EPILEPTIFORM ACTIVITY INDUCED BY LOW CHLORIDE MEDIUM IN THE CA1 SUBFIELD OF THE HIPPOCAMPAL SLICE

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NOTE:

Due to formatting, figure 5 with its legend was displayed on two different pages (39-39a) for the sake of clarity.

ABSTRACT

The present thesis is a study of the low-Cl^- model of epileptogenesis in the rat hippocampal slice maintained "in vitro".

Perfusion of the slices with low-Cl^- artificial cerebrospinal fluid (ACSF) induced short and long lasting stimulus-induced epileptiform bursts. The short lasting bursts (SB) represented a mixture of EPSP and inverted IPSP. The long lasting bursts (LB) were totally dependent on GABA-mediated chloride conductances and were probably generated by the sustained firing of interneurons synapsing on the soma of the pyramidal neurons.

NMDA antagonist could block the late part of the LB, but not the SB nor the early part of the LB. However, when the intensity of the stimulation was increased, the late part of the LB reappeared.

The present work demonstrates that the maintenance of the physiological chloride equilibrium is essential for the integrity of GABAergic inhibitory mechanisms, and that any physiopathological condition susceptible to alter the chloride equilibrium may induce epileptogenesis.

RÉSUMÉ

La présente thèse est une étude du modèle d'épileptogénèse du bas-chlore sur des tranches d'hippocampe de rat maintenues "in vitro".

La perfusion des tranches avec du liquide céphalorachidien artificiel (LCRA) à faible teneur en chlore ($LCRA-Cl^-$) induisit des activités épileptiformes stimulées de courte et de longue durée. Les activités épileptiformes de courte durée (SB) consistaient en un mélange de PPSE et de PPSI inversé. Les activités épileptiformes de longue durée (LB) se sont révélées être totalement dépendentes des conductances à chlore médiées par la GABA, et furent probablement générées par l'activité intense et soutenue des interneurones faisant synapse avec le corps cellulaire des neurones pyramidals.

Le blocage des récepteurs au NMDA a permis de bloquer la partie tardive des LB, sans bloquer les SB ni la partie précoce des LB. Toutefois, le fait d'augmenter l'intensité des stimuli a permis à nouveau l'expression de la partie tardive des LB.

Le présent travail démontre que la sauvegarde de l'équilibre physiologique du chlore est essentielle pour l'intégrité des mécanismes inhibiteurs GABAergiques, et que toute condition physiopathologique pouvant altérer l'équilibre du chlore est susceptible d'induire des états épileptogènes.

CONTENTS

PREFACE

This work has been published in the Journal of Neurophysiology and was done in collaboration with Paul Perreault, Jacques Louvel and René Pumain. Paul Perreault was involved in teaching me the technique of intracellular recordings. Jacques Louvel and René Pumain taught me the technique of ion selective microelectrode in Montreal and during a brief stay in France, in their laboratory. In the Appendix, the technique of ion selective microelectrode is briefly described. Massimo Avoli, my supervisor, wrote the original version of this paper. A general introduction that gives a brief historical background is presented as well as a final discussion.

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GENERAL INTRODUCTION

The present thesis is a study of the low Cl^- model of epileptogenesis in the rat hippocampal slice maintained "in vitro". Part of this work has been published (Avoli et al. 1990).

Epilepsy is one of the most common neurological diseases. About 1% of the population suffers from epilepsy. Epileptic seizures can be described as resulting from a large collection of neurons discharging in abnormal synchrony. This synchronous discharge produces stereotyped and involuntary movements or paroxysmal behavioral alterations that profoundly affect the life of the patient. Epilepsy can be qualified as partial or generalized. Partial seizures affect only a part of the brain and the clinical manifestations reflect the functions represented in the region of the brain involved. Generalized epilepsy involves large parts of the brain and can be subdivided, according to the traditional view, into petit mal and grand mal. Petit mal seizures are characterized by a transient loss of consciousness; grand mal seizures are characterized by a loss of consciousness associated with tonic-clonic movements.

Cases of epilepsy have been reported throughout history and has been considered for long time as the manifestation of negative forces in man. In 1881, Gowers suggested that the epileptic condition results from a temporary decrease in "resistance" that would lead to a sudden release of "nerve force" (Gowers 1881). The first scientific approach to human epilepsy was made by Hughling Jackson. According to Jackson all epileptic seizures were due to the excessive discharge of groups of nerve cells in the brain (Jackson 1931). At that time all seizures were thought to be caused by local structural or functional physiopathology of the central grey matter (Jackson 1931). Later Hunt (1931) brought in the concept of an equilibrium between inhibition and excitation. He proposed that the brain was composed of inhibitory and excitatory cells interacting with each other, and that epileptiform activity would result from an increase in the activity of excitatory cells, or a decrease in the activity of inhibitory cells. Since then, electrophysiological investigations have indeed revealed the presence of inhibitory and excitatory activity (see Sherrington 1906) in the hippocampus (Kandel et al., 1961; Andersen et al., 1964) and in the neocortex (Krnjevic et al., 1964; Avoli 1986); and in the light of recent advances in neurophysiology, both an increase in the activity of excitatory functions and a decrease in the activity of inhibitory mechanisms have been found

experimentally to contribute to neuronal hyperexcitability.

Changes in the intrinsic post-synaptic characteristics of cortical neurons were shown to lead to a larger EPSP amplitude (Lynch and Schubert 1980; Andersen et al., 1980; see also Ward 1969). An increase in the sensitivity to excitatory neurotransmitter was also demonstrated in isolated cortical slabs obtained from chronic epileptic animals (Ward 1969; Sharpless 1969). Paroxysmal depolarizing shift (PDS), an experimental model for interictal discharges, was initially considered as a giant EPSP, possibly caused by the release of a larger than normal quantity of neurotransmitter from a given input population, or impulses originating from a large number of excitatory cells converging on the bursting elements (Prince 1968; Prince and Schwartzkroin 1978). A PDS is a brief epileptiform event that exhibits a transient, large-amplitude depolarization associated with repetitive firing, and this excitation arises with virtual synchrony in the majority of cells in a neuronal population. However, the generation of the PDS was later shown to be attributed to a loss of the simultaneously occurring IPSP (Dingledine and Gjerstad 1979; Traub et al. 1987).

An increased synchrony in neuronal populations is also considered as a factor underlying the expression of epileptiform activity (Miles et al. 1984; Miles and Wong 1987a). A slight decrease or impairment in the efficacy of

the inhibitory mechanisms, experimentally obtained by bathing the neuronal tissue in very low concentrations of GABAergic antagonists, can lead to an increase in the synchrony of firing (Miles and Wong 1983,1987a; Wong and Traub 1983; Miles et al. 1984). Thus, any decrease in the efficacy of inhibition, beside the loss of inhibition itself, increases neuronal synchrony and renders the neuronal tissue hyperexcitable.

Thus the integrity of inhibition is important for the maintenance of the normal behavior of neurons. And indeed, several models of epileptogenesis based on a decrease in the efficacy of inhibitory mechanisms in the central nervous system have been developed and studied. A brief review will be presented and various models of epileptogenesis based on a decrease of inhibition will be described, prior to the detailed study of the low Cl^- model of epileptogenesis.

In the central nervous system (CNS), gamma-aminobutyric acid (GABA) is an ubiquitous amino acid that fulfils the requirements for a role as a neurotransmitter (for review see Avoli 1988). The action of a neurotransmitter is determined largely by the characteristics of the receptors to which it binds. GABA binds to two specific receptor types, the $GABA_A$ and the $GABA_B$ receptors. The $GABA_A$ receptor activates a chloride

conductance and brings the membrane potential close to the chloride equilibrium potential. Since the chloride equilibrium potential is more negative than the resting membrane potential, activation of $GABA_A$ receptors inhibits neuronal activity. In the hippocampus, $GABA_A$ receptors mediate both feedforward and feedback inhibition (Alger and Nicoll 1982), preventing multiple firing of the pyramidal neurons and the spread of the activity into local neuronal circuits (see Miles and Wong 1987a,b). The GABAergic IPSP mediated by the $GABA_A$ receptor is an early IPSP that is hyperpolarizing at the soma and depolarizing in the dendrites.

The $GABA_A$ receptor is linked via a G protein to a potassium conductance. The binding of GABA to $GABA_A$ receptors that are located in the dendrites gives rise to a late hyperpolarizing potential. Antagonists for the $GABA_A$ receptor have been discovered (Dutar and Nicoll 1988) and it was shown that the blockade of $GABA_A$ receptors does not lead to the development of epileptiform activity (Dutar and Nicoll 1988; Malouf et al. 1990).

The blockade of inhibition mediated by $GABA_A$ receptors, by the use of various drugs, has been widely studied as a model of epileptogenesis. Impairment of $GABA_A$-mediated inhibition can be achieve by i) a selective loss of GABA-releasing neurons "in vivo", ii) decreasing the synthesis of GABA i.e. inhibition of glutamic acid

decarboxylase, the enzyme that transforms glutamate into GABA, iii) pharmacological blockage of the $GABA_A$ receptors, or iv) the blockage of, or the interference with chloride conductances linked to the $GABA_A$ receptor.

Selective loss of GABA-releasing neurons

Selective degeneration of GABAergic neurons in the brain can be achieved by topical application of neurotoxic agents on the surface of the cortex. Application of alumina gel (Ribak et al. 1979) or cobalt (Ross and Craig 1981) on the exposed cortex of an animal in a chronic experiment leads to epileptic seizures. Assays revealed that the brain tissue of these animals showed a large fall in the activity of the enzyme glutamic acid decarboxylase (GAD) and in the content of GABA.

