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Ionic Mechanisms of Anoxia:

Potential Role for γ-aminobutyric Acid

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in partial fulfilment of the requirements for the degree of
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ABSTRACT

In order to examine a potential role for the inhibitory neurotransmitter, GABA (γ-aminobutyric acid) in the early ionic responses of the mammalian CNS to anoxia, ion-selective microelectrodes (ISMs) were used to record changes in $[K^+]_o$, $[Cl^-]_o$ and $[Na^+]_o$ in the CA1 region of guinea pig hippocampal slices during applications of GABA and during brief anoxia. Bath applications of 1–10 mM GABA for 5 min evoked graded changes in $[K^+]_o$ and $[Cl^-]_o$ in both stratum pyramidale (SP) and stratum radiatum (SR). EC_{50} values for $[K^+]_o$ increases in SP and SR were 3.8 and 4.7 mM, respectively; for $[Cl^-]_o$ increases in SP and decreases in SR, EC_{50} levels were 4.1 and 5.6 mM. The $[Na^+]_o$ response to 5 mM GABA was a decrease in both SP and SR.

When ionic changes induced by GABA (5 mM) were compared to those evoked by O_2 replacement by N_2 for 5 min, anoxic responses were found to be very similar in direction of change and time-course. In SP there were increases in $[K^+]_o$ and $[Cl^-]_o$ and a decrease in $[Na^+]_o$, and in SR an increase in $[K^+]_o$ and decreases in $[Cl^-]_o$ and $[Na^+]_o$. Changes in SR were greater than those in SP, except for $\Delta[K^+]_o$ evoked by GABA. To estimate changes in the extracellular space (ECS), variations in $[TMA^+]_o$ were recorded during GABA and N_2 exposures. Volume decreases were observed with both GABA and anoxia and significantly less in SP than in SR: 5.3 $\pm$ 0.03 % and 6.2 $\pm$ 0.8 %, respectively with N_2 and 4.6 $\pm$ 0.2 % and 4.8 $\pm$ 0.3 % with GABA. The ECS reduction was insufficient to account for the ionic changes

observed. The $GABA_A$ receptor antagonist, BMI (bicuculline methioidide) (100μM) reversibly attenuated all ion changes evoked by GABA and N_2. In SP BMI depressed increases in $[K^+]_o$ and $[Cl^-]_o$ with GABA by 90%, and with N_2 by 50–60%. In SR the $\Delta[K^+]_o$ evoked by GABA was blocked, and that with anoxia was attenuated by 70 %; decreases in $[Cl^-]_o$ were depressed by more than 50 %, and $[Na^+]_o$ changes in SP and SR were decreased by 20–30 %. The similarity of GABA- and N_2-induced ion changes and their attenuation by BMI suggest that $GABA_A$ receptor activation during early anoxia can contribute to the associated ionic events.

The $GABA_B$ agonist, baclofen ($10^o - 3 \times 10^3$ μM) evoked dose-dependent increases in $[K^+]_o$ in SP and SR, with EC_{50} = 40 and 39 μM, respectively. The sigmoid concentration-response curves and sensitivity of responses to the $GABA_B$ antagonists, indicate a receptor-mediated increase in $\Delta[K^+]_o$, due to an increase in g_K. The similar EC_{50} values at the soma and dendrites suggests that their $GABA_B$ receptors are identical. The higher affinity of $GABA_B$ cf. $GABA_A$ receptors supports the possibility that an early component of anoxic-evoked K^+ accumulation may be due to activation of both $GABA_A$ and $GABA_B$ receptors. The observation that $[K^+]_o$ increase with N_2 can be enhanced by $GABA_B$ antagonism may reflect a protectant action of $GABA_B$ receptors by decrease of glutamate release from terminals and postsynaptic hyperpolarization.

The results support the hypothesis that early extracellular ion changes induced during anoxia may be at least partly due to extracellular accumulation of GABA and activation of $GABA_A$ and $GABA_B$ receptors, and the possibility that agonists and (or) potentiation of GABA-ergic mechanisms might be usefully

combined with other agents for neuroprotection from excitotoxicity during and following global ischemia.

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PUBLICATIONS RELATED TO PhD PROGRAM RESEARCH

I Papers

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II Abstracts.

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LIST OF ABBREVIATIONS

ACh	acetylcholine
ACSF	artificial cerebrospinal fluid
Ag/AgCl	silver/silver chloride
AMPA	a-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
4-AP	4-aminopyridine
BMI	bicuculline methiodide
CACA	cis-4-aminocrotonic acid
CCl_4	carbon tetrachloride
$[Cl^-]_o$	extracellular chloride ion concentration
DAG	diacylglycerol
DG	dentate gyrus
DPCPX	8-cyclopentyl-1,3,-dipropylxanthine
ECS	extracellular space
EPSP	excitatory post-synaptic potential
FIM	fixed interference method
FPM	fixed primary ion method
GABA	γ-aminobutyric acid
HCO_3^-	bicarbonate ion
HPO_4^{2-}	phosphate ion

$[K^+]_o$	extracellular potassium ion concentration
ISM	ion-selective microelectrode
IP_3	inositol triphosphate
IPSP	inhibitory post-synaptic potential
LM	lacunosum moleculare
MK801	dizolcipine, NMDA channel blocker
$[Na^+]_o$	extracellular sodium ion concentration
NMDA	N-methyl-D-aspartate
PAH	post-anoxic hyperpolarization
PCr	phosphocreatine
PIP_2	phosphoinositol diphosphate
PLC	phospholipase C
PS	population spike
PT	permeability transition
R_M	resting membrane resistance
R_{N_2}	membrane resistance during anoxia
ROS	reactive oxygen species
SO	stratum oriens
SO_4^{2-}	sulphate ion
SP	stratum pyramidale
SR	stratum radiatum
SSM	separate solutions method
THIP	4,5,6,7-tetrahydroisoxazalo[5,4-C]pyridine-3-ol

TM	transmembrane
TMA	tetramethylammonium
$[TMA^+]_o$	extracellular tetramethylammonium ion concentration
TMSDMA	dimethylaminotrimethylsilane
TTX	tetrodotoxin
V_F	field potential
V_K	potassium potential
V_M	membrane potential
V_R	resting membrane potential
$\Delta V_{D(N_2)}$	depolarizing change in membrane potential during anoxia
$\Delta V_{H(N_2)}$	hyperpolarizing change in membrane potential during anoxia
ΔV_{PAH}	post-anoxic hyperpolarization of membrane potential

INTRODUCTION

I. ANOXIA.

i. Background.

The function of the mammalian brain depends on a continuous supply of O_2 and glucose. The brain has a high metabolic rate but low O_2 stores and small reserves of energy Choi 1988; Choi and Rothman 1990; Hansen 1985; Raichle 1983; Siesjo 1988) and the majority of mammalian CNS adult neurons are post-mitotic cells. Therefore any long-enough sustained injury to these cells leads to permanent consequences and results in neurological deficits. Ischemia (arrest of blood flow, which removes the supply of O_2 and glucose) occurs in such context as general organ failure (cardiac arrest and coronary artery bypass graft) or as in local insults such as thrombo-embolism of a cerebral artery or cerebral hemorrhages (Hunter et al. 1998). Hypoxia (lowered O_2 tension in arterial blood) and anoxia (complete lack of O_2 in the blood) are conditions in which oxygen deprivation, by itself, presents the sole factor responsible for neuronal injury. Situations in which a low oxygen level leads to neuronal injury, are found in instances such as carbon monoxide poisoning or during general anaesthesia when, even though mechanical ventilation maintains tidal volume and

respiratory rate, the O_2 supply fails secondary to a mistake in selection of gas lines.

Several lines of evidence suggest that the mammalian brain is more tolerant to ischemia than it was previously believed to be. Numerous studies have shown that for the first days of life, the brain of newborn rats is more resistant to hypoxia than that of adult rats (Kass and Lipton 1989). The realization that the human brain may be resistant to ischemia raised the possibility of intervening successfully before irreversible damage occurs (Choi and Rothman 1990; Raichle, 1983).

The pathophysiological changes seen with hypoxia/ischemia are quite complex. Despite this, two basic sources of or contributing factors to pathophysiological processes have been separated. One is the cerebral circulation and energy metabolism. The second is the intrinsic neuronal organization – anatomical connectivity, neurotransmitters and receptors. The latter is closely linked to the blood supply and therefore can only artificially be separated from it. Ischemic injuries can be classified as focal and global ischemia. Irreversible focal ischemia stems from interruption of the blood flow to an area of the brain and leads to brain infarction. Global ischemia is usually reversible and may last a variable amount of time. If a global ischemic period is more prolonged then a diffuse pattern of neuronal damage is seen. If, on the other hand, the duration of global ischemia is relatively short, only certain areas of the brain tend to be more susceptible.

In animal models of global ischemia experimental injury is produced in the forebrain by reversible cessation of blood flow to the brain. Then after defined survival times the animals are sacrificed and their brain is subject to extensive pathophysiological, biochemical and morphological investigations. One standard ischemic model is the four-vessel occlusion model. Firstly, the vertebral arteries are irreversibly occluded, then after a delay the carotid arteries are reversibly clamped. The animals are awake and continue to breathe. The second standard model – two-vessel occlusion combines bilateral occlusion of the common carotid arteries with hypotension in intubated and ventilated animals. This is necessary because bilateral common carotid artery occlusion alone does not lead to a critical reduction of blood flow because of intracranial anastomosis between the carotid and vertebral circulation. The hypotension required by the two vessel occlusion model is achieved by pharmacological means or by bleeding.

Other models of ischemia include combinations of unilateral ligation of one of the carotid arteries with transient global hypoxia, or of two-vessel occlusion with basilar artery occlusion, cardiac arrest, or intrathoracic vascular occlusions. In the case of the gerbil, which has the peculiarity of no anastomosis between the anterior and posterior circulation, transient unilateral occlusion of the common carotid artery alone leads to selective neuronal lesions. The complex pathophysiological parameters encountered in ischemic models, are better studied in mammals with a larger brain, such as the cat or monkey. However, for morphological, biochemical and pharmacological studies, smaller

animals such as rodents are used. In general, rodent models rely on self-sustained survival of animals. However, this may involve the bias of selecting those robust animals that do not undergo the possible complications such as fatal seizures or spontaneous death, which may occur after ischemia. The introduction of *in vitro* recording in brain slice preparation facilitates the study of transient global anoxia/ischemia. The common use of hippocampal slices stems from the fact that the hippocampus is a highly laminar structure with a well characterized pattern of connectivity and also the fact that the hippocampal CA1 area is one of the most vulnerable regions of the CNS in response to anoxic insults.

ii. Differential neuronal vulnerability.

It is well established that although all neurons are sensitive to anoxic/ischemic insults, among them there is a hierarchy of susceptibility to ischemic injury (Brierly 1977; Brown 1977). Neurons of the reticular nucleus, CA1 pyramidal cells in the hippocampus and the Purkinje cells in the cerebellum appear to be the most vulnerable – followed in order by striatal medium-sized neurons, neocortical neurons of layers III, V, and VI, and the neurons of the entorhinal cortex and thalamus (Choi and Rothman 1990; Pulsinelli 1985; Schmidt-Kastner and Freund 1991; Shimizu et al. 1996; Siesjo 1988). According to our current knowledge, these neurons are the "hardware" of some of the highest brain functions, including memory and learning, cognition processes, and consciousness and awareness.

The peculiarities of the local vascular supply (Spielmeyer 1925) were thought to account for the differential vulnerability of specific brain regions (Scholtz 1963). Blood flow is supplied to the anterior portions of the cerebral hemispheres and the retina by the carotid (anterior) circulation and to the posterior cerebral hemispheres and hindbrain by the vertebrobasilar (posterior) circulation. Hippocampal and mesial temporal structures are supplied mainly by the posterior circulation. In episodes of severe hypotension, when the process of autoregulation of cerebral blood flow fails, the areas between the major arterial territories (watershed zones) are affected first (Brierly 1977). The cortical watershed between the two circulations takes place at the temporo-parieto-occipital junction and the deep watershed at the level of the thalamus.

However, there is a variety of highly focal ischemic lesions of brain which are clearly independent of the vascular distribution and which can only be explained by the unique physical and chemical properties of the specific neuronal groups (Vogt and Vogt 1937). One characteristic, which separates neurons into subpopulations with different vulnerability to hypoxic/ischemic insults, is their ability to synthesis, release and respond to different neurotransmitters. The selective ischemic damage to neurons could in part be influenced by the neurotransmitter input and/or by differences in the balance between the excitatory and inhibitory input to each region. For example, a comparison of the densities of glutamate to $GABA_A$ receptors yields some interesting observations (Zilles 1992). The ratio of the two densities is highest in

the hippocampus, favouring glutamate by 4.3 times, a feature which correlates well with the fact that the hippocampus has the highest susceptibility to ischemic damage (Collins et al. 1989). The excitotoxicity theory proposes that continuous exposure of neurons to high levels of glutamate can produce delayed toxic effects and even cell death. The high levels of glutamate trigger elevation of free cytosolic Ca^{2+} by cellular influx and release from intracellular stores. The calcium accumulation then seems to play a key role in propagation of the process which leads toward irreversible neuronal damage by the activation of a series of neurotoxic events, e.g. lipid peroxidation, free radical generation, activation of proteolytic enzymes and pathological gene expression. The particular properties of different neuronal groups in handling this cellular accumulation of calcium are likely to be an important factor in their susceptibility to anoxic injury (Choi 1988; Choi and Rothman 1990; Graham et al. 1990; Graham et al. 1993; Kass and Lipton 1982; Kass et al. 1989; Mody and MacDonald 1995; Rothman 1984; Szatkowski and Attwell 1994). When the excitotoxicity theory was first proposed by Olney et al. (1971), they speculated that elevated levels of endogenous amino acids might play a role in the genesis of certain human neurological or psychological disorders. The evidence is most convincing in diseases involving acute onset, such as stroke, trauma, and hypoglycemia. But neurotoxicity due to excitatory amino acids may also be involved in slowly progressive degenerative conditions, such as Huntington's Chorea, Parkinson's Disease, Alzheimer's Disease and others (Choi 1988).

Neuronal necrosis following an ischemic insult was regarded as the sole mechanism for neuronal loss. However, this type of cell death does not explain the higher susceptibility of neurons from vulnerable areas to ischemic insult. For some years a binary classification of cell death suggests that physiologically appropriate death is due to apoptosis and that pathological mechanisms involve necrosis. Necrotic cell death is characterized by severe and sudden injury associated with an inflammatory response. In contrast, neuronal apoptosis is not associated with inflammation and involves the participation of specific genetic programs. Programmed cell death seems to be initiated before the appearance of morphological indications that are associated with necrosis. This suggests that the choice of dying by necrosis or apoptosis may depend on the degree of injury (the dose and duration). Apoptosis is now believed to be one of the possible mechanisms underlying delayed neuronal cell death in the regions that show "selective vulnerability" (Linnik et al. 1993; Thompson 1995). The rise in $[Ca^{2+}]_i$ triggers a series of events, including activation of immediate early genes, that might activate programmed cell death. C-jun immunoreactivity is increased in the vulnerable regions following ischemic injuries (Dragunow et al. 1993, 1994). Increased expression of c-jun appears to be a marker for commitment to apoptosis. The apoptotic process has been divided into four stages (1) cell cycle arrest, (2) capacitation for apoptosis and proliferation, (3) irreversible commitment to death and (4) nucleolysis and proteolysis (Kroemer et al. 1995).

In the whole cascade of events the DNA fragmentation appears to be preceded by activation of caspase-3. This proteolytic enzyme is part of a family

of cysteine-proteinases linked to apoptosis (Nagata 1997; Thornberry et al. 1997; Gillardon et al. 1997; Gagliardini et al. 1994). Activation of caspase-3 in hippocampal neurons is necessary for apoptosis and can be prevented by protein synthesis inhibitors (Gillardon et al. 1997).

iii. Pathophysiology.

(a) Disturbed ion gradients.

Life and survival require that cells osmotically pump ions across membranes; and translocate material between central and peripheral domains, and perform biosynthetic work necessary to keep pace with spontaneous or enzyme catalyzed degradation of material. All cellular work is done at the expense of the ATP. After anoxia/ischemia, active transport and biosynthetic work are done utilizing the available stores of phosphocreatine (PCr) and ATP, and the ATP formed during anaerobic glycolysis. In the absence of ATP, ion gradients are dissipated, intracellular communication is halted and macromolecules and their assemblies are broken down in reactions that are thermodynamically spontaneous (Siesjo 1988). Energy failure triggers a series of events that may hasten the breakdown of cell structure.

Much of the data on ion distribution and homeostasis during anoxic/ischemic insults comes from the use of ion-selective microelectrodes (ISMs). Before the introduction of ISMs, no techniques were available for direct measurement of ion concentrations in brain interstitial space. Only indirect estimations could be made, with aid of electrophysiological techniques to detect

changes in membrane potential and resistance, or by inference from the CSF. The composition of the CSF is supposed to reflect the ionic composition of the interstitial space. However, at times of acute changes, the long time course of the equilibrium process between the two compartments hinders the understanding of the ionic changes that take place in the interstitium. With the event of ISM this difficulty was overcome (Hansen 1985).

Changes in the ionic environment may affect the synaptic transmission and functionality of neurons and lead to irreversible damage or death of brain cells. After brief periods of anoxia, 5-10 min, most neurons show signs of almost full morphologic recovery, but after 24-72 hours, unknown events trigger mechanisms that lead to irreversible injury and death of neurons located in vulnerable areas (Silver and Erecinska 1992). The resumption of cerebral blood flow after a period of ischemia leads to restoration of the interstitial ionic environment in the interstitium (Hansen 1985). However, during the phase of reperfusion and reoxygenation some reactions are triggered that ultimately lead to cellular disintegration. Recent results strongly suggest that reactive oxygen species (ROS), generated during the reperfusion period, may trigger the delayed neuronal cell death (Kuroda and Siesjo 1997). Oxygen free radicals have been also implicated in the damage of vascular endothelium. Endothelial cells have been proposed as a source of oxygen free radicals. Mitochondrial Ca^{2+} overload and a permeability transition (PT) of the inner mitochondrial membrane have been shown to play an important role in the production of ROS (Siesjo and Siesjo 1996). In an *in vitro* model of anoxia-reoxygenation using cultures of

cerebral endothelial cells, Beetsch et al (1998) showed that treatment with ROS inhibitors such as superoxide dismutase or scavengers like oxypurinol were effective in preventing the cytotoxic effect of ROS. Others have found that treatment with anti-oxidant agents prevents the apoptotic ischemic cell death (Dugan et al. 1996).

Examining the ionic changes seen with anoxia might help in understanding differences in sensitivity both within and between species. Differences between anoxia-tolerant and anoxia-sensitive tissues could involve differences in the permeability of membranes to ions and the mechanisms of ion homeostasis. There are different mechanisms of activation and control of transmembrane ion distribution in specific neurons and brain regions, and these are subject to change during life and/or secondary to different insults. For example, it is well established that the immature brain is highly resistant to anoxia (Adolph 1971; Fazekas et al. 1941; Hansen 1985). Glial cells are less susceptible to ischemic injuries than neurons, possibly because pathways for ion movements/dissipation are more abundant than for nerve cells and therefore more ions can leave/enter the cell (Silver et al. 1997). The changes in the ionic composition of the extracellular space (ECS) during anoxia are initially of a lesser magnitude and delayed in the newborn mammalian brain than in the mature animal (Hansen 1977; Hansen 1985; Morris and Trippenbach 1993).

(i) Extracellular K^+ accumulation.

When the mature rat brain is exposed to an ischemic insult (e.g. cardiac arrest) extracellular K^+ begins to rise slowly. This initial phase I lasts for about 2 min and can be extended by manipulations of the energy supply, for example with hyperglycemia (Hansen 1985; Siesjo 1988). In the newborn mammalian brain phase I is prolonged (Hansen 1977; Morris and Trippenbach 1993). After the initial increase in $[K^+]_o$ a second and steeper rise (phase II), that lasts only a few seconds, occurs. This is then succeeded by a progressive increase over about 10 min to reach a constant level of $[K^+]_o$ (phase III) (Hansen 1977; Siesjo 1988).

The progressive increase in extracellular potassium is a multifactorial event that involves a reduction of ATP and disturbance of energy-dependent processes, e.g. the functioning of Na^+/K^+ ATP-ase and other co/counter ion transport mechanisms and/or uptake systems. The early efflux of potassium has been implicated as a cause of the initial hyperpolarizing electrophysiological response observed in some/many neurons exposed to anoxia. There is evidence that at least in medullary neurons, the activation of ATP-sensitive K^+ channels attenuates the onset of anoxia-induced depolarization and can therefore play a role in cell survival (Haddad and Jiang 1994). Evidence also strongly points towards a role for elevated levels of intracellular Ca^{2+} and the activation of a Ca^{2+}-dependent K^+ conductance, especially in the CA1 region of the hippocampus (Krnjevic and Xu 1990; Martin et al. 1994). During anoxia accumulation of neurotransmitters in the synaptic clefts and the ECS can lead to

activation of pre- and post-synaptic receptors. Both excitatory amino acid (EAA) receptors and $GABA_B$ receptor-linked channels are permeant to K^+ and could contribute to the rise in $[K^+]_o$.

(ii) Na^+ and Cl^- influx.

