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Cellular Mechanisms of the Neurotoxicity Caused by Hyperammonemia and Hypoglycemia

by

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ABSTRACT

Hyperammonemia and hypoglycemia are metabolic disorders which interfere with the function of the central nervous system. Cellular mechanisms of disturbed neuronal function in these two conditions were investigated in rat hippocampal slices with the use of extracellular and intracellular recording.

Synaptic transmission from Schaffer-collaterals to CA1 pyramidal neurons was diminished by as little as 0.5 mM NH_4Cl. During the presence of 1-4 mM NH_4^+, brain concentrations seen in hepatic encephalopathy, the membrane potential of CA1 neurons depolarized with no significant change in input resistance. Quisqualate-induced inward currents, which were sensitive to CNQX, were blocked, glutamate-induced currents were slightly reduced, while NMDA-induced currents were greatly facilitated.

Low glucose concentrations (0.2-1 mM) seen in hypoglycemia caused mostly biphasic membrane potential changes: a small initial hyperpolarization followed by a large depolarization. Occasionally only one of these potential changes was observed. Input resistance of the neurons always decreased. Low glucose also interfered with synaptic transmission: first the generation of action potentials by EPSPs was inhibited, while later the size of EPSPs decreased. Quisqualate and NMDA currents were first potentiated, then inhibited.

Results suggest that NH_4^+ depresses transmission by eliminating quisqualate-induced responses that are mainly responsible for EPSPs, while low glucose probably shunts EPSPs so that they can no longer generate action potentials at the axon hillock. This impairment in synaptic transmission is likely to contribute to neurological abnormalities seen in hyperammonemia and hypoglycemia.

Symbols and Abbreviations

A/D	Analog-to-Digital
AMPA	α-Amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
AHP	Afterhyperpolarization
APH	2-Amino-7-phosphonoheptanoate
APV	2-Amino-5-phosphonovalerate
CNQX	6-Cyano-7-nitroquinoxaline-2,3-dione
DGG	γ-D-Glutamylglycine
DNQX	6,7-Dinitroquinoxaline-2,3-dione
EAA	Excitatory Amino Acid
EEG	Electroencephalogram
E_K	K^+ equilibrium potential
EPSP	Excitatory Postsynaptic Potential
EPSPs	Excitatory Postsynaptic Potentials
fEPSP	Field Excitatory Postsynaptic Potential
GABA	γ-Aminobutyric Acid
gK^+	K^+ conductance
gNH_4^+	NH_4^+ conductance
I_{AHP}	Afterhyperpolarization current
IPSC	Inhibitory Postsynaptic Current
IPSP	Inhibitory Postsynaptic Potential
IPSPs	Inhibitory Postsynaptic Potentials
$[K]_o$	Extracellular Potassium Concentration

KYN	Kynurenic Acid
Mohm	Mega-ohms
NMDA	N-methyl-D-aspartate
pH	Negative Logarithm of the Hydrogen-ion Concentration
SE	Standard Error
TTX	Tetrodotoxin

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INTRODUCTION

Synaptic transmission from Schaffer-collateral/ commissural fibers to CA1 pyramidal neurons in the hippocampal slice has been widely used to study factors that influence synaptic transmission in the central nervous system (Dingledine, 1984). The two main advantages of this preparation are its stability, which facilitates extracellular or intracellular recording, and a ready control of the composition of the extracellular milieu through the perfusion system. However, brain slice preparations have a number of drawbacks (Schwartzkroin, 1984): the slice is separated from its normal inputs and will, sooner or later, deteriorate; inevitably, some of the tissue will be damaged by the slicing process; the slice is also subject to a period of anoxia during preparation.

In spite of its disadvantages, this preparation was chosen for the study presented in this thesis, which is to investigate the effects on synaptic transmission of changes in the extracellular environment which simulate two metabolic disorders, hyperammonemia and hypoglycemia. Hyperammonemia is seen mostly in disorders involving hepatic insufficiency, and hypoglycemia results most frequently from an excess of insulin. It has been shown that in both conditions excitatory synaptic transmission

in hippocampus is depressed. However, previous work from this laboratory indicated that neither of these conditions causes a reduction in the stimulus-evoked release of the transmitter, glutamate. For this reason work presented here was focussed on postsynaptic factors that may be responsible for an inhibition of synaptic transmission.

Chapter 1 SYNAPTIC TRANSMISSION IN THE CA1 AREA OF THE HIPPOCAMPUS

Excitatory and inhibitory synaptic transmission is essential for the normal function of the central nervous system. In the CA1 area of hippocampus, the major excitatory input is the terminals of Schaffer-collateral/commissural fibers which are the axons of CA3 pyramidal neurons and of the contralateral CA1 pyramidal neurons, respectively. Inhibitory synaptic transmission is mediated by local inhibitory interneurons (Amaral, 1987).

1.1 Excitatory synaptic transmission from Schaffer-collateral/commissural fibers to CA1 pyramidal neurons

1.1.1 Neurotransmitter(s)

Glutamate and aspartate are considered to be the neurotransmitters in this pathway, although aspartate is probably only of minor importance under normal conditions. Glutamate satisfies the criteria that define a neurotransmitter (for review, see Fonnum, 1984):

a. Ca^{2+}-dependent glutamate and aspartate release from Schaffer-collateral/commissural fibers has been established.

b. glutamate and glutaminase (the enzyme for glutamate synthesis) have been found in presynaptic terminals;

c. postsynaptic actions of glutamate, postsynaptic glutamate receptors, their agonists and antagonists have been identified;

d. there exist high and probably low affinity uptake mechanisms which quickly terminate the postsynaptic action of glutamate.

1.1.2 The glutamate receptors

Glutamate, when functioning as a neurotransmitter, has ionotropic and metabotropic effects in CNS and there exist ionotropic and metabotropic receptors, respectively (Baskys, 1992). Activation of the ionotropic receptors directly opens ligand-gated cation channels and changes neuronal membrane permeability (Mayer & Westbrook, 1987). Three ionotropic glutamate receptors have been identified and named after their preferential agonists, AMPA (α-amino-3- hydroxy-5-methyl-4-isoxazolepropionate) (or previously quisqualate), kainate and NMDA (N-methyl-D-aspartate) (Watkins & Olverman, 1987; Collingridge & Lester, 1989). The quisqualate receptor was renamed because AMPA is a more selective agonist than quisqualate, the latter having both ionotropic and metabotropic actions. The NMDA receptor is blocked by its

antagonists such as APV (2-amino-5-phosphonovalerate) and MK-801. CNQX (6-cyano-7- nitroquinoxaline-2,3-dione) and its analogues abolish both AMPA and kainate receptor activated responses (Collingridge & Lester, 1989).

The metabotropic effect of glutamate involves the activation of metabolic processes such as an increased hydrolysis of membrane phosphoinositides, production of the second messengers diacylglycerol and inositol 1-4-5-triphosphate, and mobilization of intracellular Ca^{2+} (Baskys, 1992). The metabotropic receptor also has an influence on ion conductances (Stratton et al., 1989; Baskys et al., 1990; Charpak et al., 1990). Metabotropic receptor agonists include trans-ACPD (trans-1-amino-1,3-cyclopentanedicarboxylic acid), quisqualate, ibotenate and glutamate. Neither NMDA nor non-NMDA antagonists block metabotropic receptors. In high concentrations 2-amino-3-phosphonopropionate (AP3) and 2-amino-4-phosphonopropionate (AP4) block the metabolic processes induced by quisqualate, ibotenate or trans-ACPD. Because of the high concentrations required, the selectivity of the blockade by AP3 and AP4 is questioned (see Baskys, 1992).

Glutamate also acts on a so-called L-APB (L-2-amino-4-phosphonobutyrate) receptor with L-APB as one of the agonists. The L-APB receptor is likely to be a presynaptic autoreceptor which functions to inhibit transmitter release (Collingridge & Lester, 1989).

1.1.3 Conductances activated by ionotropic glutamate receptors

Activation of ionotropic glutamate receptors causes membrane depolarization, accompanied by an increased membrane conductance. Under voltage clamp conditions, currents activated by quisqualate and kainate have a relatively linear voltage-current relationship. Reversal potential is at about 0 mV for both quisqualate and kainate-induced responses (MacDonald & Porietis, 1982; MacDonald & Wojtowicz, 1982; Mayer & Westbrook, 1984). Studies with whole cell voltage-clamp recording revealed that AMPA and kainate channels are equipermeant to Na^+ and K^+ (Ascher & Nowak, 1988a); neither of them have an appreciable Ca^{2+} permeability (Mayer & Westbrook, 1987b). Kainate-induced responses last longer than those induced by quisqualate and are depressed in the presence of quisqualate (Kiskin et al., 1986; Trussel et al., 1988). Both AMPA and kainate can activate the AMPA receptor expressed in human embryonic kidney cells, suggesting that quisqualate and kainate share the same receptor, the AMPA receptor, and therefore the same channel (Keinanen et al., 1990; Sommer et al., 1990).

NMDA activated responses also reverse at about 0 mV. Due to a voltage-dependent blockade by Mg^{2+}, the voltage-current relation of the NMDA mediated conductance is

highly non-linear under physiological conditions (MacDonald et al., 1982; Flatman et al., 1983; Mayer & Westbrook, 1984). The NMDA channel is permeable to Na^+, K^+ and Ca^{2+} with ten times greater permeability to Ca^{2+} than to Na^+ (Ascher & Nowak, 1988b; Mayer & Westbrook, 1985; Mayer & Westbrook, 1987). Activation of NMDA channels needs the presence of low concentrations of glycine (Kleckner & Dingledine, 1988).

When physiological concentrations of Mg^{2+} (1-4 mM) are present, a highly non-linear voltage-current relation of the NMDA induced currents is revealed. In the membrane potential range of -100 mV to around -30 mV, NMDA-induced inward current increases with membrane depolarization. Maximal NMDA current occurs when membrane potential is at -30 mV. At membrane potentials more positive than -30 mV, NMDA current decreases with depolarization and reverses direction at 0 mV. If Mg^{2+} is not added to the extracellular medium, the voltage-current relation of NMDA current becomes almost linear (MacDonald et al., 1982; Flatman et al., 1983; Mayer & Westbrook, 1984; Hestrin et al., 1990). Studies with sirgle channel recordings have demonstrated that in Mg^{2+}-free medium, membrane potential change has no effect on NMDA channel activity. Adding Mg^{2+} reduces the duration of NMDA channel openings which has an inverse relationship with extracellular Mg^{2+} concentration. The effect of Mg^{2+} on NMDA channel is highly

voltage-dependent: inhibition is maximal at more negative membrane potentials, and decreases as the membrane is depolarized (Nowak, et al., 1984; Ascher & Nowak, 1988b). Other divalent cations such as Co^{2+} and Mn^{2+} also block the NMDA channel in a fashion similar to Mg^{2+} (Ascher & Nowak, 1988b).

NMDA responses can be greatly potentiated by even a very low concentration of glycine (10 nM) (Johnson & Ascher, 1987). Glycine probably acts directly on a specific site at the NMDA receptor (Kleckner & Dingledine, 1988; Collingridge & Lester, 1989). It significantly increases the frequency of channel openings and slightly prolongs the mean open time but does not affect channel conductance (Johnson & Ascher, 1987).

By combining the use of whole cell patch-clamp technique and of a Ca^{2+} indicator, Arsenazo III, MacDermott et al. (1986) showed that Ca^{2+} entry through NMDA channels increased free intracellular Ca^{2+} concentration in cultured spinal cord neurons. This conclusion was supported by the observation that the increase of free intracellular Ca^{2+} was abolished when NMDA channels were blocked by Mg^{2+} or by holding the membrane potential at +60 mV, so that both the NMDA currents and the voltage-dependent Ca^{2+} currents were outward. In CA1 hippocampal neurons and striatal neurons, increasing intracellular Ca^{2+} concentrations by NMDA receptor activation has also been demonstrated with the

use of fluorescent Ca^{2+} indicator, Fura-2 (Connor et al., 1988; Murphy et al., 1987). Studies with single channel recordings confirmed that the NMDA channel is permeable to Ca^{2+} (Ascher & Nowak, 1988b). The Ca^{2+} entry through NMDA channels probably plays a role in certain physiological processes such as neural plasticity and in the neurotoxicity of excitatory amino acids (Choi, 1987; Collingridge & Lester, 1989).

1.1.4 Conductance affected by metabotropic glutamate receptors

The metabotropic action of glutamate causes depolarization in hippocampal neurons. In contrast to the ionotropic effect, depolarization mediated by the metabotropic glutamate receptor is associated with a decreased membrane conductance (Stratton et al., 1989). Afterhyperpolarization (AHP) that follows a burst of action potentials is significantly reduced by the metabotropic effect of glutamate (Stratton et al., 1989; Baskys et al., 1990; Charpak et al., 1990). Voltage clamp studies showed that activation of metabotropic glutamate receptors blocks I_{AHP}, the Ca^{2+}-dependent late potassium current underlying the AHP (Baskys et al., 1990; Charpak et al., 1990). The connection between the ionic conductances suppressed by, and the metabolic processes activated by, the

metabotropic receptor has not yet been well established (Baskys, 1992).

1.1.5 Synaptic transmissions mediated by glutamate receptor subtypes

Stimulation of Schaffer collateral/commissural fibers evokes a series of postsynaptic responses: a two-component EPSP, followed by a hyperpolarizing IPSP which also has two components, a fast IPSP and a slow IPSP (Collingridge & Lester, 1989; Sivilotti and Nistri, 1991). Action potentials triggered by an EPSP may be followed by an even slower hyperpolarization, AHP (Alger & Nicoll, 1980). Usually, activation of the Schaffer-collateral pathway produces a fast EPSP which mediates the normal synaptic transmission (Collingridge et al., 1983; Mayer & Westbrook, 1987a). Increasing stimulus intensity produces a second EPSP which has small amplitude, is long-lasting and its peak has a longer latency than that of the first EPSP (Davis & Collingridge, 1989; Hestrin et al., 1990). The role of the AMPA receptor in the production of the fast EPSP is suggested by the observation that concentrations of APV (an NMDA receptor antagonist), which block the slow EPSP and the responses to NMDA, do not affect synaptic transmission either from Schaffer-collaterals to CA1 pyramidal neurons (Collingridge et al.,

1983) or in other hippocampal pathways investigated so far (Collingridge & Lester, 1989). In contrast, in the presence of APV, CNQX (an AMPA receptor antagonist) can completely block the fast EPSP and synaptic transmission in hippocampal pathways with no effect on resting membrane potential or input resistance (Fletcher, et al., 1988; Blake et al., 1989; Davis & Collingridge, 1989). This indicates the essential role of AMPA receptor in synaptic transmission.

The NMDA receptor is a receptor/channel complex. As mentioned above (Chapter 1.1.3), with normal Mg^{2+} concentration (1-4 mM), NMDA channel is inhibited in a voltage-dependent manner by Mg^{2+} ions. Therefore, synaptically released glutamate mainly acts on non-NMDA receptors and blocking of NMDA receptors by APV has no effect on synaptic transmission (Collingridge et al., 1983; Collingridge & Lester, 1989). However the involvement of the NMDA receptor in synaptic transmission may appear when this Mg^{2+} blockade is removed under the following conditions (for review, see Mayer & Westbrook, 1987; Collingridge & Lester, 1989):

a. a reduction in extracellular Mg^{2+} concentration;

b. membrane depolarization caused for instance by the removal of local GABAergic inhibition or by high frequency activation of presynaptic fibers.

It is worthwhile to note that in many CNS pathways a

brief train of high frequency stimulation of presynaptic fibers creates a long-lasting increase in the efficacy of excitatory synaptic transmission, called long-term potentiation (LTP). This phenomenon is thought to underlie certain forms of neuroplasticity, such as learning and memory (Kennedy, 1989). With a few exceptions (Aniksztejn & Ben-Ari, 1991; Grover & Teyler, 1990), activation of NMDA receptors is essential for the induction of LTP (Collingridge & Lester, 1989).

Activation of metabotropic and L-APB receptors (see Chapter 1.1.4) depresses excitatory synaptic transmission in hippocampal pathways probably through inhibition of glutamate release (Collingridge & Lester, 1989; Baskys, 1992). The observations supporting this conclusion are the following. The suppression of synaptic transmission induced by metabotropic glutamate receptors is not due to its effect on postsynaptic K^+ conductance because the inhibition persists when postsynaptic K^+ conductance is blocked by intracellular Cs^+ (Baskys & Malenka, 1991). Neither do agonists of the metabotropic glutamate receptor affect the postsynaptic responses to ionophoretically applied AMPA. The fast and slow excitatory postsynaptic currents (EPSC) are reduced at the same time (Baskys & Malenka, 1991). Furthermore, percentage of the facilitation of EPSC by the second of paired-pulses increases in the presence of metabotropic receptor

agonists. This implies that increasing transmitter release by paired-pulse stimulation can overcome the inhibitory effect of trans-ACPD on EPSC. These results suggest that activation of metabotropic glutamate receptors inhibits transmitter release.

Similar results have been seen in L-APB induced depression of synaptic transmission (see Collingridge & Lester, 1989). In the presence of L-APB, excitatory amino acid induced postsynaptic responses are not inhibited; paired-pulse facilitation increases; the size of presynaptic fiber volley decreases and there is no change in the amplitude of spontaneous miniature EPSP, suggesting that L-APB does not change the postsynaptic responses to the released transmitter. Therefore, L-APB is likely to reduce transmitter release by a presynaptic action.

1.2 Inhibitory synaptic transmission in the CA1 Area of the hippocampus

1.2.1 $GABA_A$ and $GABA_B$ receptors

Several lines of evidence suggest that GABA is the main inhibitory transmitter in the hippocampus. GABAergic terminals and fibers from inhibitory interneurons form a dense plexus around the CA1 pyramidal neurons (Ribak et al., 1978; Woodson et al., 1989). Autoradiographic studies

demonstrate the presence of GABA receptors in the somatic and dendritic region of CA1 pyramidal neurons (Bowery et al., 1987). Iontophoretically applied GABA produces a Cl^- dependent, hyperpolarizing IPSP in the somata of CA1 pyramidal neurons and a depolarizing IPSP in the dendrite (Andersen et al., 1980).

Biochemical and electrophysiological experiments have identified at least two categories of GABA receptors, A and B (see Sivilotti & Nistri, 1991). The $GABA_A$ system is defined on the basis of its sensitivity to agonists such as muscimol (MUS), 4,5,6,7-tetrahydroisoxazolo(5,4-c) pyridin-3-ol (THIP) and isoguvacine (IGV) and its inhibition by antagonists, bicuculline, picrotoxin and securinine. $GABA_A$ receptor activation opens a Cl^- channel and increases the membrane permeability to Cl^-. The influx of Cl^- along its electrochemical gradient produces a hyperpolarization which is suppressed by the competitive $GABA_A$ antagonist, bicuculline, acting on the GABA recognition site. On the other hand, the non-competitive antagonist, picrotoxin, suppresses the probability of channel activation in rat hippocampal neurons in an all-or-none manner so that the channels are either not activated at all or function as normal with no change in conductance or kinetics of Cl^- channels (Segal & Barker, 1984a; Barker et al., 1983).

The $GABA_B$ receptor was first detected presynaptically on

nerve terminals where it acts to inhibit transmitter release (Fox et al., 1978). It was later found that the postsynaptic $GABA_B$ receptor in mammalian CNS, including hippocampus, is linked to a K^+ channel by a GTP-binding protein (G protein) (Newberry & Nicoll, 1984; 1985; Andrade et al., 1986). Baclofen, a $GABA_B$ agonist, activates a hyperpolarizing K^+ conductance which is not affected by bicuculline or picrotoxin, but is blocked by Ba^{2+} and by the $GABA_B$ antagonist, phaclofen. Impairment of G protein function by pertussis toxin or GTP-γ-S (a GTP analogue which cannot be hydrolyzed) also depresses the baclofen-induced K^+ conductance (Andrade et al., 1986).

In summary, GABA has $GABA_A$ and $GABA_B$ receptors. Both of them are inhibitory but have different ionic mechanisms.

1.2.2 Inhibitory potentials in CA1 pyramidal neuron

Activation of the Schaffer-collateral pathway usually excites both CA1 pyramidal neurons and GABAergic interneurons. The latter release GABA and create an hyperpolarizing IPSP in pyramidal neurons. This hyperpolarizing IPSP has a fast and a slow component. The fast one is accompanied by an increase in Cl^- conductance which has an identical reversal potential with that of responses induced by iontophoretically applied GABA (Andersen et al., 1980; Alger & Nicoll, 1982; Knowles et

al., 1984). The $GABA_A$ antagonists bicuculline and picrotoxin block this fast IPSP (Alger & Nicoll, 1982; Knowles et al., 1984). Bormann et al. (1987) and Kaila et al. (1989) showed that the Cl^- channel activated by GABA was also permeable to HCO_3^- which, by having an equilibrium potential more positive than the membrane potential, produced a depolarizing IPSP in mouse spinal neurons and crayfish muscle fibers.

An increase in K^+ conductance is involved in the slow IPSP (Newberry & Nicoll, 1984; Dutar & Nicoll, 1988a). Bicuculline and picrotoxin have no effect on the slow IPSP, but the $GABA_B$ receptor antagonist, phaclofen inhibits it (Dutar & Nicoll, 1988a). Blocking the connection between the $GABA_B$ receptor and K^+ channel by pertussis toxin and GTP-γ-S also suppresses the slow IPSP (Dutar & Nicoll, 1988b; Thalmann, 1988).

The firing of CA1 pyramidal neurons can also activate several other K^+ currents which include the Ca^{2+}-dependent K^+ current; the muscarine-sensitive K^+ current (M current) and another Ca^{2+}-dependent K^+ current, I_{AHP}. These K^+ currents produce AHP (see Storm, 1990; Knopfel et al., 1990). Normally, this AHP may be contaminated by IPSP (Alger & Nicoll, 1980).

In summary, the hyperpolarization that follows synaptically evoked action potentials involves increased Cl^- and K^+ conductances.

Chapter 2 CELLULAR MECHANISMS OF AMMONIA TOXICITY

2.1 The physical chemistry and metabolism of ammonia

Ammonia is normally present in low concentrations in human blood (70-113 μM) and cerebrospinal fluid (20-100 μM) (Cooper & Plum, 1987). Ammonia can arise as a byproduct of biochemical reactions in the intestine and other organs. Most of the ammonia absorbed through the gut is converted to urea in the liver; the fraction that is not captured by the liver enters the blood and is metabolized in organs such as brain, muscle and heart (for review, see Summerskill & Wolpert, 1970; Haussinger, 1986; Cooper & Plum, 1987). In the brain, where there is no urea cycle, ammonia is removed in glia by the synthesis of glutamine from glutamate and ammonia. Since glutamine synthetase is almost exclusively located in astrocytes (Martinez-Hernandez et al., 1977), glial cells have a critical role in ammonia detoxification in the central nervous system. Ammonia is also the byproduct of many metabolic reactions. For instance, glutaminase produces glutamate and ammonia from glutamine (Kvamme et al., 1970; Benjamin & Quastel, 1975; Benjamin, 1981), while glutamate dehydrogenase catalyzes the oxidative deamination of glutamate with

ammonia as one of the products (Chee et al., 1979).

Ammonia in its neutral form is NH_3 which is a weak base. In biological fluid, NH_3 quickly protonates to become ammonium ion (NH_4^+) (Kleiner, 1981). The equilibration of NH_3 with ammonium ions ($NH_3 + H^+ \rightleftharpoons NH_4^+$) is determined by the dissociation constant (pKa) which at physiological temperature is 9.15. Therefore 99% of the total ammonia is NH_4^+ at physiological pH of 7.2-7.4 (Bromberg et al., 1960). NH_3 is lipid soluble and can diffuse across biological membranes (Kleiner, 1981), while as an ion, NH_4^+ can cross membranes through K^+ and Na^+ channels and can replace about 30% of K^+ conductance in maintaining the resting membrane potential in the squid giant axon when K^+ is replaced by NH_4^+ (Binstock & Lecar, 1969; Luttgau, 1961). NH_4^+ also passes through the acetylcholine-activated end-plate channels at the vertebrate neuromuscular junction and has an even greater permeability than Na^+ (Dwyer et al., 1980).

Ammonia enters the brain from the blood by simple diffusion. During steady arterial ammonia levels there is a linear correlation between arterial and CSF ammonia concentrations, even under conditions of extreme hyperammonemia (Cooper & Plum, 1987). During experimental hepatic failure in rats arterial and brain ammonia levels increase to 1-5 mM (Mans et al., 1979; Zeneroli et al., 1988; Swain et al., 1991) and the severity of behavioral

symptoms correlate with brain ammonia levels. In human chronic liver disease arterial and CSF ammonia levels are similar to those seen in rats (Plum, 1971). Hyperammonemia probably mediates most of the symptoms of hepatic encephalopathy (Benjamin, 1982; Butterworth et al., 1988a). For instance, acute administration of ammonium acetate to rats at a dose of 7.8 mmol/Kg produced unresponsiveness in 5-10 min and after a few more minutes, the rat became convulsive (Cooper & Plum, 1987). Several mechanisms by which ammonia interferes with neuronal functions have been proposed, such as the inhibition of transmitter release, conduction block of action potentials and suppression of inhibitory hyperpolarizations. These are considered in more detail in the following sections.

2.2 Effect of ammonium ions on membrane potential, ionic concentrations and pH.

2.2.1 Depolarization caused by ammonium ions

Ammonium ions depolarize the resting membrane potential in many different preparations (Binstock & Lecar, 1969; Lux et al., 1970; Nicoll, 1978; Hino, 1979; Iles & Jack, 1980; Alger & Nicoll, 1983; Golby et al., 1990). The amplitude of the NH_4^+-induced depolarization varies from preparation to preparation and so does the effect of NH_4^+ on membrane

conductance. For instance, in hippocampal slices, 4 mM NH_4Cl depolarized hippocampal CA1 neurons by 2-5 mV (Alger & Nicoll, 1983), while depolarization caused by intravenous injection of ammonium acetate (100-300 mg/kg bodyweight) was 2-15 mV (Lux et al., 1970). However, the concentration of NH_4^+ surrounding the neuron in vivo cannot be calculated. Iles and Jack (1980) demonstrated that the NH_4^+ induced depolarization was not associated with a consistent change in the input resistance of spinal motoneurons, while results from other laboratories using different preparations indicated an increase in input conductance (Nicoll, 1978; Hino, 1979; Alger & Nicoll, 1983). The duration of the neuronal action potential was prolonged and amplitude slightly reduced when ammonium ion was administrated (Lux et al., 1970; Alger & Nicoll, 1983). Ammonium ions also inhibited a Ca^{2+}-dependent late hyperpolarization mediated by K^+ and shifted its reversal potential in the depolarizing direction (Alger & Nicoll, 1983).

Both neurochemical and electrophysiological observations suggest that NH_4^+ reduces the cross membrane gradient of K^+. The study of Benjamin et al. (1978) demonstrated that in neocortical slices, NH_4Cl in 1 mM or higher concentrations significantly reduced intracellular K^+ content, while at the same time increasing the intracellular content of Cl^-. Intravenous administration of

ammonium acetate in vivo produced a loss of brain K^+ as revealed by an increase in the potassium content of the plasma draining the brain (Hawkins et al., 1973). With the use of potassium-sensitive electrodes, Alger & Nicoll (1983) observed an increase of extracellular K^+ activity in the presence of 4 mM NH_4Cl. Since NH_3 can diffuse across the cell membrane and NH_4^+ can be taken up by neurons through K^+ channels (Binstock & Lecar, 1969; Kleiner, 1981), accumulated intracellular NH_4^+ is likely to replace intracellular K^+ and therefore will reduce the electrochemical gradient of K^+. This would thus result in net depolarization of the membrane.

2.2.2 The effect of ammonium ions on intracellular pH

Changes in intracellular pH caused by NH_4^+ differ depending on experimental preparations. In the squid giant axon, ammonium ions increase the intracellular pH, but upon the removal of ammonium ions intracellular pH drops below control level (Boron & De Weer, 1976). The explanation suggested by Boron and De Weer is as follows: Since $NH_4^+ \rightleftharpoons NH_3 + H^+$, the direction of this equation is different when NH_4^+ is applied and removed. Exposing the squid giant axon to NH_4Cl first induces a rapid influx of the lipid soluble NH_3. The subsequent protonation of NH_3 forms NH_4^+, thereby reducing the intracellular hydrogen ion concentration;

upon the removal of extracellular NH_4Cl, NH_3 quickly moves out of the cell, which increases the production of NH_3 from the remaining intracellular NH_4^+ and releases H^+.

In cortical slices, however, ammonium ions cause a drop in intracellular pH (Brooks et al., 1989; Kauppinen et al., 1991). The drop in intracellular pH caused by 1 mM NH_4^+ was as great as that produced by 20 mM NH_4^+, indicating that the degree of acidification is not dependent on NH_4^+ concentrations in this range (Kauppinen et al., 1991). Similar intracellular acidification by ammonium ions was observed by Erećinska et al. (1987) in synaptosomes and by Fitzpatrick et al. (1989) in the cerebral cortex in vivo. This intracellular acidification is probably seen because the mammalian neuronal membrane has a much greater density of ion channels than the squid giant axon and therefore there is a greater initial intracellular accumulation of NH_4^+ than that of NH_3. After entering, NH_4^+ will keep producing H^+ through the mechanism suggested by Boron and De Weer (see Szerb & Butterworth, 1992). This pH change constitutes a possible mechanism for the effect of ammonium ions on synaptic transmission (see next chapter).

2.3 The effect of ammonium ions on inhibitory synaptic transmission

The first indication that ammonium ions affect synaptic transmission was the observation that ammonium ions reduce the hyperpolarizing inhibitory postsynaptic potential (IPSP) in cat spinal motoneurons (Lux & Schubert, 1969). The phenomenon was also found later in the neocortex (Raabe & Gumnit, 1975), the hippocampus (Alger & Nicoll, 1983), trochlear motoneurons (Llinas et al., 1974), crayfish stretch receptor neurons (Hino, 1979) and frog spinal motoneurons (Nicoll, 1978). These studies showed that ammonium ions shifted the reversal potential of the Cl^--dependent IPSP towards resting membrane potential, without changing the conductance increase involved in IPSP (see also Lux et al., 1970; Lux, 1971; Iles & Jack, 1980). In other words, the driving force of the IPSP (i.e. the electrochemical gradient of Cl^-) was reduced by ammonium ions which appear to interfere with the extrusion of Cl^- from the cytoplasm into the extracellular space.

The concentration gradient of Cl^- across the membrane is maintained by a Cl^- pump. One of the possible mechanisms through which ammonium ions may affect the function of Cl^- pump is by reducing the potassium gradient either by increasing extracellular K^+ or by the depletion of intracellular K^+ (see section 2.2.1 of introduction). For

instance, the effect of ammonium ion on IPSP can be simulated by a rise of extracellular potassium which decreases the extrusion rate of intracellular Cl^- (Wallin, 1967; Russell & Brown, 1972; Thompson et al., 1988) and shifts the IPSP reversal potential towards resting membrane potential (Alger & Nicoll, 1983; Thompson & Gähwiler, 1989). Thompson & Gähwiler (1989) further hypothesize that the major driving force of Cl^- extrusion is via potassium/chloride co-transport. Therefore an increase of extracellular K^+ (Alger & Nicoll, 1983) and a decrease of intracellular K^+ (Benjamin et al., 1978) caused by ammonium ions should inhibit the potassium/chloride co-transport system. Supporting this conclusion is the observation by Benjamin et al. (1978) that NH_4^+ induced an increase of intracellular Cl^- content at the time when intracellular K^+ was decreased.