Hypoxic treatment also leads to the almost specific degeneration of symmetrical synapses (type II) (Sloper et al. 1980) that are believed to be inhibitory in nature (Eccles 1969; Ribak et al. 1979). Several epilepsies are believed to be the consequence of severe hypoxic episodes in early infancy.

Decrease in GABA synthesis

A direct impairment in the synthesis of GABA can be achieved by pharmacological blockage of GAD. This enzyme is

responsible for the decarboxylation of glutamic acid to synthesize GABA. Thiosemicarbazide (Killam and Bain 1957) and methoxypyridoxine (Ozawa and Okada 1976) both decrease the activity of GAD and thus decreases the production of GABA. In "in vitro" studies in the hippocampal slice preparation, a significant correlation was made between the decrease in GABA content by methoxypyridoxine treatments, and the appearance of paroxysmal depolarizing shift and epileptiform activity (Ozawa and Okada 1976).

Mice exposed to oxygen at several atmospheres also developed seizure activities that were attributed to an inactivation of GAD (Wood et al., 1966 of 9).

Pharmacological blockade of $GABA_A$ receptors

The most studied "in vivo" model of epileptogenesis involves the pharmacological blockade of the $GABA_A$ receptor with penicillin. Topical application of penicillin on the cortex (Walker et al., 1945; Prince and Wilder 1967) have been shown to induce focal spike discharges as well as full blown ictal activities. As demonstrated by experiments performed in the hippocampal slice preparation, at small concentration, penicillin impairs dendritic inhibition whereas somatic inhibition remains intact (Dingledine and Gjerstad 1980; Avoli 1988).

The bicuculline model of epileptogenesis has also been extensively studied (for review see Alger 1984 and

Avoli 1988). Bicuculline is a specific antagonist of $GABA_A$ receptor. Systemic application of bicuculline leads to generalized convulsions (Meldrum 1975) whereas bath application of bicuculline in the "in vitro" hippocampal slice leads to the appearance of paroxysmal depolarizing shifts and burst discharges (Dingledine et al. 1986; Wong and Traub 1983).

Chloride conductances

Similar to bicuculline is the action of picrotoxin that is thought to block the chloride conductances associated with the $GABA_A$ receptors (Olsen and Leeb-Lundberg 1981). Application of picrotoxin induces the appearance of epileptiform activity similar to that induced by bicuculline (Dingledine et al. 1986; Hablitz 1984). Penicillin was also reported to block non-synaptic chloride channels in crab muscle (Hochner et al., 1976).

The "in vitro" preparation allows the modification of the ionic composition of the extracellular space of the nervous tissue. Substitution of chloride ions was demonstrated to induce epileptiform activity (Yamamoto and Kawai 1968), probably by shifthing the chloride equilibrium in the depolarizing direction. This model of epileptogenesis was the first "in vitro" model of epilepsy to be reported in the literature. However no detailed

description of the epileptiform activity was made and the mechanisms underlying the expression of epileptiform activity were not investigated.

The work reported here was aimed at studying the type of epileptiform activity obtained following the substitution of most of chloride ions with impermeant anions, and to work toward a better understanding of human epileptogenesis.

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EPILEPTIFORM ACTIVITY INDUCED BY LOW CHLORIDE MEDIUM IN THE CA1 SUBFIELD OF THE HIPPOCAMPAL SLICE

INTRODUCTION

METHODS

RESULTS

DISCUSSION

INTRODUCTION

The use of *in vitro* preparations of mammalian brain has allowed the characterization of several cellular mechanisms that otherwise would not have been amenable to be studied *in situ* (for review see Dingledine 1984). One of the advantages of the *in vitro* preparation resides in the possibility of modifying the extracellular microenvironment by changing its ionic composition. By doing so one can infer the physiological role of a given ion by perfusing the neuronal preparation with artificial cerebrospinal fluid (ACSF) containing various concentrations of the ion under study, a blocker that prevents the entry of ions through specific channels, or in which a given ion has been replaced with an impermeant ion or molecule of the same charge.

Modifying the composition of the extracellular microenvironment has also been used to analyze *in vitro* some basic mechanisms of epileptogenesis. Epileptiform discharges can be readily observed in hippocampal slices during perfusion with ACSF containing low $[Ca^{2+}]$-high $[Mg^{2+}]$ (Haas and Jeffreys 1984; Yaari et al. 1987), low $[Mg^{2+}]$ (Mody et al. 1987; Tancredi at al. 1988; Tancredi et al. 1990), high $[K^+]$ (Korn et al. 1987; Rutecki et al. 1985), or low $[Cl^-]$ (Chamberlin and Dingledine 1988;

Yamamoto 1972; Yamamoto and Kawai 1967, 1968, 1969). Interestingly, low Cl^- epileptogenesis represents the first *in vitro* model of epilepsy reported in the literature (Yamamoto and Kawai 1967). In this type of medium epileptiform discharges are presumably caused by the decreased efficacy of Cl^- potentials mediated through the -aminobutyric acid $(GABA)_A$ receptor (for review see Avoli 1988). To date, however, little was known about the cellular mechanisms that characterize low Cl^--induced epileptogenesis. Furthermore none of the previous studies had dealt with the activity generated by hippocampal pyramidal cells in the CA1 subfield.

This series of experiments was undertaken to fill these gaps. By the use of standard electrophysiological techniques, we analyzed some cellular and pharmacologic properties of the epileptiform discharges generated by CA1 pyramidal cells during perfusion with ACSF where most NaCl had been replaced with either Na-isethionate and Na-methylsulfate. A preliminary report of this work has appeared (Pumain et al. 1988).

METHODS

Preparation and incubation of the slice

Male Sprague-Dawley rats (150-300g) were decapitated under ether anesthesia and their brain quickly removed from the skull. The hippocampi were dissected free, and transverse slices (400-500 µm thick) were cut using a McIlwain tissue chopper. The slices were then transferred into a tissue chamber where they laid at the interface between oxygenated ACSF and humidified gas (95% O_2, 5% CO_2) at 34±1°C (SE). The composition of the normal 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 at pH 7.4. Low Cl^- ACSF was prepared by replacing 124 mM of NaCl with equimolar Na-isethionate or Na-methylsulfate. Because the concentration of cations decreases in the presence of impermeant anions (Chamberlin and Dingledine 1988; Dani et al. 1983; Louvel et al. 1988), in some experiments the low Cl^- ACSF contained 3.5 and 2.8 mM of $CaCl_2$ and KCl, respectively. When necessary, bicuculline methiodide (BMI, 5-20µM), picrotoxin (100µM), 3-3(2carboxy-piperazine-4-yl)propyl-1-phosphonate (CPP,1-5µM), or DL-2-amino-5-phosphonovalerate (APV,10-100µM) were also

added to the ACSF. All the chemicals were acquired from Sigma with the exception of Na-methylsulfate and CPP, which were obtained from ICN and Tocris Neuramine, respectively.

Extracellular and intracellular recording

Field potential and intracellular recordings were performed in the subregion b of the CA1 subfield. For intracellular recordings glass microelectrodes were filled with 4 M K-acetate (resistance, 60-80MΩ), whereas for extracellular recordings microelectrodes were filled with 2M NaCl (resistance, 2-10MΩ). The intracellular signals were fed to a high-impedance, negative- capacitance DC amplifier with bridge circuit that allowed current to be passed through the intracellular microelectrode. The bridge balance was carefully checked throughout the experiment and adjusted if necessary. In three slices field potentials were recorded simultaneously in the CA1 and CA3 subfield. Intracellular and extracellular data were displayed on an oscilloscope and/or on a Gould pen recorder. In the latter case a 20-MHz wave-form digitizer was often used to avoid distortion of the signals because of slow time response of the pen. In some instances data were recorded on FM tape for later analysis. In normal ACSF, pyramidal cells in the CA1 subfield displayed resting membrane potential more negative than -60mV (-67.4±5.2 mV, mean±S.D., n=12), apparent input resistance

of at least 15 MΩ (28.5±10.7 MΩ, n=16) and action potential amplitude larger than 85 mV (94.5±14.2 mV, n=16). Most recordings were stable for periods of 1 to 5 h.

Measurements with ion selective electrodes

Ion selective microelectrodes were prepared according to the methods described in Lux (1974) (see Annex for further details on ion selective microelectrodes). Double barreled pipettes were pulled and broken to a tip diameter of 1-2 µm. The reference channel was filled with NaCl (1M). The other channel had the tip silanized and was then filled with a resin selective for K^+. Ion selective microelectrodes were prepared shortly before the experiment. Each ion selective microelectrode was calibrated by using solutions of known ionic composition and was considered suitable if: (i) it was capable of giving a stable signal over a period of several minutes, and (ii) it was characterized by a near-Nernstian slope response (i.e.≥52 mV during a tenfold change in the concentration of K^+). The calibration was usually repeated at the end of the experiment to correct any possible loss of sensitivity of the ion selective microelectrode during the experiment. The signals were fed via Ag/AgCl wires to a Meyer and Renz (Frankfurt) amplifier and displayed on a Gould pen recorder. In agreement with

previous experiments performed in our laboratories (Louvel et al. 1988), the base line value of $[K^+]_o$ in low Cl^- ACSF was approximately 2.5 mM.

Stimulation and data analysis

Orthodromic stimuli (10-500 μA, 30-90 μs) were delivered through sharpened and insulated monopolar tungsten or bipolar stainless steel electrodes placed in stratum (s.) radiatum. Antidromic stimuli were delivered to the outer portion of the alveus, and a minimal intensity of current was employed to avoid current spread into s. oriens. In some experiments the CA1 subfield was separated from the CA3 region by a cut that extended from the alveus up to and including the lower part of the granule cells layer of the dentate area.