It is believed that at least one of the anoxic mechanisms of brain damage involves acute neuronal swelling (Hansen and Olsen 1980; Jiang and Haddad 1991; Van Harreveld 1966). This swelling is presumed to be due to entry of Cl^- and Na^+, because extracellullar Na^+ and Cl^- decrease during ischemia in mammalian brain (Hansen and Zeuthen 1981) and also, because it has been shown that Na^+ and Cl^- are necessary for cell swelling to occur (Choi 1985; Hartley and Choi 1989; Rothman 1985). It has been hypothesized that anoxia induces this Na^+ influx by Na/K-ATP-ase inhibition (Silver et al. 1997) and release of EAAs in the brain. Influx of Na^+ and Cl^- into the cell via receptor-activated mechanisms and/or failure of the energy dependent uptake systems, ion pumps, co/counter transport mechanisms, or simply by slow leakage into the cells leads to cell swelling (Hansen 1985). If this is not opposed, it is followed by lysis and cellular death of neurons, endothelial cells and glia. In experiments carried out in cultured neurons, where Na^+ and Cl^- were lowered by substitution in the superfusing solution, the ensuing neuronal loss that occurs with exposure to various EAA agonists was prevented (Rothman 1985; Rothman and Olney 1986). Also, the removal of $[Na^+]_o$ and replacement with an impermeant cation, N-methyl-D-glucamine ($NMDG^+$) completely protects freshly dissociated CA1

neurons during and after severe anoxia, for up to 90 min (Friedman and Haddad 1994a).

(iii) Intracellular Ca^{2+} accumulation.

The single, most important event that has been linked to selective and delayed neuronal damage is an intracellular accumulation of Ca^{2+} that occurs during and following ischemic insults. There are several potential sources for increased $[Ca^{2+}]_i$ namely agonist-activated calcium channels (NMDA and non-NMDA), voltage-dependent calcium channels (L-type), reverse operation of the Na^+-Ca^{2+} exchanger (Nachsen et al. 1986), channels activated by membrane stretch (Yang and Sachs 1989) and membrane leak conductances. More recently, the possibility of calcium being released from internal stores has been recognized as a mechanism of significant importance (Belousov et al. 1995; Kass and Lipton 1982; Mody and MacDonald 1995; Simpson et al. 1995). Some studies show that the L-type Ca^{2+} channels may not be so important, since during anoxia L-type Ca^{2+} currents and low-threshold transient currents are blocked, possibly due to high $[Ca^{2+}]_i$ or ATP depletion (Krnjevic and Leblond 1987; 1989). Others show that L-type Ca^{2+} channels may contribute to the increased intracellular calcium levels during the induction of neuronal damage during cerebral ischemia. In an *in vitro* model of oxygen/glucose deprivation, Pisoni et al. (1998) showed that in neocortical neurons the rise in $[Ca^{2+}]_i$ is prevented by nifedipine or nimodipine, Ca^{2+} channel blockers, but not by dantrolene, an agent that blocks calcium release from the sarcoplasmic reticulum. Ca^{2+} accumulation in the cell

leads to activation of several enzyme families – including protein C kinases (PKC), calmodulin-regulated enzymes, calpains, and phospholipases. Phospholipase C breaks down Phosphoinositol diphosphate (PIP_2), that in turn generates diacylglycerol (DAG), a messenger restricted to the membrane and inositol triphosphate (IP_3), that diffuses through the cytosol and may induce release of Ca^{2+} from intracellular stores. Phospholipase A_2 leads to formation of arachidonic acid and to formation of substantial amount of oxygen free radicals (Chan et al. 1985; Siesjo et al. 1989). This cascade of events has the final result of activation of lipases and proteases that leads to breakdown of cellular membranes and cell death. Preventing calcium accumulation in the cytosol by enhanced expression of calbindin, a calcium buffering protein, such as in brief stress, is beneficial (Rami et al. 1998).

(b) Loss of synaptic transmission.

Cerebral hypoxia causes cessation of spontaneous cortical activity in 10–30 s in mammals (Mayevsky and Chance 1975). Within 1–3 min there is a block of evoked responses. Numerous research studies have attempted to establish the basis for the evoked potential failure. Some suggest that membrane depolarization rather than membrane hyperpolarization or disruption of neurotransmitter release is responsible for failure of evoked potentials during hypoxia (Lipton and Whittingham 1979). Others, suggest that the suppression of neuronal activity by the fall of pO_2 is due to a block of the voltage-gated calcium

channels that are required to induce synaptic responses (Krnjevic and Leblond 1987, 1989).

Recent studies have shown regional and developmental differences in the vulnerability of postsynaptic currents to anoxia (Cherubini et al. 1989; Hershkowitz et al. 1993). There are also observations suggesting that excitatory glutamate-mediated synaptic currents are more resistant to anoxia than inhibitory, GABA-ergic ones since the inhibitory component is depressed more rapidly during anoxic episodes and recovers with a longer delay upon reoxygenation (Fujiwara et al. 1987; Hershkowitz et.al. 1993; Krjnevic et.al. 1991; Rosen and Morris 1993). Also, attributed to the higher sensitivity to anoxia of the inhibitory postsynaptic potentials (IPSP) is the transient increase in excitability that occurs in the neocortical cells during early anoxia and/or recovery (Rosen and Morris 1993). It is now known that the monosynaptic $GABA_A$ receptor mediated currents are resistant to anoxia, while the late monosynaptic $GABA_B$ receptor-activated component is strongly depressed. The inhibition of polysynaptic IPSPs by anoxia is attributed to loss of the glutamate-mediated excitation of interneurons (Khazipov et al. 1993).

(c) Hippocampal circuitry.

The hippocampal neurons are interconnected by a chain of excitatory synapses: granule cells of the dentate gyrus receive excitation from the entorhinal cortex via the perforant path; granule cells send their excitatory axons, the mossy fibers, to the CA3 region; and the CA3 neurons in turn send their axons, the

Schaffer collaterals, to CA1 neurons (Amaral and Witter 1989; Lacaille et al. 1987; Lacaille and Schwartzkroin 1988). Thus the vulnerable CA1 neurons are at the end of a tri-synaptic chain of excitatory synapses. In addition, principal cells of each subfield possess extensive local networks. From research on epilepsy models in the hippocampus, it is known that CA3 neurons provide an excitatory burst-like output via the Schaffer collaterals to CA1 neurons. The perforant pathway from the entorhinal cortex, in addition to the tri-synaptic loop, sends a direct projection to CA1 cells (Garcia-Ugalde et al. 1992). Additional afferent fibers to the hippocampus include the commissural fibers and subcortical projection system using acetylcholine, noradrenaline, serotonin, histamine and GABA as transmitters. Different types of inhibitory interneurons, most of which are GABA-ergic are present in all subfields of the hippocampus. Based upon the concept of the tri-synaptic chain of excitatory connections, lesion experiments that produce deafferentiation of CA1 region from the excitatory inputs (perforant path or the entorhinal cortex) protect the CA1 neurons against ischemic injury (Benveniste et al. 1989).

(d) Role of excitatory amino acids (EAAs) and their receptors.

(i) Glutamate release during ischemia.

The mechanism by which glutamate is released in ischemia has been controversial. Some reports have shown that the release is Ca^{2+}-dependent (Drjer et al. 1985), while others found that is was partly or wholly Ca^{2+}-independent, implying a non-exocytotic process, such as reversal of glutamate

uptake (Attwell et al.1993; Szatkowski et al. 1990) or release through a swelling-activated anion transport mechanism. It is unlikely that significant release of glutamate via exocytosis would occur in an ischemic core, since the decrease in ATP concentration inhibits this process. In this situation much of the release appears due to reversal of glutamate uptake system. The direction of glutamate transport by the uptake carrier is determined by the concentration of the other ions that it transports besides glutamate. Normally, for each glutamate ion this involves the influx of two Na^+ while one K^+ and one OH^- or HCO^-_3 are transported out, with one net positive charge transfer into the cell with each cycle.

However, the changes in transmembrane ion and voltage gradient that occur in ischemia – a ΔpH towards acidosis, increase in extracellular K^+ and decrease in extracellular Na^+, with accumulation of Na^+ ions inside the cell, plus membrane depolarization tend to make the carrier operate in the opposite direction to uptake. The carrier therefore will pump glutamate out of neurons and glia into the extracellular space until a new equilibrium is achieved. The extracellular increase in K^+ will be the driving force that raises the extracellular concentration of glutamate to neurotoxic levels (Szatkowski et al. 1990).

(ii) Excitatory amino acid receptors.

Glutamate acts on two types of receptors – NMDA and non-NMDA. It was shown that Ca^{2+} enters the cell through NMDA receptor-operated channels (Cotman and Iversen 1987; Huntley et al. 1994) and that in the post-ischemia

phase there is a potentiation of the NMDA receptor component of the synaptic current (Szatkowski and Attwell 1994). The different ways by which NMDA receptor currents may be enhanced by ischemia involve:

- depolarization by $[K^+]_o$ increase, with upregulation of the expression of NMDA NR2A subunits,
- arachidonic acid,
- rise in $[Ca^{2+}]_i$ with activation of PKC,
- elevated levels of $[glycine]_o$.

Initially it was thought that only NMDA channels are permeable to Ca^{2+}; however, it is now postulated that especially in abnormal conditions, even non-NMDA channels (such as those activated by AMPA receptors) may allow the passage of Ca^{2+}. When GluR2 subunit is expressed in the AMPA receptor-linked channel the complex is impermeant to Ca^{2+}. In anoxia/ischemia, and also other conditions (LTP, epilepsy) there is downregulation of the GluR2 subunit synthesis and AMPA receptors become permeable to Ca^{2+} and can therefore contribute to excitotoxicity and neuronal death (Huntley et al. 1994; Mody and MacDonald 1995).

It has been shown in the hippocampus that with ischemia glutamate binding to the non-NMDA metabotropic (mGLU) receptors induces (via a GTP-binding G protein, coupled to phospholipase C (PLC)) the formation of intracellular DAG, followed by intracellular Ca^{2+} mobilization (Brown and Reymann 1995). However, other experiments that have tested both agonists and antagonists to mGLU receptors found a lack of effects of these compounds on

the anoxic events which occur in the CA1 region of the hippocampus (Tan et al. 1995).

(iii) The excitotoxic hypothesis of ischemic cell death.

During anoxia extracellular levels of glutamate and aspartate are known to increase in the hippocampus (Benveniste et al. 1984; Hagberg et al. 1985). A functional correlate of this is that ten hours after ischemia there is an increased firing rate of the neurons in the hippocampal CA1 region. As previously mentioned, the excitotoxicity theory proposes that glutamate and aspartate are potent neurotoxins (Olney et al. 1978) and when released during ischemia, can cause neuronal death in selectively vulnerable regions by activation of post-synaptic EAA receptors (Jorgensen and Diemer 1982). The imbalance between excitation and inhibition seems to play a key factor in the delayed neuronal death. Direct or indirect modulators of the glutamatergic activity, such as adenosine (Rudolphi et al. 1992) or glycine (Johnson and Ascher 1987; Kleckner and Dingledine 1988) can also play a role.

Measurements of glutamate, glycine and GABA obtained after repetitive episodes of brief forebrain ischemia, which are associated with CA1 hippocampal cell damage, show that glycine levels remain higher then the baseline, even after reperfusion, while glutamate and GABA levels eventually return to normal. An excitotoxic index, which reflects the neurotransmitter response, is defined as [glutamate] X [glycine]/ [GABA], and appears to be a

biochemical marker of selective vulnerability (Globus et al. 1991; Lin et al. 1992).

(e) Role of other neurotransmitters.

(i) Adenosine.

There is some evidence that adenosine is an endogenous neuroprotective agent. Probably several mechanisms can contribute to this effect and the relative importance of these mechanisms may depend on the type of ischemia. An important caveat is that acute and long-term effects of adenosine receptor agonists and antagonists differ widely. This effect inversion suggests that drugs that affect adenosine receptors also induce important adaptative events in the CNS. For example, acute treatment with theophylline or caffeine generally aggravates the damage induced by ischemia, but long term treatment with these adenosine antagonists has been reported to have the opposite effect (Rudolphi et al. 1989). Similarly long term treatment with the A1 receptor selective antagonist DPCPX was shown to be neuroprotective (von Lubitz et al. 1994), probably because of upregulation of adenosine receptors following prolonged blockade.

To date there are three known adenosine receptors. Adenosine acting on the presynaptic A1 receptors inhibits the release of many neurotransmitters, especially excitatory, such as glutamate. This effect is mediated via G-proteins to both Ca^{2+} and K^{+} ion channels (Rudolphi et al. 1992; Heron et al. 1993). In the postsynaptic neuron, A1 receptor activation helps to maintain the intracellular

Ca^{2+} homeostasis. There is evidence that adenosine decreases the ischemic and post-ischemic increase in calcium in vulnerable regions of the brain *in vivo* (Andine 1993) and *in vitro* (Dux et al. 1992).

Activation of A2 receptors leads to vasodilation and decreased free radical formation. For example adenosine has been shown to increase cerebral blood flow rate during anoxia, probably as an emergency response until metabolic depression sets in (Hylland et al. 1994). The source of free radicals formed during the reperfusion state is not clearly understood. The activated microglia and the neutrophil leukocytes contribute to the formation of free radicals. Adenosine and adenosine analogues prevent the oxidative bursts in the microglia and macrophages and also the accumulation of neutrophils in vascular beds after anoxia.

Finally it has been suggested that adenosine acts as an endogenous activator of cellular antioxidant defence systems (Maggiarwar et al. 1994) and this effect may be exerted via A3 receptor.

(ii) GABA.

The release of multiple neurotransmitters into the extracellular space can contribute not only to disruption of ion homeostasis, but also to an imbalance between excitation and inhibition, that may constitute a major determinant of selective vulnerability. GABA actions in the CNS are mostly inhibitory and are mediated via ionotropic and metabotropic receptors. The distribution of GABA receptors is ubiquitous and heterogeneous.. The density of the GABA receptors,

as well as the ratio between the densities of EAA and GABA receptors, appears to play a role in the selective vulnerability. Higher densities of GABA binding are found in the cerebellum and striatum, with lesser incidence in the septum and an intermediate range in the hippocampus and cortex (Zilles 1992; Zukin et al. 1974).

During ischemia GABA release and accumulation in the extracellular space occurs via three different mechanisms (Phillis et al. 1994; Torp et al. 1993):

- exocytotic calcium-dependent release of the vesicular pool,
- reversal of the uptake system, which releases the non-vesicular pool,
- release of both the vesicular and non-vesicular pools, as the cell disintegrates under the action of calcium activated phospholipases and oxygen-free radicals.

The temporal profile of neurotransmitter dynamics in the ischemia-reperfusion model shows an initial increase, during the ischemic period, for all amino acid neurotransmitters – excitatory (such as glutamate and aspartate) and inhibitory (such as GABA, glycine, alanine and taurine). The largest amplification of all amino acids, when compared to basal levels is seen in GABA (Graham et al. 1990). During ischemia GABA increases from barely detectable levels to quite high levels in the cerebral hemispheres (Graham et al. 1990; Matsumoto et al. 1996), striatum (Shuaib et al. 1994) and hippocampus (Ravindran et al. 1994; Torp et al. 1993; Andiné et al. 1991). Ischemic synaptic release and

accumulation of GABA proceeds by different mechanisms than for glutamate and aspartate. For example there are different transporters and the Na^+ channel blocker, tetrodotoxin (TTX) reduces ischemia-evoked GABA release but has no effect on glutamate efflux (Phillis et al. 1994). Also, there is evidence that glutamate stimulates GABA release (Schatzki et al. 1990). This, as well as the anaerobic insensitivity of glutamate conversion to GABA can enhance the increase in GABA as compared to glutamate and aspartate.

It appears that the accumulation of GABA in the extracellular space may be protective against the development of delayed neuronal death, and that GABA actions counterbalance the physiological and toxic effects of glutamate. Recently, agonists of the receptor have been shown to provide neuroprotection during ischemia. In cultured neurons the agonists appear to block kainate-stimulated cell death, although GABA itself appeared to accelerate cell death (Erdo et al., 1991). In the 4-vessel occlusion model of ischemia there is selective loss of glutamatergic neurons and relative sparing of GABA-ergic neurons. The function of these neurons during and after ischemia appears to protect target neurons from excitotoxic death (Johansen and Diemer 1991). Some studies show that GABA-ergic neurons in the hippocampus show resistance to ischemia (Nitsch et al. 1991). Others find the existence of two subtypes of GABAergic neurons, one that shows resistance to ischemia and a second that is less resistant to injury (Shuaib et al. 1993). Support for GABA-ergic neuroprotection comes from other sources as well. In adrenalectomized animals the high GABA levels which were observed were protective against the hippocampal cell loss that occurred in

non-adrenalectomized animals, where lower GABA levels were seen (Ravindran et al. 1994). This suggests that the vulnerability of hippocampal cells could be altered by modulation of GABA release with steroids.

During reperfusion after an initial normalization of transmitter levels there is a secondary elevation of all amino acid neurotransmitters. There is evidence however, that during the reperfusion phase GABA, after this increase, gradually declines. This exacerbates even further the imbalance between excitation and inhibition, with the ultimate effect being delayed neuronal death (Shuaib et al. 1994).

II. γ-AMINOBUTYRIC ACID (GABA).

GABA (γ-aminobutyric acid) is widely known as the major inhibitory neurotransmitter in the mammalian central nervous system (Krjnevic 1974; Kaila and Thompson 1994; Mody et al. 1994; Nistri and Constanti 1979; Sivilotti and Nistri 1991). In general, it has been considered that activation of GABA-ergic neurons inhibits neuronal activity and is brought about by membrane hyperpolarization (Krnjevic and Schwartz 1966; 1967). The two known mechanisms responsible for this effect relate to GABA binding to a receptor coupled to an anion conductance ($GABA_A$ receptor), or (via G proteins) to K^+ or Ca^{2+} channels ($GABA_B$ receptor) (Bormann 1988). Activation of $GABA_A$ receptors results in Cl^- influx, whereas that of $GABA_B$ receptors causes K^+ efflux and/or inhibition of Ca^{2+} currents. The fast component of the inhibitory post-synaptic potential (IPSP) is mediated via the $GABA_A$ receptors, while the late, slow IPSP component appears to be mediated by the $GABA_B$ receptors (Bormann 1988). Also, although the affinity for GABA is higher for the $GABA_B$ receptors the evoked slow IPSP is seen only with higher stimulation frequencies. This suggests that these receptors are located more extrasynaptically and respond only when the agonist reaches sites more remote from the synapse (Bowery et al. 1987; Sivilotti and Nistri 1991).

Electrophysiological and pharmacological studies have yielded another type of GABA receptor ionophore, which has been labelled $GABA_C$. The identification of multiple GABA receptor types in the mammalian brain has led to proposals that GABA receptors be classified on the basis of their pharmacology

and regional and cellular localization (Nistri and Constanti 1979; Bowery, 1984; Sivilotti and Nistri, 1991; Bormann and Feigenspan, 1995).

i. GABA Receptors.

(a) Pharmacology and molecular biology of $GABA_A$ receptor.

The $GABA_A$ receptor belongs to the super-family of ligand gated ion channels and is sensitive to specific agonists (eg. muscimol, isoguvacine, THIP (4,5,6,7-tetrahydroisoxazalo [5,4-c] pyridine-3-ol)) as well as antagonists. It is a heteromeric complex, with separate binding sites for GABA and its allosteric modulators (anxiolytic-benzodiazepines, barbiturates, and steroids), together with an integral ion channel (Sivilotti and Nistri 1991). Inverse agonists, (β-carbolines, bind at the allosteric binding site for benzodiazepines and are anxiogenic drugs, compared with the anxiolytic effect induced by benzodiazepines (Sivilotti and Nistri 1991). Bicuculline, a competitive antagonist binds at the specific recognition site for GABA, while picrotoxin acts by direct block of the channel.

The $GABA_A$ receptor is a pentamer formed by integral membrane proteins belonging to at least five different classes – α, β, γ, δ, and ρ and (Nakayasu et al. 1995). At least six subtypes of the α, four of β, four of γ, and two of the ρ subunits have been cloned. The transmembrane (TM) II domain is believed to form the ion channel pore. The channel is permeant to organic and inorganic anions such as Cl^- and HCO^-_3 (Eccles et al. 1977; Bormann 1988). The ratio of permeability of HCO^-_3 vs. Cl^- is 0.2 (Bormann et al. 1987).

(b) Pharmacology and molecular biology of $GABA_B$ receptor.

The $GABA_B$ receptors differ from the $GABA_A$ receptors in that their action is mediated via intracellular second messenger systems and/or a G protein linked direct membrane pathway. Baclofen, a specific agonist for these receptors, was initially shown to have a pre-synaptic effect to decrease neurotransmitter release in the CNS (Bowery et al. 1980; Hill and Bowery 1981; Bowery et al. 1982). It was later demonstrated with intracellular recordings that baclofen can reduce synaptic transmission by actions at both pre- and post-synaptic sites (Allerton et al. 1989; Fox et al. 1978; Inoue et al. 1985). $GABA_B$ receptors also generate the late IPSP, which arises from activation of K^+ channels (Bowery 1993; Nistri and Constanti 1979). This K^+ conductance is relatively insensitive to bicuculline and can be antagonized by phaclofen and related compounds (Inoue et al. 1985b; Misgeld et al 1995; Newberry and Nicoll 1984, 1985; Premkumar and Gage 1994; Soltesz et al. 1988). In general pre- and post-synaptic $GABA_B$ receptors may be coupled to both K^+ and Ca^{2+} channels through a G protein linkage either directly or indirectly via second messenger systems, such as inositol phosphate, adenylyl cyclase and arachidonic acid (Bowery 1993; Mintz and Bean 1993; Misgeld et al. 1995; Pfrieger et al. 1994; Nakayasu 1995; Scanziani et al. 1992; Thompson et al. 1993). The ability of baclofen to block Ca^{2+} channels has been shown most clearly in dorsal root ganglia neurons (Dolphin and Scott 1986), but may be an important mechanism at presynaptic sites in the CNS.

