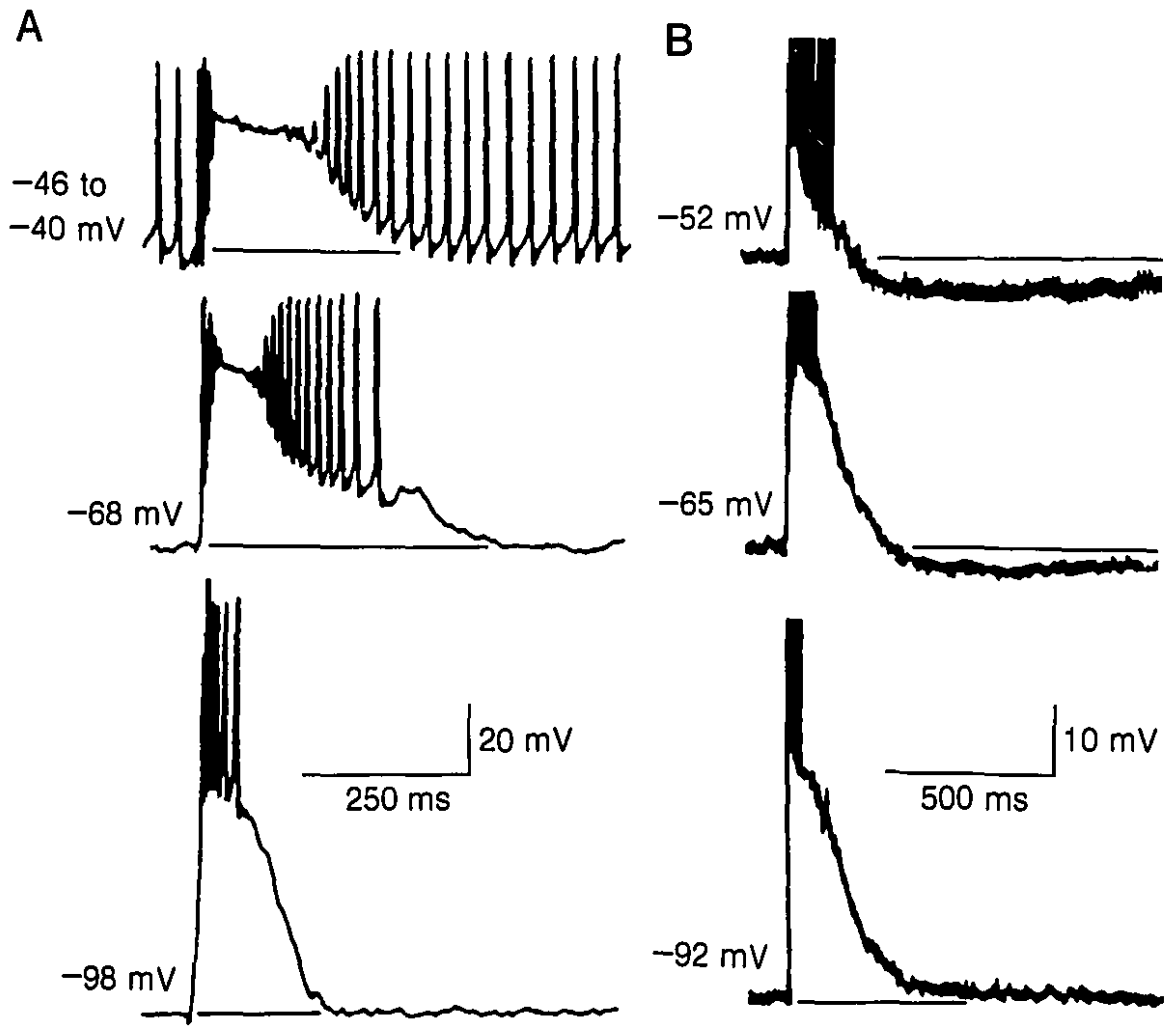

Figure 3

Figure 4. Effects of membrane potential (Vm) changes on the amplitude of the stimulus-induced response evoked in 2 neurons injected with QX-314 (A, B). Variation of the Vm was achieved by intracellular injection of steady, DC current of positive or negative polarities. A. The voltage behaviour of the PDS measured at three different latencies (60, 300, 1000ms) from the stimulus artifact. The response evoked at each membrane level consists of a depolarization shift with no overriding action potentials (insert). The PDS evoked in this neuron was over 1000ms in duration, and its resting Vm was -86mV (arrow). Note that the voltage behaviour of the entire response, which includes its peak (60ms) and falling (300, 1000ms) phases, behaves as a monotonic function of the Vm and increases in amplitude as the membrane becomes more hyperpolarized. B. Comparison of the peak amplitudes of the PDS and its preceding early EPSP at 5 different membrane potentials (resting Vm (arrow) = -66mV). The graph shows that the peak amplitudes of both the early EPSP (circle) and the PDS (triangle) behave linearly during changes in Vm. However, the slope of the PDS (-0.489) is 500% greater than its preceding EPSP (-0.0815). This suggests that the conductance associated with the PDS is much larger.