Most of the findings reported below were obtained during perfusion with low Cl^- ACSF containing Na-methylsulfate as substitute. However, because the results obtained with Na-isethionate were similar to those induced by Na- methylsulfate, the data were pooled.

RESULTS

Synaptic responses and epileptiform discharges in low Cl^- ACSF

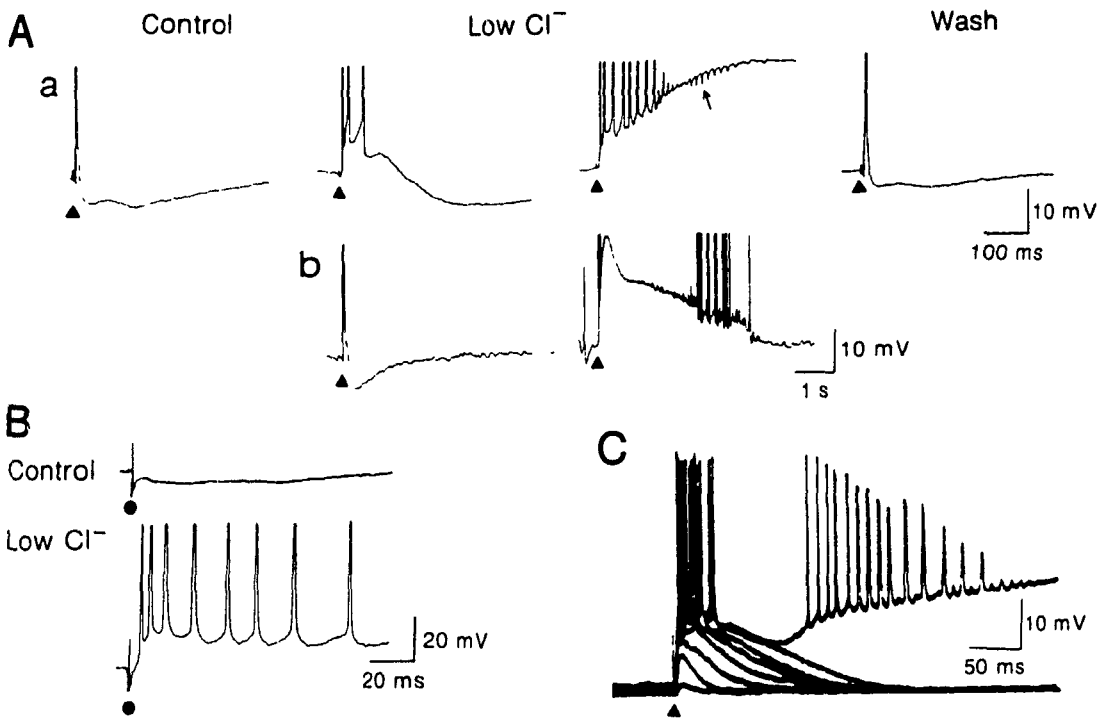
In keeping with previous experiments performed in the CA3 subfield and the dentate area of the *in vitro* hippocampal slice (Chamberlin and Dingledine 1988; Yamamoto 1972; Yamamoto and Kawai 1967, 1968, 1969), orthodromic and antidromic responses generated by CA1 pyramidal cells were transformed into epileptiform potentials during perfusion with low Cl^- ACSF. As illustrated in figure 1A, stimulation of the s. radiatum in low Cl^- ACSF evoked either a short-lasting epileptiform burst (SB) of action potentials (duration: 122±41 ms, mean±S.D., n=15) followed by a late hyperpolarization or a sustained, long-lasting epileptiform burst (LB; duration: 7.2±3.6, n=12). The latter type of epileptiform response was characterized by an initial burst of action potentials that was similar in shape to the SB but was followed by a depolarizing plateau of large amplitude. Action potentials decreased in amplitude during the onset of the plateau, and they reappeared during the repolarizing phase suggesting that they underwent a depolarization block. Similar epileptiform discharges were also evoked by antidromic stimuli

delivered in the alveus (Fig. 1B, see also Fig. 3B and 5, C and D). The effects induced by low Cl^- ACSF were fully washable within 30 minutes (Fig. 1A).

The ability of CA1 pyramidal cells to generate either a SB or a LB after electrical activation was dependent on the strength of the extracellular focal stimulus. As shown in figure 1C, stimuli delivered in s. radiatum evoked at first an excitatory postsynaptic potential (EPSP) that grew in amplitude and duration and turned into a SB as the stimulus intensity was progressively increased. LBs were induced only by stimuli of high strength. In addition, SB and LB displayed different refractory periods -an interstimulus interval longer than 20s was required to consistently induce a LB. Conversely, the SB could be evoked by stimuli delivered up to 1 Hz. When a high-intensity, orthodromic stimulus occurred during the refractory period of the LB, only a SB could be elicited (see for instance figure 1A).

The shape and amplitude of both SB and LB were modified by hyperpolarizing the membrane potential with intracellular injection of steady, DC negative current through K-acetate filled microelectrodes (not illustrated). However, the occurrence of action potentials (which in turn would activate repolarizing conductances) during the stimulus-induced epileptiform responses hampered any detailed analysis of the changes evoked by modifying the membrane potential. Therefore we repeated this type of

Figure 1


A
Control
Low Cl⁻
Wash
a
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B
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10 mV
50 ms

Figure 1 - A: Intracellular responses to orthodromic stimuli delivered in s. radiatum before (**Control**), during (**Low Cl^-**) and after washout (**Wash**) of low Cl^- ACSF. The epileptiform responses shown in **b** are the same as in a but at slow time base. Note that orthodromic stimuli in low Cl^- ACSF induce two types of burst: short-lasting epileptiform burst (**SB**, *left* in Low Cl^- panel) and long-lasting epileptiform burst (**LB**, *right* in the Low Cl^- panel). In this case the SB was evoked by an orthodromic stimulus that was delivered 11 s after a stimulus-induced LB (i.e. during the LB's refractory period). **B:** Intracellular responses induced by alvear stimuli before (**Control**) and during (**Low Cl^-**) perfusion with low Cl^- ACSF. **C:** occurrence of LB is dependent upon the intensity of the orthodromic stimulus. Stimuli of increasing strength induce an EPSP which grows in amplitude and is capable of generating a SB and a LB. In this and the following figures stimuli delivered in the alveus and stratum radiatum are indicated by filled circles and triangles, respectively.

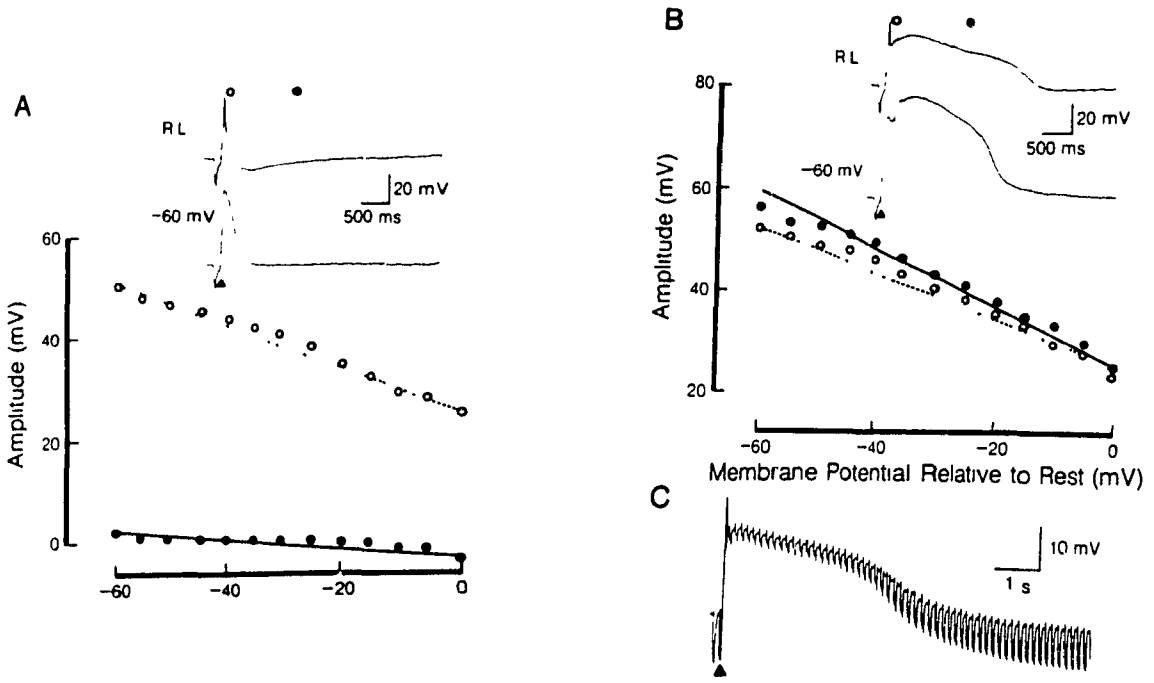

Figure 2 - Effects induced by varying the resting membrane potential upon the amplitude of the SB (A) and the LB (B) generated by a CA1 pyramidal cell that was recorded intracellularly with a microelectrode filled with QX-314. The samples on the top of each panel are actual responses recorded at resting potential (R.L.) and 60 mV hyperpolarization; in each panel the plot shown below was obtained by measuring the amplitude of the SB (A) and LB (B) 140 and 1400 ms following the orthodromic stimulus; the abscissa values indicate the membrane potential relative to rest. C: Changes in membrane conductance during a LB as measured by injecting hyperpolarizing pulses of intracellular current of constant intensity.

experiments by using microelectrodes filled with the lidocaine derivative QX-314, which blocks voltage-dependent Na^+ currents (Connors and Prince 1982). As shown in figure 2 (A and B), the amplitude of both SB and LB increased as a continuum during hyperpolarizations of the membrane up to 60 mV more negative than the resting potential value. Furthermore, this type of linear behavior could be appreciated when measuring the amplitude of both the early and late component of the LB. The membrane conductance tested by injecting intracellular pulses of hyperpolarizing current increased by 65-80% (n=3) during the long-lasting depolarizing plateau associated with the LB (Fig. 2C).