The relatively complex actions of different pharmacological agents (agonists antagonists) on $GABA_B$ receptors has demonstrated a high degree of receptor heterogeneity (Kerr et al. 1987). Some antagonists/other compounds selectively block the pre- or post-synaptic effects of baclofen (Dutar and Nicoll 1988). This suggests that and effector systems for baclofen may differ depending on their pre- or post-synaptic location. With the recent development of more selective agonists and antagonists for these receptors a clearer picture should emerge (Bowery 1993). The pharmacological distinction of receptor subtypes will be further supported by the characterization of the molecular structure. The initial identification has found that the receptor is an 80kDa protein that shows similarity to the metabotropic glutamate receptors (Nakayasu et al. 1995; Kaupmann et al. 1997).

(c) Pharmacology and molecular biology of $GABA_C$ receptors.

Several lines of evidence now indicate the existence of a third class of GABA receptor, which is distinct pharmacologically from $GABA_A$ and $GABA_B$ receptors and is found predominantly in the vertebrate retina. These novel $GABA_C$ receptors are Cl^- conducting pores that are selectively activated by partially folded GABA analogs such as cis-4-aminocrotonic acid (CACA, and also by non-selective agonists such as GABA and muscimol. The receptors show higher affinity for GABA than the $GABA_A$ receptors (Johnston 1995). They do not show allosteric modulation by benzodiazepines, barbiturates and steroids, are insensitive to bicuclline and baclofen, and antagonized by picrotoxin. $GABA_C$

receptors are comprised from the novel subunits ($GABA_A$) designated ρ1 and ρ2 (Bormann and Feigenspan 1995). The subunits form a pentameric structure with a central pore, with an open channel diameter of 5.1 A, which is very similar to that of 5.6 A for the $GABA_A$ receptor. Analysis of channel gating times shows that $GABA_C$ receptors display a mean open time about 5 times greater than that of $GABA_A$ receptors. Also, the $GABA_C$ receptor activation shows less desensitization than those of $GABA_A$ receptors (Bormann and Feigenspan 1995). Whilst the ρ1subunit appears to be restricted to the retina, the ρ2 subunit is expressed significantly in other parts of the CNS, most notably in the hippocampus and the cortex (Bormann and Feigenspan 1995). However, little is known about the pharmacology and the functional significance of the latter locations.

ii. Hyperpolarizing and depolarizing responses to GABA.

As described above, the major inhibitory neurotransmitter, GABA activates three types of receptor – $GABA_A$, $GABA_C$ and $GABA_B$ in the CNS. The first two of these are linked to ligand-gated ion channels which are permeant to anions; the third is coupled to K^+ and Ca^{2+} channels via G protein linkage. The changes in membrane potential seen with synaptic release and exogenous application of GABA are not only a hyperpolarization but also a depolarization. The latter occurs either as a pure phenomenon or part of a biphasic shift (Alger and Nicoll 1982; Andersen et al. 1980; Avoli and Perreault 1987; Ballanyi and Grafe 1985; Inoue et al. 1985c). The major mechanism of GABA-mediated inhibition,

first recognized, involved post-synaptic membrane hyperpolarization and a chloride- dependent increase in conductance of cerebral cortical neurons *in vivo* (Krjnevic and Schwartz 1967). Due to the fact that most neurons have resting membrane potential (V_R) values very close to the Cl^- equilibrium potential (E_{Cl}) (about 70 mV), the change in membrane potential (V_M) induced by the action of GABA at the $GABA_A$ receptors may easily vary in sign. Therefore, small deviations of V_M around E_{Cl}^- may direct the driving force for Cl^- either inward or outward, and thus, the Cl^--dependent "fast" IPSP can be hyperpolarizing or depolarizing ("inverted"), respectively. This is often seen in brain slice preparations, (e.g. in the hippocampus and especially in the neocortex, in which neurons tend to have more negative V_M values (= 80 mV) than *in vivo* and the evoked fast IPSP or membrane response to focal application of GABA is frequently depolarizing at rest. The above-mentioned GABA depolarizing effects, that occur mainly via $GABA_A$ receptors are thought to be limited to V_M values below 70 mV, which represents E_{Cl} when solving the Nernst equation, for $[Cl^-]_i \approx$ 10 mM and $[Cl^-]_o \approx$ 130 mM. This figure assumes that Cl^- is the only ion species carrying current through the channels opened by GABA. However, in reality GABA accumulation or exogenous application can lead to activation of at least three types of known receptors with subsequent activation of mutiple ion conductances and/or co/counter ion transport mechanisms.

(a) GABA-induced depolarization in peripheral neurons.

The dorsal root ganglia (DRG) neurons respond to GABA application with a depolarization which is dependent on $[Cl^-]_o$ and sensitive to bicuculline and picrotoxin (Gallagher et al. 1978). E_{GABA} was determined to be ≈ 24 mV in these neurons. This value, together with the assumption that Cl^- is the only carrier for the GABA induced current led the authors to assume that $[Cl^-]_i$ in DRG neurons would be about 53 mM. Such a high concentration could not be produced solely by passive diffusion and it was suggested that an inward directed Cl^- pump must be present in these cells.

An elegant study, combining extra- and intracellular ion measurements during GABA application with current-clamp and pharmacological tests, elucidated the mechanisms of the depolarization and maintainenance of high $[Cl^-]_i$ of rat sympathetic neurons (Ballanyi and Grafe 1985). In these cells, at mean V_R values of about 50 mV $E_{Cl} = E_{GABA}$ = 30 mV and the measured mean $[Cl^-]_i$ was 30 mM, a smaller value than that calculated for DRG neurons (Gallagher et al. 1978). The depolarization evoked by GABA at V_R was accompanied by decreases in $[Cl^-]_i$ and $[K^+]_i$; while increases in $[Cl^-]_i$ and $[K^+]_i$ were measured during GABA-induced hyperpolarization, obtained in cells held at a V_M depolarized above 10 mV. The authors suggested that the mechanism responsible for Cl^- influx was Cl^--K^+ co-transport and showed that Na^+ was also necessary.

(b) GABA-induced depolarization in pyramidal neurons.

During the last decade numerous *in vitro* studies using slice preparations have investigated GABA's influence on hippocampal pyramidal neurons. Intracellular recordings showed that although the responses to somatic application of GABA in CA1 and CA3 and the antidromically evoked IPSP in CA1 are hyperpolarizing, the application of GABA in the dendritic regions of CA1 and CA3 neurons can produce depolarization and/or biphasic hyperpolarizing/depolarizing responses (Alger and Nicoll 1982; Andersen et al. 1980; Misgeld et al. 1986; Muller et al. 1989; Thalmann et al. 1981). These fast IPSP depolarizations are promoted or enhanced by barbiturates and GABA uptake inhibitors, as well as lowering the temperature and are blocked by dendritic iontophoresis of BMI or TTX (Alger and Nicoll 1982).

Under usual conditions the fast IPSP is hyperpolarizing, because GABA uptake limits diffusion. However, during conditions with higher concentrations of extracellular GABA accumulation, such as excess stimulation or interference with uptake, depolarizing responses may be generated at extrasynaptic receptors (Sivilotti and Nistri 1991). The ionic mechanism determining the depolarizing IPSPs is uncertain. It may reflect a mixed Cl^-/cationic conductance (Alger and Nicoll 1982) or a mixed Cl^-/HCO^-_3 conductance (Kaila 1994; Kaila et al. 1994; Misgeld et al. 1986) or a Cl^- conductance in the dendrites with an outward current. In the neocortex dendritic GABA application has been shown to have similar depolarizing effects on pyramidal neurons from superficial (II-III) and deep (V) layers of the neocortex (Connors et al. 1988; El-Beheiry and Puil 1990;

Scharfman et al. 1985). However, the response appears more complex and it has been far less examined than in the hippocampus.

(c) Dendritic/somatic GABA effects: two pumps.

Looking at the mechanisms responsible for GABA depolarization in the CA1/ CA3 region of the guinea pig hippocampal slices, Muller et al. (1989) measured with ISMs the ionic changes in $[Cl^-]_o$ that occur during localized GABA applications to the somatic or dendritic layer. They observed mainly decreases in $[Cl^-]_o$ stratum pyramidale (SP) and a decrease followed by an increase in stratum radiatum (SR). The decrease in $[Cl^-]_o$ was blocked by microdrop application of BMI 100 μM to the SP, suggesting that it was due to the spread of GABA to the somatic layer. Muller et al. (1989) concluded that there are differences in the distribution of $[Cl^-]_i$ between the soma and the dendrites. According to the two-pump hypothesis, two separate Cl^- pumping processes operate in CA1 pyramidal neurons. A Cl^- extrusion mechanism, active at the soma would locally maintain a low $[Cl^-]_i$, whereas in the dendrites an inward pump would be responsible for higher levels of $[Cl^-]_i$. This would lead to opposite effects of GABA – somatic application of GABA causing neuronal hyperpolarization, with dendritic application causing depolarization.

(d) Dendritic/somatic GABA effects: two receptors.

Other studies examining membrane potential changes evoked by GABA in the two layers, SP and SR show that application at the soma elicited

hyperpolarization, but at the apical dendrites caused mixed hyper- and depolarization (Alger and Nicoll 1982). Although bicuculline methiodide (BMI) 100 μM blocked both hyper- and depolarizing responses, small or intermediate concentrations (1-30 μM) appeared to preferentially inhibit the depolarizing response and uncover dendritic hyperpolarization. This finding is not compatible with a dendritic pump maintaining an outward Cl^- gradient through a single type of GABA-activated channel. It seemed reasonable to suggest that at least in the dendrites there are two GABA receptorsGABA – "depolarizing" ones, which are more abundant and more susceptible to BMI (and other GABA antagonists e.g. picrotoxin and penicillin), and "hyperpolarizing" ones.

The original two receptor theory postulated the existence of two receptor subtypes, one of which may activate a mixed ionic conductance (Alger and Nicoll 1982). However, recent voltage clamp experiments show that the depolarizing response is not due to a non-specific cation current; rather its reversal potential is dependent on $[HCO^-_3]_i$ (Perkins 1996; Perkins and Wong 1997).

(e) The chloride accumulation model.

Why a larger amount of GABA is required to elicit the depolarizing response than the hyperpolarizing one may be explained by the chloride accumulation model (Staley et al. 1995). This is based on the observation that the $GABA_A$ receptor channel is permeable to both Cl^- and HCO^-_3 (Bormann et al. 1987). Intracellular Cl^- acccumulation during the hyperpolarizing component of the GABA response produces a loss of Cl^- driving force and net flux of Cl^- during the late part. The

gradient for HCO^-_3 is maintained throughout by the action of carbonic anhydrase ($CO_2 + H_2O \rightarrow H_2CO_3 \rightarrow H^+ + HCO^-_3$) and this gives a late depolarization by sustained HCO^-_3. The question that remains unanswered is whether the depolarizing response is mediated by the same receptor channel as the hyperpolarizing one or by a separate channel that has a greater permeability to HCO^-_3.

III. WORKING HYPOTHESIS.

The major targets of ischemia research are to find methods to prevent or alleviate glutamate neurotoxicity, uncontrolled cytosolic calcium overload and free radical formation. Several lines of research have shown the putative neuroprotective role of calcium channel blockers, such as nifedipine (Varming et al. 1996; Schurr et al. 1995). Other studies have found that during anoxia the L-type Ca^{2+} channels are blocked, secondary to run down of the ATP supply or the elevation of $[Ca^{2+}]_i$ and that this may not be a major source of calcium overload of the cells (Krnjevic and Leblond 1987; 1989). Depolarization-induced calcium uptake has been shown to be decreased by anoxia, and so this represents a mechanism by which neurons may be partially protected against damage (Kass et al. 1994). Although NMDA (and AMPA) receptor activation promotes Ca^{2+} influx, intracellular Ca^{2+} release and accumulation with failure of extrusion may be a more critical cause of overload (Krnjevic and Xu 1989).

The excitotoxic hypothesis postulates a central role for the excitatory amino acids and their receptors in the neuronal damage that ensues following cerebral ischemia/ hypoxia. A major premise is that neuronal protection can be achieved via blockade of the EAA receptors with specific antagonists. However, some evidence points to the fact that glutamate exposure may not be a completely valid model for the direct action of anoxia on the brain and that antagonists to these amino acids do not offer complete protection (Friedman and Haddad 1993).

Several other studies indicate that hypoxic neuronal damage cannot solely result from an excitotoxic mechanism, and that the involvement of other neurotransmitters and/or processes is likely (e.g. Schurr et al. 1995). Sodium influx (unrelated to glutamate receptor-operated channels) and ATP depletion are potential causes for anoxic damage (Friedman and Haddad 1993). It apppears that sodium channel properties are altered during anoxia, so that a low concentration of Na channel blocker that would not normally affect the population spike during the pre-anoxic period is likely to affect the Na channel during anoxia. Although some studies have shown that Na channel blockade is not protective against anoxia (Varming et al. 1996), others demonstrate neuroprotection (Boening et al. 1989; Fried et al. 1995; Obrenovitch 1997).

Tissue acidosis is also postulated to contribute to neuronal loss in cerebral ischemia. In cultured hippocampal slices it has been shown that brief intracellular acidification (pH 6.62) results in cell loss in the stratum pyramidale of CA1 and the hilar region. Removal of molecular oxygen greatly attenuated the cell loss, which suggested that the generation of ROS could underlie the acidosis-induced toxicity (Shen et al. 1995).

In anoxia the largest concentration amplification of neurotransmitters is for GABA, with a seven-fold increase of total tissue levels (Graham et al. 1990). Enhancement of GABA-ergic transmission has been shown to protect against neuronal ischemic death (Abramowicz et al. 1991; Green and Cross 1997; Johansen and Diemer 1991; Kass et al. 1992; Lyden 1997; Schwartz et al. 1995; Shuaib et al. 1993; Thompson 1994). However, a GABA agonist

promotion of excitotoxicity has also been demonstrated (Walden et al. 1990). One mechanism by which GABA might induce neuronal death following ischemia is that massive release of GABA could have strong depolarizing effects, which would support cation influx and elevate excitability. The fact that both polysynaptic $GABA_A$- and $GABA_B$-mediated IPSPs (at least in the hippocampus) are sensitive to anoxia (Khazipov et al. 1995) indicates that a protective action of GABA through activity-related synaptic transmission does not operate during the anoxic event. However, spontaneous release and extra-vesicular release of GABA can activate synaptic and extra-synaptic receptors, as post-synaptic sensitivity to GABA is sustained (Khazipov et al. 1993; Rosen and Morris 1993; Zhu and Krnjevic 1994).

Understanding the potential role for GABA and the interplay of activation of different receptors during hypoxia/ischemia could be of help in devising efficient and safe therapeutic measures against brain injury. The present study aimed to define a potential role of the inhibitory neurotransmitter, γ-aminobutyric acid in the generation of ionic changes associated with the early response of the brain to anoxia. ISMs have been used to measure and compare changes in extracellular $[K^+]$, $[Cl^-]$ and $[Na^+]$ and osmotic shifts that occur during anoxic exposure and GABA application. Recordings were carried out in the cell body layer, stratum pyramidale (SP) and the apical dendritic layer, stratum radiatum (SR) in the CA1 region of guinea pig hippocampal slices.

METHODS

I. Preparation of Tissue.

i. Animals.

Experiments were carried out using brain slices from 200–300 g male or female albino guinea pigs. The animals were obtained from Charles River Canada Inc. and upon delivery to the Animal Care Facility of the Faculty of Medicine were separated into small groups of two or three and placed in plastic cages containing beta chip. They were maintained at room temperature (25–26°C) and received a regular diet of #5072 Purina Guinea Pig Chow and supply of water.

ii. Hippocampal slice.

The animals were restrained with a plastic cone and sacrificed by decapitation with a small animal guillotine (Ealing Scientific Ltd.) A long sagittal incision was made through the skin overlying the skull, using a scalpel. The cranium was then cut along the midline with scissors and the parietal bones together with the dura mater were removed with bone forceps. Two horizontal incisions were made: one caudal — to separate the cerebellum, and a second one rostral to separate the olfactory bulbs. The two hemispheres were then lifted upwards and forwards with a round spatula so as to sever all the cranial nerves. The hemispheres were placed on a moistened filter paper on the cover of a Petri dish which contained ice. Using a single-edge razor blade, the brain was divided into its two halves. One hemisphere

was placed into a beaker containing ice-cold oxygenated artificial cerebro-spinal fluid (ACSF). The remaining hemisphere was placed medial side up. The brain stem was lifted with forceps, while a spatula was used to gently push the occipital cortex downwards until the whole medial and inferior side of the hippocampal formation could be visualized. Both ends of the hippocampus were then freed and it was rolled upwards and backwards towards the occipital pole. The fimbria was cut and the hippocampus was transferred to the stage of a manual chopper (Frederic Haer and Co.), where it was placed on moistened filter paper with the dentate area inferior and the alveus superior. The first cut removed 2–3 mm from one end of the structure. Five to eight slices were then cut to a thickness of 400 µm using manual adjustment of a micrometer and the dropping of the blade. The slices were then transferred from the chopper stage to a pre-chamber (BSC-PC, Medical Systems Inc.) with the aid of a fine brush. In this chamber they rested on a nylon net in ACSF, equilibrated with 95%O_2/5%CO_2 for a minimum of 1–1.5 hours. Fig. 1 shows a photograph of the microscopic view of a transverse hippocampal slice, which since it was immunohistologically stained for the presence of calbindin, illustrates particularly well the cell bodies and dendrites of CA2 and CA1 and the mossy fibers and granule cells of the dentate gyrus (Baimbridge and Miller 1988; Baimbridge et al. 1991; Celio 1990).

iii. Recording chamber and ACSF delivery.

A wide mouthed pipette was used to transfer the brain slices to the recording chamber (Haas et al. 1979). The model of interface chamber used was the BSC-

HT (Brain Slice Chamber – Haas Top) from Medical Systems Inc. (Figs. 2 and 3). Slices rested on a strip of lens tissue and were superfused at a rate of 2–3 ml/min with ACSF, equilibrated with 95%O_2/5%CO_2 and passed through a heated water jacket before reaching the bath. The surface of the slice was also exposed to a flow of warm and humidified 95%O_2/5%CO_2 gas mixture.

The bath perfusion system was adapted from that originally described by Barolet et al. 1989 (see Fig. 4). It consisted of six plastic reservoirs (60 ml each), which were placed at about 75 cm – 1 m above the recording site and connected through small bore Teflon TFE tubing (ID = 0.97 mm) to an eight-way zero dead-space connector (Omnifit #N1103, Omnifit Ltd.), which provided the inflow to the recording chamber. The selection of flow was done manually with the aid of a controller circuit via switches, using a 12 V DC rechargeable battery (GC 1290, Electrosonic Inc.) to actuate open/close positions of in-line solenoid valves (Model 3-188-900, General Valve Corporation.). The perfusion flow was monitored with an in-series flowmeter (GF-2100 #11, Gilmont Instruments). In one of the two grooves in the floor of the recording chamber (see diagram of Fig.2) a sintered silver/silver chloride wire (A-M Systems, Inc.) was placed to serve as electrical ground. The other groove held a thermistor probe (Fenwall Electronics series GB 32) which was used to monitor the temperature of the bath and provide control through a feed-back system (Model BTC-12, manufactured by J. Knowles, McGill University). The recording chamber was placed on the warming bath of the base unit (BSC-BU), shown in Fig. 3. During experiments the slices were transilluminated from below with a fiber optic light source (Fiber-Lite Series 180, Dolan-Jenner Industries, Inc.)

and viewed through a dissecting microscope (Model M7A, Wild Leitz). The slices were maintained using a set-point temperature of 33°C and the fluctuation of temperature was less than one degree, peak-to-peak.

Even though it would seem best to study brain slices at or near normal body temperature, several investigators found that the preparation survives longer and healthier at lower temperatures (33–34°C). In addition it is recognized that membrane and synaptic properties of neocortical neurons *in vitro* are optimally studied at 33–34°C (Alger 1984; Rosen and Morris 1994; Schwartzkroin 1981).

In normoxic conditions both warming, ≥35°C, or cooling, ≤32°C, induce reduction in the amplitude of early and late EPSPs and disappearance of the IPSP (Rosen and Morris 1994). At 38—40°C slice excitability is increased resulting in epileptiform discharges (Alger 1984).

In anoxic conditions warming, ≥35°C, accentuates the synaptic depression, while cooling, ≤32°C, has a stabilizing effect on the membrane (Rosen and Morris 1994). This suggests that the optimal temperature in this type of preparation, *in vitro* brain slices, is 32–34°C.

II. Stimulation and recording.

i. Equipment and techniques.

Recordings which produced useful data for measurement and analysis were made in a total of 142 slices, obtained from 80 animals. In most experiments recordings were extracellular and carried out with ion-selective microelectrodes (ISMs) — with the recording chamber/bath and micromanipulators mounted in a Faraday cage. The ISM was placed in the CA1 b region in either stratum pyramidale (SP) — the cell body layer, or in stratum radiatum (SR) — the apical dendritic layer, as illustrated in the diagram in Fig. 1 (also see Fig. 5). The electrode was visually placed in SP, which was identified both by its translucence under microscopic view and the ability of afferent input to evoke a large population spike superimposed on the field EPSP (Fig. 15). Localization in the SR was made by horizontal displacement of the electrode in a direction perpendicular to the SP layer and 100–200 μm closer to the LM and DG (see Fig. 1). In some experiments intracellular recordings and simple extracellular recordings were performed.

Micro-concentric bipolar electrodes (model MCE-100, Rhodes Medical Instruments) were placed into the tissue in the stratum radiatum region on or near the axons of the CA3 neurons (Schaffer collaterals) to orthodromically stimulate the CA1 pyramidal neurons. Constant voltage pulses generated by a Digitimer (Model D4030, Digitimer Ltd.) were converted to constant current stimuli with a Neuro Log Stimulus Isolator NL800 (Digitimer Ltd.). Single pulses (100–300 μs; 20–150 μA) were applied at a frequency of 0.003–1 Hz to evoke field potentials in the SP and

SR of CA1. Stimulation was used only at the beginning of experiments to identify evoked field potentials in SP and SR and optimize selection of recording sites.