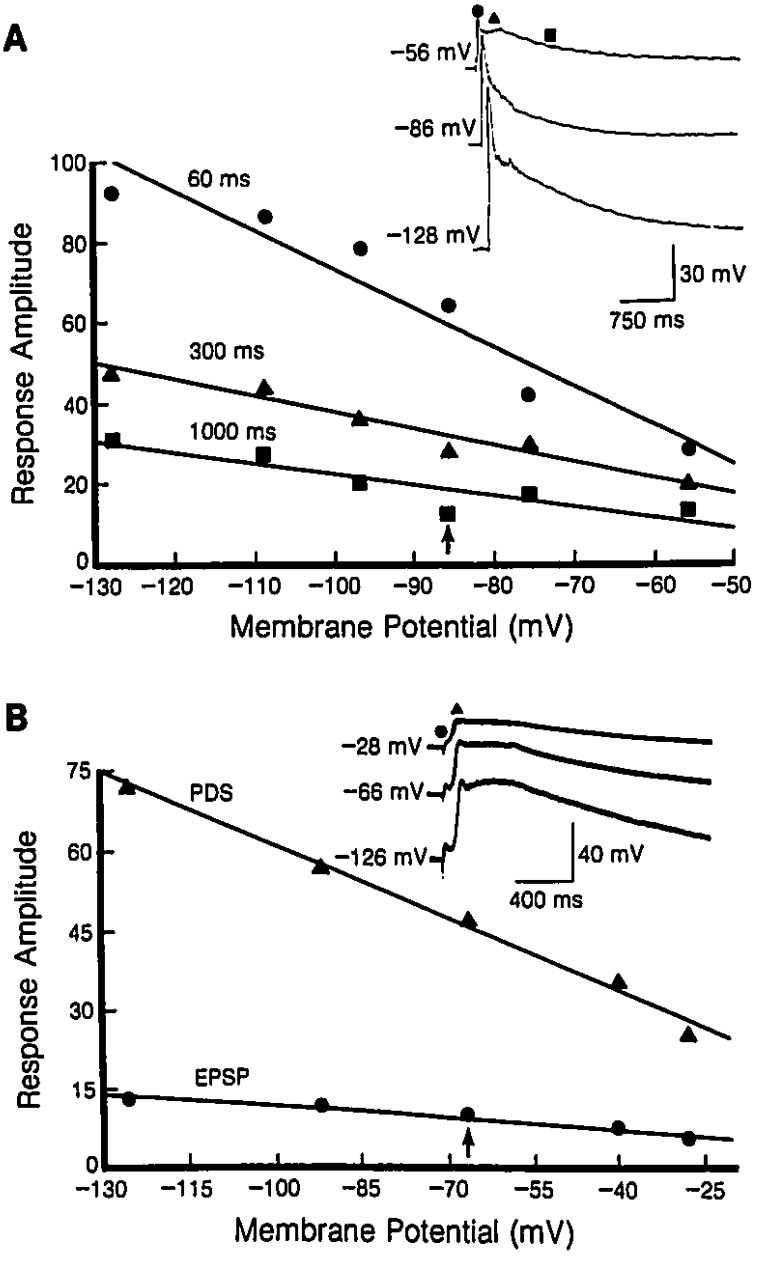
A
−56 mV
−86 mV
−128 mV
30 mV
750 ms
60 ms
300 ms
1000 ms
Response Amplitude
100
80
60
40
20
0
−130 −120 −110 −100 −90 −80 −70 −60 −50
Membrane Potential (mV)
B
−28 mV
−66 mV
−126 mV
40 mV
400 ms
PDS
EPSP
Response Amplitude
75
60
45
30
15
0
−130 −115 −100 −85 −70 −55 −40 −25
Membrane Potential (mV)

Figure 4

Figure 5. Some characteristics of the stimulus-induced synchronized bursting in human neocortical slices treated with bicuculline methiodide. Each triangle points at the stimulus artifact. A. The paroxysmal depolarization shift (upper trace) is a synchronous epileptiform event being concurrent with the field discharge (lower trace) which represents the simultaneous activities of a large population of neurons. The intracellular and extracellular recording electrodes were approximately 500 μm apart. B. A typical laminar profile of the epileptiform field potentials. The field response changes from positive going to negative going shifts as the recording electrode was moved from the pia to the middle layers (800-1200μm). However, these negative potentials gradually decrease in amplitude as the recording site approaches the white matter (2400μm). C. Refractoriness of the field bursts. The results were obtained from two different slices. Both examples demonstrate that the response will begin to fail following stimulus frequency higher than 0.20 Hz. When the frequency is further increased, the response eventually disappears. Note in Ca that at a stimulus frequency of 1 Hz, the response evoked at threshold intensity (10μs) displays a higher failure rate than the response evoked at suprathreshold strength (30μs).