The intracellular epileptiform responses recorded in CA1 pyramidal cells were associated with a synchronous, field potential and an increase in $[K^+]_o$. As shown in part Aa of figure 3, LB was accompanied by a biphasic field potential (duration 5-15s) and a large, transient increase in $[K^+]_o$ (9.7±2.9 mM from a baseline of approximately 2.5mM, n=7 experiments). On the contrary, a brief, small-amplitude field potential and a small increase in $[K^+]_o$ ($\leq$2.5 mM, n= 5 experiments) were observed during the SB evoked by stimuli of low intensity or elicited during the LB's refractory period (Fig. 3Ab). In figure 3B one can also appreciate that the field potential and the

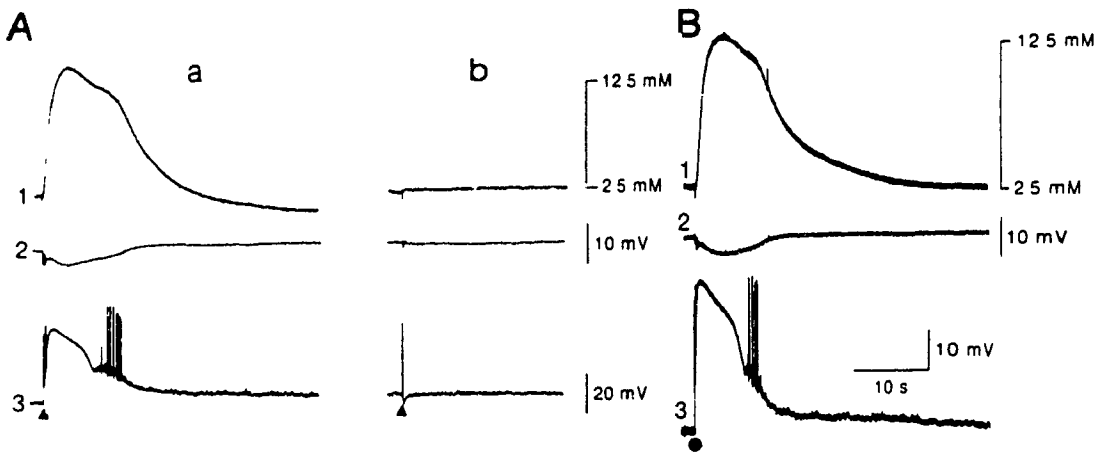

Figure 3 - Changes in $[K^+]_o$ (1), extracellular field (2) and intracellular (3) potentials simultaneously recorded in the CA1 subfield of an hippocampal slice perfused with low Cl^- ACSF following orthodromic (A) and antidromic (B) stimuli. In A, a and b show a LB and a SB respectively. Due to the slow time base, the extracellular and intracellular bursts associated with the SB are not discernible in Ab.

simultaneously recorded change in $[K^+]$ generated during an alveus-induced LB were similar to those observed after orthodromic activation.

Spontaneously occurring epileptiform discharges with shape and duration similar to the SB or the LB were observed in nearly 20% of over 90 slices perfused with low Cl^- ACSF (Fig. 4A and B, control). When field potentials were simultaneously recorded in the CA1 and CA3 subfields (n= 3 slices), similar epileptiform events were seen in both areas (Fig. 4A). The spontaneous discharges disappeared in the CA1 (Fig 4Ba, Cut) but persisted in the CA3 subfield (not illustrated) after surgical separation of these two regions. SB and LB could be elicited, however, in the isolated CA1 subfield after orthodromic stimuli (Fig. 4Bb, Cut).

Recordings performed at different sites along an axis normal to s. pyramidale (n=6 slices) revealed that the fastest and largest increases in $[K^+]_o$ during the LB occurred at the border between s. oriens and s. pyramidale (Fig. 5A). Furthermore, the field potential of the LB studied in these experiments reversed in polarity in the middle portion of s. radiatum, approximately 350-400μm from the s. pyramidale. However, the changes in amplitude of this field potential differed depending on the latency from the stimulus at which measurements were performed. The early part of the LB that coincided with the initial burst

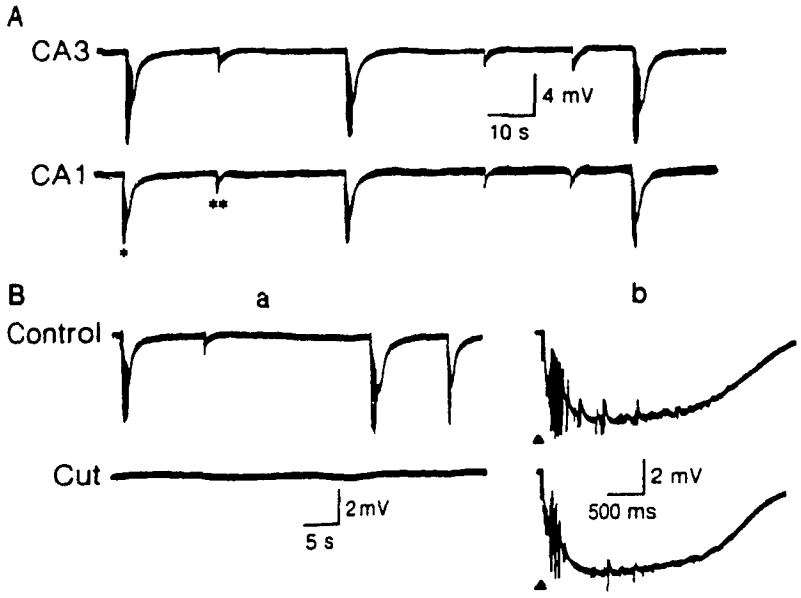

Figure 4 - **A**: Spontaneous epileptiform discharges recorded simultaneously in the CA3 and CA1 subfield by two extracellular microelectrodes placed in the s. pyramidale. Note that similar types of epileptiform events occur at the same time in the two areas. One LB and one SB are indicated in the CA1 trace by a single and double asterisk, respectively. **B**: Effects induced by separating the CA1 from the CA3 subfield (Cut) on spontaneous and stimulus-induced low-Cl^- epileptiform bursts. In **a**, note that the spontaneous epileptiform events recorded in the CA1 subfield of the intact slice (Control) disappear after the surgical separation of this area from the CA3 subfield (Cut). However stimulus-induced discharges in the CA1 area are not affected by this procedure (**b**).

FIGURE 5

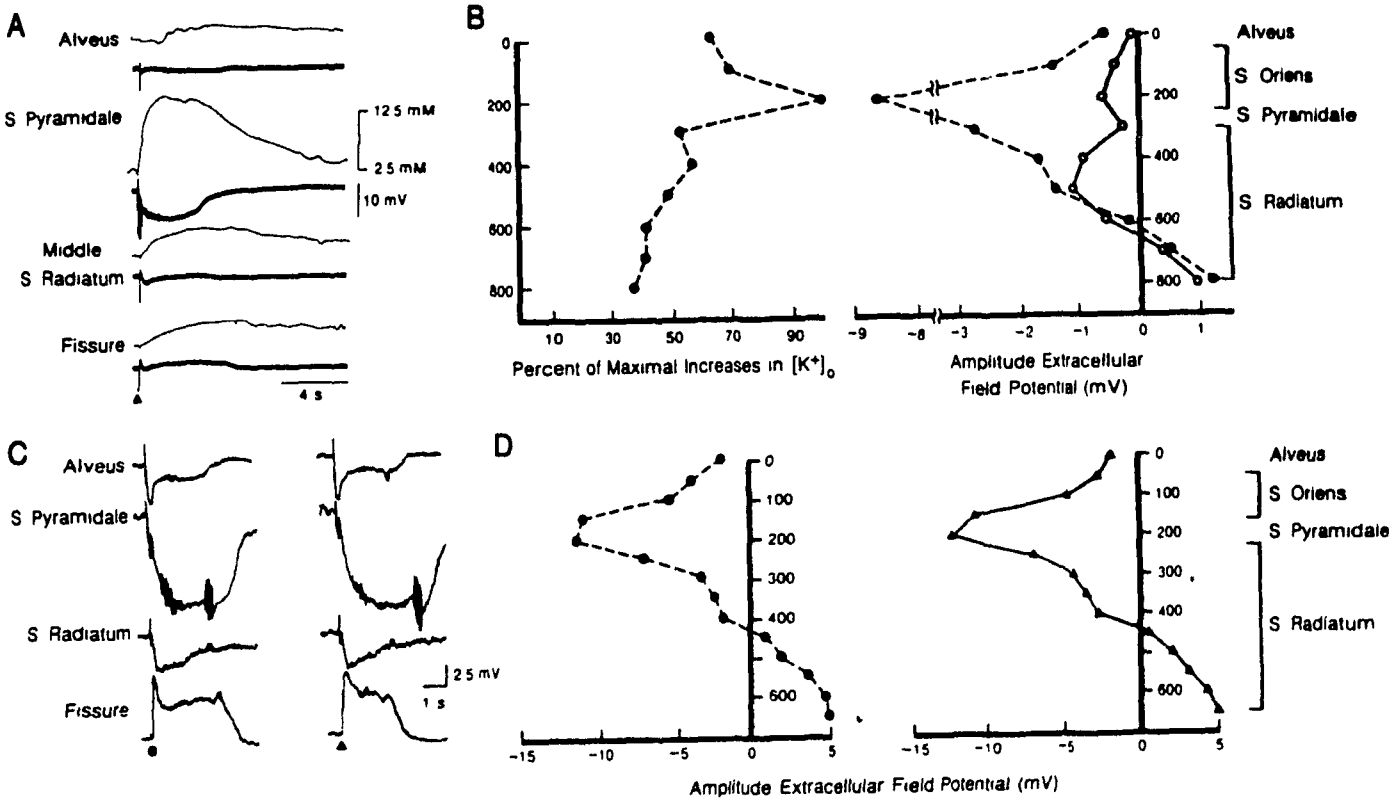
A
Alveus
S Pyramidale
12.5 mM
2.5 mM
10 mV
Middle
S Radiatum
Fissure
4 s
B
Percent of Maximal Increases in $[K^+]_o$
Amplitude Extracellular Field Potential (mV)
Alveus
S Oriens
S Pyramidale
S Radiatum
C
Alveus
S Pyramidale
S Radiatum
Fissure
2.5 mV
1 s
D
Amplitude Extracellular Field Potential (mV)
Alveus
S Oriens
S Pyramidale
S Radiatum