Either a single-barrel electrode or ISM was advanced 100 µm into the slice with a manual microdrive (Model K2C, La Précision Cinématographique). Both channels of the double-barrel ISM were connected via silver/silver chloride (Ag/AgCl) wire connectors and guarded cables to the high-impedance inputs of a differential DC amplifier (Model 604, Keithley Instruments Ltd.), as shown in Fig. 6. When a single micropipette was used it was connected to the negative input of the amplifier. The reference channel unity gain output was split to allow magnification by an amplifier (Model 3A3 Tektronix) and filtering prior to display on an oscilloscope (Model 565 Tektronix). It was also recorded along with the differential output on a polygraph (Model P7, Grass Instruments). Also, for some experiments the signal for the oscilloscope was directed after amplification to an analog-to-digital interface (TL-1 DMA-AXON INSTRUMENTS) for digitization and export to an IBM compatible computer (PEER 1630-DTK Computer). Acquisition software Axotape 1.2 or 2.0 (AXON INSTRUMENTS) was used for sampling before, after and during anoxic and pharmacological tests. In some experiments evoked field potentials were photographed using a Nihon-Koden camera (Model PC2A).

ii. Intracellular microelectrodes.

Electrodes for intracellular recordings were pulled from 0.7–0.9 mm ID/1.2 mm OD Prism™ borosilicate glass capillaries (Dagan Corporation), using a Flaming Brown Model P•80/PC micropipette puller (Sutter Instruments Co.). The microelectrodes

were filled with 3 M potassium acetate (resistance 40–80 MΩ) and connected through a Ag/AgCl wire to the probe of an intracellular amplifier (8700 Cell Explorer, Dagan Corporation). A piezo-electric microdrive (Model 6000 Inchworm Motor Controller, Burleigh Instruments Inc.) held the electrode and was mounted on a tri-dimensional manipulator (Model SM-20 Digital Micromanipulator, Narishige Scientific Instrument Laboratory). The tip of the electrode was advanced in the slice in 4–6 µm steps, until a suitable DC recording was obtained. A drop of potential of at least 40 mV and action potentials (spontaneous or othodromically evoked) signalled penetration of a cell. Steady hyperpolarizing current was injected continuously for 1–5 min and the cell was allowed to stabilize at or near resting membrane potential for several minutes. Most impalements were obtained at ≈100 µm depth of recording.

iii. Single-barrel extracellular microelectrodes.

Single-barrel recording electrodes filled with NaCl were used for initial recordings to assess the viability of slices and to determine the position of the recording electrode in stratum pyramidale (SP) or stratum radiatum (SR). These electrodes were prepared from 0.9 mm ID/1.2 mm OD Prism™ borosilicate glass capillaries (Dagan Corporation). Freshly pulled electrodes were filled with 0.9% NaCl and Ag/AgCl wires inserted and sealed with wax. Microelectrode tips were broken back to yield a final resistance of approximately 10–20 MΩ.

iv. Ion-selective microelectrodes.

(a) Fabrication.

Most recordings were carried out using ISMs. They were constructed from double-barrelled theta borosilicate glass tubing (R and D Scientific Co.), using methods previously developed and described (Morris et al. 1985; Walker 1971). One channel contained the liquid ion sensor membrane; the second, which was filled only with conducting electrolyte solution, acted as a reference channel and ordinary recording electrode. To prepare these electrodes glass blanks were first cut into 6 cm lengths. A one cm section of glass was removed from the end of one barrel with a grinding wheel (Dremel Moto-Tool Model 370, Dremel Mfg. Co.). The glass was then cleaned by soaking in phosphoric acid for 2 h, followed by 15 rinses with distilled water and 15 rinses with de-ionized water. The blanks were immersed in acetone for 2 h, and then drained and heated at 200°C for 2 h. Batches of clean glass were stored in sealed test tubes.

Electrodes were pulled to a tip diameter of 1 µm or less using a vertical Narishige PE-2 electrode puller (Narishige Scientific Instrument Laboratory). After pulling, the glass of the ion sensor channel was made hydrophobic by exposing the electrode to a hot vapour technique of silanization (Coles & Tsacopoulos 1977). This process allowed the placement and retention of the liquid ion sensor in the tip of the electrode, without any problem of displacement by water (see original description by Walker 1971). Before silanization was begun teflon tubing (0.5 µm OD, Small Parts Inc.) was inserted into both barrels of the pulled glass capillary and sealed with heated shellac (elephant brand; Thew, Arnott and Co., Ltd.). The

electrode tip was then inserted into a glass tube surrounded by a nichrome wire coil and a silane vapour mixture was allowed to pass through the ion-sensor channel, while heating the electrode tip to 240–260°C. Dry N_2 at a pressure of 40 psi was passed through the reference barrel continuously to keep it free of silane. The ionophore channel was exposed for 5 min to 40 psi N_2 that was routed over the surface of dimethylaminotrimethylsilane (TMSDMA, Fluka Chemical Corp.).

Once silanized, the tip of the ionophore channel was backfilled under microscopic control with a column several hundred µm long of the appropriate ion sensor (see Table I). Then the reference channel was backfilled with 0.15 M NaCl or, in the case of chloride electrodes with 0.15 M Na glucuronate. The reference channel was filled with an appropriate electrolyte solution that did not contain the ion to be measured, to avoid artefacts due to leakage from the tip. The ion sensor channel was then backfilled above the level of the ionophore with an electrolyte solution containing the ion (K^+, Cl^-, Na^+, or TMA^+) to be measured. Ag/AgCl wires were inserted into each barrel and sealed with shellac. Electrodes were stored at 4°C in glass jars containing distilled water. Prior to an experiment electrode tips were broken back with a glass rod under the microscope. The resistance registered by the reference channel after breaking the tip to 3–5 µm was 5–15 MΩ.

(b) Calibration.

Electrodes used to measure ion concentrations were calibrated before and after use, in solutions of varying concentrations of the ion to be measured. The effect of temperature on the electrode voltage was controlled by calibrating at a temperature

close/identical to that of the preparation (33°C) and by using the tissue bath solution as a correction reference. The specially designed calibrating chamber contained 6 wells, embedded in a water jacket and was connected to a circulating water bath (Thermomix 1441, B. Braun Melsungen AG). Calibrations were carried out by changing from lower to progressively higher concentrations and then the process was repeated in the reverse direction.

For calibrating electrodes made with the Fluka valinomycin neutral carrier K^+ sensor (Potassium Ionophore I-Cocktail B (Fluka 60398)), six different solutions with varying concentrations of KCl (1, 2, 3, 5, 10 and 20 mM) were used. The solutions were made in ACSF with adjustment of $[Na^+]$ to keep the ionic activity constant. Voltage values of the K^+-dependent potential (V_K) were read from the meter of the differential amplifier and recorded in parallel on the polygraph. The semi-logarithmic calibration curve showing the variation of V_K as a function of $[K^+]$ was characteristic to each electrode. The mean slope for the valinomycin based K^+ electrodes used in experiments was 59 ± 5 mV (n = 9) per decade of concentration. A typical calibration curve for a K^+ electrode is shown in Fig. 7.

The Cl^--sensitive electrodes were prepared with Chloride Ionophore I - Cocktail A (Fluka 24902) and calibrated in solutions of 50, 75, 100, 150 and 200 mM of NaCl that contained NaCl alone or together with Na glucuronate in quantities to balance the change in activity of the solution. The mean slope for the chloride electrodes was 50 ± 4 mV (n = 6) per decade of concentration. A typical calibration curve for a Cl^- electrode is shown in Fig. 8.

The Na^+-sensitive electrodes were filled with Sodium Ionophore II - Cocktail A (Fluka 71178) and calibrated in solutions of 50, 75, 100, 150 and 200 mM of NaCl. To keep activity constant K^+ was substituted for Na^+ in solutions of lower Na^+ concentration. The mean slope for the Na^+ electrodes was 55 ± 4 mV (n = 6) per decade of concentration. A typical calibration curve is presented in Fig. 9.

TMA^+ (tetramethylammonium)-sensitive electrodes were made with the Corning 477317 classical K^+ ion exchanger so that they would have higher selectivity for quaternary ammonium salts than for K^+ (Ammann 1986). When the superfusing ACSF solution contained TMA, the electrode lost its ability to measure K^+ and showed selectivity for TMA (see Fig. 10). Calibrating solutions for TMA electrodes were made in ACSF and contained 1, 2, 3, 5, 10 and 20 mM of TMA chloride. Na^+ substitution was used to keep the ionic activity constant. The mean slope for the Corning-based TMA electrodes was 55 ± 3 mV (n = 6) per decade of concentration. A typical calibration curve for a TMA^+ electrode is shown in Fig. 11.

(c) Preliminary tests.

The day of the experiment electrodes were tested for stability of potential, resistance and response to ion concentration change. For a freshly made electrode a period of 10–15 min was necessary for the potential to stabilize. If the potential showed no sign of stabilizing or if there was an unacceptable level of recording noise the electrode was discarded. The resistance registered by the reference channel was 5–15 MΩ. The ion sensor channel had a much higher resistance — in the order of 10^{10} to 10^{12} MΩ, although this was not routinely recorded.

Construction of the calibration curve and analysis of experimental ISM data were carried out using a specially designed computer program, ELCAL (Barolet et al. 1989). The program allowed the fitting of calibration curves by non-linear regression, conversion of chart record displacements to ion concentration, and direct calculation of selectivity coefficients using the fixed interference method (FIM). The size of the response to the test solutions approached the theoretical Nernstian value (e.g. for a valinomycin K^+ electrode a tenfold increase in concentration yielded a 54–64 mV response). The speed of electrode responses was in the order of < 1 s.

(d) Selectivity tests.

The voltage response of an ISM arises because of an asymmetry of ions across the sensor membrane. The membrane acts as a semipermeable barrier to develop a potential that is a function of the ion gradient (Ammann 1986) and is characterized by the Nernst equation :

$$E = E_o + s \log a_i \qquad [1]$$

where E = EMF, electromotive force,

E_o = a constant reference potential,

a_i = activity of the ion, *i*,

s = Nernstian slope,

and

$$s = 2.303 \frac{RT}{z_i F} \qquad [2]$$

where R = gas constant,

T = absolute temperature,

F = Faraday constant,

z_i = valence of primary ion, *i*

Equation [1] is true for a perfect ion sensor; however, most sensors are not perfectly selective for a single ion species, and the contribution of interfering ions is represented by the extended Nicolsky-Eisenman equation (Eisenman 1967; Nicolsky 1937a,b):

$$E = E_o + s \log [a_i + \sum_i K_{ij}^{Pot} (a_j)^{z_i / z_j}] \quad [3]$$

where a_j = activity of interfering ion, *j*,

K_{ij}^{Pot} = potentiometric selectivity factor,

z_j = valence of interfering ion, *j*.

Both the fixed interference method (FIM) and fixed primary ion method (FPM) (Ammann 1986) were used to determine the selectivity of the different ionophores for the corresponding ion to be measured (K^+, Na^+, Cl^- or TMA^+) over drugs or chemicals used in experiments (see Table II) with the FIM method, that recommended by the IUPAC Commission (Guilbault et al. 1976), the electrode potential is measured in varying concentrations of the relevant ion in the presence of a constant amount of the interfering ion. Because for certain compounds (e.g. bicuculline methiodide (BMI)) solutions of different concentration were used in experiments, the alternative FPM approach was applied to assess the voltage change, if any, in the presence of a constant level of the ion to be measured and increasing amounts of the interfering compound. Selectivity factors of the K^+, Cl^-

and Na^+ ISMs relative to other ions and compounds are shown in Tables II and III. For many of the values obtained from the literature the separate solutions method (SSM) (which compares 0.1 or 0.15 M solutions of the ion to be measured and the interfering ion) was that used.

III. Solutions, Drugs and Treatments.

i. ACSF and bath application of drugs.

The ACSF was prepared with Millipore-treated water and contained (in mM): NaCl 120, KCl 3.3, $NaHCO_3$ 26, $MgSO_4$ 1.3, NaH_2PO_4 1.23, $CaCl_2$ 1.8, glucose 11.0 and was saturated with 95%O_2/5%CO_2 at a pH of 7.35–7.4. If the pH of the Ringer was lower, then NaOH 0.1 N solution was used to titrate to the desired pH. In most experiments drugs were applied as bath superfusates. The GABA receptor agonists were superfused for periods of 5 min and preceded and followed by standard ACSF for control and recovery periods of at least 15 and 30 min, respectively. Only in a few cases were agonists applied for longer periods. In experiments using receptor antagonists and TMA, solutions containing these drugs were usually applied continuously for long periods (several hours) and tests with GABA agonists were made with ACSF solutions which contained both the agonist and antagonist. Table IV lists these compounds and their sources.

ii. Pressure ejection of drugs.

In some cases, drugs were applied by pressure ejection technique. The ejection electrode (2–10 MΩ resistance) was pulled with the Flaming Brown horizontal puller from glass capillary (0.9 mm ID/1.2 mm OD Prism™ borosilicate glass capillaries, Dagan Corporation). Prior to filling, the electrode tip was rendered hydrophobic by dipping it in a 2% solution of butylchlorosilane (Fluka Corporation) in CCl_4. This

procedure opposed dissipation due to the surface tension effect of the ejected microdrop on the outer wall of the microelectrode shank, as gauged by direct observation of ejections in the air under the dissecting microscope. The electrode was filled with the drug dissolved in ACSF and then broken back to a tip diameter of 2–3 μm. It was advanced at a 75° angle into the slice to a point near the recording depth, 100 μm. The solution was ejected with pulses of 15–40 psi for 100–900 ms from a Picospritzer (General Valve Corporation). No mechanical artifacts were observed as tested with pulses of ACSF only, without any drug.

iii. Anoxia.

Anoxic episodes were induced for 5 min (and only occasionally for slightly longer) by exposure of the hippocampal slice to a gas mixture of 95%N_2/5%CO_2. This was created by switching both the gas mixture bubbling in the warming bath situated beneath the recording chamber and the superfusate source, by changing from a reservoir of ACSF saturated with 95%O_2/5%CO2 to one saturated with 95%N_2/5%CO_2. The time lag necessary for the new ACSF to reach the slice was calculated from the total length of the tubing and the delivery rate. The anoxic episode was considered to start only from the time when the superfusate had reached the slice, not from the moment when the bubbling gas mixture was changed to N_2 — a decision based on earlier studies in our laboratory, using O_2 microelectrodes. Measurements showed that PO_2 drops to almost zero levels in the

first minute of the anoxic trial (Rosen and Morris 1991). After the anoxic trials a recovery period of at least 30 min was allowed before a second anoxic exposure was applied to the slice. Not more then 2–4 anoxic episodes were applied to the same slice. These brief anoxic trials were successive and the recordings were done in randomized order in the two layers of interest : SP and SR. In some groups of experiments they were alternated with applications of GABA to allow comparison of responses; in such cases the exposure to GABA preceded that to N_2 at each location.

IV. Statistical Analysis.

All calculations of mean, standard error (SE), Student t-test, Mann-Whitney U or Welch non-parametric t-test, correlation coefficient (r) and linear and non-linear regression were performed by computer using statistical analysis provided by INSTAT version 2.0 or PRISM version 2.01 (Graphpad Software, Inc.). The probability level used for statistical significance was ≤ 0.05.

RESULTS

I. $[K^+]_o$ Measurements in Stratum Pyramidale and Stratum Radiatum.

i. Resting levels of $[K^+]_o$ and changes with stimulation.

The baseline levels for extracellular potassium, measured in the superfused slice at 100 μm depth, were typically 3.46 ± 0.19 mM (n = 17) in the SP and 3.58 ± 0.23 (n = 14) in the SR. These paired values are not significantly different.

The evoked release and accumulation of K^+ during frequency stimulation in the hippocampus *in vivo* and *in vitro* in the SP is well established (Krnjević et. al. 1982; Morris et al. 1991). In the current series of experiments changes in $[K^+]_o$ evoked by stimulation (0.1 ms, 20 s duration, 1–10 Hz) were measured in SP and SR of the hippocampal CA1 region. Increases in $[K^+]_o$ were observed in both layers; the maximum $\Delta[K^+]_o$ during the repetitive stimulation was in the range 9–15 mM. In four slices paired but separate recordings (SP vs. SR measured in each slice) were carried out. The change in $[K^+]_o$ ($\Delta[K^+]_o$) with 10 Hz stimulation was significantly greater in SR when compared to SP (n = 6; $p \leq 0.05$). Fig. 12 shows the peak values of $\Delta[K^+]_o$ recorded in SP (open circles) and SR (filled circles) with progressive increase in the rate of stimulation (curves fitted by linear regression); the slope for SR (1.81 ± 0.03, n = 6) was significantly greater than that for SP (0.93 ± 0.03, n = 6) ($p \leq 0.0001$).

ii. $[K^+]_o$ changes evoked by GABA.

Bath applications of GABA (1–10 mM for 5 min), dissolved in ACSF, resulted in dose-dependent, reversible increases in $[K^+]_o$. The distribution of GABA-evoked increases in $[K^+]_o$ was initially examined by comparing changes in SP and SR of both the CA1 and CA3 regions. At all doses changes evoked by GABA appeared greater in the dendritic layer (SR) than in the somatic layer (SP). Although this observation was consistent for both CA1 and CA3, there was a more pronounced effect in CA1. Therefore this region was chosen as the focus of attention for further observations — a decision which was also influenced by the fact that CA1 is one of the most vulnerable regions in the CNS to anoxia. Fig. 13 illustrates the magnitude of the observed changes (n = 55 observations in 13 slices) in the SP and SR. Only for increases in $[K^+]_o$ evoked by 1 and 10 mM GABA were those recorded in SR significantly greater when compared with unpaired data recorded in SP ($p \leq 0.05$). From curve fitting by non-linear regression analysis (not illustrated) values of R_{max} = 1.25 ± 0.24 and 2.42 ± 0.11 mM and EC_{50} = 3.8 and 4.7 mM were obtained for SP and SR, respectively; these were not statistically different.

The initial change in $[K^+]_o$ was noticed approximately 1.5 to 2 minutes after the beginning of drug perfusion in all experiments (see examples of responses to 5 mM GABA recorded in the SP and SR, Fig. 16 A). This is in agreement with the delay due to the dead space in the perfusion system which was estimated before the beginning of the experiments and also verified by using applications of KCl solutions. Peak changes in $[K^+]_o$ evoked by GABA were usually observed approximately 2 min after the initial increase and reached a plateau level that was

sustained and followed by a gradual decline during drug washout. "Desensitization" during the GABA application was not a common feature; sometimes further or progressive increase was observed (e.g. Fig. 16C). The plateau lasted in the washout period for times similar to the onset of change before the decline, which was sometimes followed by an undershoot. This undershoot lasted between 2–5 min, had a peak ≤ 0.45 mM and was more consistently observed with higher doses of GABA (5–10mM). The effects of GABA on $[K^+]_o$ were accompanied by positive shifts in the tissue potential (≤ 2 mV), which have not been illustrated or analyzed.

The population spikes (PS) of field potentials evoked by orthodromic stimulation of the Schaffer collaterals in the CA2/CA3 region and recorded in the stratum pyramidale of CA1 were reversibly depressed or blocked in the presence of GABA. The dose-dependent nature of the depression of the PS by GABA is represented in Table V and the graph of Fig. 14. The PS recorded in SR showed a similar depression. The time course of the observed changes in the population spikes, including their recovery, was comparable to the time course of the $[K^+]_o$ changes.

iii. Comparison of changes in $[K^+]_o$ evoked by GABA and anoxia.

In a further series of experiments responses to GABA (5 mM for 5 min) and brief anoxic exposure (replacement of O_2 by N_2 for 5 min in both the superfusing solution and the bubbling gas mixture in the bath chamber) were compared with recordings in both SP and SR in each slice. Recording was from only one site during first an application of GABA, and then an exposure to N_2; the site of recording was then

changed and a second application of GABA made, followed by a second exposure to N_2. The order of location was changed for alternate slices within a single group of experiments. The response evoked by 5 mM of GABA was arbitrarily selected as a standard to be compared with the effect of anoxia since the EC_{50} for $\Delta[K^+]_o$ in SP and SR was ≈ 4–5 mM and the $[K^+]_o$ accumulation with GABA was smaller than that with N_2, but of sufficient magnitude for assessment and comparison (see examples of Fig. 16). This protocol was followed for all subsequent comparisons for all other ions.

Fig. 15 shows examples of the reversible depression of the field potential and PS induced by 5 mM GABA and by anoxia and recorded in the SP. Fig. 16 illustrates for comparison typical $[K^+]_o$ changes evoked by GABA (A and C) and N_2 (B and D) in SP and SR. The onset of $[K^+]_o$ increase was approximately 20–30 s after the change to ACSF with 95% N_2, while a peak or plateau was usually reached at one to two minutes later. Sometimes further increases occurred (e.g. Fig. 16B and D). Undershoots were frequently seen during recovery (not illustrated). Table VI summarizes and compares the mean data (baseline, peak, and $\Delta[K^+]_o$) for n = 12 slices, while the mean changes are graphically illustrated in Fig. 17. The increases in $[K^+]_o$ in SP were 0.49 ± 0.08 mM for GABA and 1.01 ± 0.22 mM for N_2. Even larger increases in $[K^+]_o$ were observed in the SR — 0.65 ± 0.07 mM and 3.18 ± 0.17 mM with GABA and N_2, respectively. The $[K^+]_o$ changes observed in SR vs. SP were significantly greater for N_2 ($p \leq 0.001$), but not for GABA.

In a separate series of control experiments in 5 slices responses to anoxia were recorded with no antecedent exposure to GABA in order to determine any

impact of the order of treatment. $\Delta[K^+]_o$ evoked in SP and SR, respectively was 1.06 ± 0.36 nM (n = 5) and 2.1 ± 0.92 mM (n = 5). These changes were not significantly different from those recorded at either site with the standard protocol of an initial application of GABA — 1.01± 0.22 mM (n = 9) for SP and 3.18 ± 0.17 mM (n = 12) for SR.