The finding that HCO_3^- also passes through the GABA-gated Cl^- channels involved in the IPSP (Bormann et al., 1987; Kaila & Voipio, 1987, section 1.2.2) raises the question whether intracellular pH change can be a mechanism for the suppression of the hyperpolarizing IPSP by ammonium ions. Activation of GABA-gated Cl^- channels produces a fall in intracellular pH as a result of the efflux of HCO_3^-, and the latter depolarizes the membrane potential. According to Kaila et al. (1989), a postsynaptic intracellular alkalosis is expected to produce a positive shift in IPSP

reversal potential by increasing the intracellular concentration of, and hence the efflux of, HCO_3^-. Consequently, there will be an alkalotic decrease in the efficacy of inhibition, while a postsynaptic acidosis would have an opposite effect. Since NH_4^+ causes an intracellular acidification in cerebral cortex neurons (Brooks et al., 1989; Kauppinen et al., 1991), it is unlikely that NH_4^+ suppresses IPSP in mammalian preparations by increasing the efflux of HCO_3^-.

It has been observed by Alger and Nicoll (1983) in hippocampal slices that NH_4^+ reduced the increased conductance involved in IPSP but not that induced by exogenously applied GABA. In the initial stages of NH_4^+ application, the depression of IPSPs could be counteracted by increasing the excitatory synaptic transmission, i.e. by increasing the stimulus strength on presynaptic fibers. Therefore, the effect of ammonium ions on inhibitory synaptic transmission might partly be secondary to the depressive effect of ammonium ion on excitatory synaptic transmission. This is because the production of the IPSP is a multi-synaptic process in the hippocampal CA1 area in which inhibitory interneurons have to be excited first (Kandel et al., 1961; Alger and Nicoll, 1982).

2.4 The effect of ammonium ions on excitatory synaptic transmission

2.4.1 Elevated ammonium ion concentrations inhibit excitatory synaptic transmission in CNS.

Intravenous infusion of a 20% ammonium acetate solution reduced excitatory mono- and poly-synaptic transmission in the spinal cord in vivo (Raabe, 1989; Raabe, 1990). Field potentials reflecting action potentials in intraspinal presynaptic terminals (but not presynaptic fibers) were also decreased. Raabe (1989, 1990) proposed that, while lower concentrations of ammonium ions block hyperpolarizing IPSPs, higher concentrations, due to their depolarizing effect on membrane potential, decrease and then block conduction of action potentials to presynaptic terminals and prevent the release of the excitatory transmitter.

NH_4^+-induced suppression of excitatory synaptic transmission has also been shown in the hippocampus, both in vitro and in vivo (Alger & Nicoll, 1983; Théorêt et al., 1985; Théorêt & Bossu, 1985). In hippocampal slices, 3-8 mM ammonium ions reversibly depressed the orthodromically evoked EPSPs and population spikes (Alger & Nicoll, 1983; Théorêt et al., 1985). A similar effect was observed in vivo when 10-30 mM of ammonium salts was

perfused for 20 min through the lateral ventricle (Théorêt & Bossu, 1985). Compound presynaptic action potentials and glutamate-induced postsynaptic firing were slightly reduced by ammonium ions but antidromically evoked spikes were not affected, indicating that there was little change in the excitability of postsynaptic neurons (Théorêt et al., 1985; Alger & Nicoll, 1983). Therefore, Théorêt et al. concluded that the suppression of excitatory synaptic transmission by ammonium ions is due to a decreased presynaptic transmitter release.

The amount of released transmitter which produces EPSPs in postsynaptic neurons is assumed to be proportional to the amplitude of the presynaptic compound action potential which is determined by the number of presynaptic fibers activated by the stimulus. The input-output relationship between the amplitude of the compound presynaptic action potentials and the resulting postsynaptic EPSPs shifted to the right during ammonium ion administration, i.e. a given amplitude of presynaptic volley produced a smaller field EPSP in the presence of NH_4Cl than it did under control conditions (Alger & Nicoll, 1983; Théorêt et al., 1985). This observation suggests that ammonium ions interfere with EPSP production. Such an effect can be due either to decreased transmitter release or to a decrease in postsynaptic responses to the released transmitter.

2.4.2 The effect of ammonium ions on presynaptic transmitter release.

Metabolic studies have shown that the brain has at least two compartments of glutamate: a large one in neurons and a small one in glia (van den Berg & Garfinkel, 1971; van den Berg et al., 1975; Benjamin & Quastel, 1975). The two glutamate compartments are connected by the so-called glutamate-glutamine cycle (Benjamin & Quastel, 1975). Glutaminase converts glutamine to glutamate in nerve terminals. Glutamate is then released by presynaptic nerve terminals and is inactivated by uptake mainly into astrocytes where glutamine synthetase converts glutamate and NH_3 into glutamine. Glutamine formed in astrocytes is then returned by diffusion to presynaptic terminals for glutamate formation. This glutamate-glutamine cycle is largely responsible for the synthesis of transmitter glutamate (Szerb & Butterworth, 1992). Ammonia adversely affects this cycle. Acute administration of toxic concentrations of NH_4^+ considerably increases the glutamine content and slightly decreases that of glutamate in brain tissue (Matheson & van den Berg, 1975; Bates et al., 1989; Fitzpatrick et al., 1989).

Theoretically either a decrease in the activity of glutaminase, which is the major enzyme for glutamine degradation, or an increase in glutamine synthesis may be

responsible for the increase in glutamine content by NH_4^+. However, an increase of glutamine synthesis by toxic concentrations of NH_4^+ could not be found by Matheson & van den Berg (1975) who used the incorporation of [^{3}H]acetate into brain glutamine in vivo as a measure of glutamine synthesis. In control conditions, radio-labeled glutamine content reached maximum at about 10 min after injection of [^{3}H]acetate and decayed thereafter due to degradation. In the NH_4Cl-treated animals, the rate of increase of [^{3}H]glutamine was not changed but the decay (i.e. the degradation of glutamine) disappeared. Instead, ammonium salt caused a further increase in labeled glutamine content. The reason why NH_4^+ does not increase glutamine synthesis is probably because glutamine synthetase is nearly saturated with ammonia at physiological concentrations, having a Km value for ammonia between 41 μM to 180 μM (van den Berg, 1970; Pamiljans et al., 1962; Waniewski, 1992).

These observations suggest that the increased glutamine content is probably due to the effect of ammonium ions on glutamine degradation. Indeed, Benjamin (1981) and Kvamme & Lenda (1982) have shown that NH_4^+, being one of the products of glutaminase, inhibits glutaminase activity, and hence glutamate synthesis in brain cortex slice, synaptosome and brain homogenate preparations, with 0.5 mM NH_4^+ reducing glutaminase activity by about 40%.

Similar results have been observed in chronic hyperammonemia. Glutamine content was greatly increased and that of glutamate was decreased in autopsied brain tissue from cirrhotic patients with hepatic encephalopathy and in the brain from portacaval shunted animals (Lavoie et al., 1987a; Butterworth et al., 1988b). However, as with acute hyperammonemia, overall glutamine synthesis in the brain was not increased significantly (Cremer et al., 1975; Lavoie et al., 1987a; Ukida et al., 1988) and in the neocortex glutamine synthetase activities were even reduced in clinical and experimental hepatic encephalopathy (Lavoie et al., 1987b; Butterworth et al., 1988b). A reduction in glutamine synthetase activity in chronic hyperammonemia is an indication that the ability of the brain to remove ammonia is impaired. Since glutamine synthesis takes place almost exclusively in astrocytes (Martinez-Hernandez et al., 1977), the decrease of glutamine synthetase activity may be caused by pathological changes in glial cells seen in chronic hyperammonemia (Norenberg, 1981; Gregorios et al. 1985a,b; Ganz et al., 1989).

Inhibition of glutaminase by ammonium ions can be expected to reduce glutamate formation in nerve terminals and therefore transmitter glutamate release. Several studies have shown that ammonium ions decrease glutamate release and an inhibition of glutaminase was proposed to

be the mechanism (Hamberger et al., 1979; Hamberger et al., 1982; Bradford et al., 1989). For instance, glutamate release evoked by high concentrations of KCl was significantly inhibited by 2-5 mM NH_4Cl in hippocampal slices (Hamberger et al., 1979; Hamberger et al., 1982). Veratrine-induced glutamate and aspartate release from synaptosomes was also reduced by 4 mM NH_4Cl. The glutaminase inhibitor, 6-diazo-5-oxo-L-norleucine, had an effect similar to that of NH_4Cl on veratrine-induced glutamate and aspartate release (Bradford et al., 1989). However, the Ca^{2+}-dependence of glutamate release was not established in these studies.

In nerve terminals, glutamate is located in cytoplasmic and synaptic vesicle pools (Nicholls, 1989; Wilkinson & Nicholls, 1989). Glutamate constantly leaks from the cytoplasmic pool into the extracellular space and is taken up by a Na^+-dependent transport (Bradford et al., 1987). Any event that reduces the electrochemical gradient of Na^+ will causes a net loss of glutamate from the cytoplasm, and this leakage is Ca^{2+}-independent. On the other hand, synaptic vesicles release glutamate by exocytosis which is Ca^{2+}-dependent and is responsible for synaptic transmission (Dingledine & Somjen, 1981; Nicholls, 1989). Nicholls (1989) suggested that newly synthesized glutamate first enters the cytoplasm and then is transported slowly into synaptic vesicles. Therefore inhibition of glutamate

synthesis by ammonium ions should first affect the Ca^{2+}-independent glutamate release, while Ca^{2+}-dependent glutamate release from vesicles should not be inhibited until the glutamate content of synaptic vesicles starts to be depleted.

Neurochemical studies in Szerb's laboratory (Butterworth et al., 1989) showed that in slices of hippocampus the large Ca^{2+}-dependent release of glutamate evoked by elevated $[K^+]_o$ or by electrical-field stimulation was not reduced by 5 mM NH_4Cl. The field stimulation-evoked glutamate release was completely blocked in the presence of 1 μM TTX, suggesting that this release originated from synaptic vesicle and was evoked by conducted action potentials. On the other hand, 5 mM NH_4Cl significantly reduced the smaller, Ca^{2+}-independent glutamate release probably originating from cytoplasm. This decrease is likely to be the initial manifestation of the inhibition of glutamate synthesis by ammonia.

In addition to the glutamatergic and GABAergic systems, ammonium ions are known to affect other transmitter systems, namely the serotoninergic and catecholaminergic systems. Brain 5-HT turnover increases in both acute and chronic liver disease (Young et al., 1975; Bergeron et al., 1989). There is also evidence to suggest an increased synthesis and turnover of catecholamines in the brain stem of rats following hepatectomy (Bugge et al., 1986;

Bergeron et al., 1991). The mechanism of the action of ammonium ion has not yet been established.

In conclusion, insensitivity of the Ca^{2+}-dependent release of glutamate to ammonium ions suggests that the inhibition of the Ca^{2+}-dependent transmission by NH_4^+, at least initially, is not due to a reduction of glutamate release but to its postsynaptic action. The purpose of the first part of the present study was to investigate further the postsynaptic mechanisms by which ammonium ions affect excitatory synaptic transmission. In particular, this study was aimed at establishing the effects of NH_4^+ on the membrane properties of postsynaptic neurons and their responses to exogenously administrated glutamate and its analogues.

Chapter 3 MECHANISMS OF HYPOGLYCEMIA INDUCED NEURONAL INJURY

3.1 Brain glucose supply and its regulation

The major substrate for brain energy is glucose which supports about 90% of the total oxidative metabolism. In the rat, normal blood glucose concentration is 6 μmole/ml and 2.5 μmole/ml in brain tissue. (Lund-Andersen, 1979). Glucose used for brain metabolism is transported from blood to glial and neuronal cells via a carrier mediated system located at the blood-brain barrier. The system is saturable (Lund-Andersen, 1979; Siesjö, 1978). When estimating the rate of glucose transport, blood-brain barrier and cell membrane can be considered as a single membrane (Lund-Andersen, 1979). Therefore, the observed glucose content in brain is the glucose content in both extracellular and intracellular space. Since there is no active glucose transport which concentrates glucose in the brain, blood glucose concentration determines the supply of the main energy substrate of the brain (Mans et al., 1987; Siesjö, 1978). For instance, when plasma glucose level was reduced by 44% (from 8.83 mM to 4.94 mM) during starvation and drug-induced tremor, glucose in the cerebellum decreased by 75% (from 2.82 μmole/g to 0.7

μmole/g) (Cunningham, 1986). Starvation alone also reduced the plasma glucose level (from 7.73 μmole/ml to 5.52 μmole/ml), causing a 20% decrease of the glucose content in brain tissue (from 1.28 μmole/g to 1.03 μmole/g) (Mans, et al., 1987). These observations suggest that brain glucose content can be reduced to about 1 μmole/g even in conditions other than insulin-induced hypoglycemia. On the other hand, with increasing blood glucose levels, the transport of glucose from blood to brain rises until saturation occurs when plasma glucose concentration reaches about 2.5 mg/ml (Lund-Andersen, 1979).

3.2 Hypoglycemia-induced encephalopathy

3.2.1 Hypoglycemia-induced changes in EEG, energy metabolism,ion homeostasis and pH in vivo

It is well known that severe hypoglycemia evokes brain dysfunction and abnormal behaviour. Drowsiness, stupor, coma and convulsions occur. The electroencephalogram (EEG) first becomes slower, convulsive polyspike waves appear and then the EEG becomes silent (isoelectric) (Tews et al., 1965; Ferrendelli & Chang, 1973). Profound changes in energy metabolism, ionic homeostasis and irreversible neuronal necrosis take place (Tews et al., 1965; Hinzen & Muller, 1971; Wieloch et al., 1984; Harris et al., 1984;

Auer, 1986; Ikeda et al., 1987). According to Lewis et al. (1974a), intracellular glucose concentration approaches zero when blood glucose level is below 3 μmol/g. The concentration of other energy substrates such as pyruvate, lactate, glucose-6-phosphate and glycogen decrease. It is not possible to distinguish whether these changes occur in neurons or glial cells. When the blood glucose levels fall further, below 1 μmol/g, pyruvate, lactate, glycogen and glucose-6-phosphate are depleted. High-energy phosphates, such as ATP and phosphocreatine levels also fall and irreversible neuronal damage begins to occur (Lewis et al., 1974a; Auer et al., 1984a).

Early studies on the energy metabolism of the brain during hypoglycemia have provided conflicting results. While a reduction in the concentrations of ATP and phosphocreatine has been reported by Tews et al. (1965), Goldberg et al. (1966) and Hinzen & Muller, (1971), others found no changes in ATP and phosphocreatine levels during hypoglycemia (Tarr et al., 1962; King et al., 1967; Ferrendelli & Chang, 1973). Goldberg et al. (1966) showed small but significant decreases in the brain concentrations of ATP and phosphocreatine when convulsions appeared during insulin-induced hypoglycemia. A later study from the same laboratory found no changes in ATP and phosphocreatine levels in convulsing animals (King et al., 1967). A similar picture arose from the studies of

Ferrendelli's group. No changes in the concentrations of ATP and phosphocreatine during insulin-induced hypoglycemia were demonstrated even when the EEG became isoelectric (Ferrendelli & Chang, 1973; Gorell et al., 1976). A subsequent study from the same group showed that ATP and phosphocreatine levels were normal until the EEG was isoelectric, and then decreased in all regions of the brain except cerebellum (Ratcheson et al., 1981). The observation by Hinzen & Muller (1971) that there were no changes in the concentrations of brain ATP and phosphocreatine until blood glucose concentration was reduced below 1 μmol/g was confirmed by a more recent study (Lewis et al., 1974a). It is now generally accepted that when the EEG becomes isoelectric, a failure in energy production also occurs, i.e. brain ATP and phosphocreatine concentrations decrease significantly (Lewis et al., 1974b; Wieloch et al., 1984; Behar et al., 1985; Ikeda et al., 1987).

During hypoglycemia-induced isoelectricity, first there is a sudden 80% decrease of extracellular Ca^{2+} and a 4-14 fold increase of extracellular K^+ concentration. Then Ca^{2+} and K^+ concentrations return to normal for a short period, followed by a second shift of similar amplitude to the first one with no recovery. The fall of ATP and phosphocreatine levels actually starts after the initial extracellular Ca^{2+} and K^+ change. This fall suggests that

mechanisms other than energy failure may trigger the initial shifts in extracellular Ca^{2+} and K^{+} concentrations (Wieloch et al., 1984; Harris et al., 1984).

Recently, it was shown that hypoglycemia first produces a small alkaline and then a large acid shift of extracellular pH (Bengtsson et al., 1990). Bengtsson et al. (1990) proposed that these changes were produced by movement of H^{+} and HCO_3^{-} ions in and out of the cell.

3.2.2 Hypoglycemia-induced neuronal necrosis and the overflow of amino acids

When, in the early stages of hypoglycemia, blood glucose levels fall, EEG and behavioral changes appear before significant changes in brain energy status are seen, as defined by the content of ATP and phosphocreatine (Lewis et al., 1974b; Ratcheson et al., 1981; Wieloch et al., 1984). However, hypoglycemia-induced neuronal necrosis only occurs after the EEG becomes silent (Auer et al., 1984a,b; Auer, 1986). The severity of the neuronal necrosis is positively related to the duration of EEG isoelectricity, once blood sugar level falls below a critical level (Auer et al., 1984a). Several brain regions, such as the cerebral cortex, hippocampus, caudate nucleus, spinal cord and cerebellum are affected. Different cell types have different susceptibility to

necrosis. For instance, hippocampal CA3 neurons are more resistant than CA1 neurons (Auer et al., 1984a,b; Auer, 1986). Glial cells, when exposed to hypoglycemia and energy failure, do not show necrosis in vivo (Auer et al., 1985; Auer, 1986). However, during glucose deprivation, there is a significant membrane depolarization in cultured astrocytes, as indicated by a cyanine dye, and a dramatic fall in ATP/ADP ratio, in transmembrane K^+ and in glutamate gradients (Kauppinen et al., 1988).

Based on several lines of evidence, it has been proposed that the necrosis of neurons in hypoglycemia is not simply due to glucose starvation but to endogenous excitotoxins which are released during hypoglycemia (Auer et al., 1984a,b; Auer, 1986).

Initially, during hypoglycemia-induced EEG isoelectricity, ATP levels in the brain fell to one third of the normal values (Hinzen & Muller, 1971; Feise et al., 1976; Lewis et al., 1974a). A further decrease in ATP levels by reducing blood pressure did not increase neuronal necrosis (Auer et al., 1986). On the other hand, the analysis of hypoglycemia-induced necrosis revealed that the location of the necrosis was related to cerebrospinal fluid pathways, suggesting that cerebrospinal fluid-borne neurotoxin(s) may have been responsible for the hypoglycemia-induced necrosis (Auer et al., 1984b). Indeed, when the EEG becomes silent,

extracellular concentrations of some well known excitotoxins, such as aspartate and glutamate, were markedly increased. The increase of aspartate was much more than that of glutamate (Sandberg et al., 1986; Butcher et al., 1987a). Similar findings have been found in in vitro studies when the extracellular glucose concentration was reduced (Szerb & O'Regan, 1987; Szerb, 1988; Burke & Nadler, 1988).

The involvement of NMDA receptors in hypoglycemic necrosis was suggested by both in vivo and in vitro studies. Intravenous and local injections of NMDA antagonists into caudate nucleus indicated a significant protective effect against hypoglycemia-induced neuronal death (Wieloch, 1985; Papagapiou & Auer, 1990). The in vitro studies using cultured cortical and cerebellar neurons (Novelli et al., 1988; Monyer et al., 1989; Facci et al., 1990) showed that both competitive and non-competitive NMDA antagonists, and glutamate-degrading enzymes (glutamate dehydrogenase and nicotinamide adenine dinucleotide) substantially reduced the glucose deprivation-induced neuronal injury. The broad spectrum glutamate antagonist kynurenate had the same effect. In contrast, non-NMDA receptor antagonists provided little protection (Monyer et al., 1989; Facci et al., 1990). However, Sheardown et al.(1990) demonstrated that a highly selective non-NMDA antagonist, NBQX (2,3-Dihydroxy-6-

nitro-7-sulfamoyl-benzo(F)quinoxaline) protected against the global ischemia-induced cell loss, although this neuronal death had been thought to be mediated by NMDA receptors (Gill et al., 1987). Information about the effect of NBQX on hypoglycemia-induced necrosis is not yet available. NMDA antagonists affect neither the overflow of glutamate and aspartate (i.e. the released transmitter that escapes inactivation and appears in extracellular space; Butcher et al., 1987b) nor the increase of extracellular K^+ concentration and decrease of extracellular Ca^{2+} concentration caused by hypoglycemia (Zhang et al., 1990).

Other observations also support the concept that hypoglycemia-induced neuronal necrosis is due to excitotoxins. The corticostriatal pathway is believed to be glutamatergic (Fagg & Foster, 1983). Ablation of frontal cortex, which damages the glutamatergic innervations of the striatum, reduced the increase in brain aspartate levels caused by hypoglycemia and protected against the hypoglycemia-induced neuronal necrosis (Engelsen & Fonnum, 1983; Wieloch et al., 1985). This observation suggests the importance of synaptic transmission in hypoglycemic encephalopathy.

Neuronal death in hypoglycemia starts at the dendrites, where the glutamate receptors are located, and spreads to the soma (Auer et al., 1985). Neuronal damage induced by

excitotoxins (Schwarcz et al., 1983) has a very similar pattern to that induced by hypoglycemia.

3.3 Glucose deficiency and synaptic transmission

3.3.1 Glucose deficiency suppresses excitatory synaptic transmission in hippocampus

Granule cells in the dentate gyrus of the hippocampus receive glutamatergic innervation from the entorhinal cortex via the perforant pathway (Amaral, 1987). When blood glucose was reduced below 1 mM by insulin-induced hypoglycemia, excitatory transmission from the perforant pathway to dentate gyrus granule cells was completely blocked (Butcher et al., 1987c). The NMDA receptor antagonist APV had no effect on the blockade but enhanced the recovery of field potentials from hypoglycemic episodes (Butcher et al., 1987c).

In vitro studies using hippocampal slices also revealed that low extracellular glucose suppressed synaptic transmission from the perforant pathway to the dentate gyrus granule cells (Cox & Bachelard, 1982; Bachelard et al., 1984) and from Schaffer-collaterals to CA1 pyramidal cells (Burke & Nadler, 1989; Krnjević & Walz, 1990). Results from these studies demonstrated a reversible attenuation of field excitatory postsynaptic potentials

(fEPSPs) and population spikes evoked by stimulation of presynaptic fibers. Population spikes were much more sensitive to low glucose concentrations than fEPSPs, suggesting an impairment in cell excitability (Bachelard et al., 1984). The suppression of synaptic transmission in CA1 region of hippocampal slices was accompanied by a slow alkaline shift in extracellular pH (Krnjević & Walz, 1990). Spuler et al. (1987) also reported an extracellular alkalinization caused by glucose deficiency.

During low glucose concentrations, the energy state of the brain, as defined by the high-energy phosphate content, appears not to be related to the suppression of synaptic transmission. Hypoglycemia-induced blockade of synaptic transmission occurred before the cessation of EEG activity (Butcher et al., 1987c). Energy state was reported to be remarkably resistant to glucose deprivation in the guinea pig hippocampal slice (Cox et al., 1983). Glucose levels of 2 to 5 mM are sufficient to support normal cell respiration in vitro (McIlwain, 1953), yet evoked field potentials in guinea pig hippocampal slice were significantly suppressed by lowing glucose content from 10 mM to 5 mM and 2 mM glucose (Cox & Bachelard, 1982; Bachelard et al., 1984). Tissue levels of phosphocreatine and ATP were unchanged when synaptic transmission was suppressed by low extracellular glucose in hippocampal slices (Bachelard et al., 1984). There is

evidence to suggest that lactate, metabolized directly via pyruvate, can replace glucose in supporting synaptic transmission in hippocampal slices (Schurr et al., 1988), but substitution of pyruvate plus malate for glucose, which is known to maintain a normal energy status in vitro (Woodman & McIlwain, 1961), strongly depresses fEPSPs and population spikes (Cox & Bachelard, 1982). It appears therefore that glucose deprivation itself and not a loss of energy supply is responsible for the depression in synaptic transmission, although lactate can be a substitute for glucose.

3.3.2 Glucose deficiency inhibits synaptic transmission by postsynaptic mechanisms

The overflow of excitatory amino acids during hypoglycemia in vivo (Sandberg et al., 1986; Butcher et al., 1987a) and low glucose condition in vitro (Szerb & O'Regan, 1987; Szerb, 1988; Burke & Nadler, 1988) has been well documented. Insulin-induced hypoglycemia caused 12-15 fold increase of aspartate and 3-6 fold increase of glutamate efflux in vivo (Sandberg et al., 1986; Butcher et al., 1987a). Although it is impossible to determine the Ca^{2+} dependence of the increased glutamate and aspartate efflux in vivo, Engelsen & Fonnum (1983), and Butcher et al. (1987a) were able to show that prior destruction of

glutamatergic/aspartatergic corticostriatal neurons attenuated the increased aspartate and glutamate release induced by hypoglycemia. This observation suggests a synaptic contribution to the hypoglycemia-induced overflow of excitatory amino acids. In vitro studies have shown a Ca^{2+}-dependence of the increased release of aspartate and glutamate from the synaptic transmitter pool during glucose deprivation (Szerb & O'Regan, 1987; Szerb, 1988; Burke & Nadler, 1988). It has also been demonstrated that the increased glutamate release and at least part of the increased aspartate release during low glucose are due to the impairment of glial function, i.e. the uptake of glutamate and aspartate by glia is reduced (Szerb & O'Regan, 1988). In any case, glucose deficiency does not inhibit presynaptic transmitter release.

Several lines of evidence suggest that the excitability of postsynaptic neurons is decreased when less glucose is available. Extracellular studies have shown that in the dentate gyrus of the guinea pig hippocampus, the effectiveness of fEPSPs to produce field population spikes was reduced or blocked (Cox & Bachelard, 1982; Bachelard et al., 1984). Even when fEPSPs were barely affected in the presence of 5 mM glucose, the amplitude of population spikes was decreased by 60-80% (Cox & Bachelard, 1982). The input-output relationship between fEPSPs and the population spikes shifted to right, which indicates that

the same amplitude of fEPSPs produced smaller population spikes in low glucose (Bachelard et al., 1984).

The studies of Spuler et al. (1988; 1989) showed that glucose deprivation hyperpolarized the membrane potential of guinea pig hippocampal CA3 neurons and significantly increased membrane conductance. Under voltage clamp, 0 mM glucose activated an outward current with a reversal potential similar to K^+ equilibrium potential. The involvement of G proteins and of the Na^+/K^+ pump was excluded because the G protein inhibitor, pertussis toxin and the Na^+/K^+ pump blocker ouabain had no effect on the outward current induced by glucose deprivation (Spuler et al., 1988; Spuler & Grafe, 1989).

In isolated mouse dorsal root ganglion neurons, depletion of glucose and other metabolic blocking agents such as cyanide, significantly decreased the voltage-dependent Ca^{2+} currents (Duchen, 1990), but the intracellular Ca^{2+} concentration was increased by up to 220% (Duchen et al., 1990). It was therefore concluded that Ca^{2+} is released into cytoplasm from intracellular stores when energy supplies are reduced and the regulatory mechanisms which maintain a constant intracellular Ca^{2+} concentration are impaired (Duchen et al., 1990). On the other hand, removal of glucose or addition of cyanide increased a Ca^{2+}-dependent K^+ conductance (Duchen, 1990). This Ca^{2+}-dependent K^+ conductance was not related to ATP

levels but could be sufficient to shunt action potentials. In the hippocampal CA3 cells, glucose deprivation also produced a rise in intracellular Ca^{2+} concentration. However, the K^+ conductance induced by glucose deprivation did not appear to be Ca^{2+} dependent because it preceded, not followed, the increase of intracellular Ca^{2+} (Knopfel et al., 1990).

Glucose metabolism and the subsequent changes in ATP levels regulate the activities of a specific K^+ channel, the ATP-sensitive K^+ channel. Manipulations that decrease intracellular ATP, also activate the ATP-sensitive K^+ channel, while those that increase intracellular ATP close the channel (For review, see Ashcroft, 1988). There is evidence that ATP-sensitive K^+ channels modulate both glutamate release and the membrane potential changes induced by anoxia (Grigg & Anderson, 1989; Ben-Ari, 1990). Yet the glucose deprivation-induced outward current in hippocampal CA3 neurons was not affected by glibenclamide, an ATP-sensitive K^+ channel blocker (Knopfel et al., 1990). The involvement of ATP-sensitive K^+ channels in blocking synaptic transmission in low glucose environments has not been investigated.

In summary, evidence suggests that the suppression of excitatory synaptic transmissions by glucose deficiency is probably due to postsynaptic mechanisms, the nature of which is far from clear. Therefore, the second part of the

present project was initiated to investigate synaptic transmission under low glucose conditions. With the use of both extra- and intra-cellular recordings, the effect of low glucose concentration on the relationship between the presynaptic volley, EPSP and population spikes (or othodromically evoked action potentials) was investigated in order to establish the site of transmission block. The effect of glucose deprivation on membrane potential, resistance and the responses induced by exogenous glutamate agonists were also included in this study.

MATERIALS AND METHODS

Chapter 4 PREPARATION of SLICES AND SUPERFUSION SOLUTIONS

4.1 Animals, anaesthesia and slicing

Adult, male Sprague-Dawley rats (Canadian Hybrid Farms,150-300g) were anaesthetized with 15 ml ether in a 2L glass jar, and were kept under ether for 1 min after they lost consciousness and stopped moving in order to obtain a deep anaesthesia. Then they were killed by cervical dislocation with an iron bar and decapitated. The exposed skull was cut with scissors along the right and left margins and the bone flap was removed with forceps. The brain was placed in Krebs solution cooled in ice. The Krebs solution had the following composition (in mM): NaCl 120; $CaCl_2$ 2.6; KCl 3.5; NaH_2PO_4 1.2; $MgSO_4$ 1.2; $NaHCO_3$ 25; glucose 5.0. After separating the two hemispheres, the hippocampus was carefully dissected out and placed on a filter paper moistened with Krebs solution. The hippocampus was then cut at high speed and with strong force with a McIlwain tissue chopper at 0.4 mm intervals. The 0.4 mm thick, transverse slices were separated by washing with a stream of Krebs solution and were transferred to a recording chamber, where the totally submerged slices were

superfused at 30-32 °C with Krebs solution at a rate of 1-3 ml/min. The time between the death of the animal and the placing of the slices in the chamber was 3-4 minutes.

4.2 Superfusion and the superfusion chamber

The superfusion chamber (Fine Science Tools Inc., North Vancouver. B.C.) consists of two parts, an inner bath where the slices are incubated and an outer bath filled with distilled water. A thermistor regulated the temperature of the inner bath through a heater in the outer bath. The outer bath was bubbled with 95% O_2 and 5% CO_2, thereby providing warm and humidified gas for the atmosphere above the inner bath while stirring the water in the outer bath.

A 30 cm long polyethylene tube (inner diameter 2-3 mm) through which the Krebs solution flowed was coiled in the outer bath so that the solution was heated to the desired temperature before it reached the inner bath. Well-oxygenated Krebs solution (with 95% O_2 and 5% CO_2) flowed from a bottle placed about 1 m above the chamber, at 1-3 ml/min. Solution changes were achieved by combining the use of a 4-way-valve and two 2-way-valves. After flowing through the inner bath, the Krebs solution was collected in a draining well and removed by suction. Fluid level in the inner bath was adjusted by changing the vertical

position of the suction needle. Flow rate was measured by counting the drops flowing through a Travenol i.v. set.

Drugs used in the experiments were added to the Krebs solution or were introduced by iontophoresis. In several cases, sucrose was added to replace the reduced glucose concentration or NaCl was reduced by the equivalent of K^+ or NH_4Cl added. The pH of a well gassed Krebs solution was 7.28 and 5 mM NH_4Cl reduced this to 7.24.

Slices were allowed to equilibrate during the initial 60-90 min of superfusion. Following the equilibration period, experiments using extracellular recording consisted of 15-30 min superfusion with control solution followed by 15-30 min superfusion with different concentrations of glucose or NH_4Cl and a final 30 min superfusion with no NH_4Cl or with normal glucose concentration.