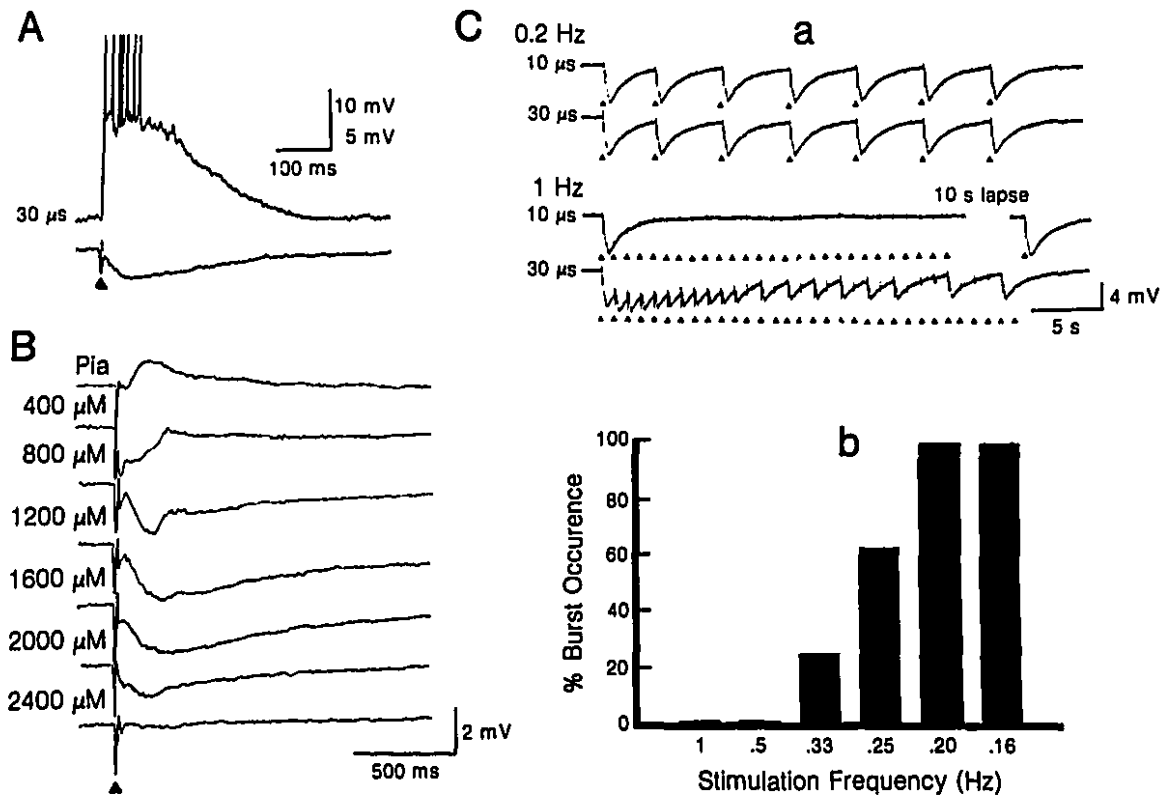
A
10 mV
5 mV
100 ms
30 µs
B
Pia
400 µM
800 µM
1200 µM
1600 µM
2000 µM
2400 µM
2 mV
500 ms
C
0.2 Hz
a
10 µs
30 µs
1 Hz
10 s lapse
10 µs
30 µs
4 mV
5 s
b
% Burst Occurence
100
80
60
40
20
0
1
.5
.33
.25
.20
.16
Stimulation Frequency (Hz)

Figure 5

Figure 6. Glial cells responses during paroxysmal events. A. Single shock stimuli (triangle) evoke the paroxysmal field discharges (lower traces) and the simultaneous depolarizations of the intracellularly recorded glial cell (upper traces). The amplitude and the degree of depolarization of the glia vary in unison with the field discharge. B. Successive glial depolarizations evoked by repetitive stin uli show a tendency to decrementally summate (insert). This type of glial behaviour is a monotonic semi-log function of the stimulus order (see graph).