An evaluation was also made of whether changes evoked by GABA or N_2 at each location (SP or SR) were influenced by the order of application or exposure — early or later. There was no significant difference observed between first and second applications of GABA in SP (0.50 ± 0.04 mM, n = 6 and 0.45 ± 0.02 mM, n = 4) or in SR (0.70 ± 0.19 mM, n = 4 and 0.71 ± 0.09 mM, n = 7). Responses to first and second exposures to anoxia showed differences — the first response being smaller in SP (0.63 ± 0.05 mM, n = 5 cf. 0.95 ± 0.11 mM, n = 4; $p \leq 0.05$) and larger in SR (3.51 ± 0.20 mM, n = 4 cf. 3.03 ± 0.09 mM, n = 8; $p \leq 0.05$). However, changes evoked by anoxia were still significantly greater in SR than in SP for comparisons within first and second exposure groups.

II. $[Cl^-]_o$ Measurements in Stratum Pyramidale and Stratum Radiatum.

i. Resting levels of $[Cl^-]_o$ and changes with stimulation.

The baseline resting level for extracellular chloride in the superfused slice measured at 100 μm depth in initial experiments was 131± 4.3 mM (n = 9) in the SP and 137± 5.0 mM (n = 9) in the SR. For these paired values, recorded from both locations in each slice, there was no significant difference.

Changes in $[Cl^-]_o$ evoked by frequency stimulation (0.1 ms, 20 s duration, 1–10 Hz) were measured in SP and SR of the CA1 region in paired recordings in four slices. These results showed increases in $[Cl^-]_o$ in SP and decreases in $[Cl^-]_o$ in SR. Fig. 18 shows the peak values of $\Delta[Cl^-]_o$ recorded in SP (open symbols) and SR (filled symbols) with increase in the rate of stimulation.

ii. $[Cl^-]_o$ changes evoked by GABA.

In initial experiments the distribution of GABA-evoked changes in $[Cl^-]_o$ was examined along the dendritic tree and soma of the pyramidal neurons, by comparing $[Cl^-]_o$ changes recorded in stratum oriens (SO), stratum pyramidale (SP) and stratum radiatum (SR) of the CA1 region of the hippocampus and evoked by bath application of 5 mM GABA for 5 min.

The chloride changes evoked by GABA were of a dual nature (increases and/or decreases). From these preliminary observations it was concluded that in the dendritic layers of the SO and SR (apical and basal dendrites, respectively) $[Cl^-]_o$ changes during GABA application were mainly decreases. In one of five experiments in SR an accompanying increase in $[Cl^-]_o$ occurred as an overshoot

during recovery, and in one of four experiments in SO there was an increase just before the expected decrease (not illustrated). In the latter case, it was possible that the recording site was closer to the somal layer (SP), where only increases in $[Cl^-]_o$ were recorded.

Bath applications of GABA (1–10 mM for 5 min) evoked dose-dependent and reversible changes in $[Cl^-]_o$. In the SP during GABA exposure an increase in the extracellular chloride levels was measured while in the SR there was a decrease. Fig. 19 shows data collected in six slices from four animals. For all doses of GABA applied (1, 2, 3, 5 and 10 mM) the same effect was observed: increases in $[Cl^-]_o$ in SP and decreases in $[Cl^-]_o$ in SR — opposite changes, which were, of course, statistically significant ($p \leq 0.0001$) and suggest the presence of different receptor/transport mechanisms. Sigmoid dose/response analysis yielded EC_{50} values of 4.1 and 5.6 mM and R_{max} values of 18.19 ± 1.3 mM and –20.87 ± 1.3 mM for SP and SR, respectively.

In a typical experiment, for example with 5 mM GABA (see Fig. 20 A and C), the initial change in $[Cl^-]_o$ was observed approximately 2 min after the beginning of drug perfusion, while peak changes (of increase in SP and decrease in SR) were observed 2 to 3 min later and reached a plateau that was followed by a gradual decline during washout. The level was sustained in the washout period for 1–2 min; then $[Cl^-]_o$ concentrations returned abruptly to baseline. In only 2 out of 14 experiments undershoots in $[Cl^-]_o$ in SP were recorded; while in SR in one of 8 experiments, the GABA-evoked decrease was followed by an increase (overshoot) in $[Cl^-]_o$ that lasted 5 min (not illustrated).

iii. Comparison of changes in $[Cl^-]_o$ evoked by GABA and anoxia.

The extracellular chloride changes evoked by 5 mM GABA were compared to those induced by anoxic exposure in SP and SR. Both treatments evoked similar changes in $[Cl^-]_o$ (see Fig. 20 A cf. B and C cf. D, as well as Table VII, which summarizes resting and evoked levels and the changes for n = 14 slices). As observed for $\Delta[K^+]_o$, changes in $[Cl^-]_o$ in response to N_2 were usually slightly earlier in onset cf. those with GABA (see Fig. 20). The mean changes evoked by GABA and anoxia are displayed in the histogram of Fig. 21. In SP increases in $[Cl^-]_o$ with GABA were 21.3 ± 2.0 mM (n = 14) and with N_2 they were 25.1 ± 2.7 mM (n = 10). The decreases recorded in SR were –14.7 ± 0.9 mM (n = 8) for GABA and –33.8 ± 5.0 mM (n = 9) with N_2. Differences between SR and SP for each treatment were, of course, statistically significant ($p \leq 0.0001$).

In an additional group of three experiments responses to anoxia were again recorded without prior exposure to GABA. $\Delta[Cl^-]_o$ evoked by N_2 in SP and SR, respectively was 16.2 ± 2.9 (n = 3) and –44 ± 11.0 (n = 3). These changes were not significantly different from those recorded with the usual protocol of an initial application of GABA — 25.1 ± 2.7 (n = 10) for SP and –33.8 ± 5.0 (n = 9) for SR.

Comparison of $\Delta[Cl^-]_o$ evoked by a first or second application of GABA showed a larger decrease for the later response in SP (18.4 ± 2.0, n = 4 cf. 13.0 ± 1.1, n = 9; $p \leq 0.05$) but no difference in SR (20.3 ± 1.4, n = 4 cf. 20.4 ± 1.1, n = 4; $p \geq 0.05$). Responses to first and second N_2 exposures were not significantly different for either SP or SR. Responses in SR remained significantly different ($p \leq 0.0001$) from those in SP within each group of first and second exposure.

III. $[Na^+]_o$ Measurements in Stratum Pyramidale and Stratum Radiatum.

i. Resting levels of $[Na^+]_o$ and changes with stimulation.

Since both GABA and anoxia are known to elicit changes in the extracellular sodium concentration, measurements and comparison of changes in $[Na^+]_o$ that occur in SP and SR were made in response to anoxic exposure and GABA application. Some of these changes are likely linked to the functioning of amino acid transmitter uptake systems, ligand and voltage-activated channels, and co-transport/counter-transport mechanisms.

Baseline resting levels for sodium measured at 100 µm depth of recording in the slices in initial experiments were 156 ± 11 mM (n = 6) in the SP and 159 ± 13 mM (n = 9) in the SR. There was no significant difference between the two levels for paired SP and SR data.

ii. $[Na^+]_o$ changes evoked by GABA.

In this series of experiments (n = 12 slices) the effects of GABA on $[Na^+]_o$ were limited to observations using only a single concentration, 5 mM GABA. Bath applications of GABA induced decreases in $[Na^+]_o$ in both SP and SR. In typical experiments (see Fig. 22 A and C) where 5 mM GABA was applied for 5 min to the slice, the initial change in $[Na^+]_o$ was observed approximately 2 min after the beginning of the drug perfusion, while the nadir was observed 2 to 3 min later and followed by a plateau for 1 to 2 min, with gradual recovery to baseline during washout.

iii. Comparison of changes in $[Na^+]_o$ evoked by GABA and by anoxia.

When the sodium changes evoked by 5 mM GABA were compared with those induced by anoxic exposure in SP and SR of the CA1 both treatments produced quite similar changes (direction of change and time-course) in extracellular sodium (see Fig. 22 A cf. B and C cf. D). The mean data from n = 9 slices for baseline, peak and $\Delta[Na^+]_o$ are summarized in Table VIII, while Fig. 23 shows the mean changes graphically. Looking at the distribution of changes across the dendritic and somatic layers, both GABA and N_2 produced consistent and more pronounced drops in $[Na^+]_o$ in SR compared to SP. The $[Na^+]_o$ changes in SP of –17.0 ± 0.7 mM (n = 8) for GABA and –25.2 ± 0.52 mM (n = 9) for N_2, when compared to those in SR of –23.5 ± 2.2 mM (n = 8) with GABA and –32.3 ± 2.6 mM (n = 9) with N_2, showed significant differences ($p \leq 0.05$).

In a further series (n = 3 slices) responses to anoxia were recorded with no earlier exposure to GABA. $\Delta[Na^+]_o$ evoked by N_2 in SP and SR, respectively was –25.6 ± 0.5 mM (n = 3) and –41.3 ± 1.5 mM (n = 3). These changes were not significantly different from those observed with the usual protocol of an initial application of GABA — –25.2 ± 0.5 mM (n = 8) for SP and –32.3 ± 2.6 mM (n = 9) for SR.

Evaluation of whether $\Delta[Na^+]_o$ evoked by a first or second application of GABA showed no difference in SP (–23.6 ± 0.9 mM, n = 4 cf. –22.8 ± 1.0 mM, n = 4) or SR (–32.9 ± 2.9 mM, n = 4 cf. –26.7 ± 2.3 mM, n = 4). However, with anoxia there was a larger decrease in SP for an initial exposure (–34.0 ± 1.1 mM, n = 4 cf. –30 ± 0.6 mM, n = 4; $p \leq 0.05$), but no difference in SR (–40.2 ± 3.3 mM,

n = 4 cf. –39.3 ± 3.2 mM, n = 5). Finally, within similar treatment groups (first or second GABA or N_2 application) responses in SR remained significantly greater than those in SP only for the first application of GABA (–32.9 ± 2.9 mM, n = 4 cf. –23.6 ± 0.9 mM, n = 4; $p \leq 0.05$) and the later exposure to anoxia (–39.3 ± 3.2 mM, n = 5 cf. –30.1 ± 0.6 mM, n = 4; $p \leq 0.05$).

IV. Changes in Extracellular Space during GABA Application and Anoxia. Ion selective microelectrodes were used to evaluate the degree of osmotic shift which might be evoked by GABA and by anoxia. Changes in extracellular space (ECS) were assessed by recording changes in the concentration of an ion restricted to the extracellular space — tetramethyl-ammonium (TMA). Recordings were carried out at three different recording depths: 50, 100 and 150 µm in SP and SR in the CA1 region in n=4, 7 and 3 different slices, respectively, during perfusion with ACSF containing 3 or 5 mM TMA.

Since the Corning 477317 K^+ ion-exchanger is highly sensitive to quaternary ammonium salts (e.g. TMA), changes in extracellular TMA were measured with ISMs prepared with this membrane. In the presence of TMA in the superfusing ACSF the K^+ Corning electrode behaves as a TMA electrode (see Fig. 10). Increases in $[TMA]_o$ reflect volume decreases of the extracellular space and movement of water into the intracellular space (neuronal and/or glial components).

All ion changes seen with both N_2 and GABA will be affected by this water shift, in that absolute increases in the numbers of extracellular K and Cl ions would be overestimated, while decreases in Cl and Na ions would be underestimated. Changes in the ECS were measured and calculated as previously described by others (Dietzel et al., 1980; Ransom et al. 1985) and using the following equation:

$$\% \text{ age shrinkage of ECS} = \left(1 - \frac{[TMA]_1}{[TMA]_2}\right) \times 100 \qquad [4]$$

where $[TMA]_1$ = $[TMA^+]_o$ before GABA or N_2,

$[TMA]_2$ = $[TMA^+]_o$ during GABA or N_2.

Each slice was exposed to 5 min applications of 5 mM GABA alternating with anoxia (N_2 replacement of O_2), while recording in SP and then in SR, or first in SR and then in SP. Fig. 24 illustrates typical recordings of $[TMA]_o$ in the SP and SR during N_2 and GABA exposures in two different slices (A and B). The onset and offset of increases and the presence of a steady plateau corresponded reasonably well with the time course of the evoked changes observed for $[K^+]_o$, $[Cl^-]_o$ and $[Na^+]_o$ (cf. with Figs. 16, 20 and 22). Peak/maximal $[TMA]_o$ increases evoked by both N_2 and GABA were significantly greater in SR than in SP for the volume decreases induced at 100 µm by N_2 of 5.28 ± 0.28 and 6.16 ± 0.80 % for SP and SR respectively, and by GABA of 4.64 ± 0.23 and 4.75 ± 0.25 % (see Table IX).

The relatively greater changes evoked in dendritic compared to somal layers may reflect differences in the surface area/volume ratio which influence the magnitude of trans-membrane ion shifts. There was also a significant gradient observed across the slice. At 50 µm changes in $[TMA]_o$ were minimal: in SP for GABA and N_2 increases were 1.17 ± 0.13 % and 1.5 ± 0.28 % respectively, while in SR they were 1.45 ± 0.25 % and 1.83 ± 0.44 %. As the recording depth was increased from 50 to 100 µm and 150 µm, these changes became greater. $[TMA]_o$ increases were approximately 3–4 times greater at depths of 100 and 150 µm compared to 50 µm. This might reflect cellular damage at the cut surface of the slice. Since 100 µm was the recording depth at which all other recordings were carried out, changes in $[TMA]_o$ at this depth were used for correction and interpretation of the ion changes evoked by GABA or anoxic exposures.

All previously recorded ion levels were individually adjusted to account for a shrinkage of ECS, with the justifiable assumption that peak/plateau changes for $[TMA]_o$ and the other ions occurred and were measured at the same times. Table X summarizes the uncorrected ion changes from Tables VI–VIII, to facilitate comparison with the corrected values shown in Table XI. For example, the N_2-evoked $[K^+]_o$ increase in SP of 1.01 mM, when corrected for shrinkage overestimation, becomes 0.77 mM.

When the individual values used in the paired data evaluation of changes from baseline for each ion in Tables VI, VII and VIII were corrected for ECS shrinkage, changes all remained significantly different ($p \leq 0.005 - 0.0001$). As well, statistical comparisons for the influence of first or second exposures to GABA or N_2 and of differences between SR and SP remained similar after correction.

It is concluded that the reductions in the ECS are insufficient to account for the changes in $[K^+]_o$, $[Cl^-]_o$ and $[Na^+]_o$ that occur during anoxic exposure or GABA application to the slices. Finally, it should be noted that actual recorded levels of ions indicate real ECS concentrations to which cellular elements are exposed. However, the cause of such changes reflects both osmotic shift and active/passive ion transport/movement.

V. Effects of Bicuculline Methiodide on Ion Changes evoked by GABA and Anoxia.

In order to further compare the effects of GABA and anoxia and define the potential role of $GABA_A$ receptor activation during anoxia, experiments were carried out to assess the sensitivity of the evoked ion changes to the $GABA_A$ receptor antagonist, bicuculline methiodide (BMI). In the presence of stimulation BMI caused a pronounced increase in the magnitude of population spikes, which was accompanied by an increase in the baseline level of extracellular potassium of 1.5–2 mM. In the absence of stimulation, however, BMI evoked no change in activity other than some infrequent spontaneous discharges. BMI did not alter $[K^+]_o$ in the absence of stimulation or increased spontaneous activity.

i. BMI effect on the $[K^+]_o$ changes.

In the presence of BMI the $[K^+]_o$ increases elicited by both GABA and N_2 in SP and SR were reversibly attenuated. In SP, in a typical experiment (see Fig. 25 A–C), 5 mM GABA induced an increase in $[K^+]_o$ from a resting level of 3.0 mM to a peak level of 4.1 mM, a $\Delta[K^+]_o$ of 1.1 mM. Pretreatment of the slice with 100 µM BMI for 30 min attenuated this GABA-evoked increase in $[K^+]_o$; from the same resting level of 3.0 mM $[K^+]_o$ increased to a peak level of only 3.2 mM. Expressed as percentage of the control, the GABA-evoked $[K^+]_o$ increase was attenuated in the presence of BMI by 82 %. Similarly, anoxic exposure (see Fig. 25 D–F) evoked an increase in $[K^+]_o$ in SP from a resting level of 3.1 mM to a peak level of 5.8 mM ($\Delta[K^+]_o$ = 2.7 mM). After 30 min of 100 µM BMI, $\Delta[K^+]_o$ was only 0.8 mM. The $[K^+]_o$ increase

was attenuated by 70 %. Upon washout of BMI, ≥90 % recovery of the responses evoked by both anoxia and GABA was obtained (see lower traces, Fig. 25 C, F).

A typical experiment with recording in SR (see Fig. 26 A–C), shows that 5 mM GABA induced an increase in $[K^+]_o$ from a resting level of 2.6 mM to a peak level of 3.1 mM ($\Delta[K^+]_o$ = 0.5 mM). Pretreatment with BMI for 30 min completely and reversibly blocked this GABA-evoked increase in $[K^+]_o$ from the same resting level of 2.6 mM. Similarly, anoxia evoked an increase in $[K^+]_o$ from a resting level of 3.3 mM to a peak of 4.6 mM ($\Delta[K^+]_o$ = 1.3 mM) (Fig.26 D–F). After BMI, the $\Delta[K^+]_o$ was only 0.5 mM, an attenuation of 62 %. The recovery of responses evoked by both anoxia and GABA is shown in the lower traces (C, F).

Table XII summarizes the mean data from 6 slices and allows comparison of the BMI effect on the evoked $[K^+]_o$ changes in SP and SR. In the SP BMI depressed the increases in $[K^+]_o$ seen with 5 mM GABA by 93 % and the N_2-evoked K^+ increase by 58 %; while in the SR BMI completely blocked the changes in $[K^+]_o$ seen with GABA, and decreased the N_2-induced $[K^+]_o$ increase by 72 % (see summary of Table XV).

The dose-dependent depression of varying concentrations of BMI (1–300 μM) on the $[K^+]_o$ increases evoked by N_2 in SP and SR in n = 10 slices is illustrated in Fig. 27. As previously described, larger increases in $[K^+]_o$ were evoked by N_2 in SR than in SP. At each concentration of BMI between 10 and 300 μM, $[K^+]_o$ changes in SR were significantly greater than in SP ($p \leq 0.05$). IC_{50} values of 54 and 63 μM were obtained from curves fitted by non-linear regression analysis for SR and SP, respectively.

ii. BMI effect on the $[Cl^-]_o$ changes.

A similar series of experiments was also carried out to assess the sensitivity of the extracellular chloride changes observed during anoxic exposure or GABA application to BMI. Pretreatment with BMI (100 μM) for 30 min reversibly attenuated the $[Cl^-]_o$ changes.

In SP in a typical experiment (Fig. 28 A–C), 5 mM GABA induced an increase in $[Cl^-]_o$ from a resting level of 125 mM to 142.5 mM ($\Delta[Cl^-]_o$ = 17.5 mM). Pretreatment with BMI attenuated this increase from the same resting level to a peak of only 128 mM ($\Delta[Cl^-]_o$ = 3 mM). The GABA-evoked $[Cl^-]_o$ increase was attenuated by 83 %. Similarly, anoxic exposure evoked an increase in $[Cl^-]_o$ from 125 mM to 162 mM ($\Delta[Cl^-]_o$ = 37 mM) (see Fig. 28 D–F). After BMI $\Delta[Cl^-]_o$ was only 16 mM — a depression of 57 %. Upon washout of BMI more than 90 % recovery of the responses to GABA and N_2 was obtained.

In SR, in a typical experiment (see Fig. 29 A–C) GABA induced a decrease in $[Cl^-]_o$ from 119 mM to 104 mM ($\Delta[Cl^-]_o$ = 15 mM). BMI attenuated this decrease from 119 mM $[Cl^-]_o$ to a level of only 112 mM ($\Delta[Cl^-]_o$ = 7 mM) an attenuation by 53 %. Similarly, N_2 evoked a decrease in $[Cl^-]_o$ from 127 mM to 104 mM ($\Delta[Cl^-]_o$ = 23 mM), while pretreatment with BMI attenuated the decrease (to a $\Delta[Cl^-]_o$ = 12 mM) by 48 % (Fig. 29 D–F). Recovery of the $[Cl^-]_o$ decrease evoked by N_2 and GABA was obtained upon washout of drug.

Table XIII summarizes the baseline $[Cl^-]_o$ levels and changes in the absence and presence of BMI (100 μM) for data from 6 slices, while the percentage attenuation is shown in Table XV. In the SP BMI depressed the increase in $[Cl^-]_o$

seen with GABA by 88 % and the N_2-evoked $[Cl^-]_o$ increase by 52.4%; while in the SR BMI pretreatment attenuated the chloride decreases evoked by both GABA and N_2 by 55 %.

iii. BMI effect on $[Na^+]_o$ changes.

The sensitivity of N_2 and GABA-induced extracellular sodium changes to BMI was also tested. It was concluded that these changes were less sensitive to the specific antagonist, as might be expected.

Figs. 30 and 31 illustrate the decreases in $[Na^+]_o$ evoked by 5 mM GABA or N_2 in the SP and SR respectively, with and without exposure to 100 μM BMI, and recovery after BMI washout. Table XIV summarizes the mean resting and evoked changes observed in 3 slices, while the effect of BMI (percentage of change relative to that without BMI) is presented in Table XV.

Pretreatment with BMI attenuated the GABA-evoked $[Na^+]_o$ change in SP by 32% and the anoxic-induced fall by only 21%. In SR BMI affected changes in $[Na^+]_o$ elicited by GABA to depress them by 31% and those seen during anoxia by 27%. Upon washout of the specific antagonist, recovery of the response was obtained.