NH_4Cl, Na glutamate, quisqualic acid, DL-2-amino-5-phosphonovaleric acid (APV) and tetrodotoxin (TTX) were obtained from Sigma Chemical Co. (St.Louis, MO). Ethyl ether was from Fisher Scientific Co. (Fair Lawn, New Jersey). N-methyl-D-aspartate (NMDA) and 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX) were purchased from Research Biochemicals Inc. (Natick, MA). The concentrations for bath application were: NH_4Cl 1-4 mM; Na glutamate 2 mM; quisqualic acid 10-20 μM; NMDA 10-20 μM; APV 25-50 μM; CNQX 20-40 μM; TTX 0.3 μM. For iontophoresis,

concentrations in the electrode were: Na glutamate 1 M; quisqualic acid 20-100 mM (in 150 mM NaCl solution); NMDA 10 mM (in 150 mM NaCl solution).

Chapter 5 ELECTRODE CONSTRUCTION AND RECORDINGS

5.1 Stimulating electrodes

Monopolar (for extracellular study) or bipolar (for intracellular study) stimulating electrodes (Frederick Haer, Brunswick ME) were placed in the stratum radiatum at the CA1/CA2 border. Stimuli were applied at a rate of 0.1 Hz (6/min) for extracellular studies and 0.017 Hz (1/min) to 0.033 Hz (2/min) for intracellular studies. In extracellular studies, stimulation intensity was adjusted to give 2/3-3/4 of the maximal field potentials or to be slightly above threshold for producing orthodromic intracellular action potentials with each stimulus. To observe antidromic conduction, paired stimuli, 10 msec apart, were applied alternately to either the alveus or the Schaffer collaterals and the population spike in the pyramidal layer was measured. Paired stimuli clearly distinguished between orthodromic and antidromic excitation since only orthodromic stimulation resulted in facilitation.

5.2 Extracellular recording

Field EPSPs and population spikes were recorded according to the method described by Dingledine (1984). Two glass microelectrodes filled with Krebs solution (resistance 2-4 Mohm) were placed into the stratum radiatum and stratum pyramidale respectively. A monopolar tungsten stimulating electrode located in the stratum radiatum at CA1/CA3 border delivered constant current pulses to the Schaffer collateral/commissural fibers (Fig. 1). Potentials were digitized with a Lab Master Board and stored on an IBM PC computer, using a program kindly provided by Dr. Peter G. Aitken of the Department of Physiology, Duke University. The average of four evoked potentials was collected every 2 min and saved on diskettes for analysis. Signals obtained were compared with a calibration signal (1 mV, 1ms) generated by a square wave calibrator (Grass Instrument, Quincy, MA) delivered at the beginning of each sweep. The amplitude of population spikes was measured in records obtained with the electrode in the stratum pyramidale. Presynaptic volley and field EPSPs were recorded with the electrode in the stratum radiatum. However, instead of using the amplitude of the EPSPs, the maximal rate of depolarization of field EPSP was estimated, because fEPSPs were often reduced in size by a large population spike which appeared as a positive

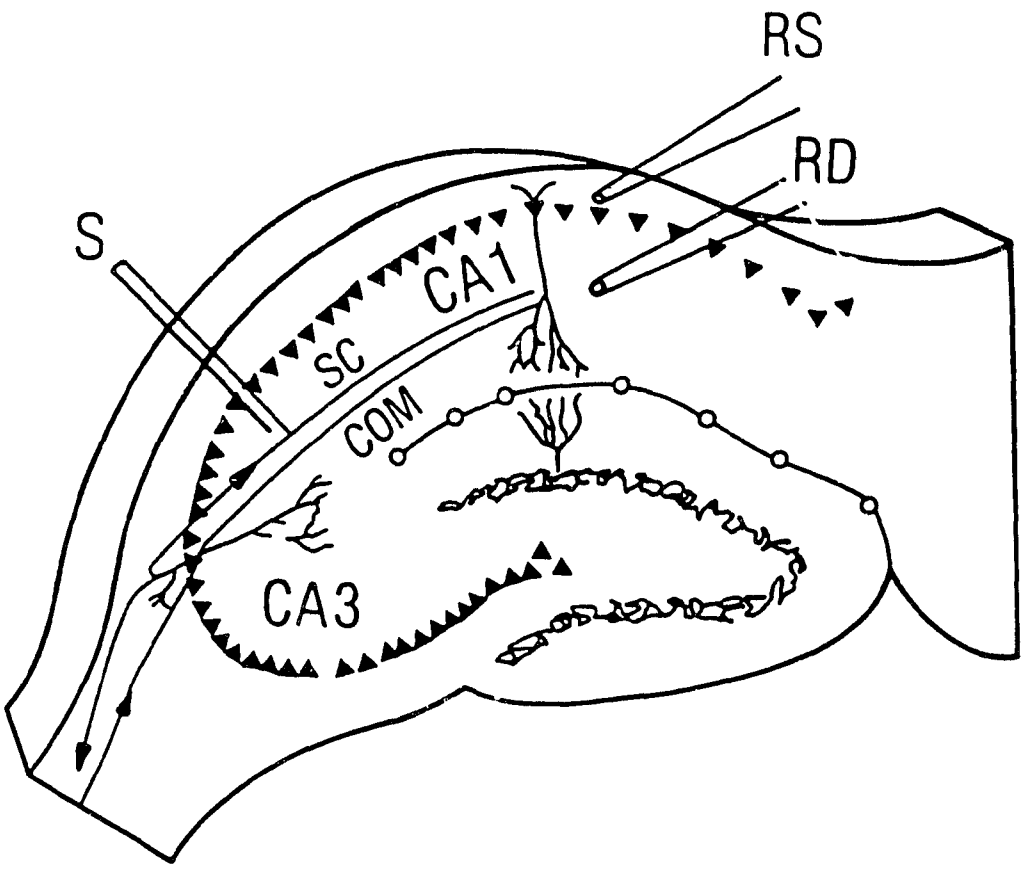


Fig. 1. Sketch of the experimental set up in the transverse hippocampal slice. Abbreviations: CA1, CA1 pyramidal cell layer; CA3, CA3 pyramidal cell layer; COM, commissural collaterals; SC, Schaffer collaterals; RD, recording electrode in dendritic layer; RS, recording electrode in soma layer; S, stimulating electrode.

deflection in the stratum radiatum (Aitken, 1985). The program determines the maximal rate of potential change between the starting point of the field EPSP and the point at which field EPSP amplitude is maximal. This is the maximal rate of rise of field EPSP. Presynaptic fiber volley results from the extracellular currents surrounding the synchronously activated Schaffer collateral/ commissural fibers. Field EPSPs reflect mainly excitatory postsynaptic currents flowing across pyramidal cell membrane. The synchronous firing of CA1 pyramidal cells produces population spikes.

Carbon filament electrodes (2-5 Mohm) made by the procedure developed by Armstrong-James and Millar (1979) contain an ultrafine carbon fiber (about 7 μM in diameter) and have a higher signal-to-noise ratio compared to the saline-filled glass electrode. They were placed in the stratum pyramidale for recording unit firings of CA1 pyramidal cells induced by the iontophoresis (Neuro Phore BH-2, Medical Systems Corp.) of glutamate, NMDA or quisqualate to the underlying stratum radiatum. Iontophoresis electrodes had a resistance of 10-50 Mohm. The number of unit firings induced by the iontophoresis was counted on a record replayed at 1/360 of the original speed.

5.3 Intracellular recording

CA1 pyramidal cells were impaled with glass microelectrodes filled with 3 M K^+-acetate, 2 M K^+-methyl sulfate or 3 M KCl (40-100 Mohm). To attempt to block the K^+ conductance, electrodes filled with Cs^+ were tested. However, neurons impaled with electrodes containing 2 M cesium chloride suddenly depolarized when exposed to ammonium salts and were consistently lost.

Changes in membrane properties were observed in either the bridge mode or the single-electrode voltage-clamp mode (SEVC) of the axoclamp II amplifier (Axon Instruments). The bridge mode was used to observe the membrane potential and resistance. For measuring input resistance the bridge balance, which compensated for the resistance of the electrode, was constantly monitored on a fast oscilloscope sweep. The balance was adjusted so that only a slow potential change, resulting from the membrane resistance, contributed to the deflection, without a fast voltage step which indicated a contribution from the resistance of the electrode.

Electrodes used to measure membrane currents in the SEVC mode had an impedance of less than 70 Mohm. In this mode the sampling frequency was set at 3-4 KHz, gain was set at 0.6-2.5 nA/mV and the duty cycle was monitored on a fast oscilloscope sweep triggered by the sample clock output of

the Axoclamp amplifier. This setting appeared to be adequate for maintaining a constant somatic membrane potential in nearly all experiments, even in those in which the experimental protocol produced large membrane currents.

After stable recording was obtained, data were collected simultaneously on a chart recorder and on a Zenith computer (Z-386/20) at a sampling rate of 5-20 KHz with Axotape (Version 1.2, Axon Instrument) or with P-Clamp (Version 5.5, Axon Instrument) programs. Data were analyzed on replayed records. Axotape program modified by Mr. Paul MacInnis of the Department of Physiology & Biophysics, Dalhousie University was used to merge individually triggered sweeps and to get analogue output of the stored data. This output was printed on a laser printer.

Statistical analysis of the data was carried out with either Student's t-test or two way analysis of variance. Differences were judged to be significant if the probability level was 5% or less ($P<0.05$).

EXPERIMENTS WITH AMMONIUM IONS

Chapter 6 Results

6.1 Extracellular studies

To determine whether ammonium ions inhibit synaptic transmission by a pre- or post-synaptic mechanism, their action on synaptic transmission was compared with their effect on glutamate release in fully submerged hippocampal slices in experiments carried out by another group of investigators in our laboratory at the same time.

6.1.1 Time course and concentration dependence of NH_4^+-induced synaptic depression

Transmission from Schaffer-collaterals to CA1 pyramidal cells, as monitored by extracellular recording in fully submerged hippocampal slices, was inhibited by 0.5 to 3 mM NH_4Cl in a concentration-dependent manner (Fig. 2A; Fig. 3), even though previous studies have shown that 5 mM NH_4Cl does not reduce the Ca^{2+}-dependent evoked release of glutamate (Szerb & Butterworth, 1991). While 0.5 mM NH_4Cl caused a reduction only of the population spike amplitude (not shown), 1-3 mM NH_4Cl also decreased the rate of rise of the fEPSP. With 1 mM NH_4Cl, the amplitude of compound

presynaptic action potentials (presynaptic volley) was reduced by about 10% (Fig. 2B), indicating that the axonal excitability and conduction were only slightly affected. The reduction in afferent volley amplitude probably is a reflection of the smaller action potentials seen with intracellular recording in the presence of ammonia (Fig. 9, Table 1). The rate of rise of EPSPs was reduced by about 35% and the amplitude of population spikes by about 80%. With 3 mM NH_4Cl, the inhibitory effect of ammonia on synaptic transmission was stronger, there was about a 20% reduction in presynaptic volley, 70% reduction in the rate of rise of EPSPs and the population spike was almost abolished (Fig. 2C; Fig. 3). The effect of NH_4Cl appeared 2-4 min after the start of superfusion and was fully reversible. Replacing Krebs solution with another identical solution or replacing NH_4Cl with equal amount NaCl had no significant effect on any of the three variables measured.

Two sets of experiments were designed to investigate further the effect of NH_4Cl on synaptic transmission.

6.1.2 Input/output relationship

The first set of experiments studied the effect of NH_4Cl on the input-output relationships between presynaptic volley and dendritic fEPSPs and between dendritic fEPSPs and

population spike amplitude. The relationship between the first two represents the action of the presynaptically released transmitter, while that between the rate of rise of dendritic fEPSPs and the amplitude of the population spikes represents the ability of fEPSPs to induce action potentials. The slope of the presynaptic volley-EPSP relationship was reduced by 1 mM NH_4Cl with no effect on the slope of the fEPSPs-population spike input/output curve (Fig. 4).

6.1.3 Iontophoresis

The second set of experiments investigated the effects of NH_4Cl on unit firing evoked by different EAA agonists: glutamate, quisqualate and NMDA. Two and five mM NH_4Cl had a weak, although significant, inhibitory effect on glutamate-induced unit firing which was reduced by 25-40% (Fig. 5). Ammonia is known to depress both excitatory and inhibitory synaptic transmissions and these opposite effects of ammonia on excitability could conceivably obscure a strong depressant action of ammonia on glutamate-induced excitation. To observe the action of ammonia on the excitatory effect of glutamate, without a change in excitability due to a reduced inhibition, the $GABA_A$ antagonist bicuculline methylchloride was introduced before and during the application of ammonia. In the

presence of 25 μM bicuculline, glutamate-induced firing was increased by about 50% and the inhibitory effect of NH_4Cl became stronger: 5 mM NH_4Cl produced a 70% inhibition (Fig. 5). Two-way analysis of variance showed the potentiating effect of bicuculline on the depressant effect of ammonia to be small but significant ($df=1/26$, $F=4.45$, $P<0.05$). It appears therefore, that the inhibitory effect of ammonia on glutamate-induced unit firing was only slightly reduced by a concurrent increase in the excitability of neurons due to a decrease caused by ammonium ions in hyperpolarizing IPSPs.

A weak inhibitory effect of 3 mM NH_4Cl was also observed on NMDA-induced firing (data not shown). In contrast, 3 mM NH_4Cl inhibited the quisqualate-induced firing by more than 80% (Fig. 6), as compared to the 40% inhibition of glutamate firing by 5 mM NH_4Cl in the absence of bicuculline. The inhibitory effect of ammonium ions on quisqualate-induced firing was significant ($P<0.01$, Student's test) at each of the 5 time points during the 30 min application of NH_4Cl.

6.2 Intracellular studies

The results from extracellular studies reported above suggest that the location of the inhibitory effect of NH_4Cl on transmission is probably on the postsynaptic neuron.

Therefore, the effect of NH_4Cl on CA1 pyramidal cells and synaptic transmission was investigated through intracellular recording.

6.2.1 Effect of NH_4^+ on membrane potential and resistance

Potential changes in 31 neurons exposed to NH_4Cl were recorded, using the bridge mode. With this technique the internal resistance of the electrode is balanced electronically, thereby allowing the measurement of the membrane resistance of the penetrated cell by observing the amplitude of voltage deflections resulting from constant current pulses. These neurons had an initial resting membrane potential of -66.0 ± 1.4 (SE) mV, a membrane resistance of 46.2 ± 2.4 Mohm and action potential amplitude of 93.0 ± 2.4 mV. After a short delay (2-3 min), 1-4 mM NH_4Cl depolarized the membrane which then remained at this depolarized level during the presence of NH_4Cl. It returned to control level when NH_4Cl was washed out (Fig. 7A). The membrane potential decreased by 15.1 ± 1.4 (SE) mV, a significant change compared to the control resting membrane potential ($P<0.01$, Student's t-test). The extent of depolarization was variable and there was no significant difference between the depolarization induced by 1-4 mM NH_4Cl, as indicated by one way analysis of

variance (df=3/27, F=1.57, P>0.22) (Fig. 7B).

No consistent membrane resistance change was observed at the time when NH_4Cl depolarized the resting membrane potential (Fig. 7A). On average, in 31 neurons, membrane resistance was reduced non- significantly by 1.1 ± 1.8 Mohm as tested by 0.2 nA hyperpolarization pulses (P>0.05, Student's t-test). To see the effect of NH_4^+ on membrane resistance at different membrane potentials, currents with different amplitudes were injected intracellularly and the corresponding membrane potential changes were observed to obtain current-voltage curves. The current-voltage relationship observed before and after the administration of NH_4Cl showed that NH_4^+ did not change the membrane resistance when membrane potential was between -40 to -90 mV (Fig. 8). However an increased resistance at higher membrane potentials was observed.

In the presence of NH_4Cl-induced depolarization, the amplitude of action potentials evoked by current injection decreased by 15-30% and action potentials became wider mostly due to a slower rate of repolarization (Fig. 9, Table 1). When the resting membrane potential was restored during the application of NH_4Cl by the injection of hyperpolarizing current, the amplitude of action potential almost completely recovered, while the rate of repolarization was still significantly slower (Fig. 9B, Table 1). In addition, NH_4^+ suppressed the 3-4 mV early

hyperpolarization which followed the control action potential.

In 11 neurons spontaneous action potentials were present during the initial control period. In 6 of them, spontaneous firing persisted during the application of NH_4Cl. In the other 5, spontaneous firing stopped 8-18 min after the introduction of NH_4Cl. Often rebound firing following the hyperpolarizing pulses appeared in the presence of NH_4^+ even when spontaneous action potentials disappeared (Fig. 11).

Although NH_4Cl had little effect on the overall input resistance, it did suppress a delayed inward rectification, similar to the Q-current. Q current is activated when the membrane potential is hyperpolarized beyond -80 mV (Halliwell & Adams, 1982). It serves to resist hyperpolarizing deviation from the resting membrane potential and its activation produces a characteristic depolarization sag in the voltage trajectory during the current injection. Slow deactivation of the Q-current contributes to the rebound depolarization and excitation when the hyperpolarizing current is removed (Fig. 10A). In 3 mM NH_4Cl, the depolarization sag and the rebound depolarization were eliminated (Fig. 10B, n=5). As a result, the steady state voltage deflections induced by large negative currents were increased by NH_4Cl, giving an apparent increase in resistance at steady state. However

there was no change in the instantaneous ohmic resistance (Fig. 10C). Due to the depolarization caused by NH_4Cl (from -67 mV to -52 mV), only one of the hyperpolarizing currents used in the presence of NH_4Cl created a hyperpolarization beyond -80 mV, i.e. -82 mV. By comparing the Q-currents at -82 mV in the absence and the presence of 3 mM NH_4Cl, results indicate a nearly complete suppression of the Q-current by ammonium ions (Fig. 10D). This is in agreement with the results shown in Fig. 8, namely that NH_4^+ decreases membrane conductance at membrane potentials more negative than -90 mV. Rebound depolarization was also eliminated by 3 mM NH_4Cl, but rebound firing often persisted probably because action potentials were inactivated by depolarization and reactivated by hyperpolarization pulses (Fig. 10B).

6.2.2 Effect of NH_4^+ on synaptic transmission

To correlate the effects of NH_4^+ on synaptic transmission with changes in the membrane properties of the post-synaptic neuron, synaptic transmission, membrane potential and resistance were simultaneously monitored with intracellular recording techniques. NH_4Cl (4 mM) gradually blocked synaptic transmission (Fig. 11,12). Orthodromic postsynaptic action potentials evoked by the stimulation of Schaffer-collaterals first became smaller in amplitude

and broader due to a slower rate of depolarization and repolarization. Before the postsynaptic action potential was blocked (about 6-8 min after the application of NH_4^+), the hyperpolarization which followed the action potentials was reduced. Then EPSPs gradually decreased in amplitude, becoming subthreshold for producing action potentials (8-10 min after the application of NH_4^+) and finally disappeared completely (about 10-12 min after the application of NH_4^+). However, rebound firing was still present with its amplitude reduced and duration prolonged (Fig. 11). Occasional transmission block appeared at the initial stage of the depolarization, but a complete block of transmission, i.e. no postsynaptic spikes, occurred only several minutes after the beginning of the depolarization plateau. Sometimes spontaneous burst firing lasting 10-15 ms and containing 3-5 action potentials during the application of 1 mM NH_4^+ was also seen (Fig. 13).

At the beginning of NH_4Cl application, an increase in stimulus intensity restored synaptic transmission, but later the block in synaptic transmission could not be overcome by stronger stimulation currents (Fig. 14). When NH_4Cl was washed out, membrane potential quickly returned to control level, and after some delay synaptic transmission also reappeared (Fig. 11,12,13). In most cases there was no significant change in membrane

resistance during the NH_4Cl administration (Fig. 11,12) but occasionally, during the application of 1 mM NH_4^+, the membrane resistance decreased and this was accompanied by a further depolarization (Fig. 13).

In summary, by recording the effects of NH_4^+ with intracellular electrodes, it could be shown that depolarization and the block in synaptic transmission followed different time courses: upon the introduction of ammonium ions, synaptic block appeared later than depolarization. After the washout of ammonium ions, synaptic block took longer to recover than the membrane potential (Fig. 11, 12). Furthermore, while NH_4^+ produced a steady level of depolarization, its inhibitory effect on transmission became progressively stronger with time.

6.2.3 Effect of NH_4^+ on excitatory amino acid-induced inward currents

Results from this laboratory previously showed that 5 mM NH_4Cl did not inhibit the evoked Ca^{2+}-dependent release of glutamate (Szerb & Butterworth, 1991). This suggested that ammonium ions blocked transmission by reducing the response of postsynaptic neurons to the released transmitter. To test this hypothesis, single electrode voltage clamp (SEVC) technique was employed in one series

of experiments to prevent the depolarizing effect of NH_4^+ on the somatic membrane potential because this depolarization may reduce the effectiveness of EAA agonists.

In agreement with the observation of a depolarizing effect of NH_4^+ seen in the bridge mode, voltage clamping revealed that NH_4^+ generally induced an inward current (Fig. 15,16,17,20), except when NMDA was applied in the presence of Mg^{2+} (Fig. 18,19). Under voltage clamp conditions 3 mM NH_4Cl reduced only slightly the glutamate-induced inward current (Fig. 15) but almost completely abolished the quisqualate current (Fig. 16; Table 2). The amplitude of quisqualate currents varied when quisqualic acid was applied for different lengths of time, but irrespective of their size, they were all depressed by NH_4Cl (Fig. 17). A result not seen in iontophoresis studies was the dramatic facilitation of NMDA current by 3 mM NH_4Cl, both in the absence (Fig. 18) and in the presence of TTX (Fig. 19; Table 2). This facilitating effect disappeared when Mg^{2+} was omitted from the superfusion solution (Fig. 20; Table 2). It appears therefore that in the presence of Mg^{2+}, NH_4^+ has opposite effects on quisqualate and NMDA-induced currents.

Since 20 to 40 mM CNQX, an AMPA receptor blocker (Honoré et al., 1988), eliminated 90 ± 5% of the quisqualic acid induced inward current (Fig. 21, n=5), quisqualic acid

must have induced an inward current by acting largely on AMPA and not through metabotropic receptors (Baskys, 1992). Inward currents induced by AMPA itself were generally inconsistent and therefore AMPA was not investigated extensively, but in two cases where the AMPA current was relatively stable, 3 mM NH_4Cl suppressed about 80% of the inward current induced by AMPA. All the effects of NH_4Cl were reversible.

To confirm and to investigate further its opposite actions on quisqualate and NMDA currents, the effect of NH_4Cl on glutamate currents was monitored in the presence of the antagonists of AMPA and NMDA receptors, CNQX and APV respectively. When NMDA receptors were blocked with APV, NH_4^+ significantly inhibited glutamate current, by more than 50%, as could be expected from its action on quisqualate current (Fig. 22, Table 2). This inhibition was somewhat less than the decrease in quisqualate current by NH_4^+. However, contrary to expectations, NH_4Cl failed to enhance glutamate current when AMPA receptors were blocked by 20-40 μM CNQX (Table 2), probably because CNQX displaced glycine from the NMDA receptor and thereby reduced the NMDA currents (Lester et al., 1989).

The opposite actions of NH_4Cl on responses evoked by quisqualate and NMDA were also observed when the membrane potential was continuously recorded. In 3 mM NH_4Cl, quisqualate-induced membrane depolarization was almost

abolished (n=6, Fig. 23) while NMDA-induced membrane depolarization was significantly increased (n=3, Fig. 24). This disappeared in the absence of added Mg^{2+} (n=3, Fig. 25). Immediately after the washout of NH_4Cl, the NMDA-induced membrane depolarization was much smaller than the depolarization produced by NMDA in the control trials (Fig. 25). This depression was not investigated any further.

In summary, the inhibitory effect of NH_4^+ on synaptic transmission from Schaffer-collateral/commissural fibers to CA1 neurons has been observed in both extracellular and intracellular studies. Postsynaptically, NH_4Cl depolarizes the membrane potential with no consistent effect on membrane resistance, reduces the amplitude and repolarization rate of action potentials and suppresses Q currents. Quisqualate induced postsynaptic responses were also significantly depressed by NH_4^+. The NMDA induced responses were, however, facilited by NH_4^+, while glutamate current consisting at least of AMPA and NMDA currents was only slightly affected.

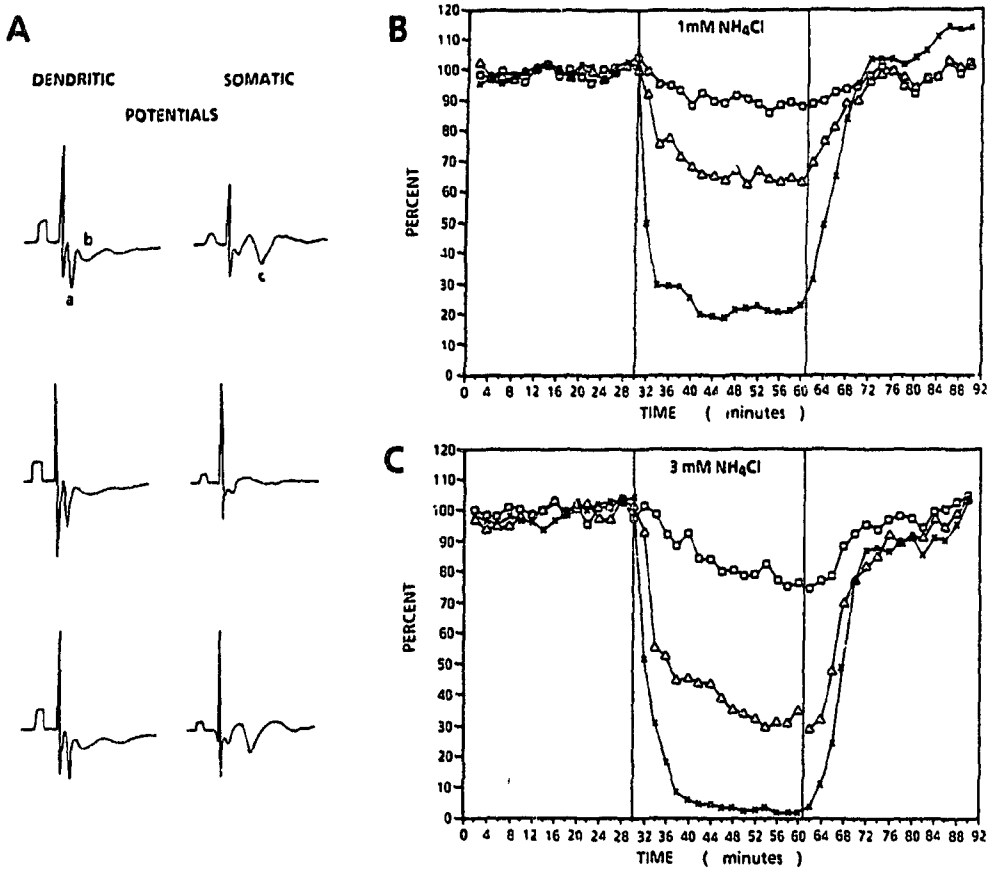


Fig. 2. A: An example of the effect of 1 mM NH_4Cl on synaptic transmission from Schaffer collaterals to CA1 pyramidal cells monitored by extracellular field potentials. a. Presynaptic volley; b. dendritic EPSP; c. population spike. Calibration pulses(1 ms, 1mV) are shown at the beginning of each trace. Top: before; middle: 20 min after the start of superfusion with 1 mM NH_4Cl; bottom: washout. Summaries of the effects of 1 mM (B) and 3 mM (C) NH_4Cl are also shown. The 15 average potentials recorded during the initial 30 min control superfusion were normalized as 100% and the other potentials were expressed as percentages. NH_4Cl was present during the middle 30 mins period; n=6 for 1 mM NH_4Cl and n=4 for 3 mM NH_4Cl. Open squares, presynaptic volley; open triangles, dendritic EPSP; crosses, population spikes.

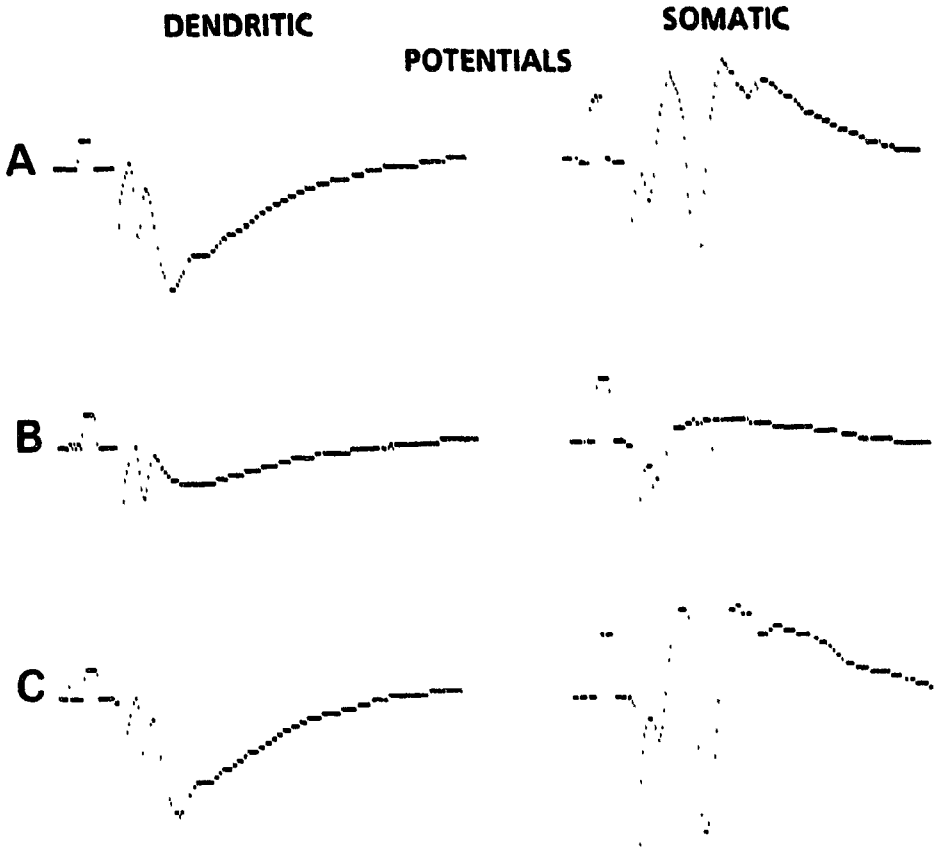


Fig. 3. Effect of 3 mM NH_4Cl on synaptic transmission. Field dendritic EPSPs and somatic population spikes are shown as indicated. A. Control. B. 25 min after the application of 3 mM NH_4Cl. Note the reduction in the size of fEPSPs and population spikes. C. Return. Calibration: 1 mV, 1 ms.