Figure 5 - A: Changes in $[K^+]_o$ (upper trace) and extracellular field potentials (lower trace) induced by orthodromic stimuli and recorded at different sites (as indicated on the left) along an axis normal to the s. pyramidale. **B:** Plot of the of the changes in $[K^+]_o$ (as percent of the maximal increase) and of the amplitudes of the extracellular field potentials during the LBs shown in **A.** Measurements were made at 100ms (empty circle) and 2000ms (filled circle) from the stimulus artifact. **C:** similar depth profiles characterize the LBs induced by electrical stimuli delivered in the alveus (*left*) and s. radiatum (*right*). **D:** plots of amplitudes of extracellular field potentials shown in C and measured 2000ms from the stimulus artifact. *Right* and *left* panels represent data obtained from LBs induced by stimuli delivered in the alveus and s. radiatum, respectively.

of action potentials, displayed maximal negativity in the middle portion of the s. radiatum, i.e., where the stimulating orthodromic electrode had been placed. Conversely, the late component attained maximal negative values near the s. pyramidale, i.e., at the site where the largest and most rapid increases in $[K^+]_o$ were seen (Fig. 5B). That stimuli delivered in the s. radiatum and in the alveus gave similar LBs is further shown in the experiment of Fig.5 (C and D). As can be appreciated there, LBs elicited by alvear stimuli had depth profiles that were indistinguishable from those induced by orthodromic activation (n=3 experiments). One should also note that the depth profile of the field potential associated with the s. radiatum-induced SB was similar to the one observed when plotting the amplitude of the extracellular potential of the early part of the LB induced by similar stimuli (not illustrated).

The electrophysiological features of the LB were different from those of spreading depression recorded during perfusion with low Cl^- ACSF. In this type of medium, spreading depression was at times seen after single-shock stimulation and could be consistently induced by train of stimuli. As shown in the experiment of figure 6A, both the extracellular and the intracellular potentials as well as the changes in $[K^+]_o$ recorded during a LB were far less intense and prolonged than those

occurring during spreading depression evoked in the same slice by a brief (10 s) train of stimuli at 2 Hz. The peaks in $[K^+]_o$ measured in the s. pyramidale of five slices during LB and spreading depression were 9.4±2.8mM and 99.7±42.4mM respectively. Furthermore, during spreading depression the input resistance measured by injecting hyperpolarizing pulses of current became immeasurably low (not illustrated). Finally, a different velocity of propagation characterized LB and spreading depression (Fig. 6B). These measurements were performed by recording in the CA1 subfield at two sites located 1.5-2.5 mm apart while eliciting LB or spreading depression by stimulating the CA3 area. The velocity was then estimated by dividing the delay between the two events (as determined by taking as beginning of the potential the half-amplitude of the onset deflection) by the distance between the two recording points. When studied in the same slices (n=2), the LB propagated at 0.016-0.16 m/s (n=9 LBs), whereas the spreading depression displayed a velocity of $1\text{-}2\cdot10^{-4}$ m/s (n=2 spreading depressions)(see also Snow et al. 1983). Furthermore, in these experiments we could also observe that SB propagated at 0.14-0.28 m/s (n=9 SBs).

Because $[K^+]$ and $[Ca^{2+}]$ appear to be lower in the low Cl^- ACSF as compared to the normal medium (Chamberlin and Dingledine 1988; Dani et al. 1983; Louvel et al. 1988),we analyzed to which extent these changes in

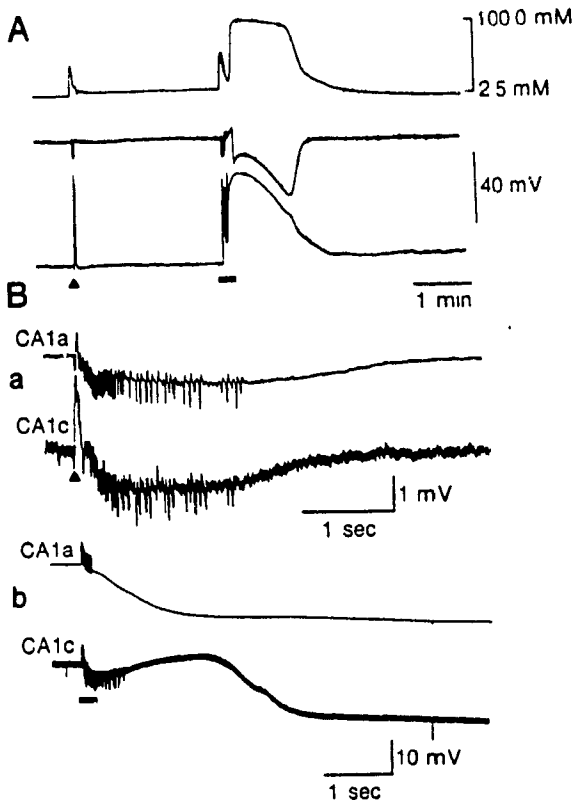

Figure 6 - A:Changes in $[K^+]_o$ (upper trace), extracellular (middle trace) and intracellular (lower trace) potentials simultaneously recorded in the CA1 subfield during perfusion with low Cl^- ACSF. Note the different amplitude and duration of the SB induced by a single orthodromic stimulus (triangle) when compared with spreading depression elicited by a train of stimuli delivered in s. radiatum at 2 Hz (bar). B: Different velocities of propagation characterize the LB (a) and the spreading depression (b) along the CA1 subfield. LB and SD were elicited by a single shock stimulus and a train at 2 Hz (bar), respectively. In both cases the microelectrodes were placed 1.5 mm apart.

ionic concentrations could contribute to the epileptiform responses seen in low Cl^- ACSF. When $[K^+]_o$ in the low Cl^- ACSF was restored to a value close to that of the normal ACSF by adding 0.8mM KCl, the LB increased in duration by 78±54% (n=5 experiments). Conversely, when $CaCl_2$ in the ACSF was brought to 3.5mM, the duration of the LB decreased by 57.6±8.6%, whereas the threshold for eliciting LB or SB increased (n=3 experiments). Finally, when the free concentration of both cations was adjusted in the low Cl^- ACSF as in normal medium, no difference in duration could be observed (n=5 experiments). The changes in duration were not accompanied by any consistent modification of the amplitude of the extracellular field potential or of the pattern of the associated synchronous firing (i.e. population spikes).

Pharmacological properties of the epileptiform responses evoked in low Cl^- ACSF.

The results obtained by analyzing the field potential profile of the epileptiform responses recorded in low Cl^- ACSF indicate that the late, negative component of the LB is maximum at the border between s. pyramidale and s. oriens. This zone is the site where GABAergic synapses between interneurons of the recurrent inhibitory pathway and pyramidal neurons are localized (Andersen et al. 1964a,b) suggesting that an outward movement of Cl^- ions

through channels coupled to $GABA_A$ receptors might occur during the prolonged depolarizing component of the LB. We studied therefore the effects exerted by pharmacologic blockade of the $GABA_A$ receptor on the low Cl^- epileptiform. This was achieved by adding BMI or picrotoxin.

As shown in figure 7A, the prolonged and sustained depolarization associated with the LB could not be elicited in medium containing BMI even following stimuli of intensity 16-fold greater than in control (n=2). In this experimental condition the initial burst of action potentials was followed by a long-lasting hyperpolarization similar to the one seen after a SB. Similar results were also obtained during application of picrotoxin (n=2 cells).