In summary, comparison is made of the effect of BMI on the three different ion species, K^+, Cl^-, and Na^+ (see Table XV). In the SP BMI depressed increases in $[K^+]_o$ and $[Cl^-]_o$ with GABA by ≃ 90% and with anoxia by 50–60%. In the SR K^+ accumulation during GABA was abolished and with anoxia was attenuated by 70%; decreases in $[Cl^-]_o$ were depressed by more then 50%. $[Na^+]_o$ changes in SP and

SR were affected to a lesser extent, being blocked by 20–30%. The effects of BMI were significantly greater ($p \leq 0.01$) in SR compared with SP for GABA-evoked changes in $[K^+]_o$ and $[Cl^-]_o$ and for the N_2-evoked increase in $[K^+]_o$.

VI. $[K^+]_o$ Changes evoked by Baclofen.

Although GABA-evoked changes in extracellular $[K^+]$ in the central nervous system have been recorded in many locations (Barolet and Morris 1991; Bührle and Sonnhof 1985; Gutnick and Segal 1981; Kudo and Fukuda 1976; Müller et al. 1989; Syková 1979) the increases have been commonly attributed to $GABA_A$ receptor activation, and there has been little or no documentation of the contribution which would be expected to arise from direct activation of K^+ channels via the $GABA_B$ receptors (Barolet and Morris 1991; Sakatani et al. 1994). Experiments were therefore carried out to assess the magnitude and time course of changes in $[K^+]_o$ in somatic and dendritic locations in the hippocampal CA1 region evoked by the $GABA_B$ receptor agonist, baclofen, and to define a potential contribution to those which accompany the action of GABA.

Valinomycin-based K^+ ISMs were used to record changes in $[K^+]_o$ and DC field potentials in SR and SP in CA1 evoked by the racemic mixture, DL-baclofen. Experiments were made using 42 slices from 22 animals. Typical baseline levels for $[K^+]_o$ in SP and SR were respectively 3.51 ± 0.12 mM ($n = 22$) and 3.56 ± 0.19 mM ($n = 22$) and not statistically different.

i. Bath applications of baclofen.

Bath applications of (±)-baclofen (1 μM – 3 mM for 5 min) evoked changes in $[K^+]_o$, which were in the majority of cases sustained throughout agonist application and reversed during wash-out.

(a) Early sustained increases in $[K^+]_o$.

Baclofen (10^0 – $3x10^3$ μM), dissolved in ACSF and superfused for periods of 4–6 min evoked in the majority of cases graded, reversible increases in $[K^+]_o$ which were usually accompanied by a positive shift in the field potential (V_F). Fig. 32 shows examples of the time course and magnitude of responses evoked by 100 μM baclofen in one slice and recorded at separate times in SP and SR. Although sometimes responses reached a peak and began to decline or plateau during the application more often there was a progressive increase in $[K^+]_o$ to a peak or an initial plateau followed by continued increase. Occasionally an undershoot in the $[K^+]_o$ level was observed during recovery, but this was never prominent or prolonged. The onset and offset of $[K^+]_o$ increase was slower than that observed for GABA.

In two experiments 10 μM baclofen evoked decreases in $[K^+]_o$ in the SP. In addition, in a number of other cases (≈ 10% of all observations) at higher concentrations (20–10^3 μM) and mainly in SP, the increases in $[K^+]_o$ appeared to be preceded by a slight decrease (cf. Fig. 32).

Table XVI shows the dose-dependence of $[K^+]_o$ changes evoked by 10^0 – 3 x 10^3 μM baclofen. At each concentration the increases were larger in SR than in SP. However, the differences were not significant. Fig. 33 illustrates the

concentration-response curves for SP (unfilled circles) and SR (filled circles) (n = 41 and 36 observations respectively, n = 3–10 observations at each concentration (n = 10 slices), data from Table XVI). The maximal values (R_{max}) for curves fitted with Hill slope = 1 were 0.59 ± 0.03 and 0.65 ± 0.03 mM, while EC_{50} values were 40 and 39 μM, respectively, with no significant difference ($p \geq 0.05$) for either statistic. The changes in $[K^+]_o$ evoked by baclofen were significantly correlated with those of the accomanying DC field potentials. Fig. 34 A and B show the relationship between peak amplitude voltage changes in potassium (ΔV_K) and the field (ΔV_F) for SP (open circles) and SR (filled circles) for 39 observations. The slopes for regression were similar: 0.74 ± 0.28 (n = 19) cf. 0.75 ± 0.33 (n = 20) respectively ($p \geq 0.05$), and each correlation was significant ($r = 0.538$, $p \leq 0.01$ for SP; $r = 0.471$, $p \leq 0.05$ for SR). Although baclofen doses for the two groups were not identical, comparison of their weighted means showed no difference; while the mean ΔV_K for SP (2.19 ± 0.32) was significantly smaller than that for SR (3.41 ± 0.44) ($p \leq 0.05$) and mean ΔV_{FS} were similar.

(b) Brief and phasic/recurrent increases in $[K^+]_o$.

In 11 slices bath application of baclofen evoked responses notably different from the early, sustained changes described in the previous section. In some cases an early but transient (unsustained) increase in $[K^+]_o$ (≤1 min) was evoked by the 5 min application of 10–50 μM baclofen (n = 5 observations). In others (n = 7 observations) much briefer and phasic increases in $[K^+]_o$ occurred either immediately or within 5–15 min following exposures to 10–50 μM baclofen for 5–30 min. These changes were large ($\Delta[K^+]_o$ = 2.7 mM ± 0.03, n = 7), short in duration

(5–8 s) and regularly repetitive at a frequency ≈ 1/12 s. They were accompanied by long-lasting field potentials, as illustrated in the example from one experiment in Fig. 35 (which also shows burst discharge at the peak of V_F and on the rising phase of $[K^+]_o$) and in some cases by more frequent inter-ictal discharges, occurring at ≈ 0.5–1/s (Fig. 35B). This recurrent activity lasted for periods of 30–60 min or longer and showed little evidence of waning of amplitude or frequency. The presence of these 'spontaneous' discharges appears to be related to the preservation of CA3 input to CA1, (as described by Perreault and Avoli 1991, 1992), as they occurred in slices from four of five dissections in which the alveus as inferior, rather than superior during chopping.

ii. Pressure ejection studies.

In 8 slices baclofen, applied by pressure ejection (constant pipette concentration of 100 µM at 50 psi) evoked in both SR and SP graded increases in $[K^+]_o$ which were dependent on the duration of release (100–900 ms) and accompanied by positive shifts in the field potential. Fig. 36 shows examples of the $\Delta[K^+]_o$ evoked in dendritic and somal layers in one experiment by a 300 ms application of 100 µM baclofen. The graph of Fig. 37 of the mean data from 4 slices demonstrates the graded increases evoked by increasing pulse durations; although increases in $[K^+]_o$ in SR (filled columns) appeared larger compared to those in SP (open columns) the differences were not statistically significant.

iii. Effects of $GABA_B$ receptor antagonists.

In three additional experiments (n = 5 slices) sustained increases in $[K^+]_o$ evoked by bath application of 50 or 100 μM baclofen could be rapidly and reversibly blocked by co-application of a $GABA_B$ receptor antagonist — either 2-OH saclofen (50 μM) or CGP 35348 (50 μM). Fig. 38 shows an example of this action.

Since $GABA_B$ antagonists decreased baclofen-evoked increases in $[K^+]_o$ (Fig. 38) an experiment was carried out to determine their possible influence on anoxic-induced changes in $[K^+]_o$. It might be predicted that a component of N_2-induced $[K^+]_o$ increase due to GABA accumulation and activation of the low-affinity, $GABA_B$ receptors should be attenuated by B receptor antagonists. However, in a single experiment (not illustrated), a bath application of 0.5 mM saclofen for 40 min, enhanced the N_2-evoked increase in $[K^+]_o$ by ≃ 80–100%, while wash-out of the antagonist was accompanied by a reversal of the action. This suggests that block of presynaptic $GABA_B$ receptors and removal of inhibition of synaptically-evoked transmitter release (GLU and/or GABA) may occur; and/or that block of post-synaptic receptors removes a hyperpolarizing, stabilizing effect of GABA to allow greater depolarization by glutamate.

VII. Intracellular Potential Changes during Anoxia.

In many earlier *in vitro* studies the effects of GABA (applied by bath perfusion and pressure ejection or iontophoresis) on membrane potential and conductance have been recorded with intracellular microelectrodes. In addition to the results from rat hippocampal slices reported by Alger and Nicoll (1982) and numerous other authors, Inoue et al. (1985 a,b) have described in considerable detail the biphasic effects evoked by GABA in the CA1 region of the guinea pig hippocampal slice. Although anoxic-induced intracellular responses of CA1 pyramidal neurons have been frequently studied in the rat hippocampus (Fujiwara et al. 1987; Hansen et al. 1982; Krnjević and Leblond 1989; Morris et al. 1995), there has been little or no examination of the changes evoked in the guinea pig. Therefore a series of experiments was carried out to assess these and provide data which might be compared with those already documented for the rat and for GABA's effects in the guinea pig.

Sharp intracellular microelectrodes were used to observe changes evoked by brief anoxia (4–5 min) in CA1 neurons in the SP of guinea pig hippocampal slices. Recordings were made from 12 cells in 10 slices (n = 10 animals). The change in membrane potential evoked by N_2 exposure was, in only one of 21 responses, a pure depolarization. In the remainder there was either (i) hyperpolarization alone (n = 7) or following a small, brief depolarization (n = 8), or (ii) a mixture of hyperpolarization and depolarization, each of which lasted more than 5–10 sec (n = 5).

Table XVII summarizes the data from 16 trials where accurate voltage and current calibrations were available. For one of 16 N_2 exposures there was a 'pure' or isolated depolarization of + 6 mV from a resting level (V_m) of – 68 mV; for the 15 others the membrane potential was hyperpolarized from V_m of –64.7± 1.8 mV by –5.1 ± 4.5 mV ($\Delta V_{H(N_2)}$). Such hyperpolarization occurred either alone or was accompanied (before, after or during) by a depolarization ($\Delta V_{D(N_2)}$,= 5.0 ± 1.1 mV, n = 10). In all cases a post-anoxic hyperpolarization (ΔV_{PAH} = –12.3 ± 2.2 mV) was observed. Although the mean change in membrane resistance (R_M) during anoxia was a decrease (from 44.9 ± 2.6 to 40.3 ± 3.2 MΩ, n = 16, $p \leq 0.05$), in 4 recordings there was an increase from R_M = 41.8 ± 4.8 to R_{N_2} = 58.5 ± 2.5 MΩ ($p \leq 0.01$); while in the remaining 12 there was a decrease from R_M = 46.0 ± 3.2 to R_{N_2} = 34.2 ± 2.2 ($p \leq 0.0001$).

In Fig. 39 recordings from two different neurons (n = 2 animals) show typical hyperpolarizing responses. In A (upper trace) anoxia evoked an initial small depolarization and then a hyperpolarization, which was accompanied by a decline in R_M and followed by a ΔV_{PAH} of ≈ 17 mV below the resting V_M level of –62 mV. In the lower trace when V_M was shifted from – 62 to – 71 mV by injection of a hyperpolarizing current (I_{hp}) the anoxic-evoked depolarization was enhanced and the hyperpolarization decreased. The trace in B shows a similar but isolated hyperpolarizing response from another neuron.

In contrast to this type of response Fig. 40 A shows a depolarization in one neuron of +10 mV from V_M = –71 mV, which was preceded by a small hyperpolarization, accompanied at its peak by a burst of action potentials, and

followed by a ΔV_{PAH} of –10 mV. In this case R_M initially declined and then increased. In Fig. 40 B another response, recorded in the same cell in the absence of resistance test pulses, demonstrates the loss of synaptic potentials (EPSP and IPSP) and early return of the EPSP.

DISCUSSION

The results of the current study have accomplished the following broad objectives: (i) establishment of the time course and magnitude of changes in the extracellular concentration of three specific ions (K, Na and Cl) that occur in the hippocampal slice during application of GABA and during brief anoxia, (ii) an assessment and evaluation of the influence of concomitant changes in the size of the extracellular space, (iii) identification of a potential role for GABA in the ionic events associated with the early phase of anoxia, and (iv) support for potentiation of GABA-ergic mechanisms as a therapeutic adjunct during and following global CNS ischemia.

I. Extracellular Ion Concentrations – Resting Levels.

Resting levels of $[K^+]_o$, $[Na^+]_o$ and $[Cl^-]_o$ recorded with ISMs in the CA1 region of the hippocampal slices of the present study are similar to those previously recorded *in vivo* and *in vitro* by other authors (Dietzel et al. 1982; Erecinska and Silver 1994; Heinemann et al. 1977; Krnjevic & Morris 1974, 1982 a,b; Morris 1974; Zetterstrom et al. 1995). Ion levels which have been recorded *in vivo* resemble those measured in the cerebrospinal fluid (Ames et al. 1964), while those of *in vitro* experiments are close to the perfusate concentrations. As expected, our resting $[K^+]_o$ levels resemble those of the ACSF (for example 3.46 mM and 3.58 mM in SP

and SR cf. 3.3 mM in ACSF). Resting $[Na^+]_o$ and $[Cl^-]_o$ levels were higher than in the ACSF (156 mM in SP and 159 mM in SR cf. 147 mM for Na^+, and 131 mM in SP and 137 mM in SR cf. 125 mM for Cl^-); this may reflect differences between the composition of the calibrating solutions and the ACSF, for which corrections were not made. Although resting levels of the individual ions in the SR appeared to be higher than in SP, statistical comparison showed no significant differences.

II. Changes in Extracellular Potassium.

i. Frequency stimulation.

The graded increases in $[K^+]_o$ recorded in response to increasing frequency of orthodromic tetanic stimulation of the Schaffer collateral pathway are similar to those recorded with ISMs in experiments in many synaptic regions in the CNS (Krnjevic and Morris 1974, 1975, 1982a, 1982b; Heinemann and Lux 1977; Kriz et al. 1974; Morris et al. 1991; Nicholson et al. 1978; Vyklicky et al. 1972). In the current experiments stimulus-evoked accumulations of K^+ in SR were larger than those in SP. This observation is consistent with some earlier recordings of quite similar $[K^+]_o$ increases over a several hundred μm span of the somatic-dendritic region, in rat CA1 *in vivo* (Krnjevic et al. 1982b) and in the dentate of guinea pig hippocampal slices (Fritz and Gardner-Medwin 1976). They also agree with the slightly larger maximum recorded in SR compared with SP during low frequency stimulation in rat hippocampal slices by Aitken and Somjen (1986), although these authors reported that the largest change they saw with tetanic stimuli was in SP. Changes recorded by Benninger et al. (1980) in guinea pig hippocampal slices were larger in SP than in SR, although they imply that the largest $\Delta[K^+]_o$ they observed was in SR. Segal and Gutnick (1980) also found larger stimulus-evoked accumulations in SP than in SR in rat hippocampal slices. In other studies Nicholson et al. (1978) measured largest increases at synaptic sites on the Purkinje cell dendrites in the cerebellum.

Not all details of methodology are included in these various reports, and the differences in results might perhaps arise from different recording techniques – e.g.

depth, distance along the dendrites, angle of electrode insertion, temperature, ACSF level of K^+, and intensity of stimulation. Our recordings were made 100–150 μm from the soma, identified by visual placement in the SP of vertically inserted electrodes, and observations were from paired recordings in SP and SR in the same slice. One possible reason for a difference between our results and those of Benninger et al. (1980) and Segal and Gutnick (1980) is that their recordings were in 5 mM KCl compared to ours in 3.3 mM; the first of these authors showed that raising K^+ in the ACSF from 3.5 to 5 mM doubled the accumulation in the SP, although they did not study changes in the SR. Another possible explanation is that our experiments used low stimulus intensity, which evoked population EPSPs rather than action potential activity in the SP (at least at low frequencies), while some others describe the use of intensities evoking near-maximal population spike response. Relevant to this are the conclusions of Aitken and Somjen (1986) that approximately half of the K^+ accumulation in SR is from presynaptic sources and the rest is from postsynaptic potentials in the dendrites, while in SP it is almost exclusively from action potentials; a similar interpretation was made by Nicholson et al. (1978). It should also be noted that some of these other studies were at higher temperatures which have been shown to evoke smaller accumulations of $[K^+]_o$ (Morris et al. 1991), so that in order to evoke measurable or larger $[K^+]_o$ increases high intensity stimulation likely would be required.

ii. $[K^+]_o$ response to GABA.

The graded changes evoked in the SP in our experiments by varying concentrations of exogenous GABA showed an EC_{50} value of 3.8 mM and R_{max} of 1.3 mM, which is similar to the respective values of 4 mM and 1.6 mM, previously reported by Barolet and Morris (1991). The earliest report of GABA-evoked K^+ change described $\Delta[K^+]_o$ of 0.2–0.5 mM in response to local droplet application of 0.5 mM GABA, larger increases in CA1 in SP and SLM compared with SR (Segal and Gutnick 1980). However, when Muller et al. (1989) used a similar technique of localized application of 10 mM GABA they found increases in $[K^+]_o$ in CA3 of 1.6 ± 0.8 mM in SP (n = 6), 1.9 ± 0.9 mM (n = 6) in SR and 1.2 ± 0.5 mM (n = 7) in the granule cell layer. They also made recordings in CA1 but they did not describe the K^+ changes. Although Barolet and Morris (1991) described a larger $\Delta[K^+]_o$ in SP than in SO and SR with 10 min applications of 1 and 5 mM GABA and no differences with 10 mM GABA, this was the result of a single experiment, their dendritic responses were measured at distances of 200 μm or more from SP, and depths of recording were less standardized than in the current study.

Our observations are consistent with the fact that depolarizing responses are more frequently recorded in dendritic cf. somatic regions in the hippocampus (Alger and Nicoll 1982b; Anderson et al. 1980; Thalmann et al. 1981) and that GABA-evoked changes in $[K^+]_o$ are believed to be largest in regions where depolarizing responses occur (Ballanyi and Grafe 1985; Kudo and Fukuda 1976; Barolet and Morris 1991). The GABA-evoked K^+ accumulations are at least partly due to activation of $GABA_A$ receptors (neuronal and glial), and may arise from co- or

counter-transport with anions, a secondary activation of g_K via membrane potential change, and to some degree Na-dependent uptake of the transmitter. A role for $GABA_B$ receptor-mediated g_K has not previously been shown. The possibility of an artefactual elevation of $[K^+]_o$ due to the ability of GABA to facilitate ACh release from cholinergic terminals (Bonnano and Raiteri 1987) is ruled out by the use of the valinomycin-based ion exchanger, which has high selectivity for K^+ over ACh^+, as well as the similarity of changes previously measured with both Corning 477317 and Fluka 60398 ISMs (Barolet and Morris 1991; Krnjevic et al. 1982a).

iii. $[K^+]_o$ response to anoxia.

The peak increase in $[K^+]_o$ of 1.01 ± 0.22 mM (n = 9), which was evoked in SP by 5 min N_2 exposure in the current study, is quite similar to the change of 0.83 ± 0.18 mM (n = 10), recorded in SP of CA1 during 3-4 min anoxia in rat slices, superfused with 3 mM KCl-containing ACSF at 33° C (Morris et al. 1991). Our finding of a much larger and significantly greater increase in $[K^+]_o$ of 3.18 ± 0.17 mM (n = 12) in SR is a novel observation, as no studies have previously looked at responses in these dendritic regions. It is consistent with the observations of Grossman and Williams (1971), who found that early anoxic events are mainly at the synapses cf. somata and, as suggested by Silver and Erecinska (1990), may reflect a larger Ca^{2+}_i increase in the dendrites and perhaps also the presynaptic terminals. $\Delta[K^+]_o$ during early anoxia have been attributed to a K^+ conductance increase rather than Na-K pump failure, as levels of ATP may decline too slowly to

be the cause (Lipton and Whittingham 1982; Martin et al. 1994). In the CA1 region Ca-activated g_K makes an important contribution (Zhu and Krnjevic 1994) cf. ATP-sensitive channels, and this is consistent with an observation that $\Delta[Ca^{2+}]_i$ and $\Delta[Ca^{2+}]_o$ precede $\Delta[K^+]_o$ and occur earlier in the hippocampus than in other regions of the CNS, such as cortex and thalamus (Silver and Erecinska 1990). Of further interest is that, in contrast to an earlier conclusion that a $[K^+]_o$ accumulation of < 1 mM would be unlikely to contribute to the early depression of synaptic transmission (Morris et al. 1991), the three-fold greater change documented here for SR cf. SP suggests that there could be some influence.

Comparison of the N_2- and GABA-evoked increases in $[K^+]_o$ showed that there was considerable resemblance — although, as would be expected, the onset and offset of change with N_2 was often more rapid. A further difference was that changes induced by GABA in SR were only slightly larger than in SP, compared to the larger difference observed for anoxia. Although there are some similarities in relation to other responses to GABA and anoxia (e.g. decrease in membrane resistance, both hyperpolarization and depolarization of membrane potential, attenuation of synaptic transmission), it is not immediately apparent that similarity of their associated ionic events might involve a causal linkage.

III. Changes in Extracellular Chloride.

i. Frequency stimulation.

Tetanic stimulation of the Schaffer collaterals evoked frequency-dependent increases in $[Cl^-]_o$ in SP and decreases in the SR. Although recordings have been made of stimulus-induced $[Cl^-]_o$ responses in the CNS, these have been exclusively in *in vivo* studies in the cerebral and cerebellar cortex (Dietzel et al. 1982; Nicholson et al. 1982b). The changes in $[Cl^-]_o$ recorded in cerebral cortex were at depths 400–500 μm apart and mainly increases, but sometimes with an initial component of decrease. Although some of the $\Delta[Cl^-]_o$ evoked by repetitive stimulation in the CNS may arise from a direct activation of $GABA_A$ receptors, it is likely that the majority is from co- or counter-transport following cation influx. The mechanisms underlying the differential responses in SP and SR are not easy to define, but one possibility is that synaptic transmission and trans-membrane ion fluxes stimulate redistribution and restoration of gradients via regional differences in Cl^- transport – with relatively greater chloride-pump extrusion at the soma and chloride-transporter accumulation at the dendrites (Hara et al 1992; Misgeld et al. 1986; Thompson et al. 1988a,b).

ii. $[Cl^-]_o$ response to GABA.