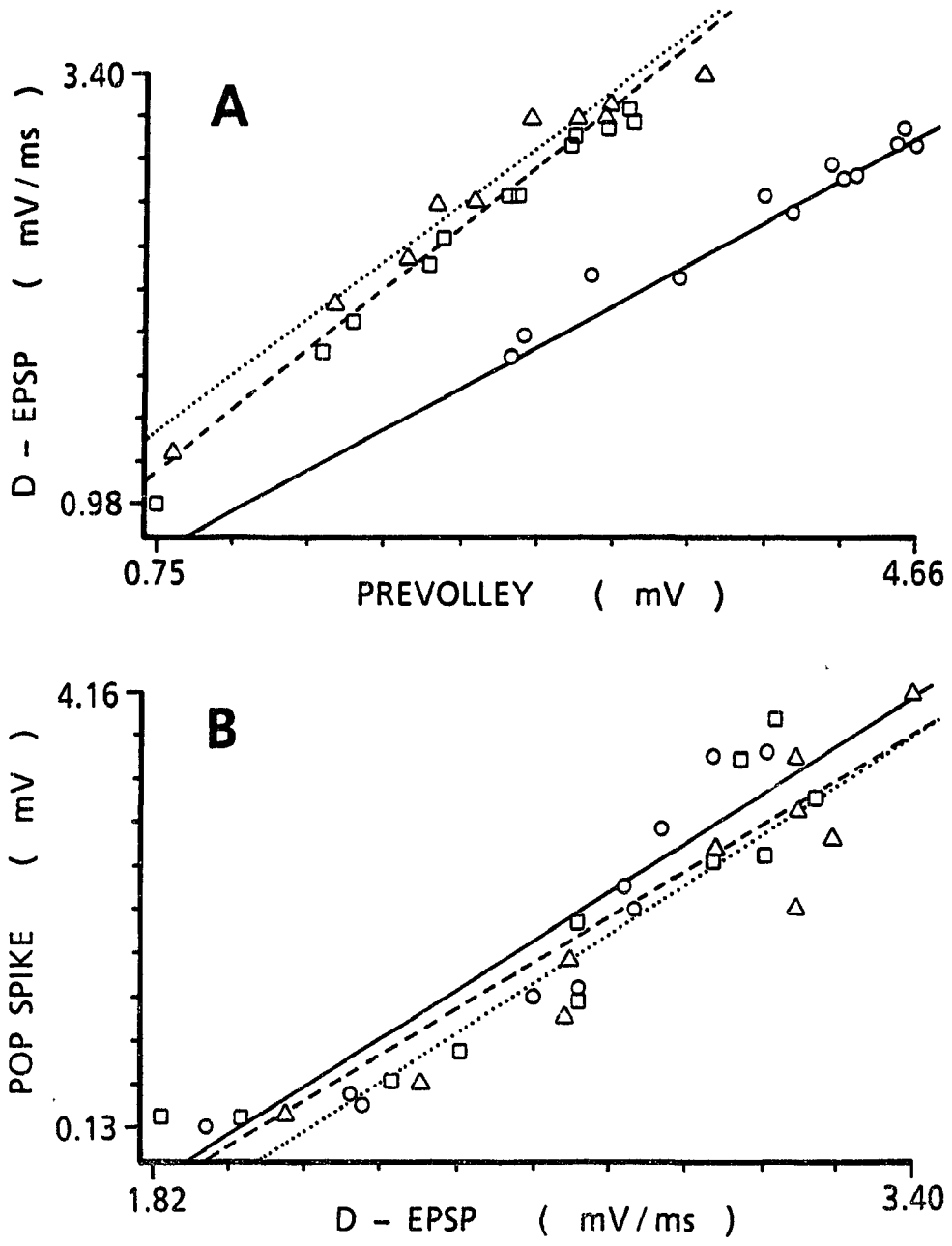


Fig. 4. An example of the effect of 1 mM NH_4Cl on the input/output relationship between the amplitude of presynaptic volley and maximal rate of rise of dendritic EPSP (A) and maximal rate of rise of dendritic EPSP and soma population spike amplitude (B) at different intensities of stimulation. Linear regression lines were drawn by the least square method. Squares and broken lines, control; circles and solid lines, 20 min after 1 mM NH_4Cl; triangles and dotted lines, washout.

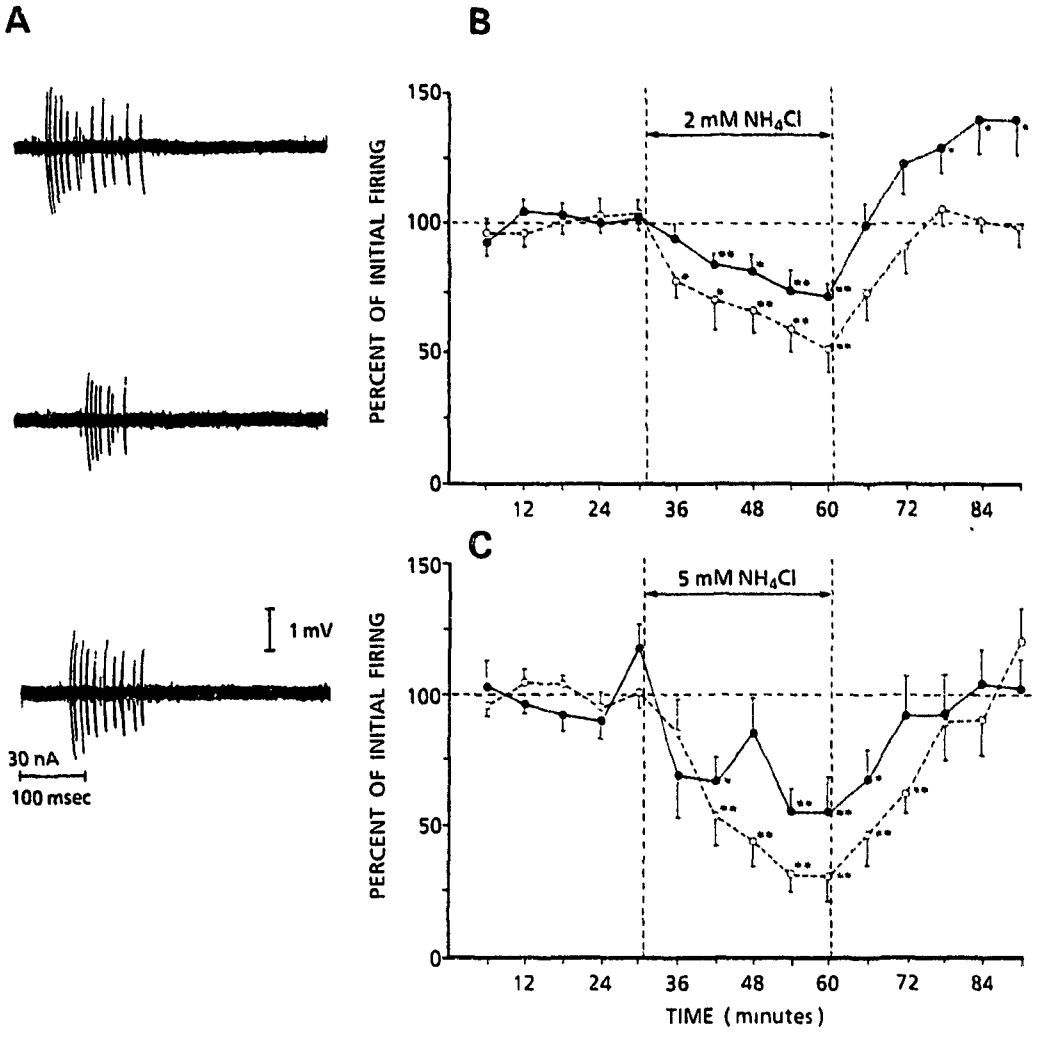


Fig. 5. A: Effect of 5 mM NH4Cl on unit firing induced by 100ms of glutamate. Top, control; middle: 20 min after the start of superfusion with 5 mM NH_4Cl; bottom: washout. Records shown were digitized, stored and replayed on a curvilinear pen recorder at 1/360 of the original speed. Summaries of the effects of 2 mM (B) and 5 mM (C) NH_4Cl are also shown. Filled circles, no bicuculline; open circles, 25 μM bicuculline. n=8 for 2 mM NH_4Cl; n=7 for 5 mM NH_4Cl. The average of the control samples was considered as 100%. Standard errors are shown. * $P<0.05$, ** $P<0.01$.

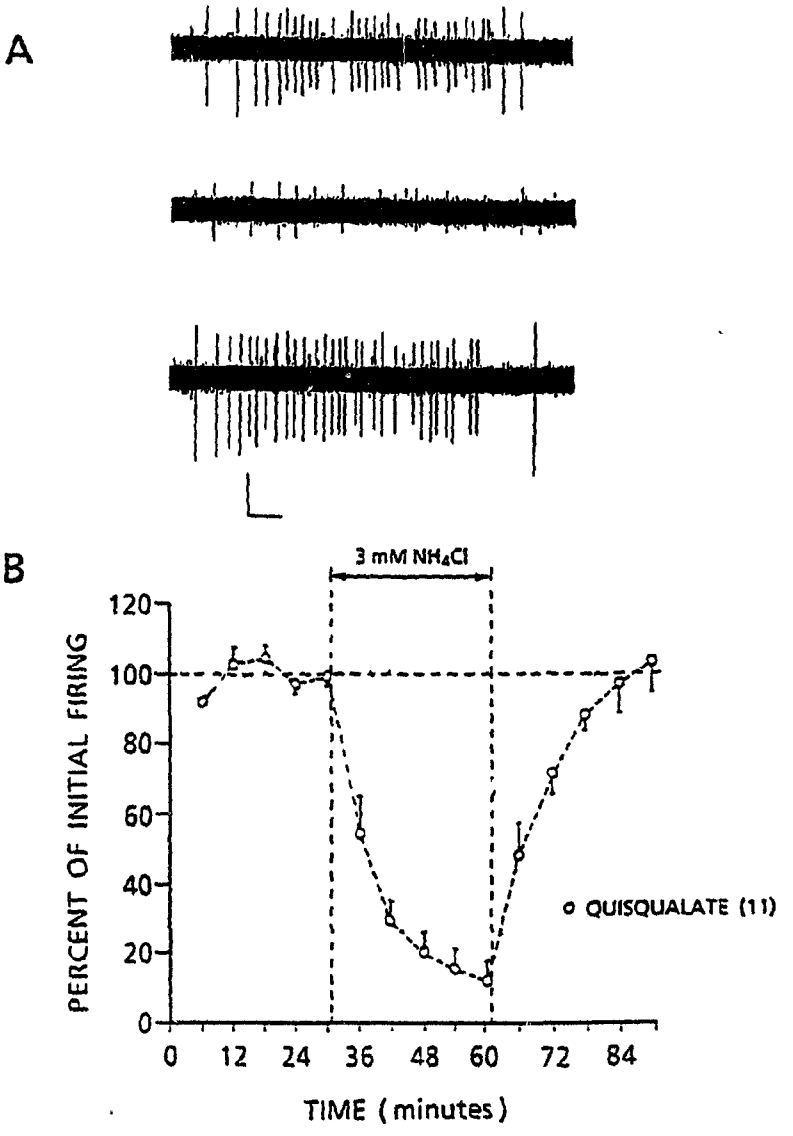


Fig. 6. The effect of NH_4Cl on unit firing induced by 500 ms iontophoresis of quisqualate. A. Top, Before; middle, during 3 mM NH_4Cl administration (12 min after NH_4Cl); bottom, washout. Calibration: 0.5 mV, 1 sec. B. Summary of the effects of 3 mM NH_4Cl on firing induced by quisqualate, n=11.

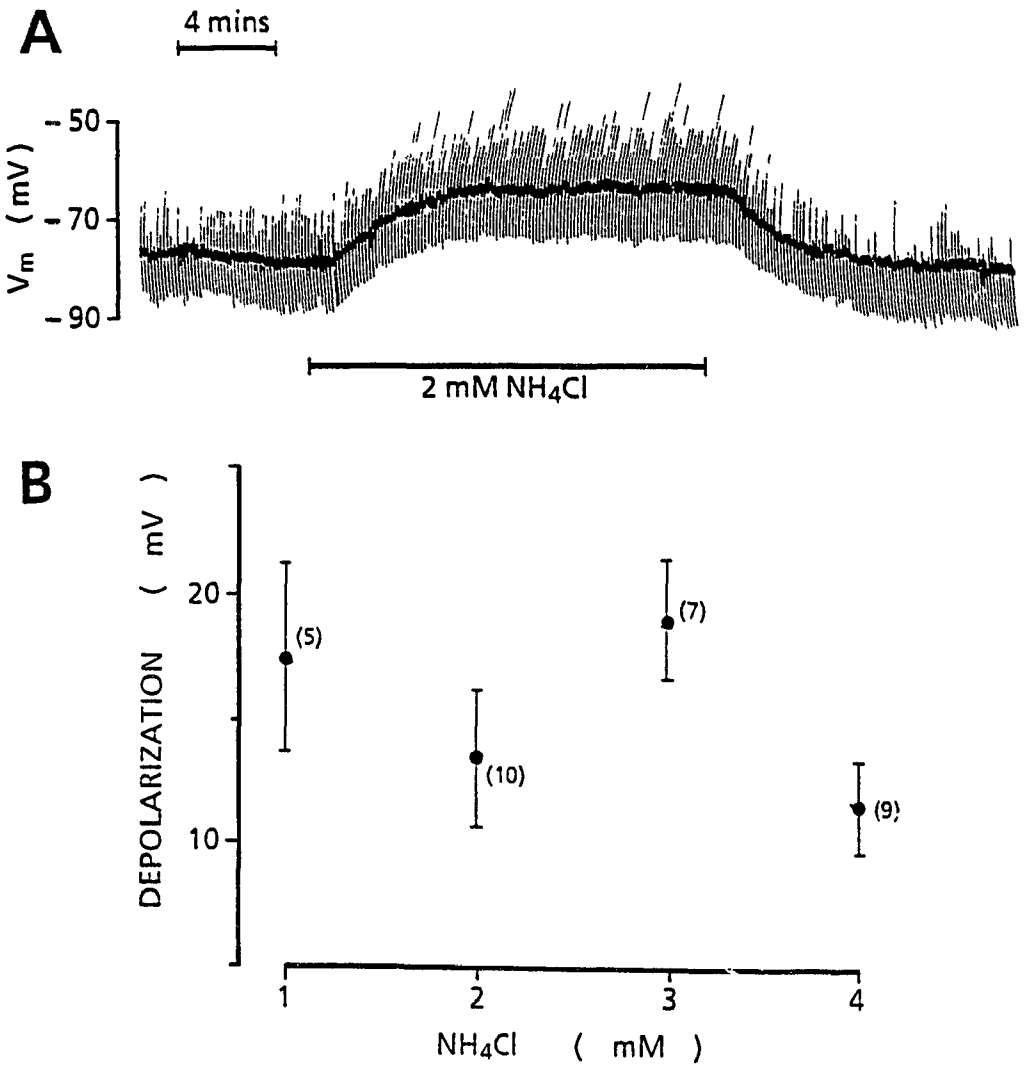


Fig. 7. A: Effect of 2 mM NH_4Cl on membrane potential and resistance. Downward deflections: potential changes due to 0.2 second, 0.2 nA hyperpolarzing pulses applied every 10 sec; upward deflections: truncated action potentials. Initial Vm= -76 mV, Rm=30 MΩ. Reversible, 15 mV depolarization during the plateau phase. B: Depolarizing effects (±SE) of different concentrations of NH_4Cl during the plateau phase. No. of observations in brackets.

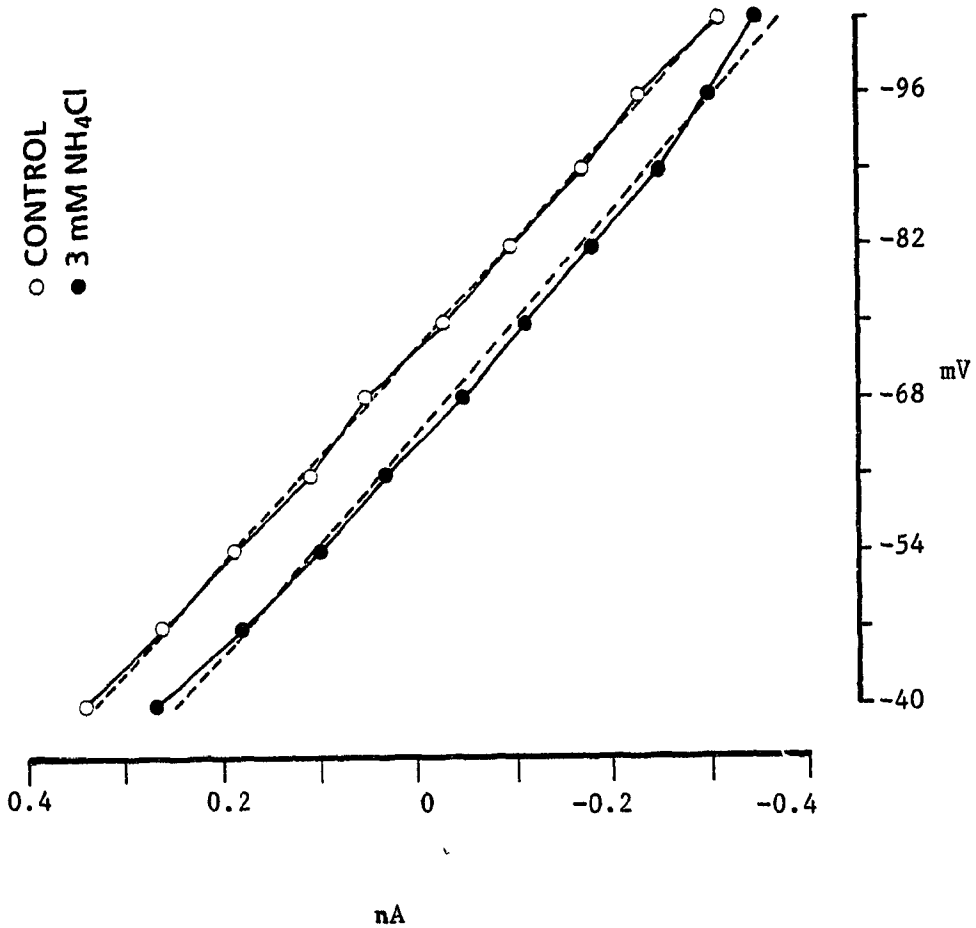


Fig. 8. Effect of 3 mM NH_4Cl on current-voltage relationship. Current-voltage curve was obtained by injecting positive and negative currents of different amplitudes for 200 ms and recording the resulting potential changes. Open symbols control, closed symbols: during NH_4Cl, which caused a 6 mV depolarization without a change in the I-V slope between -40 mV and -90 mV. At a more negative potential NH_4Cl decreased membrane conductance. Broken lines are the regression lines. The neuron had a resting Vm of 72 mV.

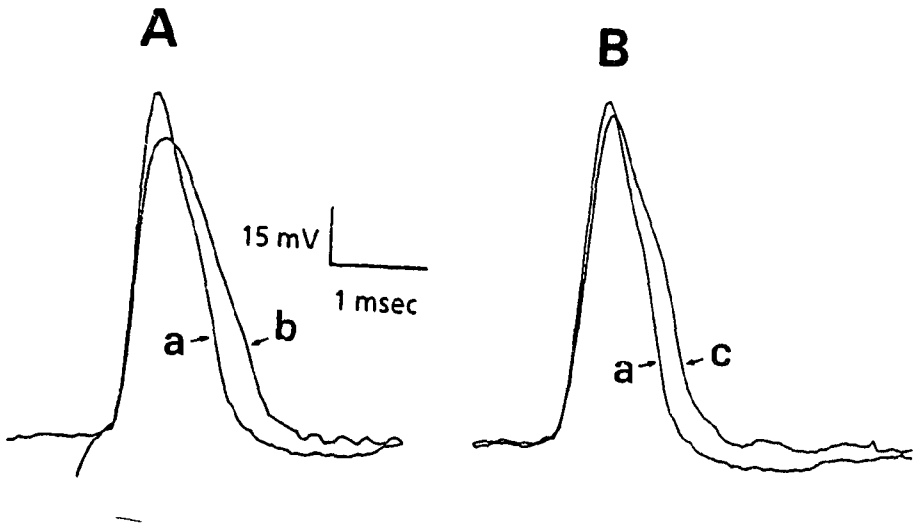


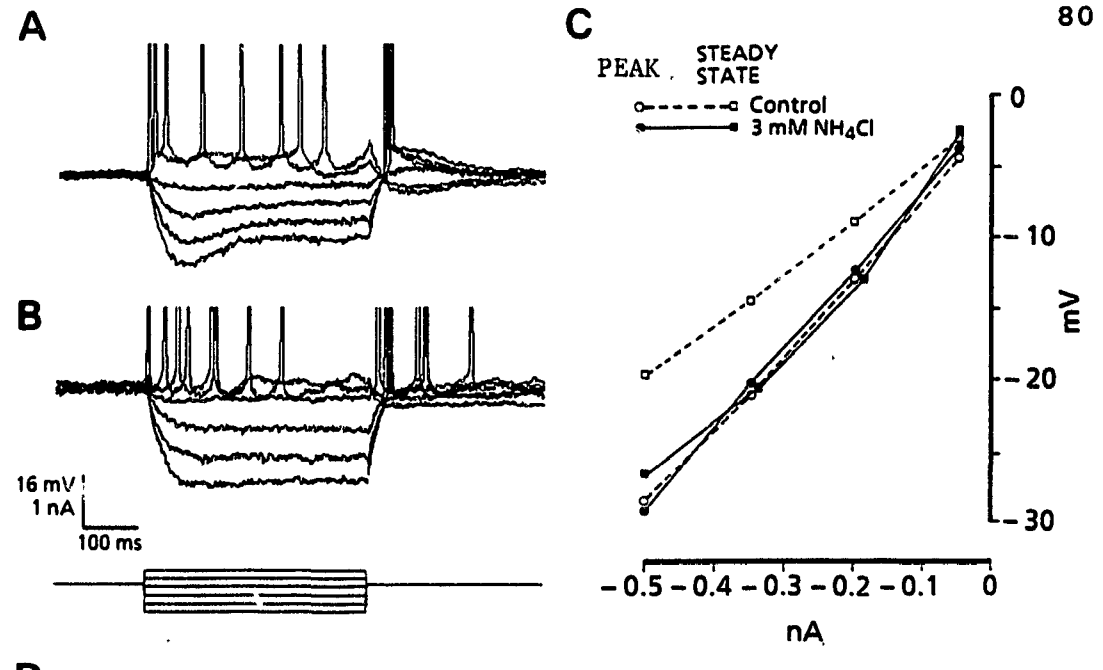
Fig. 9. Effect of 3 mM NH_4Cl on the shape of action potentials evoked by inward current applied through the recording electrode. In each case the first of a series of action potentials is shown. A. a: control, (Vm= -67 mV), b: during 3 mM NH_4Cl (Vm= -45 mV), a and b were put together in order to compare. B. a: control, c: during 3 mM NH_4Cl but membrane potential restored to approxmately control (Vm= -68 mV). Note: in A, action potentials before and during exposure to ammonia were repositioned for easier comparision, in spite of a change in membrane potential.

TABLE 1

EFFECTS OF NH^{4+} ON ACTION POTENTIAL PARAMETERS

Exp.	Condition (n)	V_m (mV)	AP (mV)	$\frac{dV}{dt}$ depol ($V.sec^{-1}$)	$\frac{dV}{dt}$ repol ($V.sec^{-1}$)
	Control (6)	-66.7 ±0.4	85.5 ±0.8	185.7 (100%) ±9.8	74.4 (100%) ±1.6
1	3 mM NH_4^+ (3)	-45.0*** ±0.1	74.3*** ±0.3	159.5 (85.9%) ±4.7	46.4 (62.4%)*** ±0.2
	3 mM NH_4^+ Hyperpolar. (5)	-68.3 ±0.1	82.2** ±0.5	191.9 (103.3%) ±6.7	51.4 (69.1%)*** ±1.6
	Control (4)	-65.5 ±0.4	83.8 ±1.1	217.1 (100%) ±8.4	77.1 (100%) ±1.6
2	3 mM NH_4HCl (2)	-55.0*** ±1.4	81.2 ±1.1	211.2 (97.3%) ±2.7	52.9 (68.6%)*** ±1.7
	3 mM NH_4HCl Hyperpolar. (4)	-64.2 ±0.2	86.0 ±0.6	215.0 (99.0%) ±1.5	56.9 (73.8%)*** ±0.5

Vm is the resting membrane potential before current injection. Action potential amplitude(AP), rate of depolarization and repolarization refer to the first action potential evoked by the intracellular injection of current. Hyperpolarization in the third row of each experiment refers to measurements made during the injection of appropriate current to restore Vm to control level. Significance of differences: ** $P<0.01$; *** $P<0.001$.



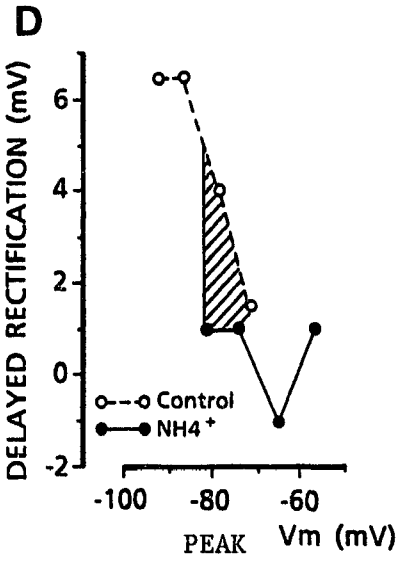


Fig. 10. Effect of 3 mM NH_4Cl on the I-V relationship and delayed inward rectification. A. In control, voltage deflections induced by injected current ranged between -0.5 and -0.05 nA activated a characteristic depolarization sag during the current injection and a rebound depolarization when the current was removed. Vm= -67 mV. B. 25 min after 3 mM NH_4Cl, the depolarization sag and rebound depolarization was abolished, but not the rebound firing. AHP following the action potentials was considerably decreased. Vm= -52 mV. C. The I-V relationship of the peak and steady state currents before and after 3 mM NH_4Cl. D. Plot of delayed rectification (Y axis) against peak membrane potential (X axis). Delayed rectification is the difference between peak and steady state membrane potential; peak membrane potential is the sum of the membrane potential before current application and the peak voltage deflection produced by the injected current. Shaded area indicates the suppression of the delayed rectification caused by NH_4^+.

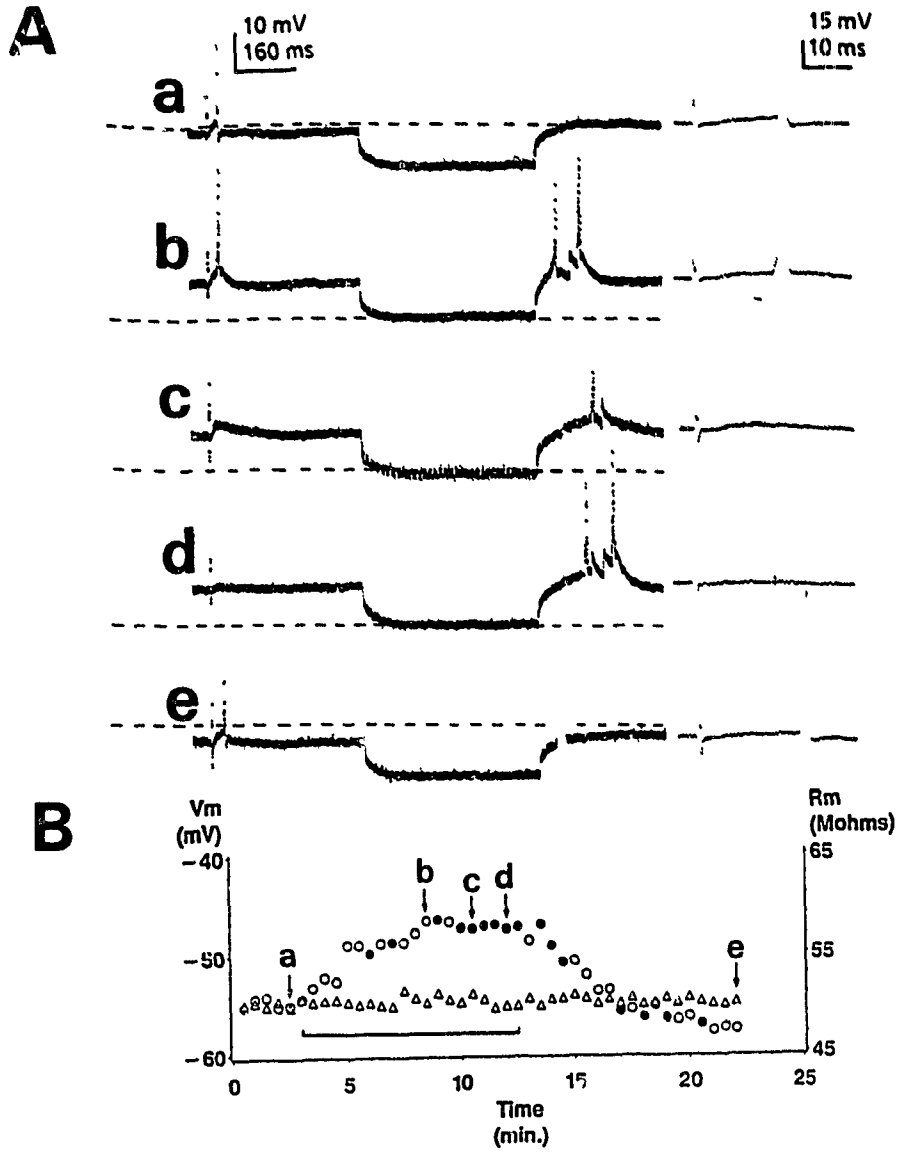


Fig. 11. Effect of 4 mM NH_4Cl on synaptic transmission, membrane potential and resistance measured simultaneously. Synaptic transmission was evoked by the 0.033 Hz stimulation of Schaffer collaterals. A: Individual sweeps (a-e) are shown on the top. On the right, the initial part of the sweeps are shown with an expanded time scale. Vm (round symbols) and Rm (triangles) are shown in B. The timing of the sweeps (a-e) shown in A are indicated. Block of synaptic transmission (no postsynaptic spike) is shown by filled circles in the bottom graph. Superfusion with 4 mM NH_4Cl is indicated by a bar at the bottom. Note the changes in action potential amplitude and shape in b and the rebound firing in b,c and d. Horizontal broken line shows the membrane potential before the application of NH_4Cl.

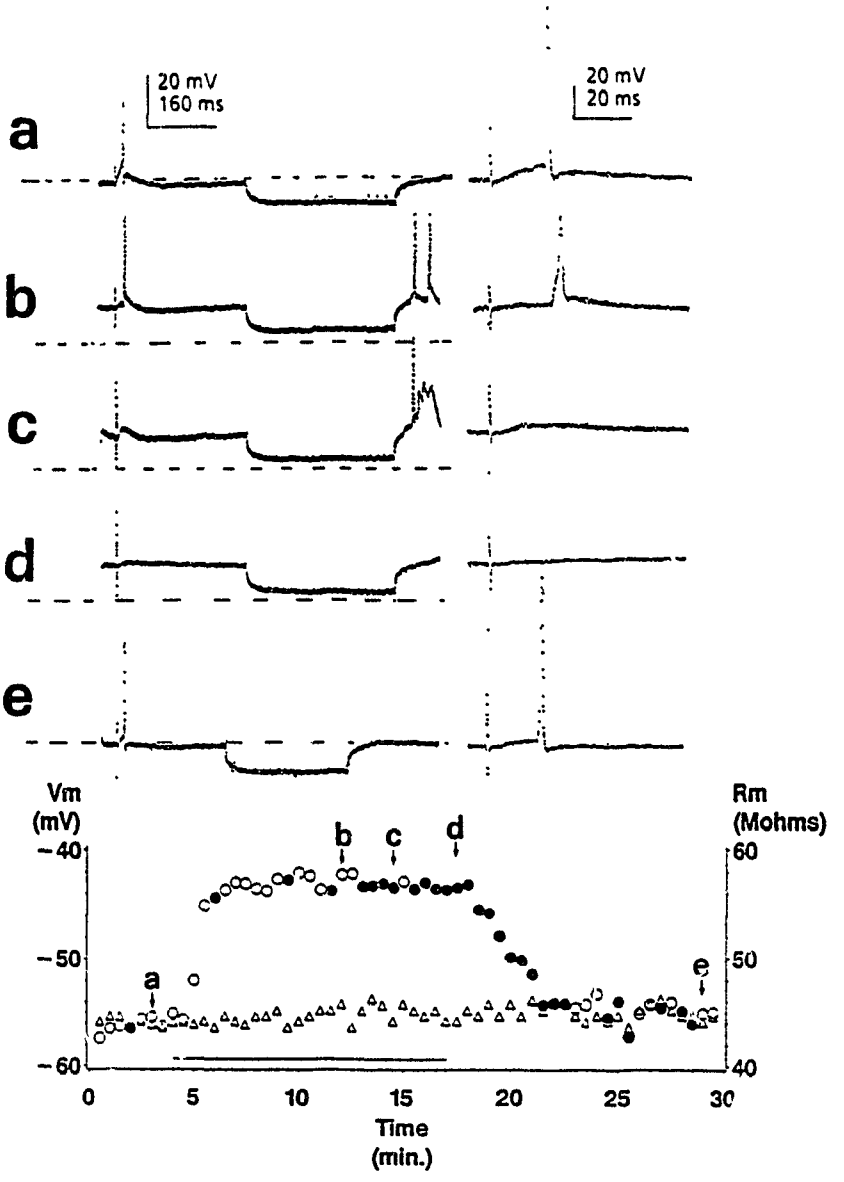


Fig. 12. Another example of the effect of 4 mM NH_4Cl on synaptic transmission, membrane potential and resistance measured simultaneously. Synaptic transmission was evoked by the 0.033 Hz stimulation of Schaffer collaterals. Individual sweeps (a-e) were collected at the times indicated in B. On the right, the initial part of the sweeps are shown with an expanded time scale. Horizontal broken line shows the membrane potential before the application of NH_4Cl. B: The time course of the effect of NH_4^+. Vm, round symbols. Rm, triangles. Block of synaptic transmission (no postsynaptic spike) is shown by filled circles. Superfusion with 4 mM NH_4Cl is indicated by a bar at the bottom. The rate of de- and re-polarization of action potentials and hyperpolarization that followed the action potentials was reduced.