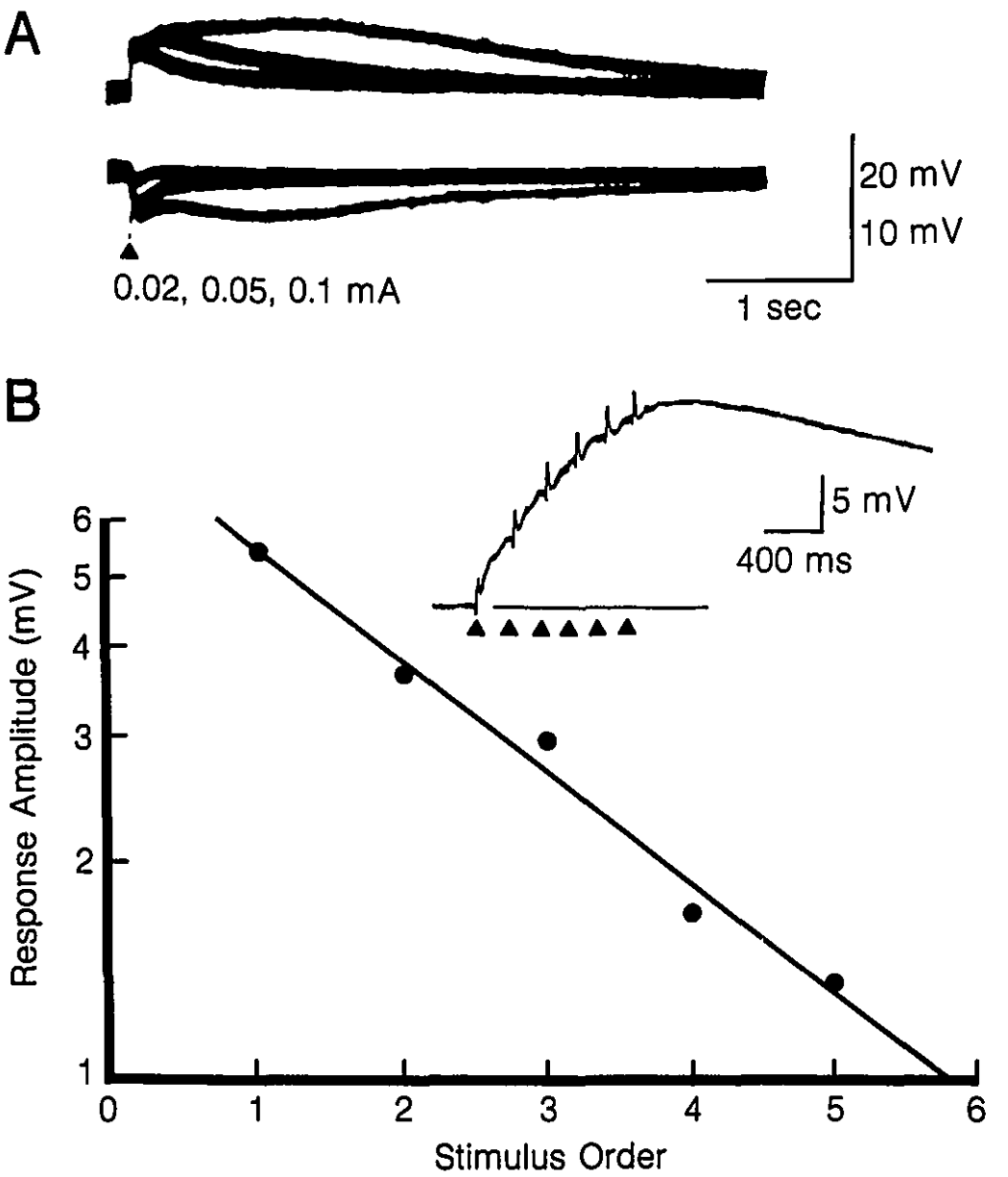
A
20 mV
10 mV
0.02, 0.05, 0.1 mA
1 sec
B
5 mV
400 ms
Response Amplitude (mV)
6
5
4
3
2
1
0
1
2
3
4
5
6
Stimulus Order

Figure 6

Figure 7. Effects of APV on the field bursts evoked in the presence of bicuculline methiodide. A. The NMDA antagonist (50 μM) reduces both the duration and the peak amplitude of the field response in a reversible manner. B. APV reduction of the field response is dosage dependent. Each bar represents the mean$\pm$S.D. (n=5) of the percent decrease in burst amplitude and duration at the designated APV dosage. By raising the APV concentration from 50 to 200 μM, an overall decrease in both the duration and amplitude can be observed. However, only the percent decrease associated with the burst duration is significant ($P < 0.005$, Student *t* test).