It was also evident in these experiments that the SB elicited by high-intensity stimuli during the refractory period decreased in duration during application of either $GABA_A$ receptor antagonist (Fig. 7B). The latter effect was further confirmed by analyzing the effects induced by BMI on the depolarizing response evoked by stimuli delivered at low intensity (Fig. 7C). As shown in figure 7D, the depressant effects evoked by BMI on the low Cl^- epileptiform responses were also appreciated by recording simultaneously the extracellular field potential

Figure 7

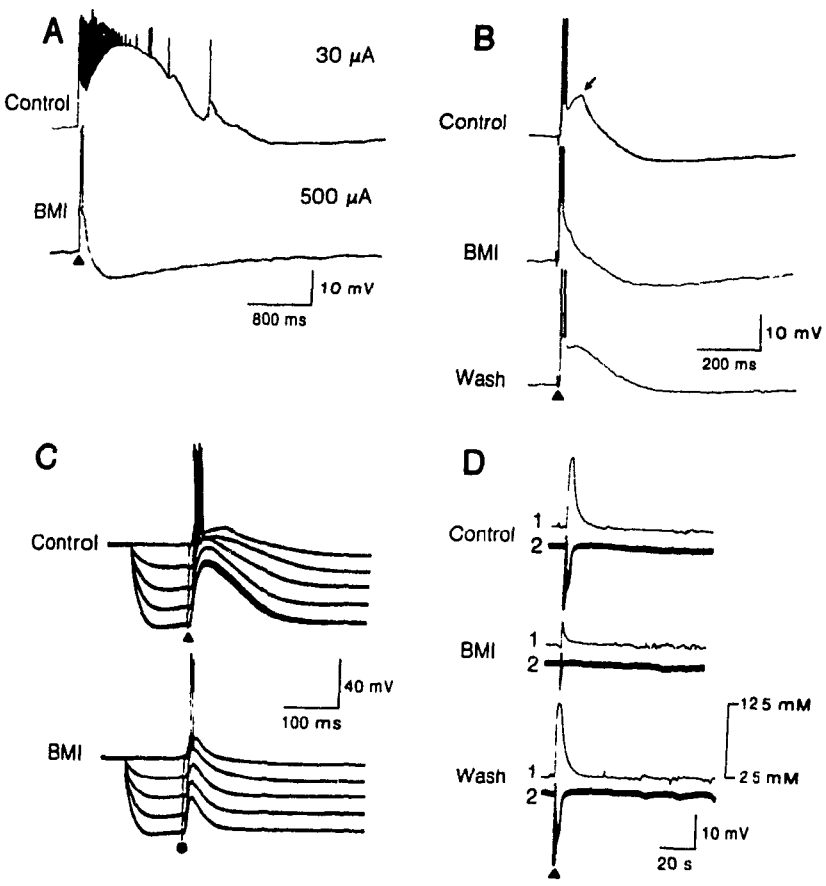
A
30 μA
Control
BMI
500 μA
10 mV
800 ms
B
Control
BMI
Wash
10 mV
200 ms
C
Control
40 mV
100 ms
BMI
D
Control
1
2
BMI
1
2
Wash
1
2
125 mM
25 mM
10 mV
20 s

Figure 7 - Effects induced by bath application of bicuculline methiodide (BMI) upon the epileptiform activity recorded in low Cl^- ACSF. A and B panels show LBs and SBs which were recorded intracellularly in the same neuron. Note in A that BMI blocks the LB which fails to occur even when higher intensity stimuli are used. In B one can appreciate that BMI reduces the depolarizing envelope of the SB (arrow in control). This effect of BMI is more clearly illustrated in C where this drug was tested on the synaptic depolarization underlying SBs evoked by low strength stimuli and recorded at different membrane potentials as obtained by intracellular injection of hyperpolarizing pulses. D: Changes in $[K^+]_o$ (1) and extracellular potential (2) simultaneously recorded in the s. pyramidale before (**Control**), during (**BMI**) and after washout (**Wash**) of 20uM BMI.

and/or the changes in $[K^+]_o$ (n=8 experiments). The effects of the two $GABA_A$ antagonists were reversible after washout with control low Cl^- ACSF.

The involvement of the N-methyl-D-aspartate (NMDA) receptor in epileptogenesis has been pointed out in several models of epilepsy (Avoli 1989; Dingledine 1986; Dingledine et al. 1986; Hwa and Avoli 1988; Mody et al. 1987; Tancredi et al. 1988; Tancredi et al 1990). We analyzed therefore the effects induced by the NMDA receptor antagonists APV and CPP on the low Cl^- epileptiform discharges. Intracellular recordings performed in three CA1 pyramidal cells revealed that the late, sustained depolarizing component of the LB induced by high-intensity stimuli was reduced and eventually blocked by either CPP or APV (Fig. 8A, 30μA). However, in the presence of these NMDA receptor antagonists an epileptiform response similar to the one elicited in control low Cl^- ACSF could still be elicited when the stimulus intensity was further increased (Fig. 8A, 200μA). Furthermore, CPP or APV did not affect the SB (Fig. 8B). In the latter case one could also appreciate that the synaptic potential underlying the SB as disclosed by hyperpolarizing the membrane potential with intracellular injection of current was not reduced (Fig. 8C). Similar results were also obtained by the use of

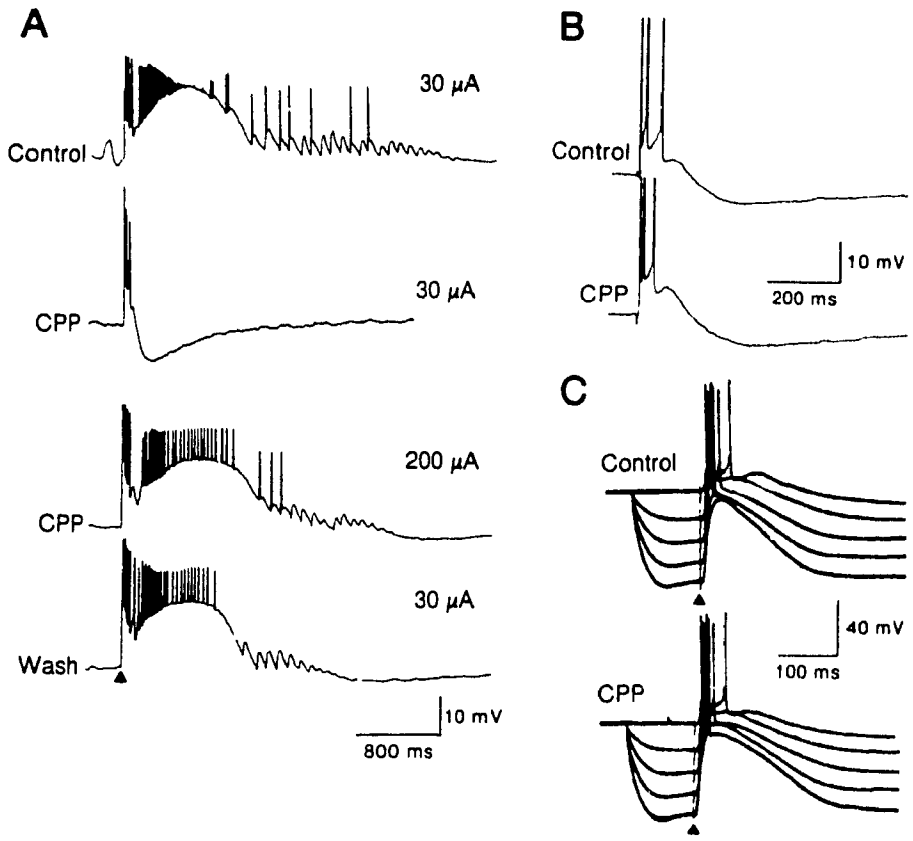

Figure 8 - Effects induced by CPP (5µM) upon LB (**A**) and SB (**B** and **C**) recorded intracellularly in two different CA1 pyramidal cells. Note that the block of the LB is dependent upon the stimulus strength since a similar LB as in control low Cl^- ACSF is evoked by orthodromic stimuli delivered at higher intensity. Note also the lack of consistent effects induced by CPP on the SB which can be also be appreciated when studying the synaptic envelope underlying the SB (**C**).

extracellular field-potential recordings in nine slices perfused with low Cl^- ACSF containing one of the two NMDA receptor antagonists.

DISCUSSION

Epileptiform discharges in low Cl^- ACSF

Two types of epileptiform responses are observed in low Cl^- ACSF after electrical activation of antidromic or orthodromic pathways. The first type, termed SB, is characterized by a brief depolarization associated with synchronous discharge of action potentials and is followed by a hyperpolarization. The second type, called LB, is composed of an early component similar to the SB but followed by a late, prolonged depolarization.

In addition to displaying similar shapes, the SB and the early component of the LB share many other features. First, they increase in amplitude during hyperpolarization of the membrane potential, behaving as expected from a conventional synaptic potential. Second, both epileptiform events are associated with a field potential that attains its maximal amplitude in the s. radiatum (namely, at the same level of the pathway activated by electrical stimuli), indicating that the excitatory input resides presumably in the Schaffer's collaterals. Third, both responses are reduced, not blocked by application of BMI or picrotoxin suggesting that they are only partially mediated by an inverted GABAergic potential such as the recurrent, somatic inhibitory postsynaptic potentials (IPSP). Fourth, blockade of the

NMDA receptor with either APV or CPP does not modify these two types of epileptiform events, indicating that a receptor subtype other than the NMDA is involved in their generation. Therefore we can conclude that the SB and the early part of the LB represent a mixture of fast EPSP because of synaptic inputs located in the apical dendrites of CA1 pyramidal cells and early IPSP.