Graded applications of GABA (from 1–10 mM) evoked concentration-dependent decreases in $[Cl^-]_o$ in SP and increases in SR, with EC_{50} values of 4.1 mM and 5.6 mM, respectively which correspond reasonably well with those which we observed

for $\Delta[K^+]_o$. The difference in direction of change in the two locations also agrees with that for the stimulation-evoked responses which we recorded. However, these changes are different from the responses to micropipette applications recorded by Muller et al. (1989), who described a bicuculline-sensitive decrease in $[Cl^-]_o$ in SP and a decrease followed by increase in SR in CA3 and CA1. The average decrease they recorded was 7.5 ± 4.9 mM and the increase 3.8 ± 2.7 mM, although their $[K^+]_o$ increases were three-fold higher than ours. Although working in the same preparation, there were some technical differences between their experiments and the current study. They likely achieved a more rapid and possibly higher concentration with brief (30 s) ejection of 10 mM GABA from electrodes than was possible with the bath application of 5 mM. In addition, their ACSF level of KCl was 6.25 mM, approximately 3 mM higher than ours. We used a more recently developed neutral ion carrier, with greater selectivity for Cl^- cf. other anions than that of the Corning 477315 exchanger used by Muller et al 1989. The exact location of recording (distance from the SP) in CA1 and CA3 is not mentioned by Muller et al. (1989), and there can be some inaccuracy in electrode placement without histological confirmation. Their applications of GABA were much more localized than ours, and our responses likely are due to a greater extent to multiple sources. Unfortunately, although we have done some experiments with GABA micro-ejection applications (Morris et al. 1995), we have only measured $\Delta[K^+]_o$, so we cannot confirm or refute their findings.

Muller et al. (1989) concluded that GABA-induced hyperpolarization is due to Cl^- influx at the soma, and depolarization at the dendrites due to Cl^- efflux and

that different Cl^- gradients are achieved in a single neuron – with outward and inward gradients being maintained by inward and outward Cl^- transport, respectively. Kaila (1994) argues that there are a number of difficulties with this interpretation: (i) the initial effect of GABA in all locations was a decrease in $[Cl^-]_o$, (ii) ionic ECS shifts caused by GABA application, especially an increase in $[Cl^-]_o$, are likely to have a significant glial component (Hoppe and Kettenman 1989), and (iii) net flux of Cl^- does not have a simple relationship to the Cl^- driving force. He points out that if the $\Delta[Cl^-]_o$ is neuronal in origin, that the initial decrease in somatic regions is not inconsistent with a depolarization, the only assumption being a current which is the sum of a hyperpolarizing Cl^- component and a strong depolarizing component, which could be carried by HCO^-_3. The interpretation by Muller et al. (1989) of differential Cl^- compartments in soma and dendrites has also been criticized by Grover et al. (1993), on the basis of the fact that both hyperpolarizing and depolarizing responses can be evoked by GABA at the soma, and that Lambert et al. (1991) have shown that CA1 pyramidal cell dendrites normally produce hyperpolarizing field IPSPs, but can also produce depolarizing IPSPs!

A few details which support these criticisms should be mentioned. Direct measurements of $[Cl^-]_i$ have shown that depolarizing actions arising from activation of $GABA_A$ receptors can cause either a net *decrease* in $[Cl^-]_i$, e.g. in sympathetic and sensory vertebrate neurons which maintain high $[Cl^-]_i$ by active Na-K-Cl co-transport (Alvarez-Leefmans et al. 1988; Ballanyi and Grafe 1985), or a net *increase* in $[Cl^-]_i$, as in the crayfish muscle fiber which has high resting Cl^- permeability, $E_{Cl} = V_M$, and a response which involves HCO^-_3 efflux accompanied by Cl^- influx (Kaila et

al. 1989). These observations led Kaila (1994) to further strongly state that "this implies that it is difficult, if not impossible, to examine the polarity of the Cl^- driving force on the basis of $GABA_A$ receptor-mediated extracellular Cl^- shifts (cf. Muller et al. 1989)".

There have, however been no measurements of $[Cl^-]_i$ in neurons of the mammalian CNS, where the situation appears to be considerably more complex, due to the differences in responses of somatic and dendritic regions and the fact that in many hippocampal pyramidal cells hyperpolarization can be converted to depolarization by sustained or repeated applications of GABA and by increasing the concentration. Passive redistribution of ions and dissipation of the Cl^- gradient could lead to a loss of the driving force, but not reversal of polarity. However, the latter can be explained by the ability of HCO^-_3 to contribute to the current, since carbonic anhydrase can maintain the anion gradient (Kaila et al. 1989; Kaila 1994), although this has been challenged by Grover et al (1993). Whether there are different types of $GABA_A$ receptors at the same and different sites, with different cation/anion conductances or differential transport mechanisms and different levels of $[Cl^-]_i$ at soma and dendrites remains highly controversial (Alger and Nicoll 1982; Grover et al. 1993; Kaila 1994; Misgeld et al. 1986; Muller et al. 1989).

Our results might be interpreted with the simple analysis of a regional difference in $[Cl^-]_i$ levels, similar but opposite to the explanation put forward by Muller et al. (1989) – i.e. that they arise from Cl^- influx to dendrites and efflux from the soma. We prefer to consider the possibility that GABA may uniformally hyperpolarize the pyramidal neuron and that the $[Cl^-]_o$ decrease in SR may reflect

this, while the $[Cl^-]_o$ increase at SP may represent a net movement of Cl^- which includes a strong component of non-neuronal Cl^- efflux (Bormann and Kettenmann 1988). The latter idea is supported by the report of Rovira et al (1984) that somatic, but not dendritic, applications of the uptake inhibitor, nipecotic acid reduced stimulus-evoked seizure activity. Another possibility which might be considered is that long applications of GABA may strongly stimulate extrusion and uptake mechanisms which over-ride receptor-activated conductance – to give increased $[Cl^-]_o$ at the soma (after influx) and decreased $[Cl^-]_o$ at the dendrites (after efflux). However, we did not see any biphasic $\Delta[Cl^-]_o$. Another reason for differences between the current study and that of Muller et al. (1989) might be that responses were recorded at different locations: if their responses were recorded not in SP, but say within 100 μm along the process (close to where we located our electrode for SR recording) the response would be like our SP response; and if their dendritic recording was much further out towards SLM, there might be a different response recorded cf. our SR response.

Finally, although it is not easy to interpret why our results are different from those of Muller et al. (1989), it is important to point out that our experiments were specifically intended to assess the effects of a prolonged and widespread accumulation of the inhibitory amino acid in the ECS, such as can happen in the anoxic/ischemic condition, rather than the basis of depolarizing and hyperpolarizing synaptic (IPSP) responses to GABA.

iii. $[Cl^-]_o$ response to anoxia.

Anoxia evoked decreases in $[Cl^-]_o$ in SP and increases in SR, somewhat more substantial but similar to those observed with GABA. There have been few studies in which changes in $[Cl^-]_i$ or $[Cl^-]_o$ during anoxia have been measured with ISMs. Hansen and Zeuthen (1981) recorded small increases of $[Cl^-]_o$ in the cortex *in vivo* during the early phase of cardiac arrest, which preceded the large decrease which accompanies anoxic depolarization and a large increase in $[K^+]_o$ and decrease in $[Ca^{2+}]_o$. An increase may be relevant to our observation of anoxic-induced increase in $[Cl^-]_o$ in the SP of CA1, but would reflect to some extent water shift. Indeed Hansen and Zeuthen calculated that there would be a net influx of Cl^- during the early ischemic period. Therefore the results of their study appear to agree more with the changes we observed in the SR. Our observation of an anoxic-induced increase in $[Cl^-]_o$ in SP (say, arising from GABA-activation of glial receptors) may importantly explain the relative preservation (or 'robustness') of $GABA_A$ monosynaptic IPSPs, which is now known to occur during anoxia (Khazipov et al. 1993; Zhu and Krnjevic 1994), despite early and more selective failure of polysynaptic IPSPs cf EPSPs (Hershkowitz et al. 1993; Rosen and Morris 1993). Chloride influx into neurons likely reflects at least in part a co-transport with Na^+, but may also have a component arising from a Ca^{2+}-activated g_{Cl} or other Cl^- conductance.

IV. Changes in Extracellular Sodium.

i. $[Na^+]_o$ response to GABA.

The time course of decreases in $[Na^+]_o$ in response to bath application of GABA (5 mM) closely resembled those observed for $\Delta[K^+]_o$ and $\Delta[Cl^-]_o$, with respect to onset of change, time to peak, plateau and recovery. The decreases were in the order of 15% and not significantly different in SR compared with SP, after correction for water shifts. There have been very few measurements of GABA-evoked change in $[Na^+]_o$ in the literature. Muller et al. (1989) recorded $\Delta[Na^+]_o$ in response to localized GABA application in CA3 (but not CA1) in some of their experiments; they found that changes in $[Na^+]_o$ closely mirrored changes in $[Cl^-]_o$ – with mainly decreases in SP and increases in SR, and that decreases of $[Na^+]_o$ tended to be larger than decreases in $[Cl^-]_o$. In an *in vivo* study of effects of amino acid applications to the cortex, no or small changes were evoked by iontophoresis of GABA; these were mentioned but not illustrated or quantified (Pumain et al. 1986). As well there have been few measurements of neuronal $[Na^+]_i$ during GABA exposure and no changes were observed (Ballanyi and Grafe 1985; Ballanyi et al. 1984; Böhrle and Sonnhof 1985). Although the possibility that GABA's depolarizing action in dendritic regions may be due to activation of a receptor-linked cation channel has received attention over a number of years (Alger and Nicoll 1982b; Alvoli 1991; Djorup et al. 1981; Perkins & Wong 1997) there has been no direct experimental support. There is, however, substantial evidence for Na- and Cl-dependent uptake for GABA into both neurons and glia and at least one GABA transporter has been cloned (Guastella et al. 1990). Muller et al. (1989) concluded

that the GABA-evoked changes of $[K^+]_o$, $[Cl^-]_o$ and $[Na^+]_o$ in their experiments were together approximately electroneutral. Although for our data $[Na^+]_o$ decreases are similar to $[Cl^-]_o$ decreases, the opposite change with $[Cl^-]_o$ increase is not easily explained, and may point to an involvement of other anions/cations and transmitter uptake and ion transport with assymetrical molecular matching.

ii. $[Na^+]_o$ response to anoxia.

Anoxia also evoked decreases in $[Na^+]_o$ in the SP and SR, with larger changes in the dendritic region and some resemblance to the changes evoked by GABA. As for $[Cl^-]_o$, there have been few measurements of $[Na^+]_o$ during anoxia; the change reported by Hansen and Zeuthen (1981) in their *in vivo* cortical recordings after cardiac arrest was an initial small increase, followed by a large decrease after several minutes, when the major anoxic depolarization occurred. As for $[Cl^-]_o$ they concluded that there was an early net influx of Na^+ into cellular elements in view of the relatively greater ECS shrinkage. In the study of Jiang and Haddad (1992) there was an early decrease in $[Na^+]_o$ in the hypoglossal motoneuron region, which they later attributed to altered activity of a Na^+-dependent transporter (Haddad and Jiang 1993). In later studies in rat central neurons anoxia increased $[Na^+]_i$ from 25 mM to 52 mM after 3-4 min (Friedman and Haddad 1994b).

V. Changes in ECS evoked by GABA and anoxia.

The changes in ECS volume assessed by measuring changes in the concentration of the membrane-impermeant cation, TMA during 5 min exposures to GABA or N_2 were decreases of 4.6 % and 4.8 % with GABA, and 5.3 % and 6.2 % for N_2 for the SP and SR, respectively at the standard recording depth of 100 μm. Overall these changes were between ≈ 5 – 6 %. This finding is in agreement with several other reports, such as that of Morris et al. (1991) in which a change of 6% was recorded for anoxia in the SP region of CA1 under quite similar conditions. Muller et al. (1989) in their studies in guinea-pig hippocampal slices found ECS shrinkage during 30 s microdrop applications of GABA (10 mM) of 5.4 % in SP and 2.6 % in the granule cell layer of the dentate and SR of CA3, but did not report any measurements in CA1. When peak ion levels recorded in the current experiments during applications of GABA or N_2 were adjusted for the average level of ECS shrinkage with each treatment at each location, it was concluded that change in the extracellular volume was insufficient by itself to produce the original deviations from resting level (cf. Tables X and XI). In view of the results of this evaluation no further tests or corrections were carried out for subsequent experiments.

VI. Effects of $GABA_A$ receptor antagonism on ion changes.

i. Responses to GABA.

The specific competitive $GABA_A$ receptor antagonist, bicuculline methiodide (BMI) markedly reduced GABA-evoked $\Delta[K^+]_o$ in both SP and SR, which points to a strong $GABA_A$ receptor-mediated contribution to the K^+ accumulation – an observation in agreement with earlier experiments (Barolet & Morris 1991). $[K^+]_o$ responses in SP appeared slightly more resistant than those in SR (see Table XV), which might be expected in view of previous documentation of a greater ability of bicuculline to block depolarizing responses to GABA. Overall, the attenuation of the GABA-evoked changes in $[K^+]_o$, $[Cl^-]_o$ and $[Na^+]_o$ can be interpreted to indicate that $GABA_A$ receptors importantly mediate the $[K^+]_o$ accumulations in SP and SR and the increase in $[Cl^-]_o$ in SP, and contribute ≥ 50 % of the decrease in $[Cl^-]_o$ in SR and less than one-third of the $\Delta[Na^+]_o$ in both locations. The conclusion that net Cl^- efflux in SP is receptor-mediated suggests an important contribution from depolarization of glia, which have high $[Cl^-]_i$; while changes in SR may mean that the net Cl^- influx reflects partly a neuronal conductance increase and also Cl^- transport uptake (Kaila 1994). Only a portion of the $\Delta[Na^+]_o$ can involve GABA-induced depolarization-dependent Na^+ influx, with the remainder probably being associated with transmitter uptake and ion-co-transport.

ii. Responses to anoxia.

The effects of BMI on ionic changes associated with anoxia are for each ion and location smaller than those seen with GABA. The greatest attenuation observed was of $\Delta[K^+]_o$ in SR, by $\approx$ 70 %, while there was attenuation of 60 % for $\Delta[K^+]_o$ in SP and for $\Delta[Cl^-]_o$ in SP and SR, but only of 20–25% for $\Delta[Na^+]_o$. Although N_2-evoked K^+ accumulations in the presence of different concentrations of BMI were consistently larger in SR cf. SP, the similarity of IC_{50} values (54 cf. 63 μM) suggests that $GABA_A$ receptors of similar sensitivity (affinity for BMI) were affected at the two sites. Even at the highest concentration (300 μM) BMI did not produce a complete block, as could occur if all of each response was mediated by $GABA_A$ receptors. Since one of the major criteria of identification of receptor involvement is response to an antagonist, the effects observed indicate that a portion of the N_2-evoked ion changes is due to activation of $GABA_A$ receptors. Since a pure $GABA_A$ agonist, such as muscimol, was not compared with GABA and there are reports of $GABA_B$ receptors showing BMI sensitivity (Inoue et al. 1985b, 1985c; Liske and Morris 1994) we cannot be certain that the observed effects of BMI are solely due to an effect on $GABA_A$ receptors, although it is likely to be so. Also, since there are GABA receptors which are insensitive to BMI – the $GABA_C$ (or rho subunit $GABA_A$ type) and the $GABA_B$ receptors – there could be a component of the BMI-insensitive residual response due to these receptors.

Of interest is the relatively large block of $\Delta[K^+]_o$ by BMI, especially in the SR. As previously mentioned, the early elevation of $[K^+]_o$ and hyperpolarization of pyramidal neurons in CA1 has been attributed mainly to activation of K^+

conductances (Martin et al. 1994). Our results suggest that part of the K^+ accumulation may come from GABA receptor activation. We propose that this may be due to one or more of the following: (i) a contribution to an increase in $[Ca^{2+}]_i$ and Ca-activated g_K (Connor et al. 1987), (ii) $GABA_A$ receptor-mediated anion conductance and co-transport of K^+, and (iii) $GABA_B$ receptor-mediated g_K.

VII. Intracellular responses to anoxia.

The observation made here of mainly hyperpolarizing responses of pyramidal cells in guinea pig CA1 to brief anoxia agrees with those emphasized by some authors in their reports of studies of rat hippocampus (Fujiwara et al. 1987; Hansen et al. 1982; Leblond and Krnjevic 1989). However, the true incidence of hyperpolarization cf. depolarization in rat hippocampus and some cortical areas appears to be lowerthan is generally believed (Luhmann 1996; Luhman and Heinemann 1992; Morris et al. 1995; Rosen and Morris 1991). In this respect it is interesting that Leblond and Krnjevic (1989) stated that for 15 hippocampal cells which had the highest spike amplitudes, anoxia evoked a net depolarization as often as hyperpolarization (10/21 trials in 15 neurons).

In other experiments, which are not described in this thesis, we have shown that there are depolarizing and hyperpolarizing responses in rat CA1 and adjacent CA2 and subicular regions and that pyramidal cells of the superficial layer of SP, which contain the Ca^{2+} binding protein, calbindin-D_{28k} (CaBP) have a higher incidence of depolarizing response to brief anoxia compared with those of the deep layer, which do not contain CaBP (Morris et al. 1995). We proposed that in the latter neurons unbuffered $[Ca^{2+}]_i$ levels activate a g_K and hyperpolarization. Whether GABA accumulation contributes to such hyperpolarization, via $GABA_A$ or $GABA_B$ receptor activation has not been determined. In agreement with the results of that study, pyramidal cells of the guinea pig hippocampus do not contain CaBP (personal communication, K. G. Baimbridge). Therefore we propose that their high incidence of hyperpolarization following anoxia reflects the absence of this

endogenous buffer. Furthermore, our recordings of hyperpolarization with N_2 and a BMI-sensitive decrease in $[Cl^-]_o$ in the SR (similar to that with GABA) support the idea that a $GABA_A$ receptor-activated Cl^- influx into neurons might also be present.

VIII. $GABA_B$ receptors and changes in $[K^+]_o$ evoked by baclofen.

Our results with brief applications of baclofen importantly extend earlier experiments in which the $[K^+]_o$ changes that would be expected to arise from activation of K^+ channels could not be demonstrated (Loeffler et al. 1982; Sakatani et al. 1994) or were seldom and inconsistently observed in the stratum pyramidale (Barolet and Morris 1991). In the present experiments, cf. those of Barolet and Morris (1991), higher rates of perfusion and shorter exposures were used and recordings were made in a much larger series of experiments and in both SP and SR locations in CA1.

i. Sustained K^+ accumulation.

The sigmoid nature of the concentration-response relationship, which reached a maximum over three logarithmic decades, and the sensitivity of responses to specific antagonists indicate that the $[K^+]_o$ increases are receptor-mediated. The fit of the data with a Hill slope of 1 agrees with the value reported by Sodickson and Bean (1996) for baclofen-evoked K^+ currents in dissociated CA3 pyramidal neurons. $[K^+]_o$ changes were detectable in response to applications of baclofen between 10 and 20 μM and maximal near 100 μM, levels quite similar to those which evoke hyperpolarization (Inoue et al. 1985a, 1985b, 1985c). The larger increases in $[K^+]_o$ in SR compared to SP, although not statistically significant, are consistent with differences in responses described by Newberry and Nicoll (1985) – a trend which may reflect the greater density of receptors in dendritic cf. somatic regions (Bowery et al. 1987; Misgeld et al. 1995). The similar EC_{50} values for SP and SR

concentration-response curves (Fig. 33) indicate that the $GABA_B$ receptors (or subtypes) at the two locations have identical affinities. The close dependence of changes in the field potential (V_F) on the potassium potential (V_K), as shown by significant correlations, with slopes of 0.74 and 0.75 for SP and SR, respectively (Fig. 34), is presumed to reflect membrane hyperpolarization mediated by K^+ outward currents.

The occurrence of decreases in $[K^+]_o$ with low concentrations of baclofen and preceding some increases evoked by higher concentrations was somewhat unexpected and paradoxical. It is unlikely that these decreases reflect inward K^+ current. Small or remotely generated increases in $[K^+]_o$ might not be measurable, because of the dead space created by the electrode tip (Krnjevic and Morris 1975; Lux and Neher 1973), but might arise from stimulated uptake and (or) diffusion gradients. The absence of recovery undershoots, even following the largest increases in $[K^+]_o$, suggests that Na^+-K^+ pump activity is not the cause. One possible explanation is that baclofen may act presynaptically to decrease spontaneous transmitter release and postsynaptic activity and thus the resting level of $[K^+]_o$ (Yoon and Rothman 1991). The heterogeneity of $GABA_B$ receptors (Bonanno and Raiteri 1993; Kaupmann et al. 1997) may also contribute different and as yet unidentified actions. Although the present experiments do not define the exact contribution of $GABA_B$ receptors to GABA-evoked changes in $[K^+]_o$, in contrast with our earlier conclusions (Barolet and Morris 1991), they suggest that there is indeed likely to be some involvement. The fact that K^+ changes evoked in the SP by GABA are much less sensitive to BMI than those induced by the specific

$GABA_A$ agonist, THIP (Barolet and Morris 1991) and that GABA-evoked increases in SR show approximately the same sensitivity to BMI as those in SP suggests that $GABA_B$ receptor activation, especially in the dendrites, contributes to the $[K^+]_o$ accumulation that accompanies sustained exposure to GABA. But it should be noted that changes in membrane potential, resistance and excitability evoked by baclofen can be blocked $\geq 10^{-4}$ μM BMI (Fox et al. 1978; Inoue et al. 1985b, 1985c; Liske and Morris 1994), so that baclofen and (or) $GABA_B$ receptor activated $[K^+]_o$ increase may be underestimated by the residual level. Since the EC_{50} values to produce changes in membrane potential and $[K^+]_o$ are similar both for baclofen and GABA, and $GABA_B$ receptors are activated at lower concentrations of GABA than $GABA_A$ receptors (Inoue et al. 1985c; Jarolimek et al. 1994), changes in $[K^+]_o$ synaptically evoked by GABA are likely to involve $GABA_B$ receptors (Misgeld et al. 1995: Premkumar and Gage 1994; Wagner and Dekin 1993).