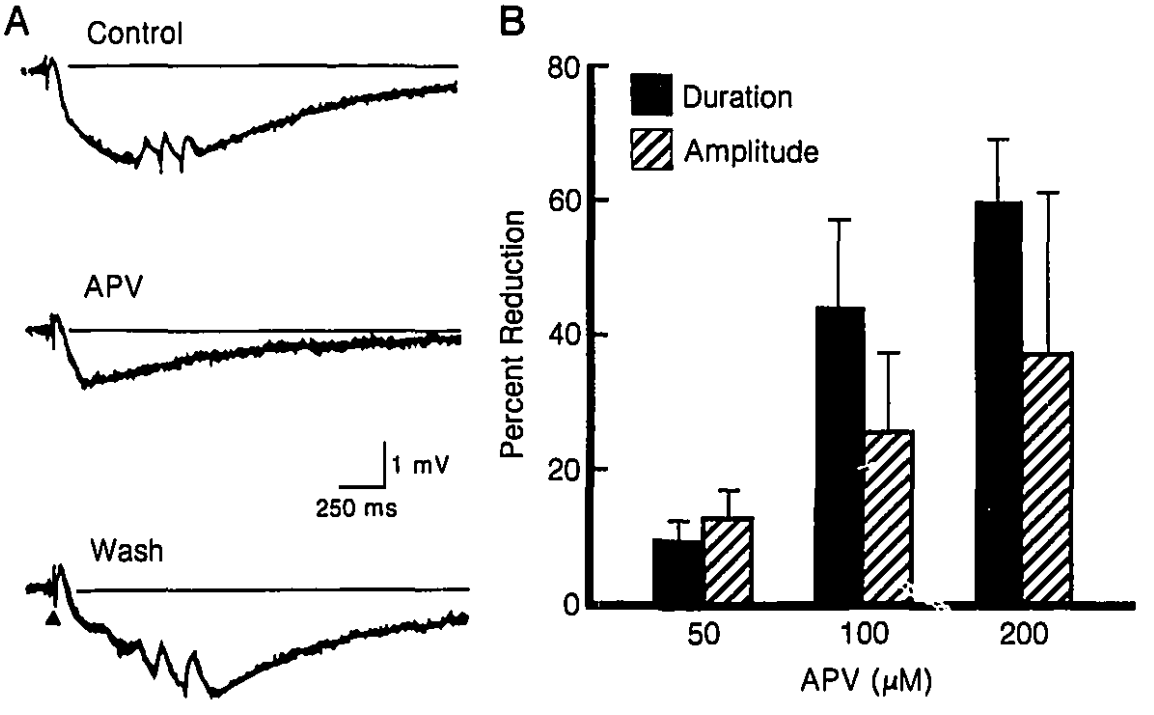
A
Control
APV
1 mV
250 ms
Wash
B
Duration
Amplitude
Percent Reduction
80
60
40
20
0
50
100
200
APV (μM)

Figure 7

Figure 8. APV attenuates the late phase of the paroxysmal depolarization shift (PDS). Recordings were obtained from two separate neurons that showed the prolonged (A) and the brief (B) discharge patterns. A. Application of APV (100 μM) reduces the late phase of the initial PDS and blocks the afterdischarge that follows it. B. Similar effects were also observed in the shorter duration burst. The response shown in Bb is evoked at a more hyperpolarized membrane level (-35 mV from the resting potential) where the overriding action potentials are inactivated. Superimposition of the pre-APV trace (control) with the post-APV trace (APV) shows that only the falling phase of the depolarization envelope is sensitive to APV (Bc). The drug concentration used in this example was 100 μM.

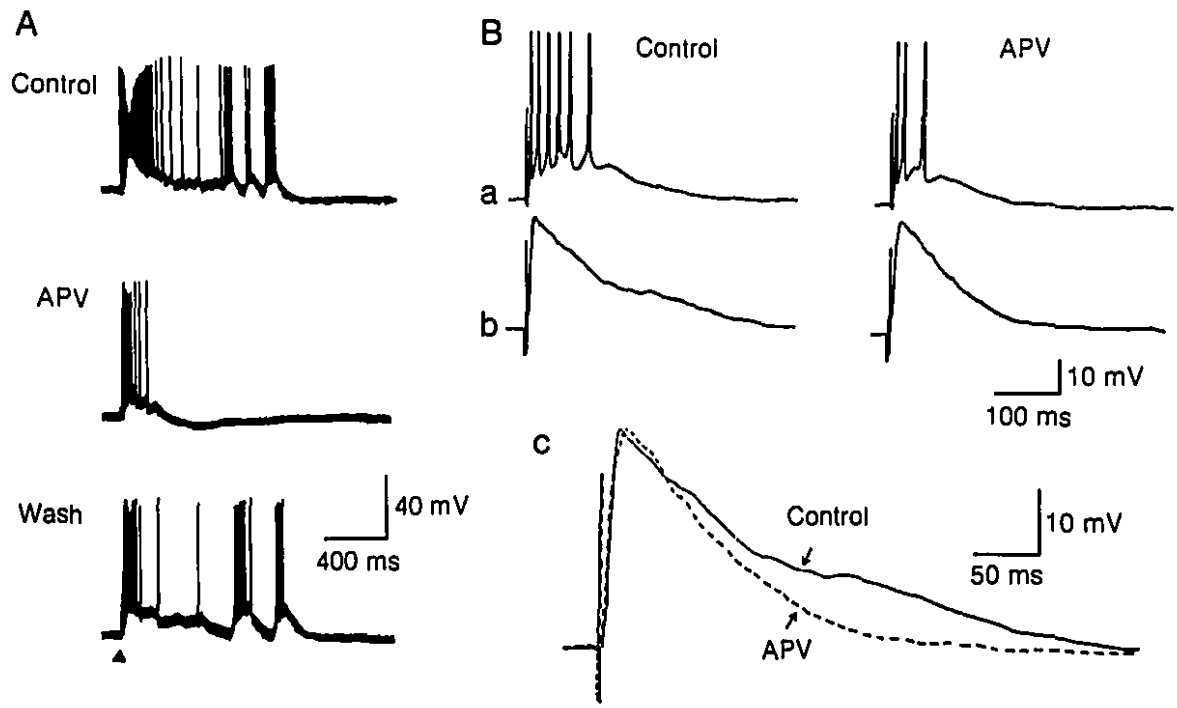
A
Control
APV
Wash
40 mV
400 ms
B
Control
APV
a
b
10 mV
100 ms
c
Control
APV
10 mV
50 ms