A different mechanism appears to be responsible for the appearance of the late, long-lasting component of the LB. Its extracellular field potential attains in fact maximal negative values at or near the border between s. pyramidale and s. oriens -not in s. radiatum as is the case for the early part of the LB or the SB. Furthermore, there is a close correlation between the maximal negativity of the field potential associated with the late component of the LB and the maximal increase in $[K^+]_o$. These findings indicate therefore that the site where the maximal depolarization occurs during the late component of the LB is localized close to the soma of CA1 pyramidal cells. Furthermore, the experiments performed with the $GABA_A$ receptor antagonists BMI and picrotoxin indicate that the generation of this component requires the functioning of GABAergic synapses. Therefore we propose that this long-lasting depolarization is caused by Cl^--mediated, GABAergic conductances that are due to the intense and prolonged firing of inhibitory interneurons forming

synapses at or close to the soma of hippocampal pyramidal cells (Andersen et al. 1964a,b). However, the firings obtained to date do not exclude that a GABAergic potential might be only necessary for triggering the LB process. Namely, it might be solely responsible for initiating a phase of increased excitability during which other synaptic, presumably reverberating, mechanisms would induce inward currents associated with the long-lasting depolarization that underlies the LB.

In the course of the present experiments we have also considered the possibility that the appearance of epileptic discharges in hippocampal slices perfused with low Cl^- ACSF might result from the lower concentration of free mono- and divalent cations that occurs in this type of medium. More specifically, we were concerned by the decrease in Ca^{2+} and K^+, because both ions have been implicated to play a role in the physiopathogenesis of epileptiform discharges (Lux et al. 1986; Oliver et al. 1978). The experiments in which one or both cations were brought close to the values normally present in the ACSF suggest, however, that this type of ionic imbalance is not sufficient to cause the appearance of epileptiform discharges. Nonetheless, our data indicate that Ca^{2+} and K^+ might play a modulatory role -increasing the $[K^+]$

in the ACSF caused a prolongation of the LB whereas, when Ca^{2+} was compensated, a decrease in the intensity of epileptiform activity was observed.

NMDA-activated conductances and low Cl^- epileptiform activity

The results obtained with CPP and APV indicate that NMDA receptors are in part involved in the triggering of the late component of the LB since both antagonists produced an increase in the threshold for the appearance of such paroxysmal event. This type of effect might suggest an action exerted by CPP or APV on the excitatory synapses that exist between the pyramidal cells and the inhibitory interneurons of the recurrent system. This conclusion is supported by recent studies indicating that in the CA1 subfield of hippocampal slices bathed in low Mg^{2+} ACSF, APV -in addition to block the epileptiform burst- also reduces in a reversible way the antidromic, somatic IPSP (Tancredi et al. 1988; Tancredi et al 1990). More recently we have observed that CPP is capable of reducing the antidromic IPSP elicited in normal ACSF (G. Hwa and M. Avoli, unpublished data).

Our experiments also show that NMDA receptors are not involved in the mechanism underlying both SB and the early component of LB. Experiments in the CA1 area have indicated that the duration of the short-lasting

epileptiform discharge generated by pyramidal cells bathed in BMI is reduced during perfusion with APV (Dingledine et al. 1986). Similar results have been observed in the rat neocortex treated with BMI (Hwa and Avoli 1988). One difference between the BMI and the low Cl^- models resides in the fact that in the latter case inhibitory conductances activated by $GABA_A$ receptors remain present, though the resulting potentials are inverted. On the contrary, after BMI application $GABA_A$ receptors and the resulting increase in conductance are decreased or blocked. Therefore in our experiments the relief of the NMDA ionophore from the gating effect of Mg^{2+} (Novak et al. 1984) during the depolarization associated with the SB might still be under the influence of the large increase in conductance caused by the efflux of Cl^-.

Is the LB a spreading depression-like event?

Spreading depression has been considered to represent an experimental model of epilepsy (Leão 1972). Furthermore, it has been recently suggested that long-lasting epileptiform discharges recorded in immature rat neocortex during perfusion with picrotoxin might share a number of traits in common with those of spreading depression (Hablitz 1987). However, the results obtained in the present experiments suggest that the latter conclusion does not apply to the LB recorded in low Cl^- ACSF. When LB and spreading

depression were compared in the same experimental condition (i.e. during perfusion with low Cl^- ACSF), marked differences could be observed between these two phenomena. First, the decrease in input resistance during spreading depression is consistently larger than the one associated with the long-lasting depolarization of LB. Second, the field-potential shift and the increase in $[K^+]_o$ are by average two to four times those seen during the LB. Measurements of $[K^+]_o$ during spreading depression in different preparations indicate values that are within the range observed in our experiments (Balestrino et al. 1989; Nicholson et al. 1978; Somjen et al. 1985). Third, the different values of the velocity of propagation of the two events in the CA1 subfield of the hippocampal slice suggest that a slow phenomenon such as K^+ redistribution (Gardner-Medwin 1983; Gardner-Medwin and Nicholson 1983; Yaari et al. 1987) might represents the main factor for the propagation of the spreading depression wave, whereas different, faster mechanisms should be responsible for the spread of the LB. Interestingly the velocity of propagation of the LB was lower that the one of the SB. The values observed in the latter case suggest that the SB spread is dependent on a mechanism of synaptic transmission with nerve conduction.

Possible additional mechanisms

Our results indicate that the main factor responsible for the appearance of low Cl^-, synchronous epileptiform discharges resides in the inversion of potentials mediated through the $GABA_A$ receptor. However, there are several additional mechanisms that are likely to contribute to the genesis of these epileptiform discharges. First, some effects observed in low Cl^- ACSF are presumably mediated through the decreased concentration of free cations that occurs in this type of medium (Chamberlin and Dingledine 1988; Dani et al. 1983; Louvel et al. 1988). Second, Cl^- substitutes can modify the intracellular and/or extracellular pH (Sharp and Thomas 1981). Third, it has been shown that voltage-dependent membrane functions are shifted in the negative direction as the result of the direct action exerted by lyotropic anions on the membrane (Dani et al. 1983; Hodgkin and Horowicz 1959, 1960; Koppenhöfer 1965). Finally, in low Cl^- ACSF one would predict that voltage- and Ca^{2+}-dependent Cl^- conductances (Madison et al. 1986; Owens et al. 1986) would be blocked or largely decreased. The exact contribution of these additional mechanisms to the changes in excitability observed in the CA1 subfield of the rat hippocampal slice still awaits further analysis.

Relevance of the present findings to epileptogenesis

Several studies have shown that during epileptiform activity the extracellular microenvironment undergoes marked changes in size as well in the concentration of ions (for review see Lux et al. 1986). Direct measurements with ion selective microelectrodes have revealed that $[Cl^-]_o$ increases by up to 7 mM. However, this increase is due to shrinkage of the extracellular space, and, in fact, it has been estimated that approximately 35mM of Cl^- could leave the extracellular space during seizure activity (Dietzel et al. 1980, 1982; Lehmenkuehler et al. 1982) Furthermore, if one considers that some of this Cl^- will enter the neuron (Deisz and Lux 1982; Lux 1974), the transmembrane gradient for Cl^- during seizure activity might approximate the one recreated experimentally in this study. Therefore some of the cellular phenomena reported here might help in understanding the changes in excitability that accompany intense epileptiform activity. Finally, some support to the possible relevance of our findings comes from studies in which the transmembrane gradient for Cl^- was modified by ammonia. In some neuronal systems ammonia block an outwardly directed Cl^- pump (Aickin et al. 1982; Llinas et al. 1974; Lux 1971), and in both humans and laboratory animals induces an encephalopathy that is accompanied by seizures (Ishida 1967; Sherlock et al. 1954).

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GENERAL CONC- USION

Although there is no such pathology as hypochloremia reported in the literature, the study of the model of low Cl^- epileptogenesis is relevant to human epilepsy. Most of the models of epileptogenesis not caused by the impairment of GABAergic functions revealed that, in spite of clear epileptiform activity, GABAergic inhibition remained functional (Chagnac-Amitai and Connors 1989; Malouf et al. 1990; Higashima 1988). That is, GABA is still present in the tissue, inhibitory post-synaptic potentials (IPSP) can be recorded, and application of GABA antagonists modulate the neuronal activity. However, the question that remains to be asked is whether inhibition always remains optimally functional and stable, or whether, in spite of the presence of GABAergic inhibition, the efficacy of the latter fluctuates generating periods of "partial inhibition" that could lead to the appearance of epileptiform activity.

For instance, we have preliminary data obtained from experiments performed on human tissue obtained from epileptic foci of patients refractory to conventional anticonvulsive therapy. Bathing the human tissue in medium containing no Mg^{++} ions leads to spontaneous epileptiform activity (Avoli et al. 1987; 1991). We found that in this preparation, in spite of clear epileptiform activity,

inhibition was still functional as shown by the presence of stimulus-induced and spontaneous IPSP's that could be blocked by GABA antagonists, and by the effects of GABA antagonists on the bursting activity. However, while recording spontaneous IPSP's, we found that the frequency of the IPSP's had a strong tendency to decrease just prior to the onset of a burst. This suggests that periodic drops in the efficacy of GABAergic inhibition could initiate bursting activity.

We demonstrated that the physiological driving force for chloride in the nervous tissue is essential for the integrity of the GABAergic inhibitory mechanisms. Thus we may conclude that any variation in the driving force for chloride ion may render the tissue hyperexcitable and prone to develop epileptiform discharges.

As mentioned above, there is no such pathology as hyperchloremia. However, punctuated variations in the chloride equilibrium may occur, for example, during episode of high intensity of firing (for review see Lux et al. 1986), or during ammoniac intoxication (Ishida 1967; Sherlock et al. 1954).

This suggests that in humans, an episode of epileptic seizure may modulate the chloride equilibrium sufficiently to decrease the efficacy of the GABAergic inhibition, beyond a critical threshold.