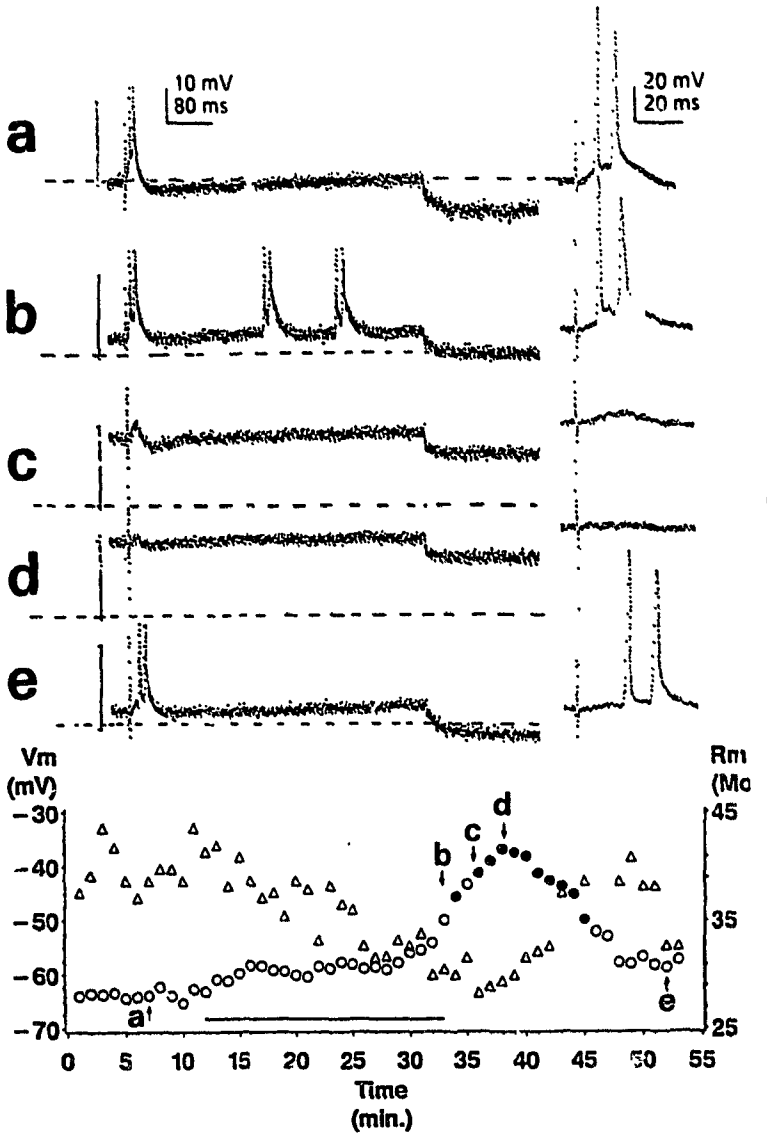


Fig. 13. Effect of 1 mM NH_4Cl on synaptic transmission, membrane potential and resistance measured simultaneously. Synaptic transmission was evoked by the 0.033 Hz stimulation of Schaffer collaterals. A: Individual sweeps (a-e) are shown on the top. On the right, the initial part of the sweeps are shown with an expanded time scale. Vm (round symbols) and Rm (triangles) are shown in B. The timing of the sweeps (a-e) shown in A are indicated. Block of synaptic transmission (no postsynaptic spike) is shown by filled circles in B. Superfusion with 1 mM NH_4Cl is indicated by a bar at the bottom. Hyperpolarizations followed the action potential was reduced (top left). Rm was also decreased in this case. Spontaneous firing appeared before synaptic transmisson failed. Horizontal broken line shows the membrane potential before the application of NH_4Cl.

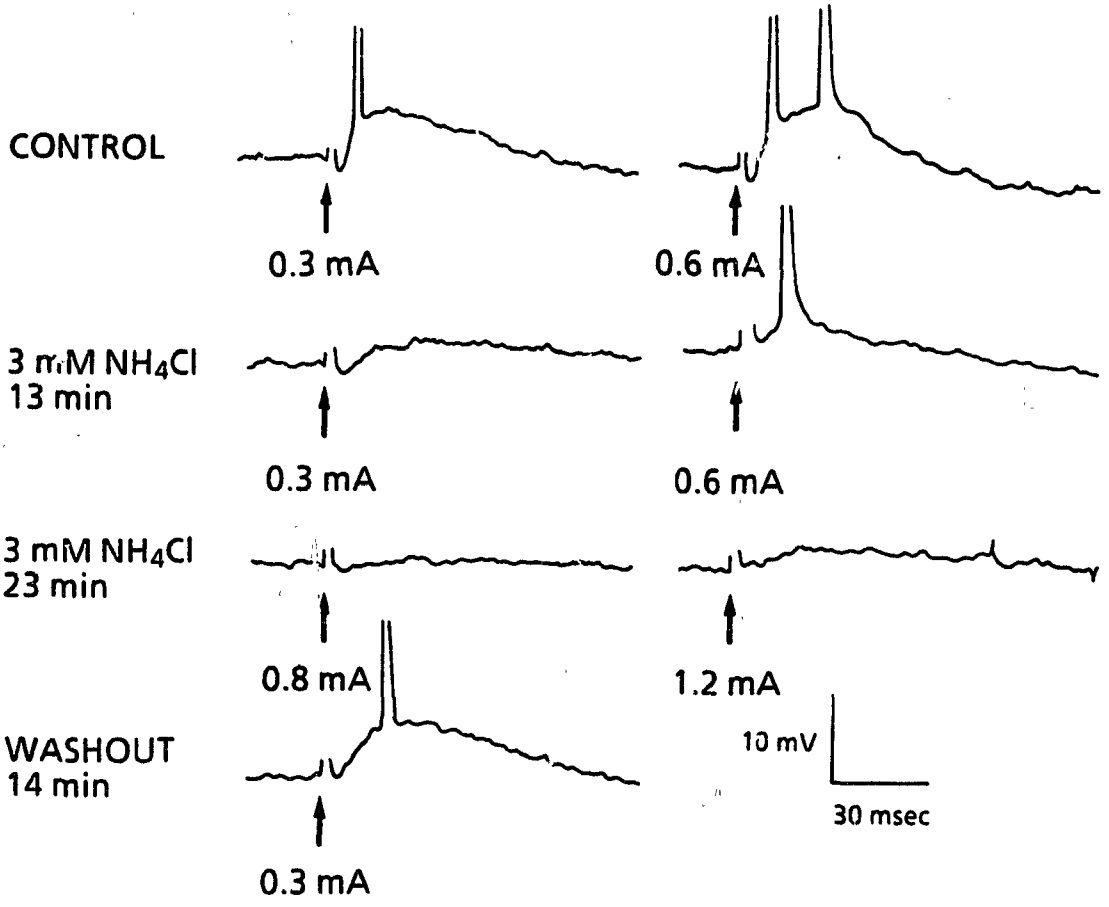


Fig. 14. Effect of increasing the intensity of presynaptic stimulation on transmission in the absence or presence of 3 mM NH_4Cl. Action potentials and stimulus artifacts are truncated. The other three experiments showed similar progressive block of synaptic transmission by 3 mM NH_4Cl.

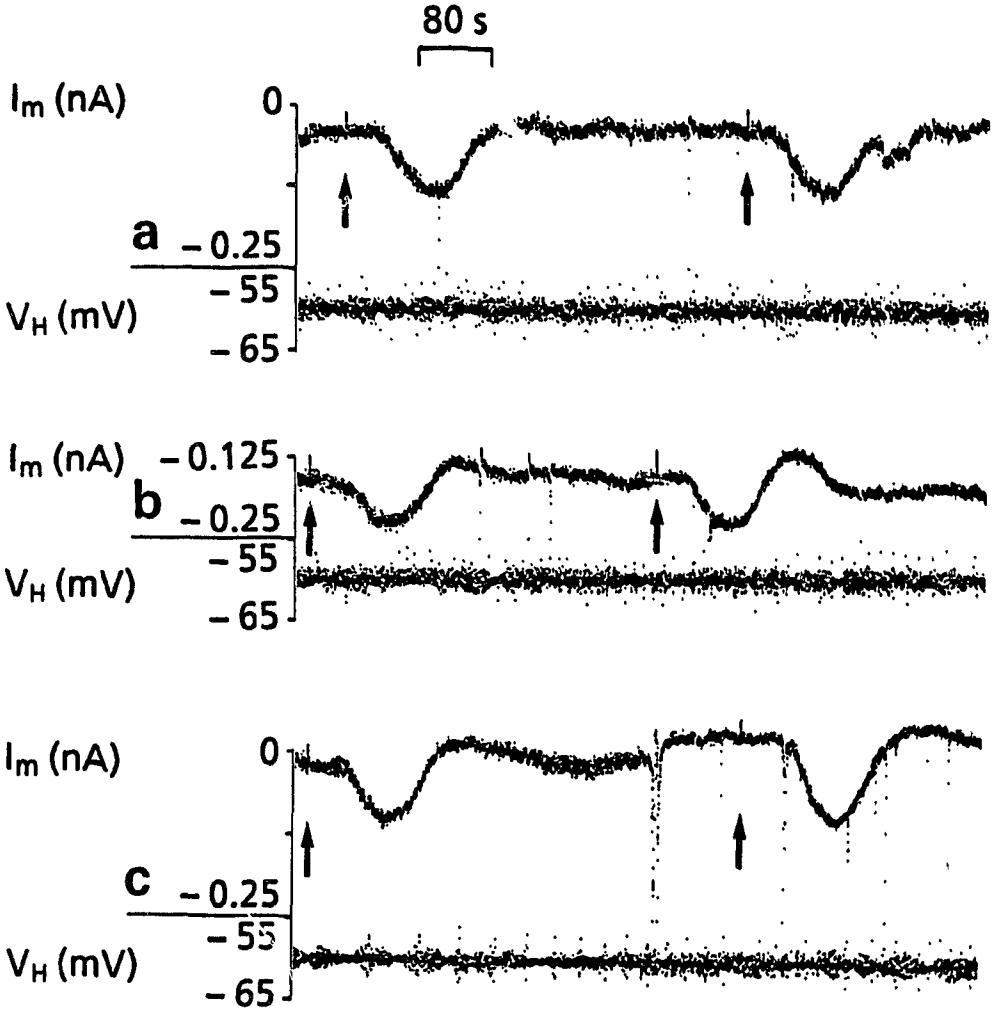


Fig. 15. The effect of 3 mM NH_4Cl on glutamate-evoked inward current. Arrows indicate superfusion with 2 mM glutamate for 55 seconds. a. continuous recording of slow inward current evoked by glutamate (upper trace). V_H is at -60 mV (lower trace). b. 12 and 17 min after superfusion with 3 mM NH_4Cl, holding current for maintaining V_H at -60 mV increased, but glutamate current is only slightly reduced. c. After washing out, both holding and glutamate currents return to normal.

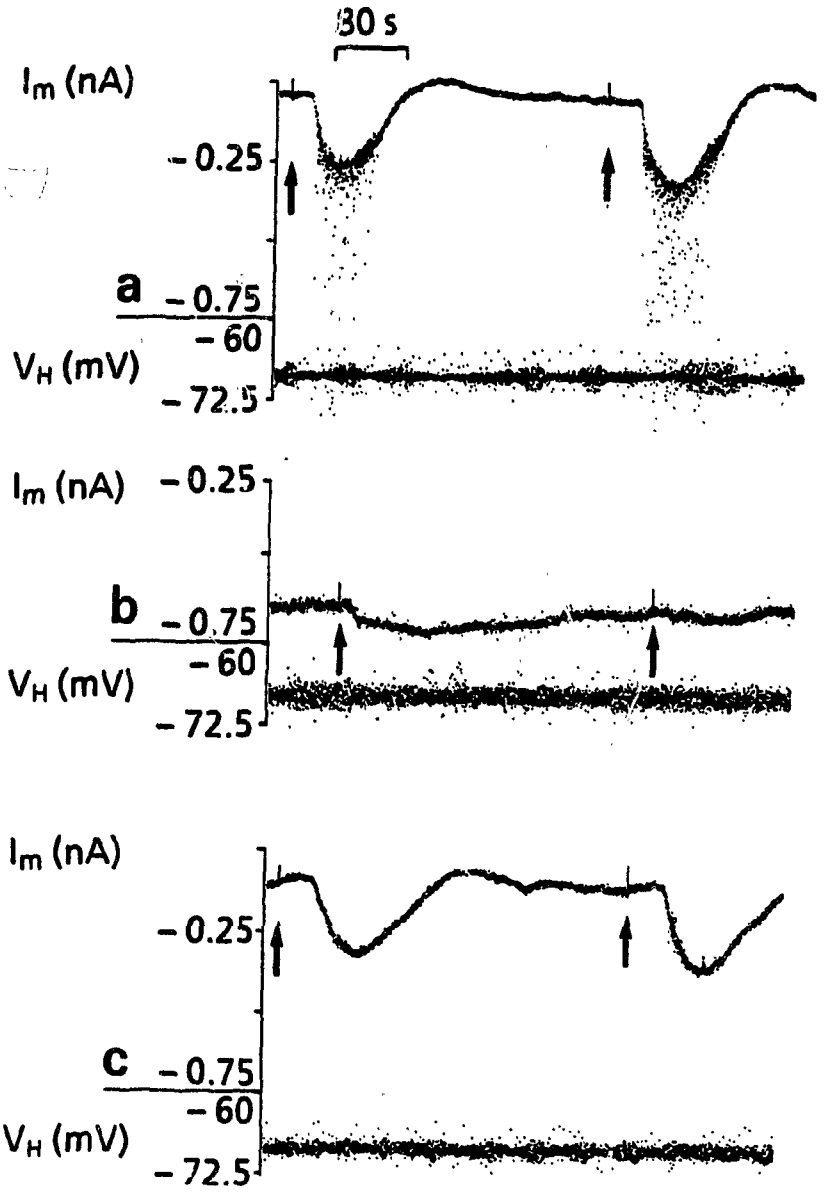


Fig. 16. The effect of 3 mM NH_4Cl on quisqualic acid-evoked inward current (upper trace). Arrows indicate superfusion with 20 μM quisqualic acid for 35 seconds. V_H set at -68 mV (lower trace). a. Control. b. 9 and 14 min after superfusion with 3 mM NH_4Cl. Note a large increase of holding current for maintaining V_H at -68 mV, quisqualic current is almost abolished. c. Following washout.

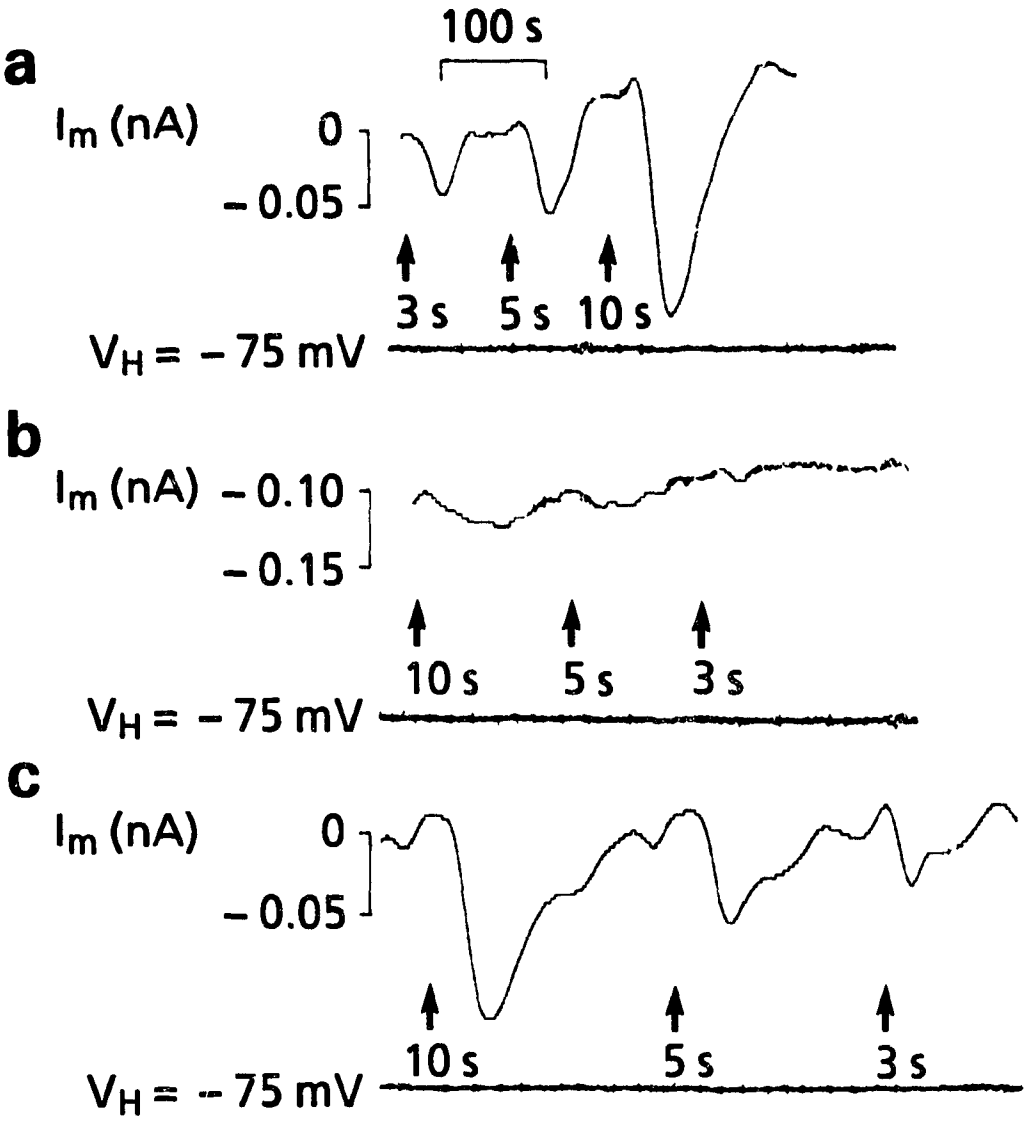


Fig. 17. The effect of 3 mM NH_4Cl on the inward current evoked by quisqualic acid applied for different lengths of time (upper trace). Arrows indicate the applications of 10 μM quisqualic acid during periods indicated in seconds. V_H set at -75 mV (lower trace). A: Control. B: 18 min after superfusion with 3 mM NH_4Cl. C. Washout.

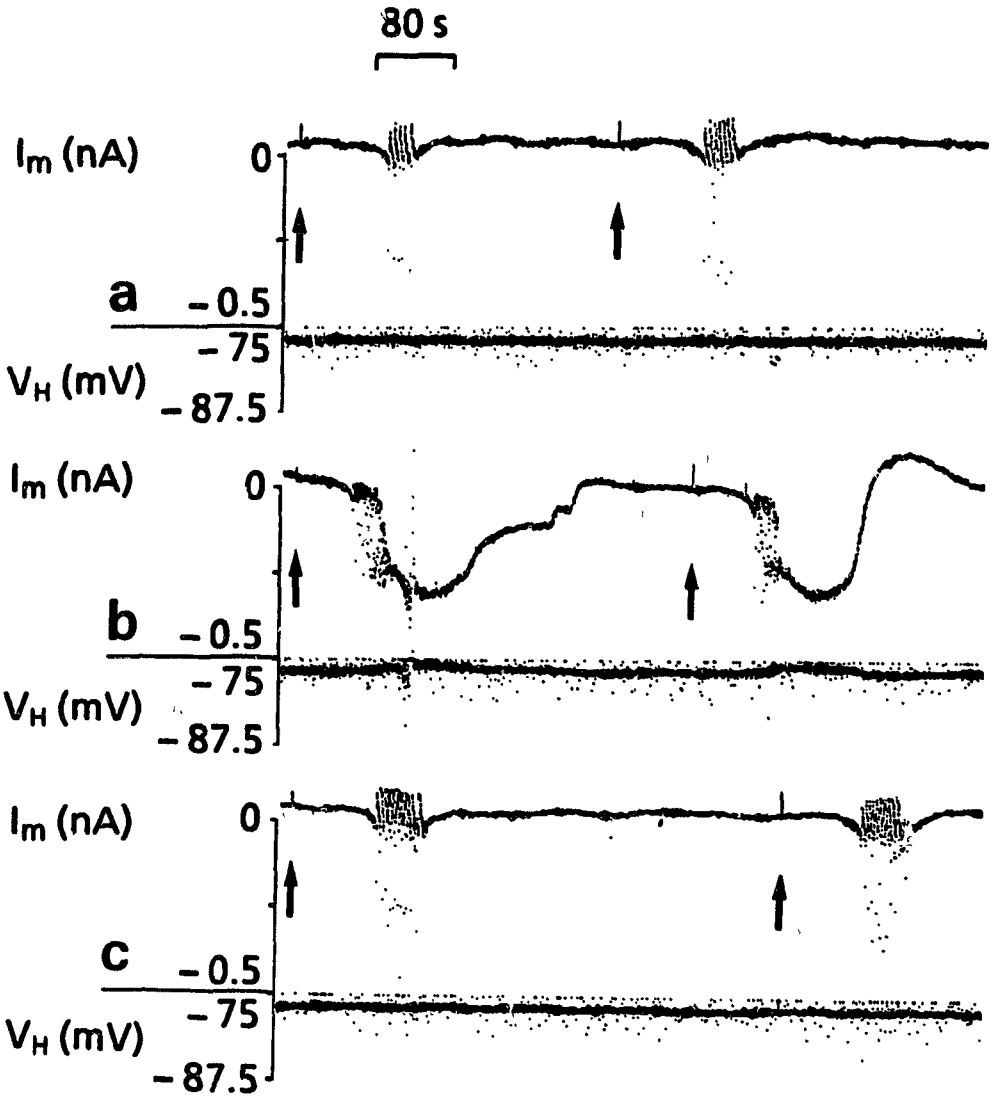


Fig. 18. The effect of NH_4Cl on NMDA-induced inward current (upper trace) in the presence of 1.2 mM Mg^{2+}. Arrows indicate superfusion with 10 μM NMDA for 70 seconds. V_H set at -76 mV (lower trace). a. Control. 10 μM NMDA induces slow inward shifts and an ongoing discharge of periodic inward currents (Krnjevic et al., 1989). b. 12 and 20 min after superfusion with 3 mM NH_4Cl. No significant change in holding current and periodic inward currents, but NMDA current is greatly facilitated. c. Washout.

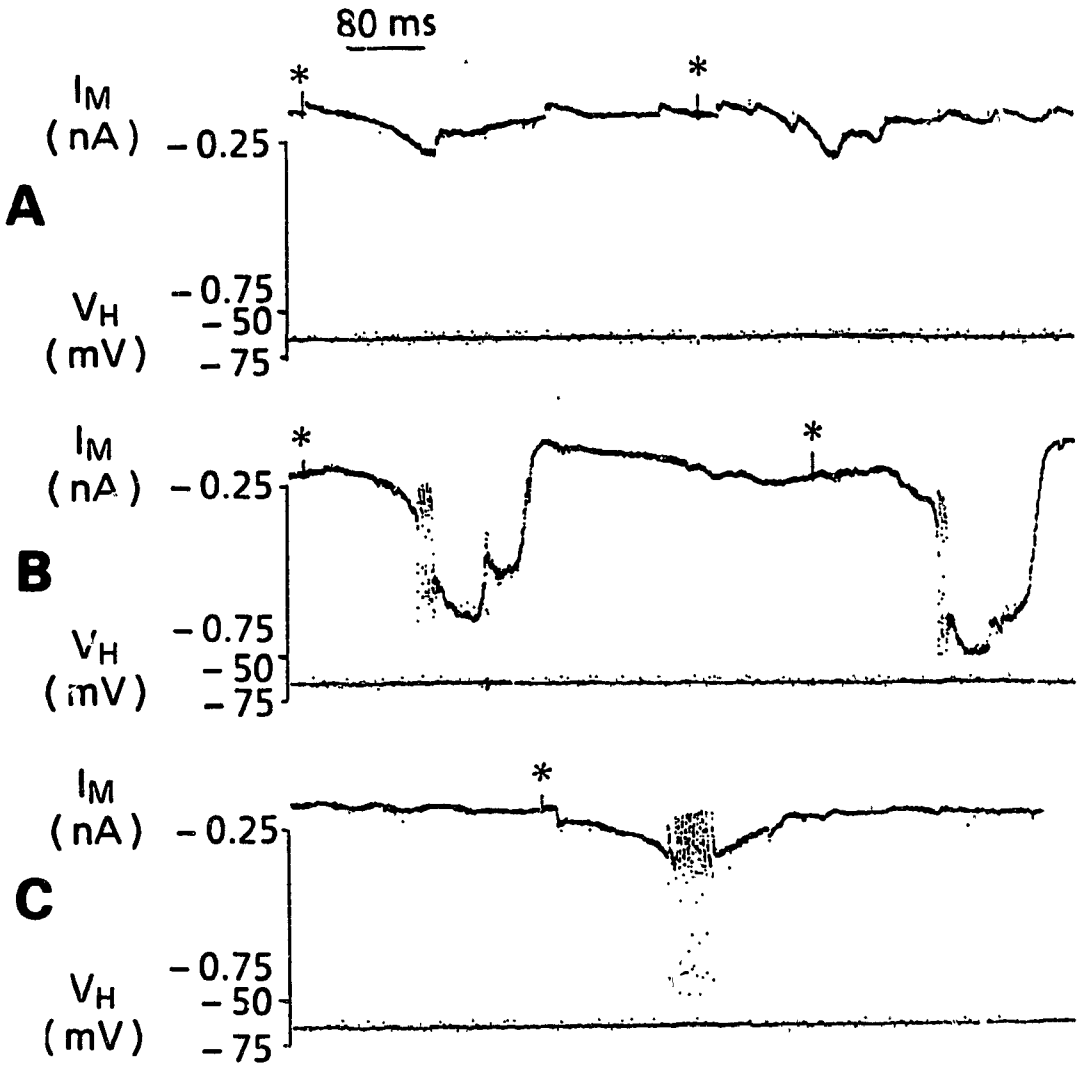


Fig. 19. The effect of NH_4Cl on NMDA-induced inward current (upper trace) in the presence of 0.3 μM TTX and 1.2 mM Mg^{2+}. * indicate superfusion with 10 μM NMDA for 90 seconds. V_H set at -65 mV (lower trace). A. Control. 10 μM NMDA induces a slow inward current. B. 10 and 19 min after superfusion with 3 mM NH_4Cl. No significant change in holding current, but NMDA current is greatly facilitated. C. Washout.

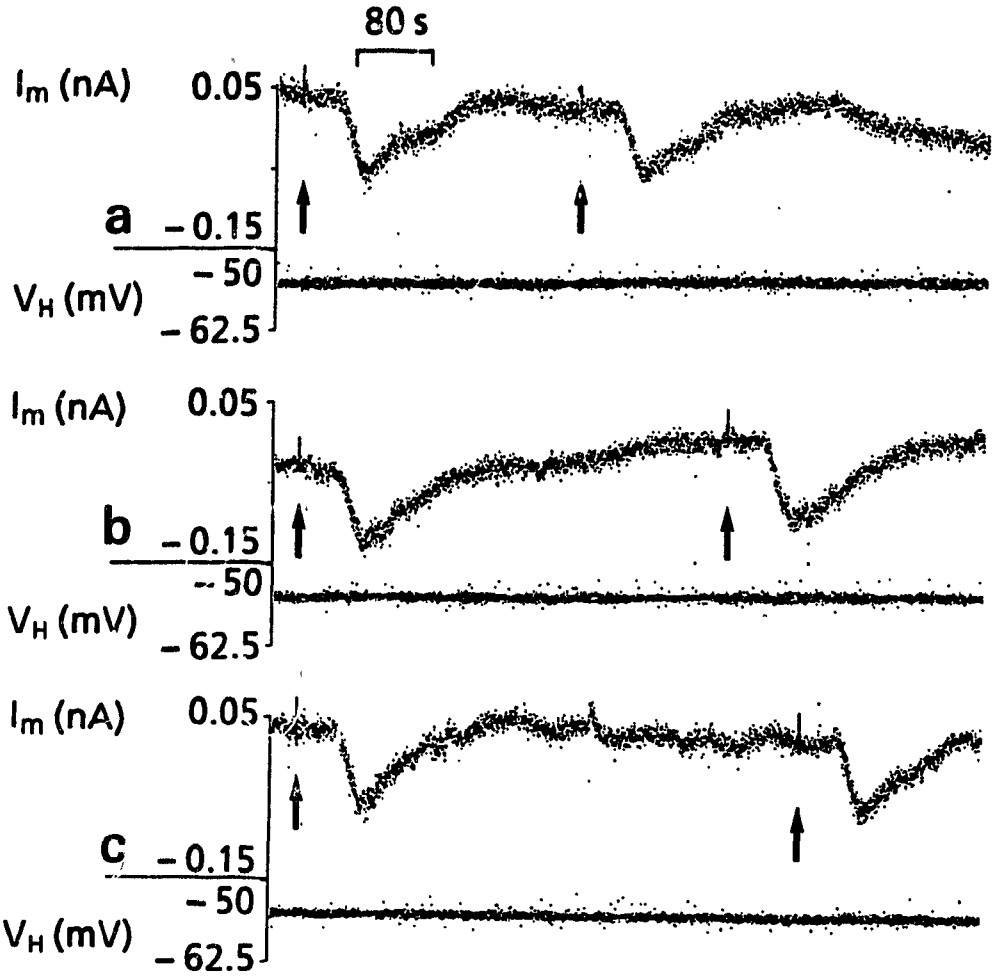


Fig. 20. The effect of NH_4Cl on NMDA-induced inward current (upper trace) without added Mg^{2+}. Superfusion fluid contained 0.3 μM TTX. Arrows indicate superfusion with 10 μM NMDA for 15 seconds. V_H set at -57 mV (lower trace). a. Control. b. 10 and 15 min after superfusion with 3 mM NH_4Cl. Holding current is increased, NMDA current is not affected. c. Washout.