Figure 8

CHAPTER 7

GENERAL DISCUSSION

"We often think that when we have completed our study of *one* we know all about *two* because'two' is 'one and one'. We forget that we still have to make a study of 'and'." - A. Eddington, *The Nature of Physics*

The work described in this thesis focused on the role of N-methyl-D-aspartate (NMDA) and non-NMDA receptors in excitatory synaptic transmission in neocortical slices of normal rats and epileptic humans. As with any other experimental method, there are pros and cons in using the in vitro brain slice technique (for review, see Langmoen and Andersen, 1981; Schwartzkroin, 1981; Andersen, 1984; Lipton and Whittingham, 1984; Reid et al., 1988). This technique was chosen for the present project because detailed electrophysiological and pharmacological analyses of human neuronal activities could be carried out in the absence of ethical constraints. However, it should be emphasized that the conclusions drawn from this type of study are based on a network of neocortical neurons in which inputs into the "neocortex" have all been severed. The absence of these inputs can attenuate cortical excitability (e.g. Sato et al., 1987,1989) and possibly the function of excitatory amino-acid receptors. Therefore, generalizations to in vivo conditions must be made with caution. For example, findings from chapter 2 (Hwa and Avoli, 1992) indicate that the NMDA receptor does not participate in excitatory transmission under the perfusion of normal artificial cerebrospinal fluid, yet in vivo studies have reported that the NMDA receptor is involved in normal neocortical function (Armstrong-James et al., 1985; Fox et al., 1989,1990; Larson-Prior et al., 1991).

1. THE REDUCED INHIBITION MODEL

An impairment in synaptic inhibition has long been recognized as a factor that can contribute to neuronal hyperexcitability. Experimentally, this condition can be achieved by the blockade of gamma-aminobutyric acid $(GABA)_A$ receptor with convulsant drugs such as bicuculline, picrotoxin or penicillin. Based on this approach, the synaptic mechanism underlying neocortical epileptogenesis was examined in chapters 3, 4 and 6. What is the relevance of this model for human epilepsy?

Many investigators have attempted to address this question by performing biochemical, autoradiographic and electrophysiological analyses of epileptic human cortical tissues. Unfortunately, no solid conclusion can be drawn. Using the first two approaches, some laboratories found that the concentrations of GABA, its metabolic enzyme or the binding of GAPA receptor are reduced in epileptic human biopsies (Lott et al., 1978; Lloyd et al., 1981; McDonald et al., 1991) while others reported a lack of change (Tursky et al., 1976; Sherwin et al., 1984,1986; Babb et al., 1989). To date, only electrophysiological studies have been able to demonstrate consistently that the inhibitory postsynaptic potential is operant in epileptogenic human neocortical slices (Schwartzkroin and Haglund, 1986; Avoli and Oliver, 1989; McCormick, 1989; Hwa et al., 1991). This, however, is not an evidence for normal GABAergic function in the epileptic human brain (see Discussion in chapter 5). In fact, several antiepileptic drugs, that are used extensively in human therapy, can exert their actions by enhancing GABAergic inhibition (see Macdonald, 1989). Therefore one might imagine a scenario in which the inhibitory mechanism is present in the brain, yet it

can become transiently ineffective (or less effective) during the occurrence of epileptic seizure - possibly through the activation of NMDA receptors (see below, section 2). In this respect, it is pertinent to use the reduced inhibition model to study the role of excitatory amino-acid receptors.