Velocity of conduction

As mentioned in the results, the SB propagated at a velocity suggesting that the spread of the SB was dependent on normal mechanisms of synaptic transmission and nerve conduction. However, the LB propagated at a velocity up to ten times slower than the SB, suggesting other mechanisms of propagation. Beside the conventional propagation of nerve impulse through synaptic transmission and nerve conduction, there are three other mechanisms of propagation that can be considered: a) fluctuations in the extracellular concentration of ions i.e. increases in the extracellular concentration of potassium and diffusion of potassium; b) transmission through electrical synapses; c) field interactions.

It is unlikely that accumulation of potassium in the extracellular space and diffusion of potassium in the tissue could account for the propagation of the LB. In our experiments, the onset of the LB was never preceded by an increase in the extracellular concentration of potassium ion. The velocity of propagation was also too high to account on the simple diffusion of potassium ions in the interstitial fluid. Spreading depression that propagates at a velocity about 1000 times slower than the LB is more likely to be dependent on the accumulation and diffusion of potassium outside the neurons (Leão 1972). Furthermore,

during spreading depression, the extracellular concentration of potassium reaches a plateau that is about ten times higher than during the LB.

However, it is reasonable to think that the increase in the extracellular concentration of potassium facilitated the propagation of the LB. This is suggested by experiments using the Low Ca" High Mg" model of epileptogenesis, where synaptic transmission is blocked. In these experiments, bursting activity was never observed in 3mM of potassium. The concentration of potassium had to be increased to 6-9mM to induce bursting (Haas and Jeffreys 1984).

In the CA1 subfield of the hippocampal slice, there is evidence for electrical coupling between pyramidal neurons. This evidence derives from experiments where intracellular dye injection revealed dye-coupling, and from paired intracellular recordings (MacVivar et al. 1982; Andrew et al. 1982). However, during dye injection, only 2-3 neurons could be stained at once. It is hard to conceive that such a limited interaction could lead to the synchronization of a large population of neurons. Indeed, computer modelling suggested that this condition is not sufficient for synchronization, at least in the Low Ca" burst (Haas and Jeffreys 1984) and in the penicillin burst (Traub and Wong 1982). Electrical coupling could however modulate the strength of synchron ation.

Field interaction takes place when extracellular current generated by the activity of a set of neurons located in a close vicinity modulates the activity of another set of neurons. The flow of the current in the extracellular space, and moreover the passage of the current from the extracellular space to the intracellular space through the membrane depend on the relative impedance of both mediums. The impedance of the medium is a function of the conductivity of the extracellular space, the tortuosity of the extracellular space and its size, the space constant of the neuronal processes in the direction of the current, and, in the present case, the laminar orientation of the pyramidal neurons in the hippocampal structure. Furthermore, in the CA1 region of the hippocampus, pyramidal neurons are densely packed with a relative absence of intervening neuroglia (Green 1964; Green and Maxwell 1961).

In theory, given that the firing threshold of pyramidal neurons is reached within 0.5-1.0ms and that CA1 pyramidal neurons are distributed approximately 20µm apart, if a group of pyramidal neurons generating a population spike recruited the immediately adjacent neurons, the highest velocity of propagation possible via field interactions can be estimated to 20mm/s. Indeed, several factors have to be considered in order to do a more precise estimation of the velocity of propagation. However, 20mm/s

is in the range of 16-160mm/s that was experimentally observed for the propagation of the LB. It is then likely that the LB may have propagated via field interactions.

NMDA Receptors

It has been observed that NMDA antagonists block the late part of the LB, but not the SB nor the early part of the LB. However, when the intensity of the stimulation was increased, the NMDA antagonist was no longer effective in blocking the late part of the LB. Thus, although NMDA receptors could modulate LB, its expression did not depend upon the activity of NMDA receptors. Since the late part of the LB was totally dependent on the activity of GABAergic inhibition, this suggests that NMDA receptors might modulate the activity of GABAergic neurons. This goes in line with evidence that NMDA receptors mediate the synaptic transmission at the synapse between the GABAergic interneuron and the soma of the pyramidal neuron (see Sah et al. 1990 and Tancredi et al. 1990). The bursting activity of the pyramidal neurons would activate the GABAergic interneurons that synapse recurrently onto the pyramidal neurons.

Increasing the intensity of stimulation could: a) overcome the blockade of the NMDA receptors since the antagonists used were competitive; b) increase the contribution of the non-NMDA receptors in the synaptic

transmission between interneurons and pyramidal neurons; and c) directly activate the GABAergic interneurons in the vicinity of the stimulating electrode, and the LB would then propagate via field interactions independently of the NMDA receptors.

Termination of the Bursts

We observed that the various substituents for chloride had the ability to decrease the free concentration of potassium and calcium in the artificial cerebrospinal fluid. However, adding the appropriate quantity of potassium and calcium, to restore a free concentration of 3.25mM and 2.0mM, respectively, we demonstrated that the decrease in the free concentration of these cations was not responsible for the appearance of the epileptiform events. Epileptiform activity developed even when the slices were bathed from the beginning of the experiment in a free concentration of potassium and calcium of 3.25mM and 2.0mM, respectively.

However, we observed that increasing both potassium and calcium concentration modulated the duration of the bursts. Increasing the concentration of potassium increased the duration of the bursts. Increasing the concentration of calcium decreased the duration of the bursts. This strongly suggests that the termination of the burst was dependent on Ca^{++}-dependent K^+ conductances (I_{AHP}; see Storm 1987; Alger

and Williamson 1988; Schwindt et al. 1988). It further suggests that the duration of the burst (5-15s) depends on the accumulated extracellular concentration of potassium and on the buffering capacity of the neuroglia for potassium. The more rapidly potassium is removed from the extracellular space, restoring the proper potassium equilibrium, the more rapidly the Ca^{++}-dependent K^{+} conductances can bring the membrane potential to resting value. We did not, however, studied the relation between the peak change in potassium concentration and the duration of the bursts.

Epileptiform Activity and pH

An increase in the intracellular pH was reported to promote bursting activity by increasing the frequency of the bursts (Haas and Jeffreys 1984). Conversely, acidification of the intracellular medium was demonstrated to uncouple cells (Orkand et al. 1981; Spray et al. 1981) and to reduce current dispersion, decreasing the bursting frequency in the Low Ca^{++} burst model of epileptogenesis (Haas and Jeffreys 1984). This is important, for substitution of chloride ions was shown to affect the intracellular pH (Sharp and Thomas 1981).

Various substituents of chloride were used in the present work. However, most of the experiments were done

using Na-methylsulfate and Na-isethionate. Methylsulfate and isethionate can be considered as anions of strong acids with a pKa smaller than 1.0 and 1.25, respectively (Sharp and Thomas 1981). Thus, it is possible that the substitution of chloride by methylsulfate and isethionate led to an intracellular acidification, facilitating the expression of epileptiform activity. However, it was reported that as a general principle, anions of strong acids have little effects on the intracellular pH (Sharp and Thomas 1981).

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Appendix

Ion Selective Microelectrode (ISM)

Essentially, the ISM are tubes of glass with an artificial selective membrane built at the tip. The membrane being selective, it behaves to various ionic concentration according to the Nernst equation:

$$E = \frac{S \log[\,]_f}{\log[\,]_i} \qquad \text{where } S = \frac{2303\ RT}{zF}$$

and E= potential (mV)

R= gas constant (8.31441 J/K mol)

T= thermodynamic temperature (K)

F= Faraday constant (96485 (C/mol)

z= valence of the ion measured

i= initial

f= final

This equation can be transformed to,

$$[\,]_f = [\,]_i \cdot 10^{E/S}$$

Finding the variable S by exposing the ISM to various known ionic concentrations allows to calibrate the ISM. Figure 1 shows an example of a calibration where an ISM selective to K^+ was exposed to concentrations of 3mM and 30mM successively. The change in potential generated was 55mV. Solving the equation for S,

$[\,]_i$ = 3mM

$[\,]_f$ = 30mM **S = 55mV / log unit**

E = 55mV

Thus to determine the concentration of K^+ in the course of an experiment using such an ISM, we use the following equation,

$$[\,]_f = [\,]_i \cdot 10^{E/55}$$

where $[\,]_i$ is assumed to be the concentration of K^+ added to the ACSF or the concentration of K^+ as measured in the solution when perfusing low Cl^- ACSF (namely 2.5mM).

The ISM were confectioned as follows. Capillary tubes (1.5 mm O.D.) were thoroughly washed in sulfuric acid and hydrogen peroxide, rinsed in distilled water and acetone, and dried in an oven. Half of the tubes were

slightly bent under the flame at one end and glued to another straight tube with dental cement or epoxy. The double-barrel electrodes were then pulled using a vertical puller.

After the ISM have been pulled they were filled with the appropriate solutions. Reference channel was filled with 100mM NaCl or ACSF, Ca^{2+}-selective and K^{+}-selective channels were filled with 100mM $CaCl_2$ and 100mM KCl, respectively. Under light microscope, the tip was broken to reach a tip diameter of 1-5µm. The ion selective channel was then connected to high and low pressure devices through a series of valves. Under the light microscope, the tip of the electrode was silanized with a solution of 10% silane in trichlorotrifluoroethane, and the ion selective resin was gently moved by suction into the tip of the channel to form a column of 100-200µm. A chlorinated silver wire was introduced into each barrel and fixed with wax. Each ISM was then calibrated according to the equation described above, using solutions of known ionic composition.

Figure 1

Calibration of an ion selective microelectrode (ISM) selective to K^+. The ISM is successively exposed to solutions of 3mM, 30mM and 3mM of KCl, in presence of $CaCl_2$ and NaCl in order to account for ionic interactions in the ACSF and the possible interaction of the various ions with the resin. The bars indicate the time of injection of the different solutions.