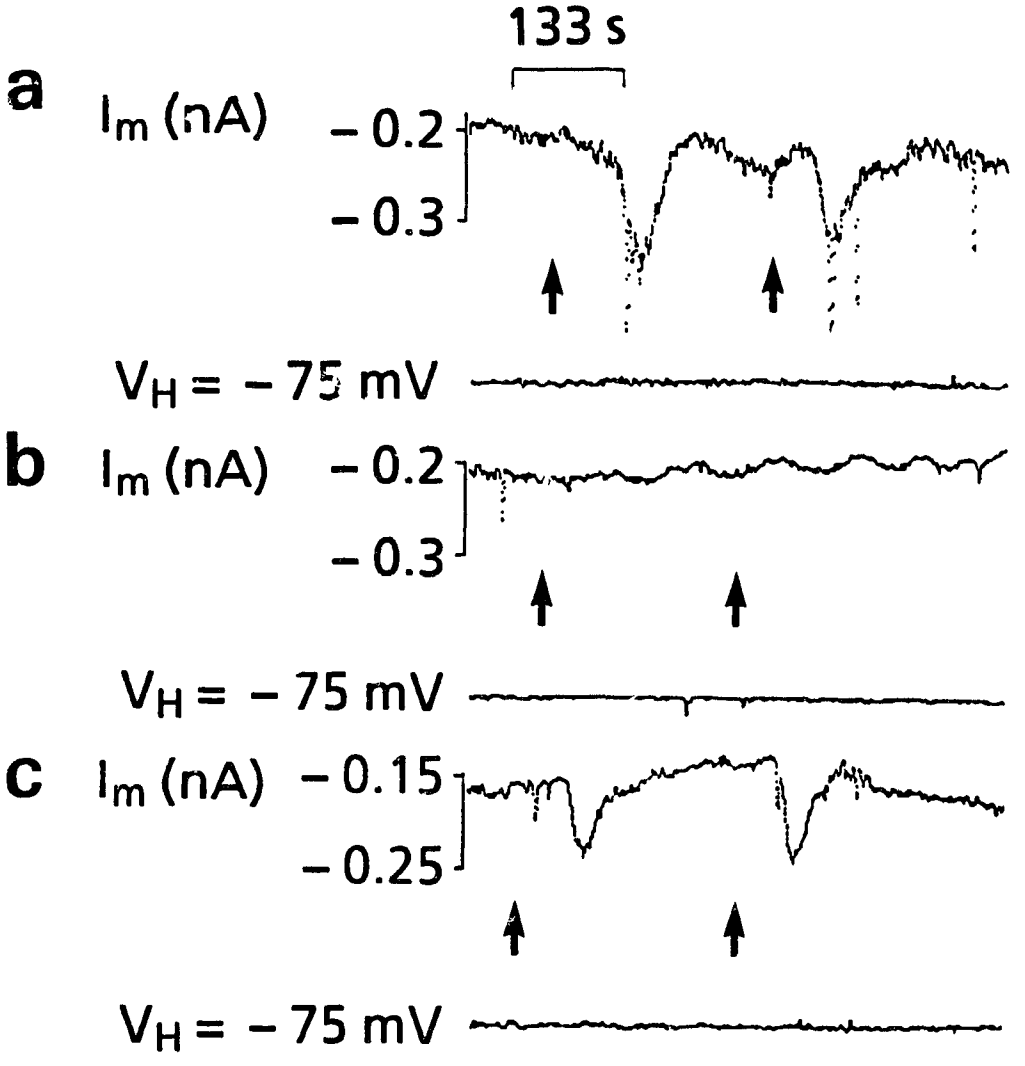


Fig. 21. The effect of CNQX on quisqualate-induced inward current (upper trace). Arrows indicate superfusion with 10 μM quisqualic acid for 5 seconds. V_H set at -75 mV (lower trace). a. Control. b. 5 mins after superfusion with 20 μM CNQX. c. Washout.

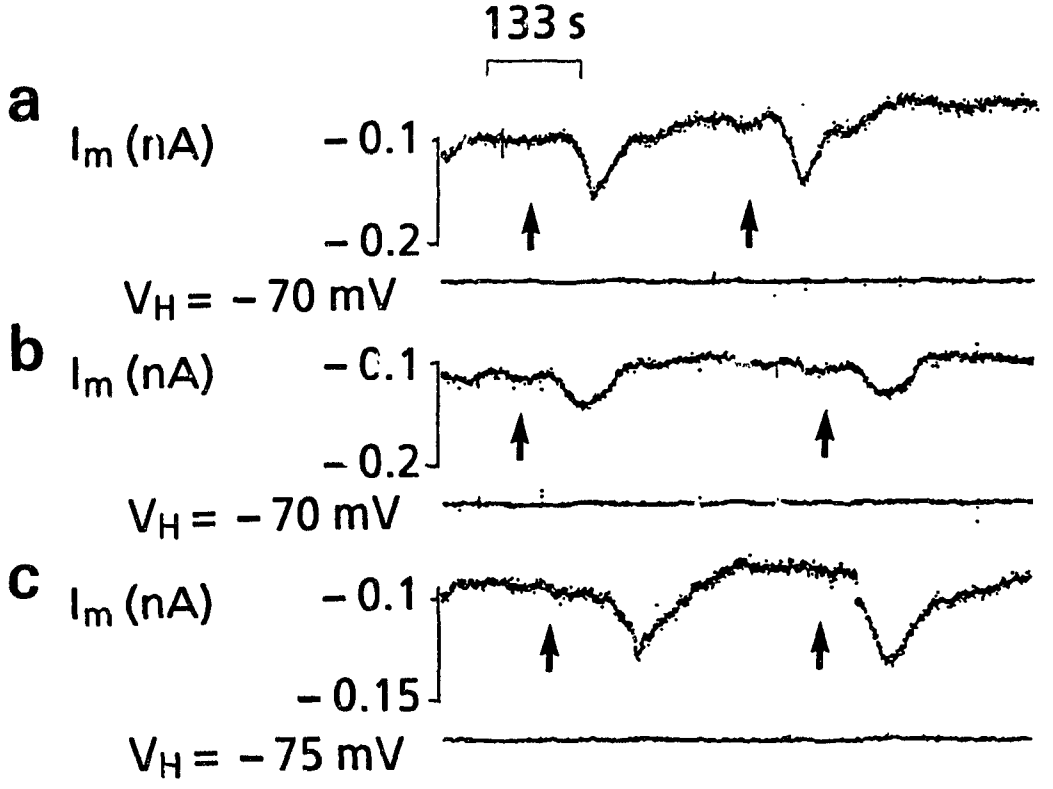


Fig. 22. The effect of 3 mM NH_4Cl on glutamate-evoked inward current in the presence of 25 μM APV (upper trace). 0.3 μM TTX was present. Arrows indicate superfusion with 2 mM glutamate for 30 seconds. V_H is at -70 mV (lower trace). a. control. b. 10 and 15 min after superfusion with 3 mM NH_4Cl. c. Washout.

Table 2.

Effect of 3 mM NH_4Cl on Excitatory Amino Acid Induced Currents.

EAA (n)	Control (pA)	3 mM NH_4Cl	% change (paired)
Quisqualate (16)	135 ±20	36*** ±8	-74*** ±11
NMDA (with Mg^{2+})# (10)	92 ±13	249** ±40	+172** ±38
NMDA (without Mg^{2+})# (5)	81 ±21	79 ±15	-2 ±9
Glutamate (9)	171 ±31	114 ±15	-33 ±16
Glutamate + 25 - 50 μM APV (6)	88 ±12	38 ±6	-58*** ±2
Glutamate + 20 - 40 μM CNQX (8)	55 ±2	54 ±7	-1 ±11

** P<0.01; *** P<0.001. # The average duration of application of 10 μM NMDA was 75 ± 10 sec in the presence of Mg^{2+} and 15 ± 2 sec in the absence of Mg^{2+}.

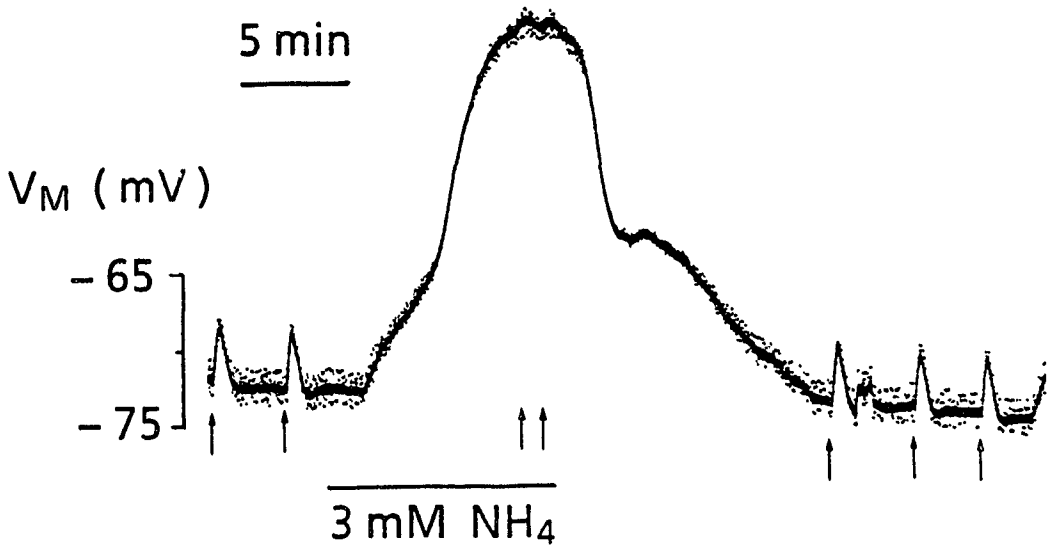


Fig. 23. Effect of 3 mM NH_4Cl on membrane potential changes induced by the bath application of quisqualic acid recorded in the bridge mode. Arrows indicate the perfusion of 20 μM quisqualic acid for 15 seconds. Initial membrane potential -72 mV. During NH_4^+-induced depolarization, effect of quisqualic acid was eliminated. After the washout of NH_4^+, quisqualic responses returned.

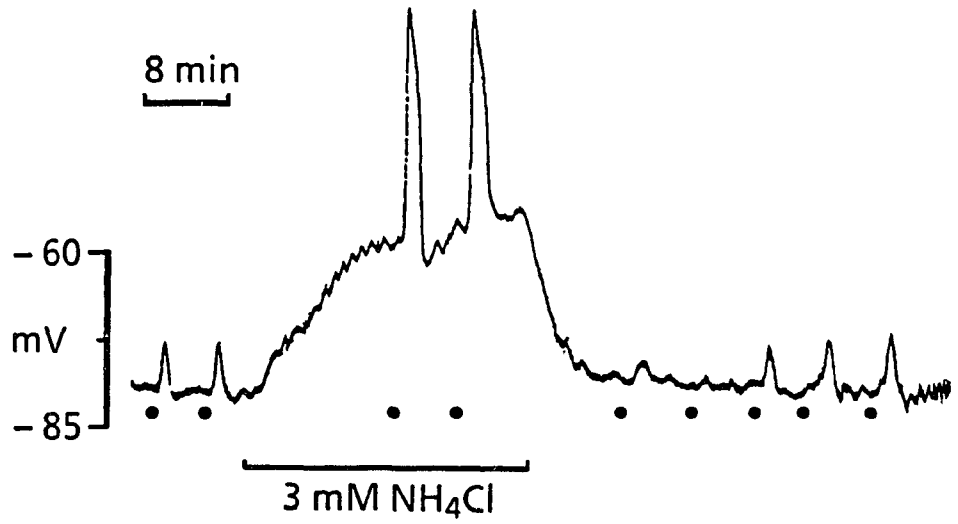


Fig. 24. Effect of 3 mM NH_4Cl on membrane potential changes induced by the bath application of NMDA recorded in the bridge mode. 1.2 mM $MgCl_2$ and 0.3 μM TTX present. Solid dots indicate the perfusion of 10 μM NMDA for 45 seconds. Initial membrane potential -78 mV. During NH_4^+-induced depolarization, effect of NMDA greatly increased, while after washout of NH_4^+, NMDA is temporarily less effective.

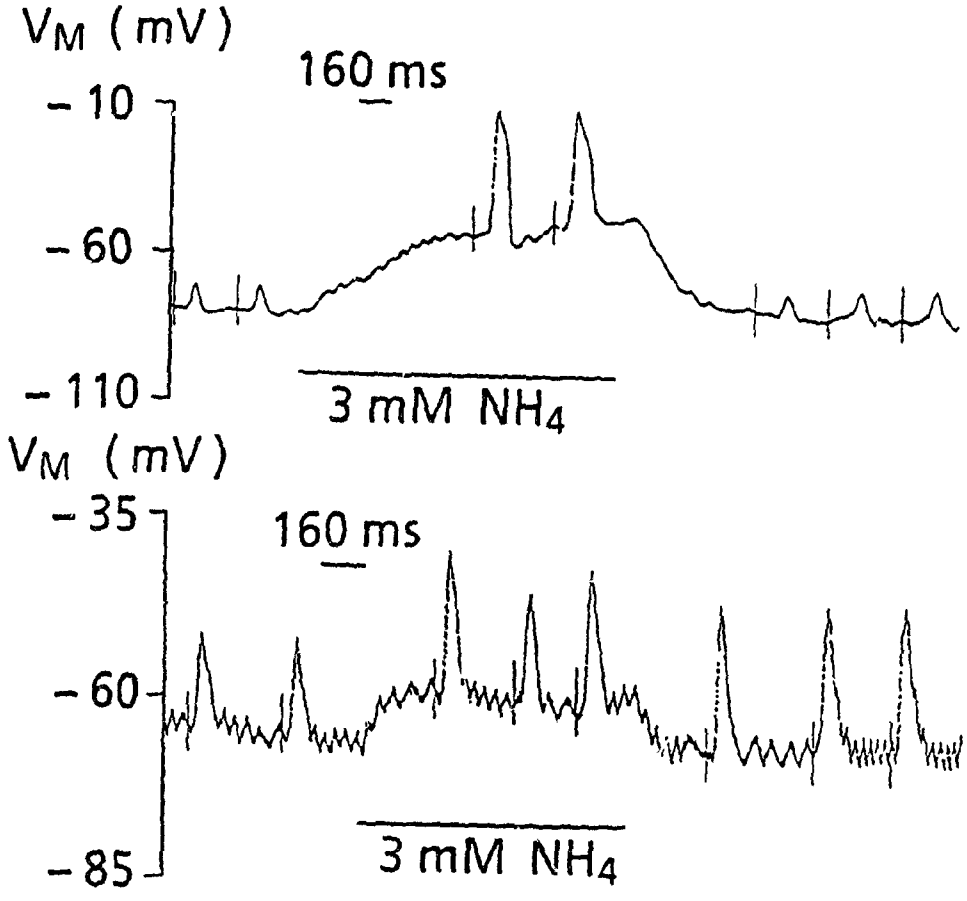


Fig. 25. Effect of 3 mM NH_4Cl on membrane potential changes induced by the bath application of NMDA recorded in the bridge mode. 0.3 μM TTX present. Mg^{2+} was absent in B. Small vertical bars indicate the start of perfusion of 10 μM NMDA for 45 seconds in A and 3 seconds in B. Initial membrane potential -78 mV for A and -66 mV for B. During NH_4^+-induced depolarization, effect of NMDA is greatly increased in the presence of 1.2 mM Mg^{2+} (A) and is not affected in the absence of Mg^{2+} (B).

Chapter 7 Discussion

7.1 The effect of NH_4^+ on CA1 pyramidal cells

7.1.1 The mechanism of the depolarizing effect of NH_4^+

Ammonium ions in a concentration of 1 to 4 mM significantly depolarized the membrane potential of CA1 pyramidal cells, a phenomenon which was first reported in hippocampal slices by Alger and Nicoll (1983). NH_4^+ also depolarized the membrane potential in other preparations (see introduction). In the present study, depolarization induced by NH_4^+ was larger than those reported previously, probably because of the use of fully submerged slices. Although Alger and Nicoll (1983) indicated that submerged and interface slices gave similar results, it is likely that fully submerged slices provide a more complete penetration of NH_4^+ than air/fluid interface slices and therefore higher concentrations of NH_4^+ are reached inside the slice. This argument is supported by the observations that NH_4^+ was also more effective in inhibiting transmission monitored with extracellular field potentials in the present study than in interface slices, as reported by Théorêt et al. (1985).

NH_4^+-induced depolarization was not accompanied by any

significant change in membrane resistance. Therefore NH_4^+ is likely to depolarize the membrane potential by mechanisms other than a change of ionic permeability. Action potentials evoked by stimulating presynaptic fibers or by direct depolarization during the NH_4^+ application had a significantly slower repolarization rate. These results, together with the observations that NH_4^+ reduced intracellular K^+ (Benjamin et al., 1978) and increased extracellular K^+ (Alger & Nicoll, 1983) suggest that NH_4^+ depletes the intracellular K^+ and therefore reduces the equilibrium potential of K^+, which will depolarize the membrane without altering membrane resistance.

The hyperpolarization which follows a synaptically evoked action potential contains a Cl^--dependent hyperpolarizing IPSP which is known to be suppressed by NH_4Cl (Alger & Nicoll, 1983). However, the suppression of the Cl^--dependent IPSP by NH_4^+ may also be due to the loss of intracellular K^+ because a reduction in the electrochemical gradient of K^+ inhibits the co-transport of K^+ with Cl^- (Thompson et al., 1988,1989), which will reduce the driving force for Cl^- and hence the Cl^--dependent IPSP.

In the presence of NH_4Cl, depolarization remained constant and its amplitude was not dependent on the concentration of NH_4Cl between 1-4 mM. The effects of ammonium ions on intracellular pH and K^+ content were not concentration-dependent either (Kauppinen et al., 1991;

Benjamin et al., 1978). These observations suggest that during the plateau phase of depolarization, an equilibrium is reached, for instance between the influx and the efflux of protonated and/or unprotonated ammonia.

According to Raabe (1989, 1990), adding NH_4^+ to the medium is equivalent to an increase in extracellular K^+ because of the similarity between NH_4^+ and K^+. One consequence of the K^+-like properties of NH_4^+ would be that NH_4^+ could enter neurons through K^+ channels and then depolarize the membrane by having a more positive equilibrium potential than does K^+. However, to maintain electroneutrality, this mechanism also requires that there be a loss of intracellular K^+, equivalent to the increase in intracellular NH_4^+ concentration. Furthermore, since gNH_4^+ is only 0.3 of gK^+ (Binstock & Lecar, 1969), depolarization due to the entry of NH_4^+ would be accompanied by an increase in membrane resistance if a large amount of K^+ was replaced by NH_4^+. This was not seen in the present experiments. Another possible mechanism would be that by entering K^+ channels, NH_4^+ interfered with the outward diffusion of K^+. However, according to the law of the independence of diffusion of ions (Hille, 1984), the movement of an ion through membrane pores does not interfere with that of another ion, unless the ion flux through the pores is near the maximal rate so that the pores are almost constantly occupied. The concentration of

K^+ used in the present study was well below that which produces maximal rates of diffusion, as evidenced from the influence of altering the K^+ concentration on membrane potential. Furthermore, increasing extracellular K^+ produces an initial hyperexcitability (Kocsis et al., 1983; present results) which is not seen with NH_4^+ (Théorêt et al., 1985; Théorêt & Bossu, 1985; present results).

In summary, the lack of change in membrane resistance during the NH_4^+ administration suggests that NH_4^+-induced membrane depolarization is probably not due to a change in ionic conductances. Rather, other mechanisms, such as the depletion of intracellular K^+, can explain the NH_4^+-induced membrane depolarization.

7.1.2 The effect of NH_4^+ on synaptic transmission

The present and previous studies have clearly demonstrated that ammonium ions inhibit excitatory synaptic transmission. Two presynaptic mechanisms have been proposed to explain the inhibitory effect of NH_4^+ on excitatory transmission. One of these is the inhibition of glutamate synthesis and release by NH_4^+ (Hamberger et al., 1979, 1982; Bradford et al., 1989). Evidence suggests that this mechanism is unlikely to be responsible for the blockade of synaptic transmission to the CA1 neurons in hippocampus because 5 mM NH_4Cl did not block the Ca^{2+}-

dependent release of glutamate induced either by elevated extracellular K^+ concentration or by conducted action potentials, whereas the same level of NH_4^+ totally suppressed the Ca^{2+}-dependent synaptic transmission (Szerb & Butterworth, 1991).

A presynaptic mechanism by which NH_4^+ may block excitatory transmission in the spinal cord is a failure of action potentials to invade presynaptic terminals due to a depolarization block of conduction (Raabe, 1989,1990). The possibility of a depolarization block of action potential conduction is supported by the present observations in the hippocampus, namely that postsynaptic somatic action potentials, recorded intracellularly, became broader and of smaller amplitude when NH_4Cl depolarized the membrane. The size of extracellularly recorded presynaptic compound action potentials was also decreased. A reduction in action potential depolarization rate and amplitude can decrease the action potential conduction and eventually may lead to conduction block in parts of axons having a low safety factor of conduction, for instance at the preterminal arborization of spinal 1A afferents (Raabe, 1989,1990). However, this does not appear to be the case in slices of hippocampus: presynaptically, the TTX-sensitive glutamate release evoked by field stimulation (Szerb, 1988) is not reduced by ammonium ions. Postsynaptically, concentrations of NH_4^+ that suppress

orthodromic transmission have no effect on antidromic firing of CA1 pyramidal neurons (Théorêt et al., 1985; Théorêt & Bossu, 1985), and the size of intracellularly recorded action potentials decreased before the block in synaptic transmission developed. Finally, synaptic transmission block caused by NH_4^+, unlike depolarization, did not reach a steady state because higher intensities of presynaptic stimulation could restore the transmission only initially. When NH_4^+ was washed out, membrane potential recovered first, well before synaptic transmission.

7.1.3 The effect of NH_4^+ on responses induced by different glutamate agonists

Ammonium ions had different effects on the inward currents induced by three types of glutamate agonists: net glutamate current was changed hardly at all, quisqualate current was almost completely suppressed, and NMDA current was greatly potentiated.

Glutamate acts on a number of receptors, such as NMDA, AMPA, kainate and metabotropic receptors. In addition, it has been recently reported that electrogenic uptake of glutamate itself is a major component of glutamate depolarization (Frenguelli et al., 1991). Further, NH_4^+ is known to reduce glutamate uptake by astrocytes and nerve

terminals (Norenberg, 1989; Mena & Cotman, 1985). Therefore, blocking one of the receptors by a specific antagonist, such as APV or CNQX, still permits the effect of glutamate on a number of other receptors, and the rate of glutamate transport into glia still has an influence on the effectiveness of glutamate. This differs from the specific effect of such agonists as NMDA or quisqualate. Consequently, it is not surprising that it was not possible to reproduce completely the effect of NH_4^+ on currents induced by specific agonists by eliminating the effect of glutamate on one of the many receptors. For instance, the reduced effectiveness of NH_4^+ in decreasing glutamate current in the presence of APV, as compared to its effect on quisqualate current, could be because some of the glutamate current was due to glutamate uptake (Frenguelli et al., 1991) not inhibited by NH_4^+. Lester et al. (1989) demonstrated that at concentrations between 10-30 μM, the non-NMDA antagonist CNQX was not entirely selective because it reduced the NMDA current by competing with glycine and therefore inhibiting NMDA current. This can explain the lack of potentiation of glutamate current by NH_4^+ when 20-40 μM CNQX was used in the present study.

Glutamate receptors which are involved in synaptic transmission from Schaffer collaterals/commissural collaterals are located on the dendrites of CA1 pyramidal cells (Dudar, 1974; Hablitz & Langmoen, 1982) where the

control of membrane potential by SEVC method is uncertain (Johnston & Brown, 1983).

This is particularly true in the present study. It was not possible to use CsCl-filled electrodes to try to block potassium conductances because neurons impaled with electrodes containing Cs^+ suddenly depolarized and then were lost when exposed to NH_4^+. This is probably because usually ammonium ions enter cells through potassium channels, the same channels that are blocked by Cs^+ (Golby et al., 1990). With potassium channels blocked, mostly undissociated and lipid soluble NH_3 molecules enter through the membrane. This would then produce a rapid intracellular alkalinization because, in the absence of NH_4^+, the reaction $NH_3 + H^+ \rightleftharpoons NH_4^+$ will proceed only to the right and $[H^+]_i$ will decrease (Boron & DeWeer, 1976). The lack of control of dendritic membrane potential may explain why potentiation of NMDA current by NH_4^+ disappeared in the absence of added Mg^{2+}. The somatic membrane potential would thus have been well controlled, but NH_4^+ could still depolarize dendrites even under voltage clamp condition and would remove the voltage-dependent Mg^{2+} block of NMDA channels.

It has been shown previously that focal application by pressure ejection of 100 μM quisqualate caused a rapid desensitization of about 70% of quisqualate current in cultured hippocampal neurons with a time constant of

around 80 msec. The remaining quisqualate current remained constant (Thio et al., 1991). However, in hippocampal slices, 143 second bath perfusion of 0.5 to 2 mM glutamate was required to observe a desensitization of the glutamate induced membrane depolarization. In contrast, simultaneous application of quisqualate, NMDA and kainate depolarized the membrane but produced no decay of the depolarization (Cole et al., 1989). In our experiments in which hippocampal slices were used, repeated applications of glutamate agonists always caused non-fading responses probably because the application was usually shorter than 60 seconds. The observation that CNQX blocked the quisqualate current suggests that NH_4Cl probably affects the function of AMPA receptors or the channels they activate.

The linear voltage-current relationship of quisqualate-induced responses (MacDonald & Porietis, 1982; MacDonald & Wojtowicz, 1982; Mayer & Westbrook, 1984) predicts that an average of 15 mV depolarization caused by NH_4Cl should reduce but not eliminate the quisqualate current. Therefore, it is unlikely that in the present study, depolarization alone is responsible for the suppression of the quisqualate current. These channels may be blocked by NH_4^+ because they are similar to those through which the Q-current passes, both allowing the passage of Na^+ and K^+ but not of Ca^{2+} (Mayer & Westbrook, 1987; Halliwell & Adams,

1982). However, a detailed analysis of the effect of NH_4^+ on receptor and/or channel functions will require a better control of membrane potential at the dendrites.

Intracellular acidification caused by NH_4^+ has been reported in cerebral cortex (Brooks et al., 1989; Fitzpatrick et al., 1989; Kauppinen et al., 1991). The most significant effect of pH on EAA agonist-induced responses is that NMDA current is inhibited by extracellular acidosis and slightly potentiated by extracellular alkalinization (Tang et al., 1990; Traynelis & Cull-Candy, 1990; Vyklicky et al., 1990). Intracellular changes of H^+ concentration have little or no effect on the NMDA-activated current (Tang et al., 1990; Traynelis & Cull-Candy, 1990). Quisqualate and kainate currents are only slightly affected by a change of extracellular pH value (Tang et al., 1990; Traynelis & Cull-Candy, 1990; Vyklicky et al., 1990). Furthermore, these observations were obtained in the absence of added Mg^{2+} while the potentiation of the NMDA current seen in the present study was observed in normal Mg^{2+} concentration and disappeared when Mg^{2+} was removed from the superfusion fluid. These considerations suggest that the effect of ammonia on NMDA current is mediated by membrane potential changes and not by changes in pH_i.

The different effects of NH_4Cl on EAA agonist-induced currents were confirmed by three other observations. When

monitoring membrane potential in the bridge mode, NH_4Cl eliminated the quisqualate-induced membrane depolarizations and facilitated those induced by NMDA. Secondly glutamate currents in the presence of the NMDA blocker APV, which were mostly due to activation of AMPA receptors, were consistently inhibited by NH_4^+. Finally, unit firing caused by quisqualate applied iontophoretically was reduced by more than 80%. However, NH_4^+ did not potentiate the unit firing evoked by iontophoretically applied NMDA, in contrast to the strong enhancement of the inward current and depolarization resulting from the bath application of NMDA. Possibly, the extracellular carbon filament electrode could not pick up all the action potentials when they became smaller due to the combined depolarization produced by NH_4Cl and NMDA.

In summary, NH_4^+ increases NMDA-induced responses, possibly through the depolarization of the poorly voltage-clamped dendritic membrane where most of the NMDA receptors are located. In addition, NH_4^+ inhibits, through a different mechanism, the AMPA receptor-mediated responses. This effect is produced by concentrations of NH_4^+ that fail to inhibit glutamate release, and is probably responsible for the suppression of synaptic transmission by NH_4^+. Increasing the intensity of stimulation of presynaptic fibers could not overcome the inhibitory effect of prolonged exposure to NH_4^+, nor did

increasing amounts of quisqualic acid applied to the bath. It seems therefore, that the inhibitory effect of NH_4^+ on AMPA receptor-mediated responses is non-competitive.

EXPERIMENTS WITH LOW GLUCOSE

Chapter 8 Results

8.1 Extracellular studies

8.1.1 Effect of low glucose on orthodromic excitation

Low glucose concentrations from 0.2-0.5 mM (normal concentration 5 mM) depressed synaptic transmission from Schaffer-collateral/commissural fibers to CA1 pyramidal cells, as indicated by the reduction by 20-55% of the maximal slope of fEPSPs and of the population spikes by 80-90% (Fig. 26). The population spike decreased 6-8 min after the onset of low glucose administration and this was followed a few minutes later by the reduction in fEPSP. When the bathing solution contained 1 mM glucose, fEPSP was not affected but the population spike was depressed. Neither the fEPSP nor the population spike appeared to be affected when the bathing solution contained 2 mM glucose. The presynaptic volley amplitude was not changed by any of the low glucose concentrations used in the experiments (Fig. 26).

8.1.2 Effect of low glucose on antidromic excitation

Orthodromically generated population spikes were much more vulnerable to glucose deficiency than the fEPSPs (Fig. 26). This suggested the possibility that low glucose interfered with the spike generation mechanism of the neuron. To determine whether this was the case, the effect of 0.2 mM glucose on population spikes evoked either by paired orthodromic or paired antidromic stimulation was compared. Under these conditions, paired stimulation facilitated the second orthodromic population spike but not the antidromic one. Glucose concentration of 0.2 mM completely blocked orthodromic population spikes but failed to affect those induced by antidromic stimulation (Fig. 27, n=6). This indicated that the ability of the axon hillock itself to generate spikes was not affected by low glucose. However, it is possible that the firing threshold of the axon hillock was increased by low glucose, which would affect othodromic population spikes but have little effect on antidromic population spikes because of the latter's high safety factor. Similar to orthodromic population spikes, the number of unit firings induced by the iontophoresis of glutamate was significantly reduced during 0.2 mM glucose (Fig. 28).

8.1.3 Effect of low glucose on the synaptic input/output relationships

When the usual stimulus intensity was applied (i.e. 2/3 of the maximal response), 1 mM glucose caused a depression of population spikes but this depression could be overcome by increasing the stimulus intensity. Glucose concentrations less than 1 mM suppressed the population spikes to an extent that even the highest intensity of stimulation could not reverse the inhibition. Therefore, superfusion with 1 mM glucose was used to investigate further the effect of glucose deficiency on the input/output relationship between presynaptic volley amplitude/maximal rate of rise of fEPSPs and maximal rate of rise of fEPSPs/population spike amplitude using different intensities of stimulation. The slope of the input-output relation between fEPSP and population spikes was reversibly depressed by more than 50% in 1 mM glucose, while the relation between presynaptic volley and fEPSPs was not affected (Fig. 29, Table 3). Changing the concentration of glucose from 5 mM to 2 mM (n=2) or to 10 mM (n=3) had no effect on either of these input/output relationships.

8.1.4 Effect of elevated extracellular K^+ and ouabain on orthodromic transmission

Severe hypoglycemia can produce an increase in the extracellular K^+ concentration and may impair the function of the Na^+/K^+ pump in the CNS (see introduction). To determine if low glucose interfered with synaptic transmission by increasing extracellular K^+ and by the subsequent membrane depolarization, the actions of low glucose were compared with the effect of elevated extracellular K^+ concentrations and that of the Na^+/K^+ pump blocker ouabain in the presence of 5 mM glucose. Superfusion with 8 mM KCl did not significantly change the amplitude of presynaptic volley from the value normally observed at 3 mM KCl, but reduced the maximal rate of fEPSPs by 60% and increased the size of population spikes 2.5 times (Fig. 30). Ten mM KCl almost completely abolished the presynaptic volley and fEPSPs, while the population spike was decreased by only 40% and was even potentiated at the beginning of superfusion with 10 mM KCl and at the beginning of the washout (Fig. 30). Ten μM ouabain irreversibly eliminated the presynaptic volley, fEPSPs and population spikes. However, there was a transient, 2.5 fold increase in the amplitude of population spikes at the beginning of superfusion with ouabain (Fig. 31). The transient increases in population

spikes seen with elevated K^+ or ouabain are opposite to the decrease in population spike amplitude seen in low glucose, although population spikes eventually fell in both cases. This suggested that neither elevation of extracellular K^+ nor block of the Na^+/K^+ pump was responsible for the effect of low glucose.