1.1. *Role of NMDA and non-NMDA receptors*

My study demonstrated that NMDA antagonists only attenuated the late phase of the bicuculline-induced interictal-like discharge (Hwa and Avoli, 1989,1991a; Hwa et al., 1991) whereas non-NMDA antagonist could abolish the discharge completely (Hwa and Avoli, 1991a). These findings are in agreement with those described in hippocampal slices (Dingledine et al., 1986; Lee and Hablitz, 1989) and entorhinal cortical slices (Jones and Lambert, 1990). Interestingly, the partial effect of NMDA antagonists observed in vitro is in sharp contrast with those described in vivo, where NMDA antagonists are found to be effective blockers of electrographic and motor seizures induced by $GABA_A$ antagonists (Celesia and Chen, 1974; Clineschmidt et al., 1982; Myslobodsky et al., 1981; Czuczwar and Meldrum, 1982; Piredda and Gale, 1986; Loeb et al., 1990; Turski et al., 1990). Can this discrepancy be a result of the difference in experimental conditions (i.e., in vivo vs. in vitro) or truly reflect the involvement of NMDA receptors in epileptogenesis (i.e., ictal vs. interictal episode)?

Answer to this question could be deduced from the following lines of converging evidence. First, the ictal-like discharge evoked in penicillin-treated hippocampal slices

(Brady and Swann, 1986) or picrotoxin-treated neocortical slices (Lee and Hablitz, 1991) of immature animals was readily abolished by NMDA antagonist. Second, electroencephalographic recordings from the penicillin focus indicated that only the ictal activity and not the interictal spike was blocked by NMDA antagonist (Celesia and Chen, 1974). Finally, in vivo study showed that NMDA antagonists displayed greater potency than non-NMDA antagonist in suppressing the bicuculline-induced convulsion (Turski et al., 1990). Thus it appears that when inhibition is reduced, seizure or ictal-like discharge is primarily mediated by the NMDA receptor while the non-NMDA receptors are crucial for the generation of interictal-like activity.

1.2. *NMDA receptor as down-regulator of excitability*

Under the condition of reduced inhibition, the excessive activation of NMDA receptors may serve as a down-regulator of excitability. For example, bath perfusion of NMDA can attenuate the interictal-like discharge in the penicillin-treated hippocampal slices (ffrench-Mullen et al., 1986) while administration of NMDA to intact animals can protect against the bicuculline-induced convulsion (Piredda and Gale, 1986; Kapetanovic et al., 1990). Although the mechanism underlying these effects of NMDA is not clearly established, the NMDA receptor can undergo use-dependent decline in responsiveness during exposure to its agonist (Fagni et al., 1983; Mayer and Westbrook, 1985). Since the recovery from this desensitization process is positively regulated by the NMDA-glycine site (Mayer et al., 1989), it would be of interest to examine the role of this site under the condition of reduced

inhibition. Studies with other models have shown that the blockade of the NMDA-glycine site is sufficient to abolish the low-$[Mg^{2+}]_o$-induced epileptiform discharge (Kleckner and Dingledine, 1989), suppress audiogenic seizure in DBA/2 mice (Singh et al., 1990) and fully-kindled seizure or its development (Croucher and Bradford, 1990,1991).

2. A CENTRAL ROLE FOR EXCITATORY AMINO-ACID RECEPTORS

Over the past few decades, experimental studies with model systems have uncovered many possible mechanisms of epileptogenesis (for review, see Dichter and Ayala, 1987; Jefferys, 1990). Although this thesis only focuses on one of these mechanisms - namely the "giant EPSP" hypothesis (Johnston and Brown, 1981), there is now little doubt that the synaptic interaction between neurons is very important for neuronal hyperexcitability. Considering that the condition which gives rise to hyperexcitability is different among the models (e.g. reduced inhibition or low $[Mg^{2+}]_o$), one might wonder how these different conditions interrelate with one another through the excitatory amino-acid transmitter system.

In an attempt to address this question, I will now direct our attention to the scheme presented in figure 1. The essence of this diagram is that different conditions can influence each others through the NMDA receptor, thereby providing many possibilities to regulate neuronal excitability. For example when synaptic inhibition is reduced, this can lead to or enhance the activation of NMDA receptors which in turn can 1) contribute to the generation of epileptiform activities (see section 2 in

chapter 1); 2) further diminish GABA-mediated inhibition (see section 4.2 in chapter 1); 3) increase the extracellular concentrations of excitatory amino acids by enhancing their presynaptic release (see section 4.2 in chapter 1). Meanwhile, the build-up of extracellular K^+, created by the occurrence of epileptiform activities, can further enhance the activation of NMDA receptors (see section 3 in chapter 1). Together these various events will produce a positive-feedback effect until the concentrations of excitatory amino acids and/or extracelluar K^+ reach a critical level at which they can then begin to negatively influence the NMDA receptor (see above, and section 3 in chapter 1). This signals the end of the cycle and the termination of epileptiform activities.