8.2 Intracellular Studies

8.2.1 Effect of low glucose on membrane potential and resistance

Forty seven CA1 pyramidal neurons were recorded intracellularly during exposure to glucose concentration of 0 mM, 0.2 mM or 0.5 mM. Before the application of low glucose, they had an average (±SE) resting membrane potential of -64.6 ± 1.08 mV, an average membrane resistance of 45.2 ± 1.75 Mohm and average action potential amplitude of 94.7 ± 1.61 mV. Most frequently, low glucose concentrations caused a biphasic membrane potential change: a small initial hyperpolarization followed by a large depolarization (Fig. 32, A). Sometimes only one of these potential changes was observed (Fig. 32, B and C). The average maximal hyperpolarization was 4.25 ± 0.64 mV (n=25). The average maximal depolarization was 25.3 ± 1.87 mV (n=22). Membrane resistance was always

reduced during low glucose regardless of the change in membrane potential (Fig. 33, C). The reduction of membrane resistance started slightly before the membrane potential was hyperpolarized and the maximal drop was 13.9 ± 1.89 Mohm or 29.9% (n=47). Spontaneous action potentials were present in 23 neurons. In all cases, their frequency decreased or they entirely disappeared during the hyperpolarization caused by low glucose. The effect of low glucose on membrane potential and resistance appeared 3-8 min after the introduction of low glucose, depending on glucose concentrations (Table 4). All the effects of low glucose were fully reversible. In about half of the experiments, to compensate for a decrease in osmolarity, the reduced glucose concentrations were replaced by equimolar sucrose, i.e. when 1 mM glucose was used, 4 mM sucrose was added to replace the missing glucose, but no obvious change in the effectiveness of low glucose was observed.

8.2.2 Effect of low glucose on synaptic transmission

The inhibitory effect of low glucose on synaptic transmission was also studied with intracellular recordings. The block in synaptic transmission developed in several phases following the start of superfusion with 0.5 mM glucose (Figs. 33 and 34). First, at the time when

the membrane started to hyperpolarize and the membrane resistance to decrease, the threshold for action potential generation progressively increased and therefore, the amplitude of EPSPs grew (Fig. 33b and c; Fig. 34b). At the same time, the hyperpolarization which followed the postsynaptic spike became smaller. After a steady increase in the amplitude of the EPSPs, their amplitude suddenly decreased, so that the EPSP could no longer reach the now elevated action potential threshold, and postsynaptic spikes were no longer generated (Fig. 33c; Fig. 34d). Eventually, when membrane resistance dropped by nearly 50%, EPSPs almost completely disappeared, irrespective of the accompanying changes in membrane potential (Fig. 33d and g; Fig. 34d and g). Upon the reintroduction of 5 mM glucose, membrane resistance started to recover along with the EPSP, but the action potential threshold remained elevated (Fig. 33e and Fig. 34e). At this time, hyperpolarizing potentials followed the EPSPs. Since the impaled neurons did not fire, the observed hyperpolarization must have been due to an IPSP, not to AHP. This suggests that transmission to inhibitory interneurons has been restored. Finally, the threshold for action potential generation returned to normal, although not necessarily at the same time as the membrane resistance. Along with the postsynaptic spikes, AHPs also fully recovered (Fig. 33f and Fig. 34f).

8.2.3 Effect of low glucose on excitatory amino acid induced inward currents

The effect of low glucose on NMDA and quisqualate-induced inward currents was investigated with the single electrode voltage-clamp technique.

Bath application of 20 μM NMDA caused an inward current whose amplitude depended on the duration of NMDA perfusion. No desensitization to repeated applications was evident in 5 mM glucose (Fig. 35, A). Low glucose initially increased and prolonged the NMDA current, and then prevented the membrane current from returning to the baseline after the end of NMDA application. When the holding current failed to return, the effectiveness of further application of NMDA was greatly reduced (Fig. 35B and C). When 5 mM glucose was reintroduced, neither the holding currents nor the NMDA currents recovered (n=10).

Repeated bath applications of 20 μM quisqualic acid also produced a non-fading inward current. Similar to its effect on NMDA current, low glucose first increased the quisqualate current and then depressed it. However, the effect of low glucose on quisqualate current was fully reversible (n=14) (Fig. 36). It appears that the suppression of quisqualate current only occurred when there was a large inward current (Fig. 36A). This conclusion is supported by the observation that there is a

highly significant correlation ($r=0.70$, $n=39$, $P<0.001$) between the change in holding current and change in quisqualate current caused by low glucose (Fig. 37).

In summary, lowering the glucose content of the medium to 0.2, 0.5 or 1 mM consistently reduced input resistance and usually produced biphasic changes in membrane potential. Intracellular recordings showed that reduced glucose concentration interfered with synaptic transmission, initially by elevating the postsynaptic firing threshold. This phenomenon was reflected in a shift to the right of the fEPSP/population spike input/output relationship recorded extracellularly. Later, EPSPs almost entirely disappeared, as observed in both extra- and intracellular recording experiments. Low glucose also interfered with postsynaptic currents induced by the application of exogeneous EAA's. Both quisqualate and NMDA currents were inhibited when glucose deficiency increased the holding current. However, while the inhibition of quisqualate current was reversed upon the reintroduction of 5 mM glucose, NMDA in the presence of low glucose produced prolonged depolarization which was irreversible upon return to the control condition.

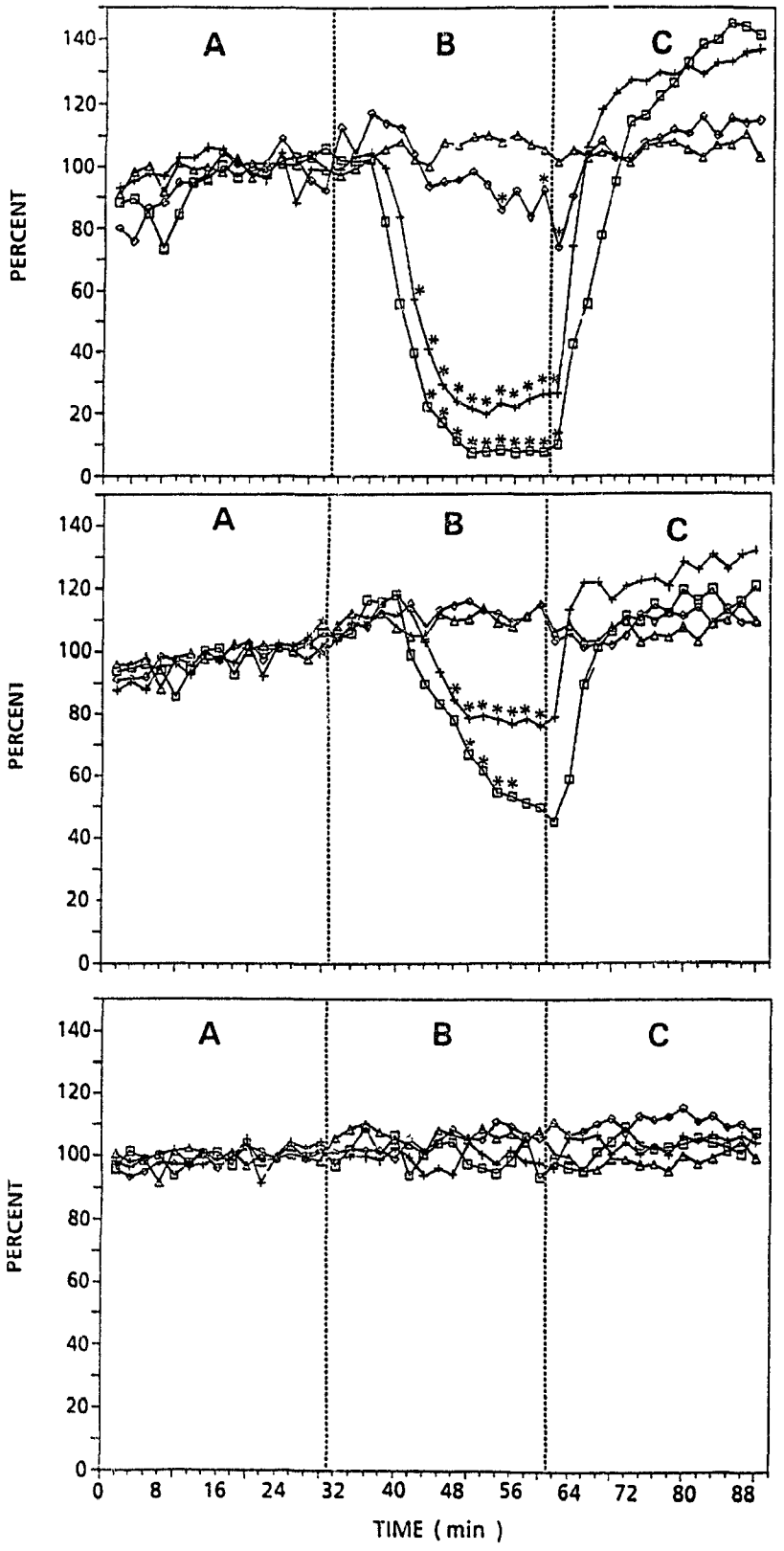
8.2.4 Effect of low glucose on glial cells

The presumed glial cells occasionally penetrated in the

present experiments (n=3) had a very stable, high membrane potential (-80.3 ± 1.33 mV) and low membrane resistance (13.5 ± 1.12 Mohm). Action potentials could not be evoked by depolarizing pulses or stimulation of Schaffer collaterals, but the latter stimulation did produce a few mV of depolarization. A glucose concentration of 0.2 mM gradually depolarized the glial membrane potential from -80.3 ± 1.33 mV to -52.2 ± 3.38 mV and increased the membrane resistance from 13.5 ± 1.12 Mohm to 20.8 ± 2.16 Mohm (Fig. 38). Glial membrane potential and membrane resistance returned to normal about 3-4 min after 0.2 mM glucose was replaced by 5 mM glucose.

Fig. 26. Effect of superfusion with reduced glucose concentration on the population spike amplitude (top), fEPSP maximal slope (middle) and presynaptic volley amplitude (bottom). During periods marked by A and C, 5 mM glucose was present. In the periods marked by B, 0.2 mM (squares), 0.5 mM (crosses), 1 mM (diamonds) and 5 mM (triangles) were present. Results were normalized by making the average of the observation during the initial superfusion with 5 mM glucose in each experiment equal 100%. Average of 6 experiments shown. * $P<0.05$.

Fig. 26


120
A
B
C
PERCENT
140
120
100
80
60
40
20
0
0
8
16
24
32
40
48
56
64
72
80
88
TIME (min)

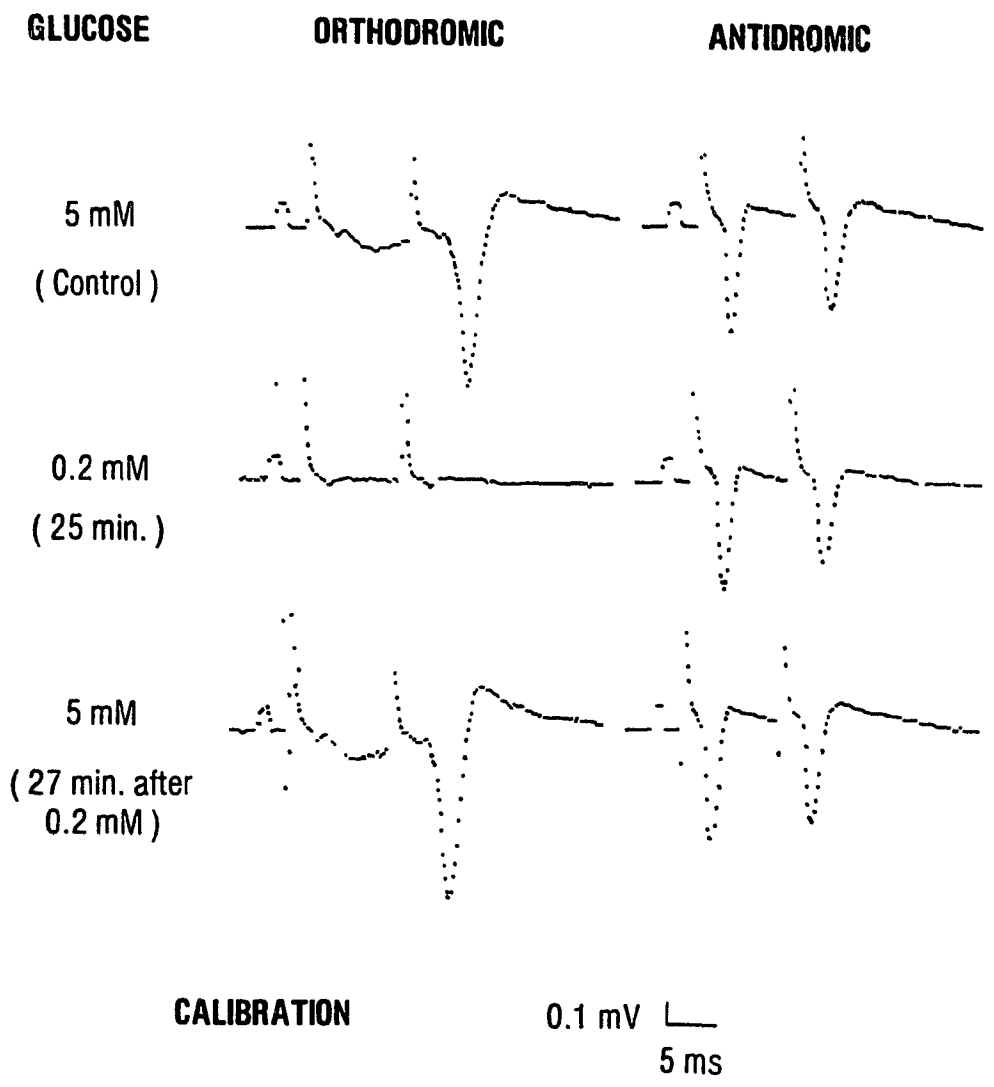


Fig. 27. Effect of 0.2 mM glucose (middle traces) on population spikes evoked orthodromically (stimulation on the stratum radiatum) and antidromically (stimulation on the alveus) by paired stimuli, 10 msec apart. Note the marked potentiation of the population spike due to the second othodromic but not due to the second antidromic stimulation.

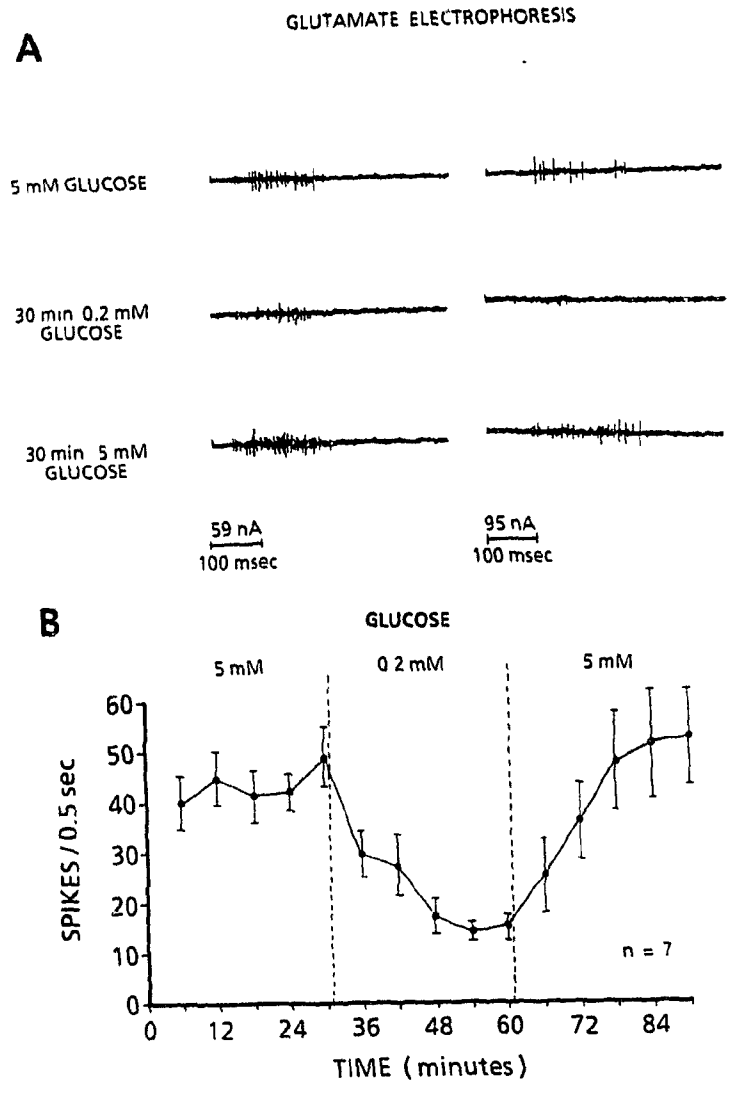


Fig. 28. The effect of low glucose on unit firing induced by 100 ms iontophoresis of glutamate. A. Top, Before; middle, during administration of 0.2 mM glucose; bottom, washout. B. Summary of the effects of low glucose on firing induced by glutamate, n=7. All differences between glutamate induced firing during low glucose are highly significant ($P<0.05$ and $P<0.01$). Bars indicate the start and duration of glutamate application.

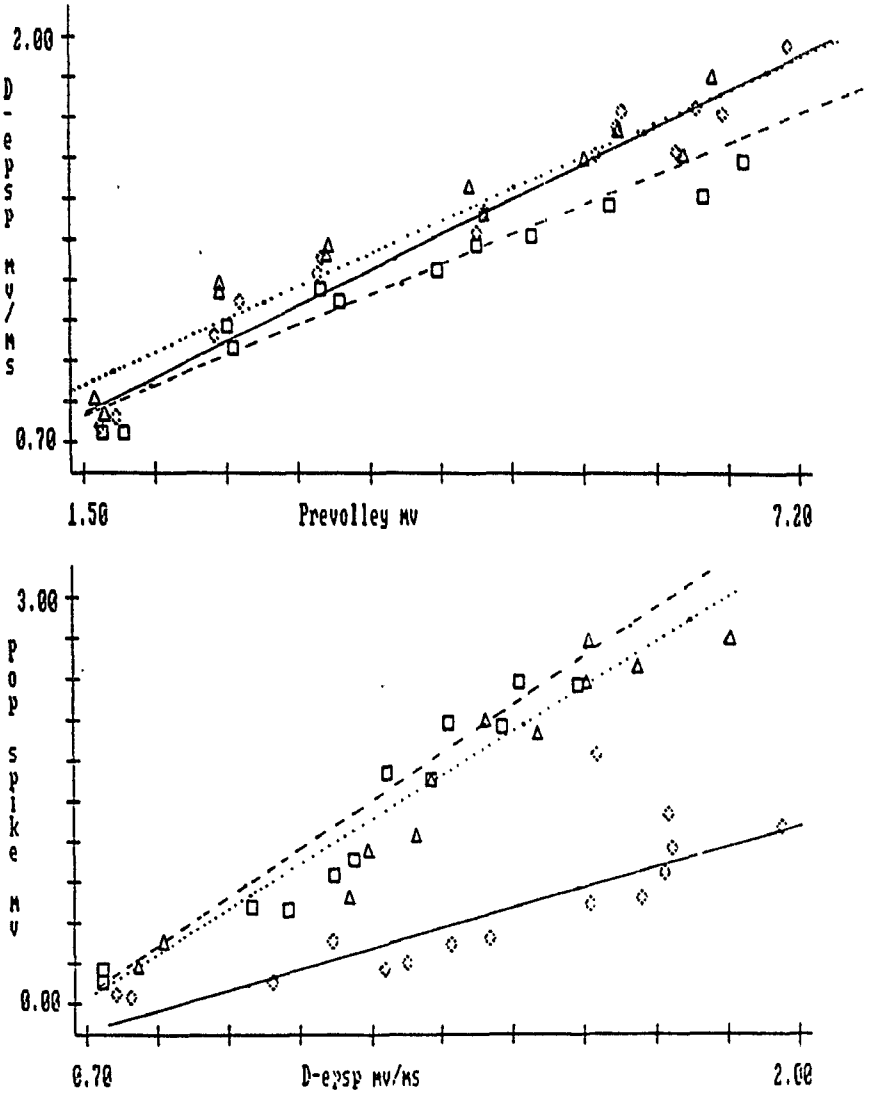


Fig. 29. Example of the effect of 1 mM glucose on the input/output relationship between presynaptic volley and dendritic EPSP (top) and dendritic EPSP and soma population spike (bottom) as measured with different intensities of stimulation. Rectangles and broken lines, control; Diamond and solid lines, superfusion with 1 mM glucose for 30 min; triangles and dotted lines, washout.

TABLE 3

EFFECT OF 1 mM GLUCOSE ON THE SLOPES OF THE INPUT/OUTPUT LINES

Glucose Concentration	Presynaptic Volley/fEPSP	fEPSP/Population Spike
5 mM (initial)	100%	100%
1 mM	89.0% ±8.6	46.5%* ±8.4
5 mM (final)	98.8% ±5.7	104.9% ±3.5

Slopes were normalized by making the slopes in the initial 5 mM glucose solution equal 100%. Averages ± s.e.m. of six observations. *Highly significantly ($p<0.01$) different from 100%.

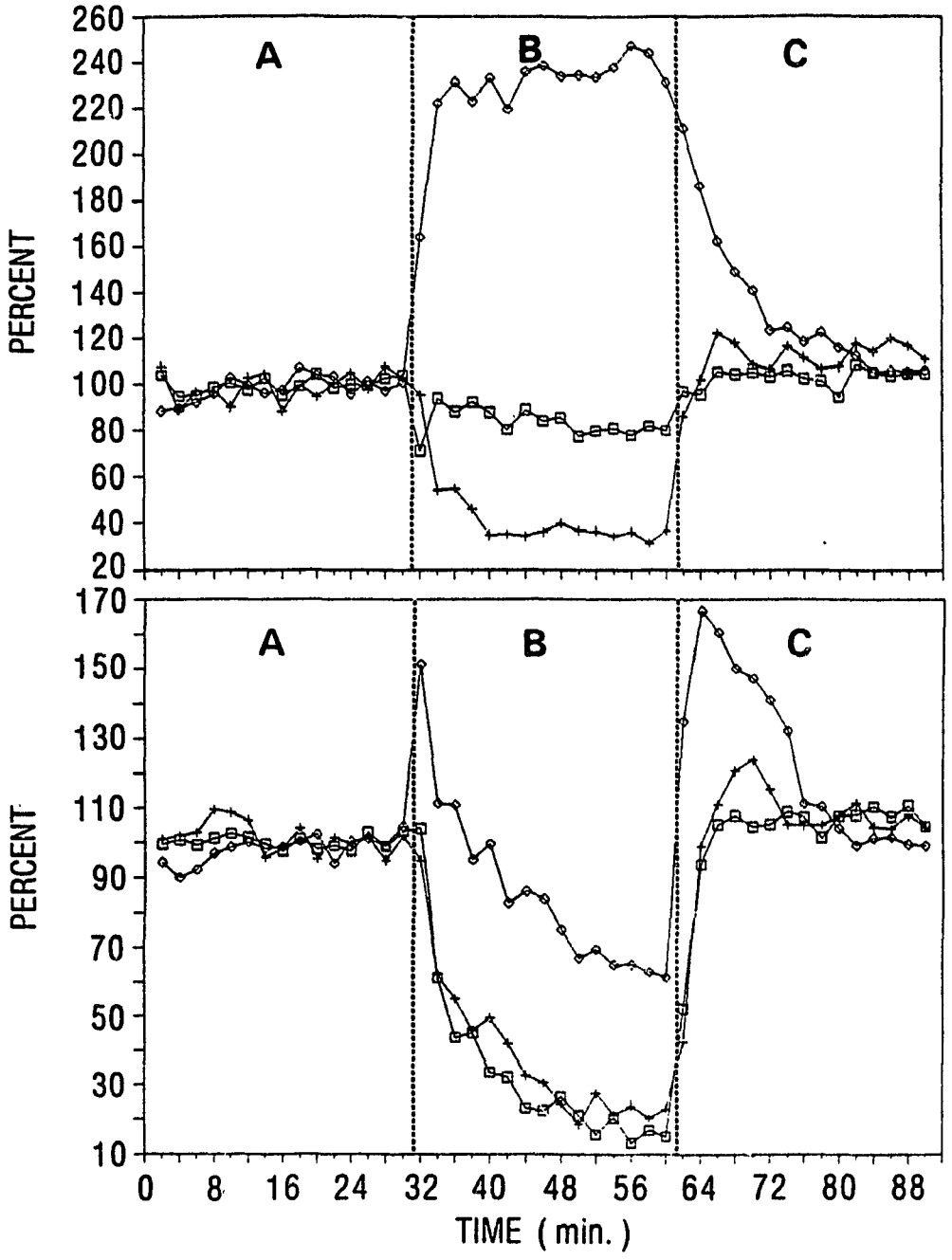


Fig. 30. Effect of 8 mM (top, n=6) and 10 mM (bottom, n=8) K^+ on the population spike amplitude (diamonds), fEPSP maximal slope (crosses) and presynaptic volley amplitude (squares). During periods marked by A and C, 3 mM K^+ was present. In the periods marked by B, K^+ concentration was changed. Results were normalized as in Fig. 26.

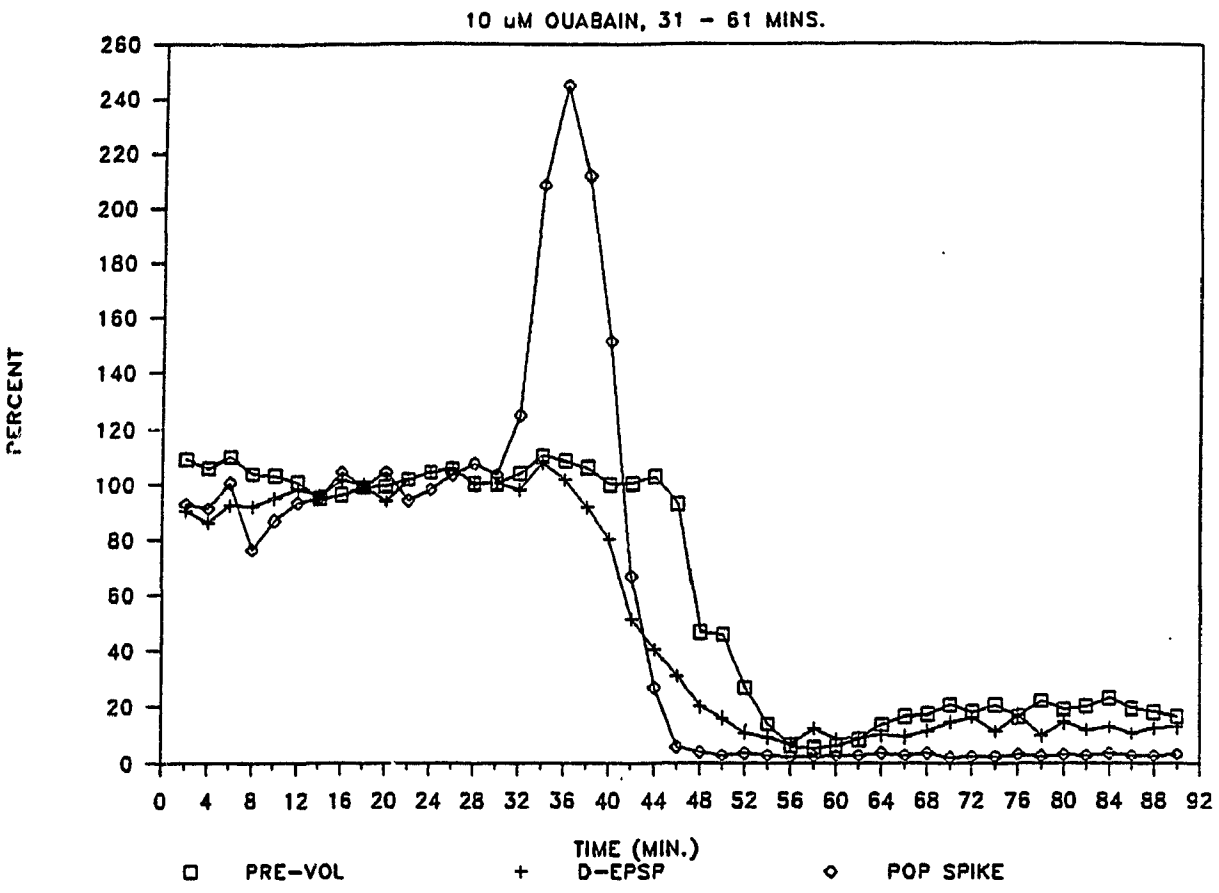


Fig. 31. The effect of ouabain (10 μM) on population spike amplitude (diamonds), fEPSP maximal slope (crosses) and presynaptic volley (squares). Results were normalized by making the average of the observation during the initial 30 mins superfusion in each experiment equal 100%. Average of 4 experiments shown.

Table 4.

SUMMARY OF RESPONSES TO LOW GLUCOSE

	0 glucose	0.2-0.5 mM glucose
Total n	10	37 (0.2 mM n=30, 0.5 mM n=7)
Hyperpolarized		
n	8	25
delay (min)	3.6±0.7	7.3±0.5
No change in Vm		
n	2	5
Depolarized		
n	0	7
delay (min)	-	6.2±1.1
Late depolarization		
n	8/10	9/30*
delay (min)	16.6±1.6	12.4±1.3
Duration of perfusion with low glucose (min)	19.1±1.9	17.1±1.1

Averages ± SEM shown. * units showing early depolarization not included.

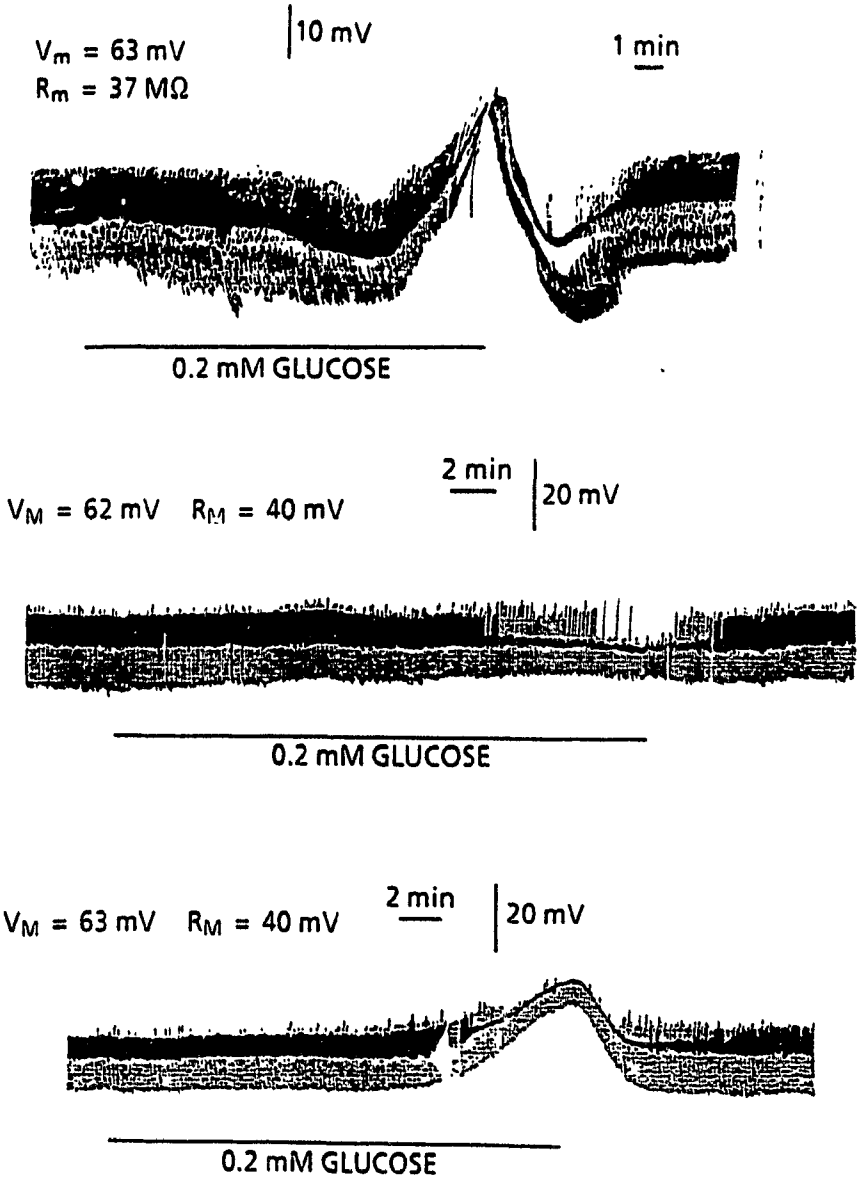
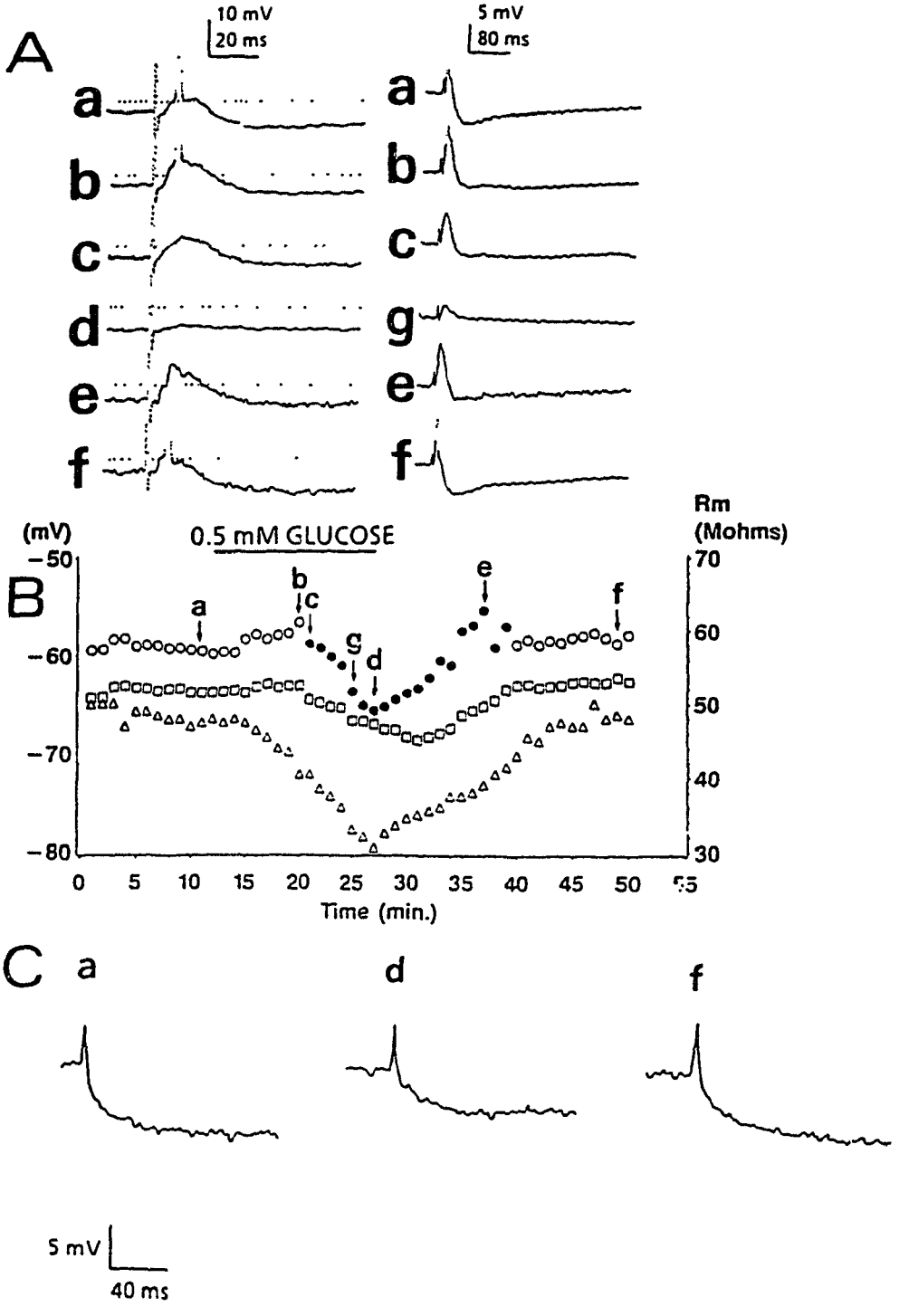


Fig. 32. Effect of low glucose (0.2 mM) on membrane potential and resistance of CA1 pyramidal neurons recorded on a curvilinear chart recorder. Downward deflections are potential changes caused by hyperpolarizing pulses (200 ms,0.2 nA,0.13 Hz); upward deflections are truncated action potentials, which appeared regularly after the hyperpolarizing pulses. The effect of low glucose on membrane potential varies. Usually, 0.2 mM glucose caused an initial hyperpolarization followed by a large depolarization (A). Sometimes, only hyperpolarization (B) or depolarization (C) was observed. Membrane resistance was significantly reduced regardless of the change in membrane potential (A,B,C).

Fig. 33. Simultaneously measurement of synaptic transmission, membrane potential and resistance in a neuron showing only hyperpolarization with low glucose. A: Letters refer to the time when the sweeps were recorded in relation to the measurements shown in B. Sweeps shown on the left and right hand side in A are the same records at different gains and speeds, except the fourth line, where d and g were taken at different times. On the left, horizontal broken line shows the initial control firing threshold which is exceeded by the EPSPs in b, c and e. On the right, in b, hyperpolarization followed the action potential was reduced. B: circles, peak of EPSPs; squares, Vm; triangles, Rm (right scale). Filled circles indicate absence postsynaptic spike. Note postsynaptic firing ceases as soon as membrane resistance drops. C: examples of potential changes due to 0.2 nA hyperpolarization pulses. Letters refer to points with the same letter in B, indicating the time when these records were taken.

Fig. 33


A
10 mV
20 ms
5 mV
80 ms
a
b
c
d
e
f
g
B
0.5 mM GLUCOSE
(mV)
−50
−60
−70
−80
Rm
(Mohms)
70
60
50
40
30
0 5 10 15 20 25 30 35 40 45 50 55
Time (min.)
C
a
d
f
5 mV
40 ms

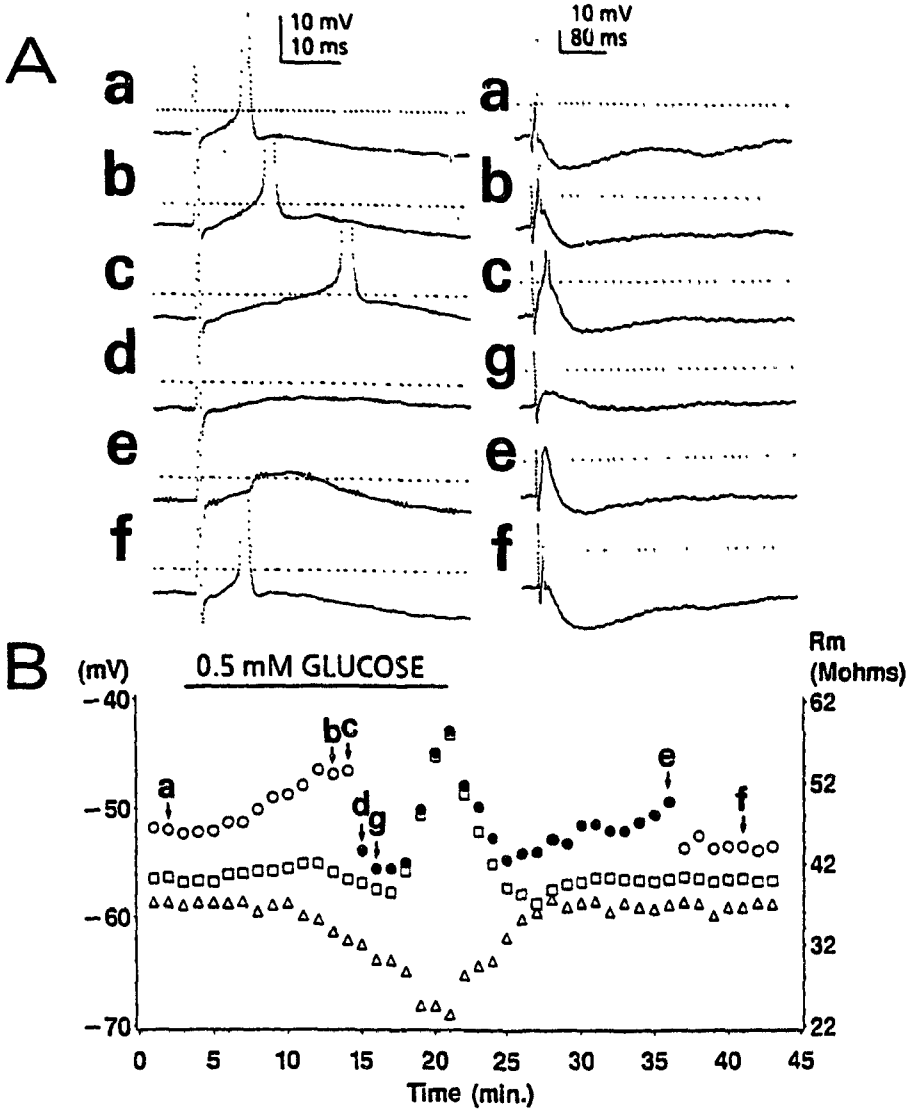


Fig. 34. Another example of the effect of low glucose (0.5 mM) on synaptic transmission. A: Letters refer to the time when the sweeps were recorded in relation to the measurements shown in B. Sweeps shown on the right and left hand side in A are the same records at different gain and speeds, except the fourth line, where d and g were taken at different times. On the left, broken lines show the initial control firing threshold which is exceeded by the EPSPs in sweeps b, c, and e. On the right, hyperpolarizations followed the actions were reduced in b and c. Vm: square symbols, action potential threshold: round symbols and Rm:triangles.

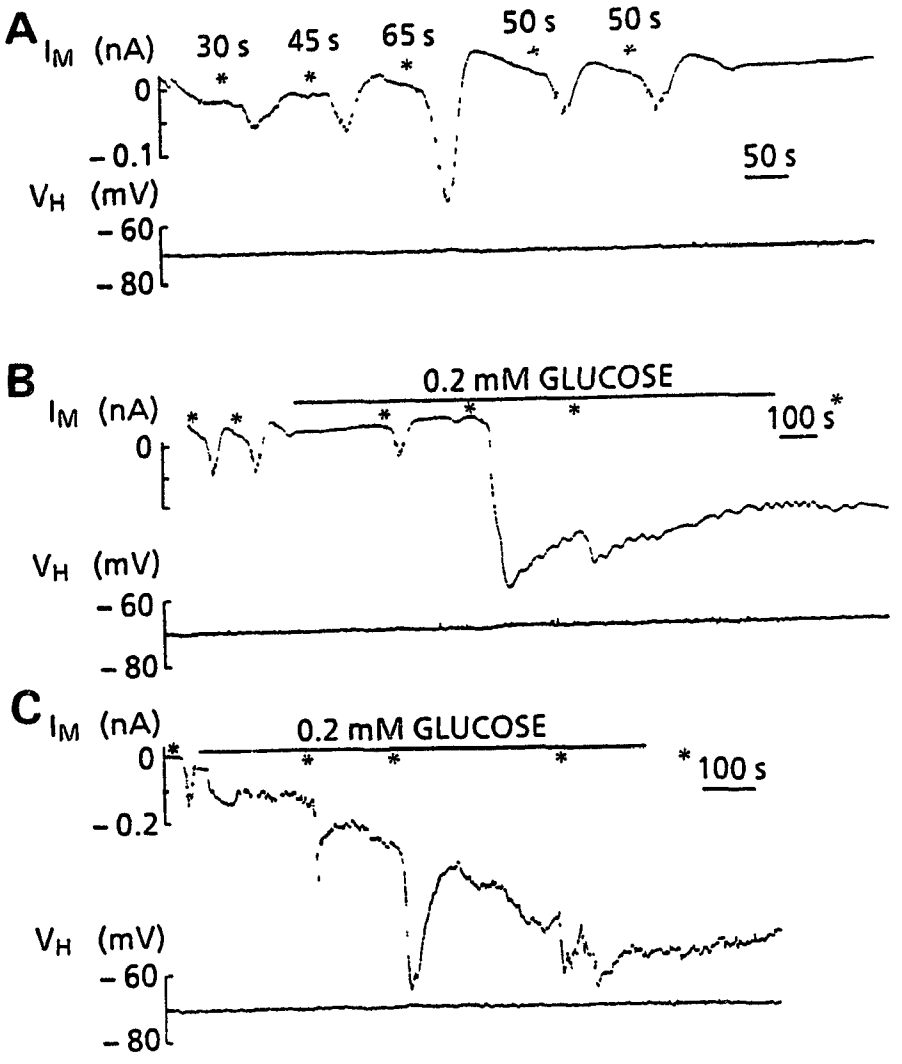


Fig. 35. The effect of low glucose concentrations on NMDA-induced inward currents in 1.2 mM Mg^{2+}. A. NMDA currents induced by repetitive bath applications of 20 μM NMDA in 5 mM glucose. Duration of application in seconds. B and C. 0.2 mM glucose caused an initial facilatation of NMDA current and then inhibited it. Superfusion with 0.2 mM glucose is indicated by a bar. The effect of low glucose on NMDA currents was not reversible, nor was the recovery of the holding current. * indicates the bath applications of 20 μM NMDA for 50 seconds in B and 30 seconds in C.

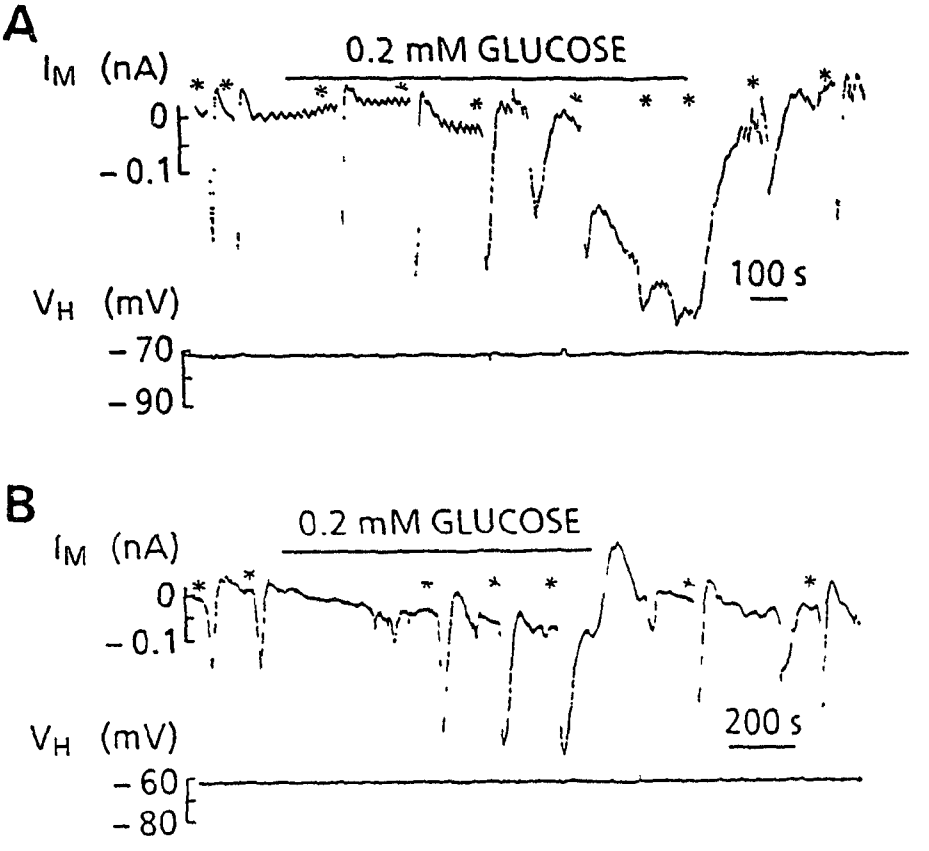


Fig. 36. The effect of low glucose on quisqualic acid-evoked inward current (upper trace). V_H (lower trace) was -72 mV for A and -61 mV for B. Superfusion with 0.2 mM glucose is indicated by a bar. Quisqualate current was first increased (A and B) and then was almost eliminated (A) when the low glucose-induced large depolarization occurred. The effect of low glucose was fully reversible. * indicates the bath application of 20 μM quisqualic acid for 4 seconds (A) and 12 seconds (B).

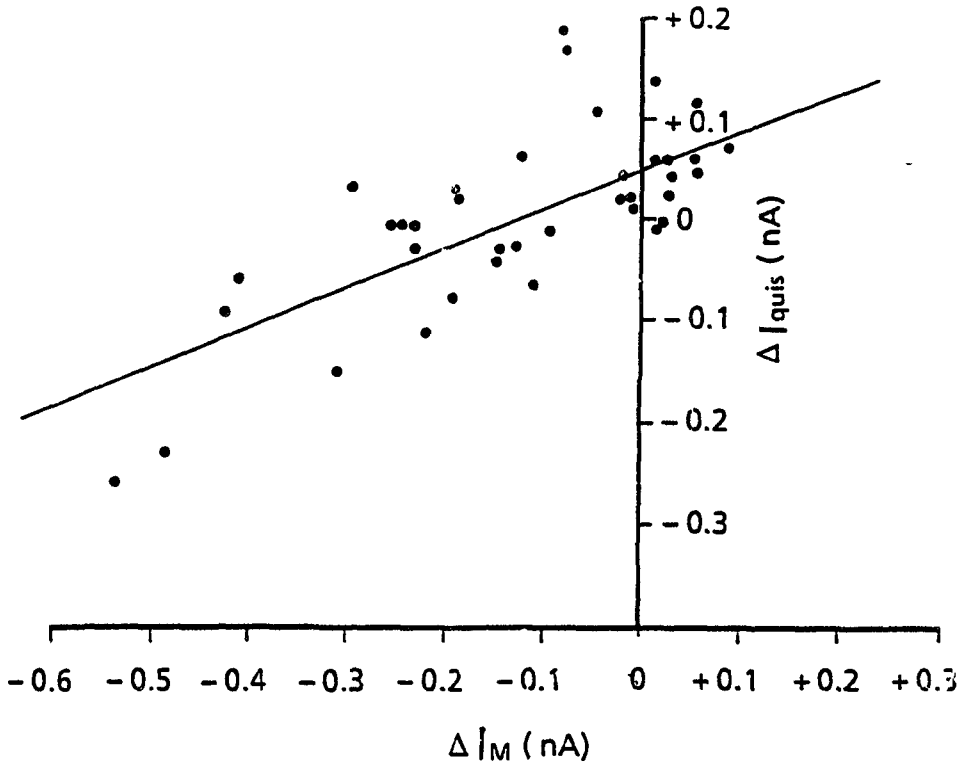


Fig. 37. Relationship between changes in membrane currents (ΔI_M) and quisqualate-induced currents (ΔI_{quis}) produced by 0.2 or 0.5 mM Glucose. There is a highly significant correlation between the two effects of low glucose (r=0.70, n=39) described by the regression equation y=0.42x + 0.05.

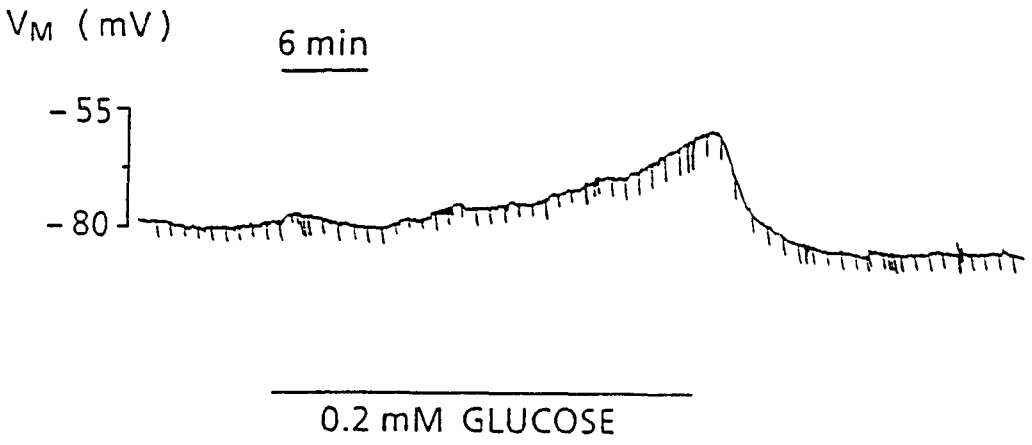


Fig. 38. The effect of low glucose on a presumed glial cells recorded on a curvilinear chart recorder. Downward deflections are potential changes caused by hyperpolarizing pulses (250 ms, 0.2 nA,0.13 Hz) 0.2 mM glucose gradually depolarized the glial cell membrane potential and increased membrane resistance. Initial Vm= -79 mV, Rm= 12 Mohm.

Chapter 9 Discussion

9.1 The effect of low glucose on CA1 pyramidal cells

Reducing the level of glucose has a hyperpolarizing effect on guinea pig CA3 neurons (Spuler et al., 1988; Spuler & Grafe, 1989; Knopfel et al., 1990), but in the present study, low glucose usually produced a small initial hyperpolarization followed by a large depolarization in rat CA1 pyramidal cells. Hyperpolarization alone or depolarization alone also occurred (Table 4). However, membrane resistance was always reduced, regardless of the direction of membrane potential change. An increase of K^+ conductance, as suggested by Spuler et al. (1988), can well explain the membrane hyperpolarization and decrease of membrane resistance as well as the blockade of postsynaptic action potential generation caused by low glucose. Since the effects of low glucose on membrane potential were the same whether KCl or potassium acetate filled electrodes were used, it is unlikely that the leakage of Cl^- from the electrode contributed to the hyperpolarizing effect of low glucose. However, the ionic mechanism for the depolarizing effect of low glucose remains unclear. A possible explanation is that, due to energy deprivation, the function of Na^+/K^+ pump was

impaired and consequently membrane potential was depolarized. However inhibition of the Na^+/K^+ pump by ouabain, at least initially, increased the excitability of the postsynaptic neuron while low glucose inhibited it. Cox et al (1983) demonstrated that more than 30 min exposure to complete glucose deprivation did not change ATP levels in brain slices. Therefore, energy failure is not likely to be the mechanism responsible for the depolarizing effect of low glucose.

There are at least 6 different K^+ conductances in CA1 pyramidal cells (Storm, 1990). The decrease in membrane resistance suggests that low glucose caused a steady increase in these K^+ conductances, although it is not clear which of these were activated. A number of channels having already been opened, synaptic activation of additional K^+ or Cl^- conductances probably had little further influence on the membrane potential and the sizes of AHPs and IPSPs were therefore greatly reduced.

9.2 The effect of low glucose on glutamate agonist-induced inward currents

Low glucose produced an initial facilitation of both NMDA and quisqualate induced inward current when membrane potential was not depolarized. This is probably due to the alkalinization of extracellular space, because glucose

depletion has been reported to create a slow alkaline shift in hippocampal slices (Krnjević & Walz, 1990), and alkalinization increases NMDA and slightly, quisqualate currents (Tang et al., 1990; Traynelis & Cull-Candy, 1990; Vyklicky et al., 1990). In addition, hyperpolarization caused by glucose deprivation will increase the electrochemical gradient of Na^+ which may also enhance the quisqualate current. Facilitation of NMDA current was also seen in the early phase of the depolarization produced by low glucose, which is probably due to the removal of the voltage-dependent Mg^{2+} blockade on NMDA channels.

There was a significant correlation between changes in quisqualate currents and changes in membrane potential caused by low glucose. When low glucose induced large depolarizations, quisqualate and NMDA currents were blocked. The reduced electrochemical gradients of Na^+ and Ca^{2+} are possible mechanisms for the suppression of quisqualate current and NMDA current as well. There was, however, a major difference between quisqualate and NMDA-induced currents following a more prolonged exposure to low glucose. When the membrane depolarized, quisqualate currents were reduced, but the membrane potential and the responsiveness of the neuron to quisqualate recovered upon the reintroduction of 5 mM glucose. In contrast, NMDA currents kept increasing in low glucose, until a permanent depolarization appeared. At this stage the neuron failed

to respond to further NMDA administration and the reintroduction of 5 mM glucose did not restore the membrane potential and the NMDA current. These observations suggest that NMDA, unlike quisqualate, causes irreversible damage in the presence of a glucose-deficient medium.

This conclusion is in agreement with previous reports on the NMDA receptor-mediated neurotoxicity of glutamate that appears in hypoglycemia and ischemia (Novelli et al., 1988; Wieloch, 1985; Gill et al., 1987). The neurotoxicity of NMDA is probably the result of an excessive influx of Ca^{2+}, because when extracellular Ca^{2+} was reduced and Mg^{2+} increased, the neurotoxicity of NMDA was nearly suppressed (Choi, 1987; Garthwaite et al., 1986; Choi et al., 1989). Although the role of Ca^{2+} was not tested in the present experiments, it is likely that here too a large influx of Ca^{2+} was responsible for the neurotoxicity of NMDA which manifested itself by an irreversible depolarization. The large Ca^{2+} influx probably occurred through NMDA channels whose Mg^{2+} block was removed by the low glucose-induced depolarization. It appears therefore that the excitatory amino acid-mediated neurotoxicity of ischemia and hypoglycemia is due to both pre- and postsynaptic factors: presynaptically these conditions cause a large efflux of excitatory amino acids, as summarized in the introduction (section 3.2.2 of introduction), and through postsynaptic

depolarization hypoglycemia and ischemia sensitize neurons to the neurotoxic effect of glutamate mediated by NMDA receptors.

9.3 The effect of low glucose on synaptic transmission

The suppression of synaptic transmission by low glucose was observed in both extra- and intra-cellular studies. Extracellular results demonstrated that glucose deprivation initially impaired the ability of the EPSP to trigger action potentials when the input-output relation between the presynaptic fiber volley and fEPSP was not affected.

In the intracellular study, both the size of EPSPs and the threshold for EPSPs to trigger action potentials are increased shortly after the application of low glucose. The initial increase in EPSP amplitude is similar to the early enhancement of quisqualate currents in low glucose, due probably in both cases to a hyperpolarization which increases the driving force of Na^+ influx. Another reason for the enhancement of EPSPs and quisqualate currents is the extracellular alkalinization caused by glucose deficiency (Krnjević & Walz, 1990; Spuler et al., 1987) which increases AMPA currents (Tang et al., 1990; Traynelis & Cull-Candy, 1990; Vyklicky et al., 1990). The subsequent transient increase in the triggering threshold

for action potential generation suggests that the electrotonic conduction of EPSPs from the soma to the axon hillock may be diminished by the increased membrane conductance. This conclusion is supported by the observation that low glucose does not depress action potential generation due to antidromic stimulation, a condition in which electrotonic conduction of depolarization is not involved.

Longer exposure to low glucose eventually abolished EPSPs. This could have resulted from a diminished release of the transmitter, glutamate, or from the shunting of EPSPs between the distant dendrites and the soma. Direct measurement of glutamate and aspartate release from hippocampal slices evoked by conducted action potentials have been shown not to decrease, however, during prolonged exposure to 0.2 mM glucose (Szerb & O'Regan, 1987; 1988), an observation which favours the latter possibility.

The effect of low glucose on quisqualate current appears to be correlated with its action on the EPSP. The initial increase of quisqualate current was similar to the initial increase of EPSP and when the glucose induced depolarization appeared, both quisqualate current and EPSP were suppressed. However, when low glucose produced a continuous hyperpolarization, EPSPs eventually disappeared, while quisqualate current was slightly increased. It is possible that low glucose failed to

reduce the quisqualate current because bath applied quisqualic acid activated receptors not only on dendrites but also on the soma, a process in which electrotonic conductance is involved to a lesser degree than in the EPSP produced by the activation of dendritic AMPA receptors.

9.4 The effect of low glucose on glial cells

Previous studies with the use of a voltage-sensitive dye showed that glucose deprivation depolarized the glial membrane potential. In addition, low glucose medium also decreased intracellular ATP level and collapsed the transmembrane potassium gradient in cultured astrocytes (Kauppinen et al., 1988). In our study, low glucose induced gradual depolarization in the presumed glial cells and this was associated with an increase in membrane resistance. Therefore, it is more likely that the depolarization observed in the present study is due to a decrease in the glial K^+ conductance, the main ion conductance in glia. Whether this change in conductance is the result of an altered energy status is not known. The depolarization of glia in a glucose deficient environment is likely to contribute to the large overflow of EAA seen in hypoglycemia because much of the released glutamate and aspartate is normally taken up by glia through a Na^+-

dependent transport mechanism (Benjamin & Quastel, 1975). Depolarization of glia, by reducing the electrochemical gradient of Na^+, would be expected to slow down this transport.

Chapter 10 OVERALL CONCLUSION

Both ammonia and low glucose inhibit synaptic transmission by postsynaptic effects. However, this is accomplished by different mechanisms, as indicated by their different effects on input/output relationship measured extracellularly and demonstrated in more detail by intracellular studies. Ammonium ions interrupt the postsynaptic action of the released transmitter that produces EPSPs, likely by blocking AMPA-mediated channels. On the other hand, low glucose prevents the triggering of action potentials possibly by increasing the membrane conductance so that EPSPs are no longer conducted electrotonically to the axon hillock. The mechanisms by which ammonium ions and low glucose suppress the hyperpolarization that follows the action potential also appear to be different. Ammonium ions decrease the hyperpolarization by reducing the electrochemical gradient of Cl^- and K^+. On the other hand, low glucose probably increases several K^+ conductances involved in the hyperpolarization so that they cannot be increased any further by orthodromic excitation.

The concentrations of NH_4Cl and low glucose used in this study have been found in clinical hyperammonemic encephalopathy, hypoglycemia and experimental starvation.

Therefore the inhibitory effects of NH_4Cl and low glucose on synaptic transmission observed in this study may contribute to the depression of central nervous system function in hepatic encephalopathy and hypoglycemic coma. Furthermore, brain glucose content can drop near or below 1 mM when the glucose supply to the brain is inadequate due to starvation and/or excessive energy utilization as mentioned in the introduction (Chapter 3.1). This glucose concentration produces a reduction in postsynaptic firing which may have a protective effect against low glucose-induced neurotoxicity, because a decrease of the rate of action potential generation, when the glucose supply is too low, will reduce energy demand and will allow the supply of glucose to catch up with its consumption.

The present study is a model for acute clinical situations, such as hyperammonemia resulting from acute hepatic failure due, for instance, to poisoning or for acute hypoglycemia due to insulin administration. Chronic metabolic changes, such as those resulting from the cirrhosis of the liver, involve additional functional disturbances, such as a pathological swelling of astrocytes. Since these swollen astrocytes are less able to detoxify ammonia, individuals with chronic hepatic failure are more prone to experience CNS disturbances due to hyperammonemia than individuals with acute hepatic failure.

By the elucidation of the mechanisms involved in the deleterious effects of these two metabolic disorders on the nervous system, it is hoped that suitable measures can be taken to prevent and reverse these toxic effects.

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