The non-NMDA receptors are not included in this diagram because little is known about their functional properties, and thus their role in the mechanism of epileptogenesis. However, as we have learned from the Cs^+-bicuculline model (Hwa and Avoli, 1991b) and from the 4-aminopyridine model (Perreault and Avoli, 1991), the non-NMDA receptors are crucial for the generation of some types of epileptiform activities. Recently, breakthrough in molecular biology studies, has resulted the cloning of non-NMDA receptor genes (see Miller, 1991). This has already triggered a tremendous amount of interest on the non-NMDA receptor system, and should provide a springboard for further investigation of excitatory amino-acid involvement in epilepsy.

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Figure 1. A schematic diagram illustrating the role of NMDA receptors during epileptogenesis. See section 2 in text for further explanation.

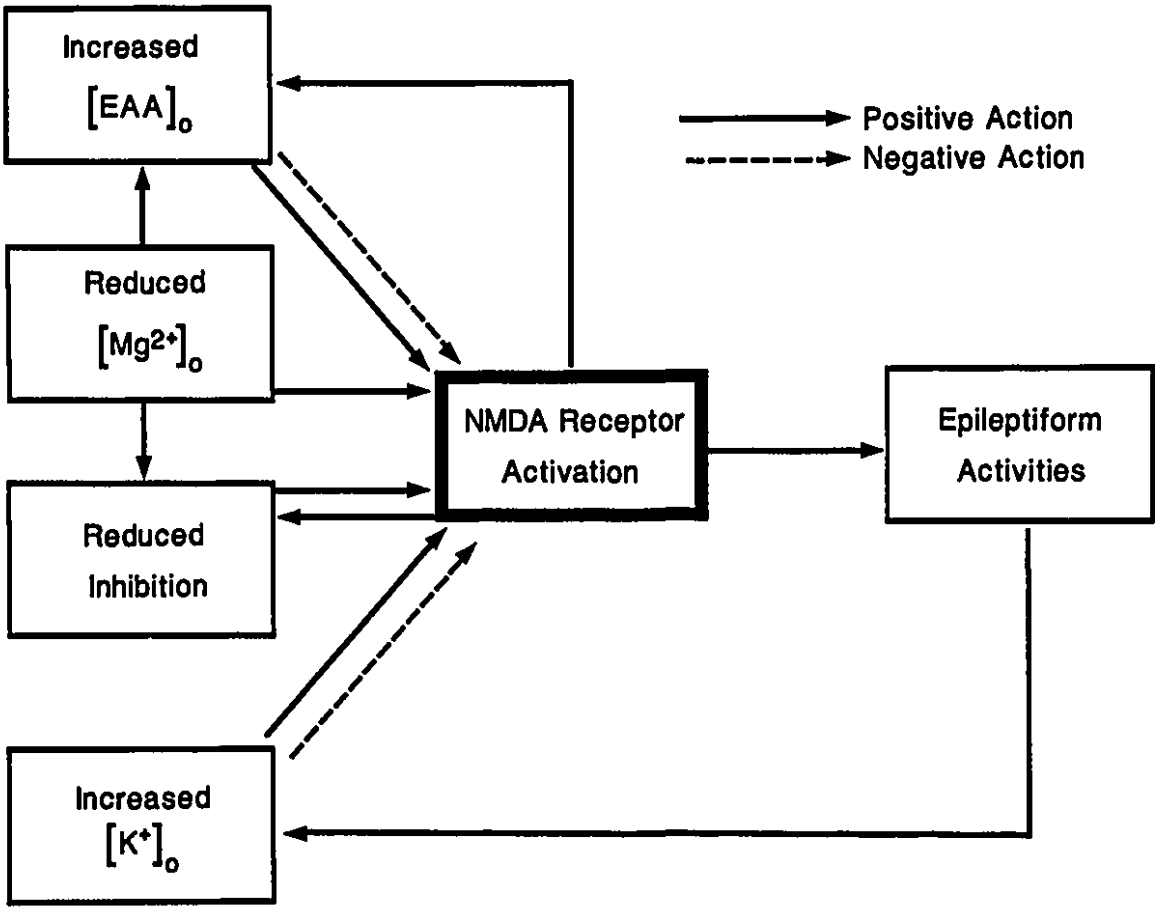
Increased
$[EAA]_o$
Reduced
$[Mg^{2+}]_o$
Reduced
Inhibition
Increased
$[K^+]_o$
NMDA Receptor
Activation
Epileptiform
Activities
Positive Action
Negative Action

Figure 1