The possibility of providing a better estimate of the contribution of $GABA_B$ receptors to GABA-evoked $[K^+]_o$ increases by using a combination of $GABA_A$ and $GABA_B$ receptor antagonists was considered. However, it seems likely that such experiments would be inconclusive. As pointed out above, baclofen effects can be antagonized by $GABA_A$ antagonists. In addition, there are the $GABA_C$ receptors which are expressed in the hippocampus (Bormann and Feigenspan 1995) and are insensitive to both $GABA_A$ and $GABA_B$ antagonists. There are also several subypes of $GABA_B$ receptor, as identified by different agonists and antagonists. Perhaps this question might be addressed by looking for additive effects using low doses of

$GABA_A$ antagonists, before or after a combination of some of the more recently developed $GABA_B$ antagonists.

ii. Recurrent K^+ potentials.

The ability of baclofen to induce synchronous, recurrent phasic field and $[K^+]_o$ potentials is, in spite of claims of anti-epileptic action (Brady and Swann 1984), in agreement with clinical reports that baclofen does not often act as an anti-convulsant and indeed, may be pro-convulsant (Bowery 1982; Rush and Gibberd 1990). Studies which suggest disinhibitory effects include those by Lewis et al. (1989) who found that low doses of baclofen ($\leq$ 1.5 μM) evoked interictal activity; while Swartzwelder et al. (1987) observed that slightly higher doses (2–20 μM) depressed interictal events but promoted ictal activity; and Watts and Jefferys (1993) reported that a low concentration decrased the frequency of 4-AP- induced short bursts but prolonged long bursts. Our results are the first to describe the ability of baclofen to evoke both interictal and ictal-like activity. The fact that there have been few descriptions of baclofen-evoked abnormal synchronous discharges may be in part due to differences in slice preparation. As well, most in vitro studies of the direct effects of baclofen have used brief applications (< 5 min) and low gain recording. In our own study of 4-AP-induced $[K^+]_o$ changes (Morris et al. 1996) we did not observe any increase in resting $[K^+]_o$ or synchronous discharges with exposure to 100 μM baclofen. However, the applications were always made after and during the 4-AP exposure and either blocked or attenuated the 4-AP-evoked activity. The ability of baclofen to block 4-AP responses (Morris et al. 1996;

Siniscalchi and Avoli 1992) and yet produce highly similar synchronous discharges, and of 4-AP to block the hyperpolarizing effect of baclofen (Inoue et al. 1985a) suggest that these compounds may compete for the same receptor or mechanism.

The experiments with baclofen focused solely on $[K^+]_o$ changes and were not extended to examine possible changes in $[Cl^-]_o$ or $[Na^+]_o$. It might have been useful to examine changes in $[Cl^-]_o$ in particular, since we would predict that for baclofen in contrast to GABA the $[Cl^-]_o$ changes might be relatively small and similar in both SP and SR, arising from co-transport with K^+ at both locations.

IX. General Comments and Conclusions.

One phenomenon we observed was that with both GABA and anoxia there were larger changes in $[K^+]_o$, $[TMA^+]_o$ and $[Na^+]_o$ in the SR in contrast to SP, although these were not always significantly different. The larger changes in SR may be attributed to the larger surface area/volume ration of the dendritic arborization as compared with that of the pyramidal somata. In addition, there are large numbers of presynaptic terminals and spine profiles in the SR. It is also worth noting that although the relative distribution of binding sites for $GABA_A$ receptors in the hippocampus is approximately equal for the SR and SP, there is a very low number of sites for $GABA_B$ receptors in the SP (Bowery et al. 1987). The low Na-K pump activity in CA1 may have greater effects at the dendrites cf. somata, to allow greater accumulation of $[K^+]_o$ and and an increased release of transmitters, especially during anoxia (Haglund and Schwartzkroin 1990). Our observations are consistent with the earliest anoxic/ischemic events being at the synapses rather than the soma (Grossman and Williams 1971; Kass and Lipton 1982) and the suggestion of Silver and Erecinska (1990) that Ca^{2+} increases at synapses are larger than in the soma, with subsequent activation of g_K.

The present study was limited mainly to measurement of changes in $[K^+]_o$ and $[Cl^-]_o$, with some observations on $[Na^+]_o$ with a view to assessing and comparing responsesto anoxia with those to exogenous GABA. Although it might have been of interest to measure HCO^-_3 since the $GABA_A$ receptor-linked channel has some permeability (≈ 0.2) to this ion, the technique requires measurement of $[H^+]_o$ and we felt that interpretation of anoxic-evoked changes would be difficult since anoxia

evokes acidosis and GABA produces an extracellular alkalosis (Chen and Chesler 1992; Kaila 1994; Kaila et al. 1992). Some studies of $\Delta[H^+]_o$ with anoxia have previously been made in CA1 in the rat hippocampus by Krnjevic and Walz (1990), who recorded a transient increase followed by progressive reduction in pH_o of 0.06 ± 0.005 unit/min at 34–35°C, similar changes in SP and SR, and little contribution from lactate transport. They suggested that an early fall in pH_i may initiate an increase in $[Ca^{2+}]_i$, which then activates g_K. Extrapolation from their data indicates that the magnitude of change in pH which we might have observed in our experiments could have been in the order of ≤ 0.1 pH unit at 33 ° C with 4–5 min of anoxia.

Although our results point out similarities of the recorded ionic responses to GABA and N_2 and attenuation by a $GABA_A$ receptor antagonist, we cannot (and did not intend to) imply or be certain that GABA accumulation in the ECS in CA1 with brief anoxia would be at the same level and evoke the same magnitude of responses as our exogenous application of 5 mM GABA. Bath applications of ≤ 1 mM GABA evoke large hyperpolarizations (and sometimes mixed depolariztion and hyperpolarization) within 1–2 min of slice superfusion (Inoue et al. 1985c). The concentration achieved at the recording site in the slice after 5 min perfusion of 1–5 mM GABA might be estimated at ≤ 100–200 μM, given the fact that steady-state equilibrium of perfused electrolytes is achieved only within 20-30 min (Morris et al. 1983). Ischemic-induced changes in GABA levels measured with microdialysis *in vivo* in mammalian hippocampus are in the order of ≤ 5–50 μM at 10–30 min – a figure, which when corrected for recovery capacity and an assumption of linear

increase with time, may represent a level of ≤ 50 μM at 5 min (Hagberg et al. 1985; Ravindran et al. 1994; Schwartz et al. 1995; Torp et al. 1993). Actual levels released at the synapse would likely be higher due to uptake and diffusion. These calculations suggest that the GABA levels with the two treatments might be somewhat comparable, but one could only know by making direct measurements.

It is widely recognized that anoxic depolarization is the critical trigger of neuronal cell death and that avoidance of its onset or attenuation can protect vulnerable regions (Martin et al. 1994). Although small ionic shifts, such as the increase in $[K^+]_o$ with brief anoxia, precede the large ion movements associated with anoxic depolarization (cf. also spreading depression) they are not necessarily the causative factor of the larger changes. The early changes may reflect compensatory mechanisms which have some protectant capacity, and it is proposed that some of this includes actions of GABA - e.g. to promote/enhance activation of g_K and g_{Cl}, cause hyperpolarization and decrease excitability and energy requirements. Although there have been some reports that GABA exacerbates glutamate-induced excitotoxicity and ischemic-induced cell death, this is limited exclusively to embryonic and neonatal culture studies (Erdo et al. 1991; Muir et al. 1996). In contrast, there is increasing evidence from *in vivo* studies of the abiiity of $GABA_A$ agonists, such as muscimol and THIP and GABA-potentiating drugs, such as steroids, benzodiazepines, barbiturates, and chlormethiazole as well as uptake inhibitors, such as tiagibine and others and several GABA-transaminase inhibitors to prevent functional changes and neuronal cell death in ischemic models (Abramowicz et al. 1991; Green and Cross 1994, 1997; Johansen and Diemer

1991; Kass et al. 1992; Lyden 1997; Schwartz et al. 1995; Shuaib et al. 1993; Sternau et al. 1988; Suzdak and Jansen 1995; Thompson 1994), while bicuculline has been shown to worsen outcome (Madden 1994). Several studies have been able to show that enhancement of GABA mechanisms can provide protection and prevent loss of GABA binding (Kato et al. 1993; Schwartz et al. 1995), even when administered at varying intervals after ischemia.

We have shown that during the very early response to anoxia GABA evokes ionic changes, which suggest a role for elevation of extracellular levels of GABA and activation of $GABA_A$ and $GABA_B$ receptors. It is proposed that these may constitute and contribute part of an initial compensatory and protectant response of the CNS, which when potentiated or sustained might delay excitotoxicity. Since the balance between excitation and inhibition appears to be a critical factor in determining selective neuronal viability (Globus et al. 1990; 1991), a role for drugs which can enhance GABA actions (including GABA-evoked ionic changes) as adjuncts with glutamate antagonists (and/or other treatments) seems an important rational direction for combination anti-ischemic therapy (Bullock and Fujisawa 1992; Lyden 1997; Lyden et al. 1995).

SUMMARY

1. The object of the research was to determine a potential role for the inhibitory neurotransmitter, GABA (γ-aminobutyric acid) in the generation of ionic responses to anoxia of one of the most vulnerable regions of the CNS, the CA1 hippocampal area.
2. Experiments were carried out *in vitro*, using ion-selective microelectrodes (ISMs) to record changes in $[K^+]_o$, $[Cl^-]_o$ and $[Na^+]_o$ evoked in stratum pyramidale (SP) and stratum radiatum (SR) of CA1 of guinea pig hippocampal slices during applications of agonists and antagonists for GABA receptors, and with anoxic episodes.
3. Bath applications of 1–10 mM GABA for 5 min caused concentration-dependent changes in $[K^+]_o$ and $[Cl^-]_o$ in SP and SR. For $[K^+]_o$ only increases were noted; with EC_{50} of 3.8 and 4.7 mM for SP and SR, respectively. For $[Cl^-]_o$ there were increases in SP and decreases in SR; with EC_{50} of 4.1 and 5.6 mM. $[Na^+]_o$ responses to 5 mM GABA were decreases in both SP and SR.
4. Ionic changes induced by GABA (5 mM perfusion for 5 min) were compared to those evoked by brief anoxia (replacement of O_2 by N_2 in the ACSF equilibration gas mixture for 5 min). The changes induced by anoxia were very similar to those observed with GABA: in SP increases in $[K^+]_o$ and $[Cl^-]_o$

and a decrease in $[Na^+]_o$, and in SR an increase in $[K^+]_o$ and decreases in $[Cl^-]_o$ and $[Na^+]_o$ and of the same or greater magnitude. Changes in SR were greater than those in SP; these were significantly different, with the exception of the $\Delta [K^+]_o$ evoked by GABA.

5. In order to estimate the magnitude and influence of water shifts, variations in the concentration of the membrane-impermeant ion, TMA^+ (tetramethylammonium ion) were recorded with ISMs during GABA and N_2 exposures. Increases in $[TMA^+]_o$ were induced by both anoxia and GABA. The changes were ≈ three-fold greater at depths of 100 and 150 µm as compared to 50 µm. Volume decreases were significantly greater in SR than in SP at the standard recording depth of 100 µm: with N_2 they were 5.3 ± 0.3 and 6.2 ± 0.8 % in SP and SR, respectively; and with GABA 4.6 ± 0.2 and 4.8 ± 0.3 %.

6. Without correction for changes in the volume of extracellular space (ECS) absolute increases in $[K^+]_o$ and $[Cl^-]_o$ may be overestimated, while decreases in $[Cl^-]_o$ and $[Na^+]_o$ would be underestimated. When corrections were applied, the reduction of ECS was determined to be insufficient to account for the ionic changes observed.

7. The $GABA_A$ receptor antagonist, BMI (bicuculline methioidide) (100µM) reversibly attenuated all ion changes evoked by both 5 mM GABA and N_2. In SP BMI depressed increases in $[K^+]_o$ and $[Cl^-]_o$ with GABA by 90 % and with N_2 by 50–60 %. In SR K^+ accumulation during GABA exposure was blocked and with anoxia attenuated by 70%; decreases in $[Cl^-]_o$ were

depressed by more than 50 %. $[Na^+]_o$ changes in SP and SR were affected to a lesser extent, being decreased by only 20–30 %. The IC_{50} values for attenuation of GABA-evoked $\Delta[K^+]_o$ by BMI (1–300 μM) were 63 and 54 μM for SP and SR, respectively.

8. The similarity of the GABA- and N_2-induced ion changes and their attenuation by BMI suggested that $GABA_A$ receptor activation during anoxic episodes may contribute to the early ionic events associated with anoxia.

9. The $GABA_B$ agonist, baclofen (10^0–3×10^{-3} μM) evoked dose-dependent increases in $[K^+]_o$ in SP and SR, with EC_{50} values of 40 and 39 μM, respectively. The sigmoid nature of the concentration-response curves and sensitivity of responses to the $GABA_B$ antagonists indicate that the $\Delta[K^+]_o$ may directly reflect a receptor-mediated increase in g_k and K^+ efflux. The similarity of EC_{50} values for SP and SR supports the idea that $GABA_B$ receptors at the two locations are identical.

10. The known higher affinity of $GABA_B$ receptors compared with $GABA_A$ receptors suggests that an early component of anoxic-evoked K^+ accumulation may be secondary to activation of both $GABA_A$ and $GABA_B$ receptors. This and the observation that $[K^+]_o$ increases induced by N_2 can be augmented by a $GABA_B$ antagonist, point to a potentially protectant action of GABA acting via its B receptors to decrease presynaptic glutamate release and produce postsynaptic hyperpolarization.

11. These data support (i) the hypothesis that early extracellular ion changes induced during anoxia may arise, at least in part, from a synaptic

accumulation of GABA and activation of both $GABA_A$ and $GABA_B$ receptors, and (ii) the potential of agonists and (or) modulators of GABA-ergic transmission in pharmacological interventions for global ischemia.

Table I. Ion-selective microelectrodes and ionophores used in experiments.

Electrode / Ion Measured	Ionophore	Supplier / Catalogue Number	Membrane Composition (Wt %)
Sodium	Sodium Ionophore II - Cocktail A	Fluka 71178	10.0 % ETH 157[a] 89.5 % o-NPOE[b] 0.5 % sodium tetraphenylborate
Potassium	Potassium Ionophore I - Cocktail B	Fluka 60398	5.0 % valinomycin 93.0 % 1,2-dimethyl-3 nitrobenzene 2.0 % potassium tetrakis(4-chlorophenyl)borate
Chloride	Chloride Ionophore I - Cocktail A	Fluka 24902	5.0 % Mn(III)TPPCl[c] 4.0 % 1-decanol 1.0 % ETH 500[d] 90.0 % o-NPOE
Tetramethyl-ammonium	Potassium Ion Exchanger	Corning 477317	potassium tetrakis(p-chlorophenyl)borate 2,3-dimethyl-nitrobenzene

[a]ETH 157 - Eidgenössische Technische Höchschule formulation #157 - N,N[1]-dibenzyl-1,2-phenylenedioxydiacetamide.

[b]o-NPOE - 2-nitrophenyloctylether.

[c]Mn(III)TPPCl - 5,10,15,20-tetraphenyl-21H,23H-porphin manganese (III) chloride.

[d]ETH 500 - tetradecylammonium tetrakis(4-chlorophenyl)borate.

Table II. Selectivity factors (log K_{ij}^{Pot}) of ion-selective microelectrodes for different ions.

Interfering Ion ($_j z_j$)	Measured Ion and Ionophore			
	K^+ Corning 477317	K^+ Fluka 60398	Na^+ Fluka 71178	Cl^- Fluka 24902
Na^+	–1.9[a] –2.8[b]	–3.6[b] –3.9[c]		
K^+			–0.4[d]	
H^+	–0.5[a]	–4.4[a]		
Mg^{2+}	–2.7[a] –3.0[b]	–3.0[b] –4.6[c]	–3.4[d]	
Ca^{2+}	–2.1[a] –3.1[b]	–3.4[b] –4.9[c]	–1.3[d]	
acetylcholine$^+$	2.7[a] 3.1[b]	–1.3[b] –3.5[c]	–1.6[d]	
$TMA^+[(CH_3)_4N^+]$	2.7[a]	–3.5[a]		
HCO^-_3				–1.5[e]
HPO_4^{2-}				–2.9[e]
SO_4^{2-}				–2.6[e]
acetate$^-$				–1.3[e]
gluconate$^-$				–2.4[e]
lactate$^-$				–0.96[e]

[a] Oehme and Simon 1976
[b] Krnjevic et al. 1982
[c] Ammann et al. 1987
[d] Ammann and Anker 1985
[e] Kondo et al. 1989

Selectivity testing for *b* was carried out with the fixed interference method (FIM) (with 0.1 or 0.15 M chloride solution or sodium salt of interfering ion); while for *a,c,d* and *e* the separate solutions method (SSM) (with 0.1 M chloride or sodium salt solutions for ion to be measured and interfering ion) was used.

Table III. Selectivity factors (log K_{ij}^{Pot}) of ion-selective microelectrodes for different compounds.

Compound ($_j{}^{z_j}$)	Ion and Ionophore			
	K^+ Corning 477317	K^+ Fluka 60398	Na^+ Fluka 71178	Cl^- Fluka 24902
APV	-	< - 3.0	-	-
Baclofen	< - 2.0[a]	< - 3.0	-	-
BMI	+ 0.8[a]	< - 3.0	< - 3.0[b]	< - 3.0[b]
CGP 35348	-	< - 3.0[b]	-	-
CNQX	-	< - 3.0	-	-
GABA	< - 3.0[a]	< - 3.0[b]	< - 3.0[b]	< - 3.0[b]
Kynurenate	-	< - 3.0[b]	-	-
Saclofen	-	< - 3.0[b]	-	-
THIP	< - 3.0[a]	-	-	-

[a] - Data from Barolet and Morris 1991.

[b] - Fixed primary ion method (FPM); all other values obtained with fixed interference method (FIM).

Table IV. List of compounds used in experiments and their sources.

Drug	Supplier
ACSF constituents	Fisher Scientific
DL-baclofen	Ciba-Geigy
bicuculline methiodide (BMI)	Sigma
CGP 35348	Ciba-Geigy
gamma-aminobutyric acid (GABA)	Sigma
kynurenate	Sigma
saclofen	Ciba-Geigy
2-OH saclofen	Ciba-Geigy
tetramethylammonium chloride (TMA)	Sigma

Table V. **Depressant effects of GABA (bath application for 5 min) on population spikes evoked by 1/s stimulation of Schaffer collateral fibers and recorded in stratum pyramidale (SP).**

GABA Concentration (mM)	% Depression in SP
1	9.1 ± 0.0 (n = 2)
2	23.0 ± 2.4 (n = 5)
3	29.0 ± 4.0 (n = 2)
5	58.4 ± 3.5 (n = 4)
6	66.0 ± 4.6 (n = 5)
10	100.0 ± 0.0 (n = 6)

Values are mean ± SE; n = number of observations, recorded in a total of 6 slices.

Depth of recording 100 µm.

Table VI. Effects of 5 mM GABA and anoxia on $[K^+]_o$ in stratum pyramidale (SP) and stratum radiatum (SR).

	GABA		N_2	
	SP	SR	SP	SR
Baseline $[K^+]_o$ (mM)	3.41 ± 0.14 (n = 10)	3.53 ± 0.21 (n = 11)	3.55 ± 0.31 (n = 9)	3.67 ± 0.37 (n = 12)
Peak $[K^+]_o$ (mM)	3.90 ±0.14† (n = 10)	4.18 ± 0.35† (n = 11)	4.56 ± 0.48† (n = 9)	6.86 ± 0.51† (n = 12)
$\Delta[K^+]_o$ (mM)	0.49 ± 0.08 (n = 10)	0.65 ± 0.07 (n = 11)	1.01 ± 0.22 (n = 9)	3.18 ± 0.17* (n = 12)

Values are mean ± SE; n = number of observations, recorded in a total of 12 slices.

Depth of recording 100 µm.

$^\dagger p \leq 0.0001$ for comparison between baseline and peak for each column (paired t-test).

$^* p \leq 0.001$ for comparison of $\Delta[K^+]_o$ between SR and SP within a single type of treatment (unpaired t-test).

Table VII. Effects of 5 mM GABA and anoxia on $[Cl^-]_o$ in stratum pyramidale (SP) and stratum radiatum (SR).

	GABA		N_2	
	SP	SR	SP	SR
Baseline $[Cl^-]_o$ (mM)	131.0 ± 9.3 (n = 14)	138.0 ± 10.9 (n = 8)	132.4 ± 16.2 (n = 10)	138.0 ± 9.9 (n = 9)
Peak $[Cl^-]_o$ (mM)	153.0 ± 7.5† (n = 14)	123.0 ± 12.7† (n = 8)	157.4 ± 17.0† (n = 10)	105.0 ± 14.9† (n = 9)
$\Delta[Cl^-]_o$ (mM)	21.3 ± 2.0 (n = 14)	−14.7 ± 0.9* (n = 8)	25.06 ± 2.7 (n = 10)	−33.8 ± 5.0* (n = 9)

Values are mean ± SE; n = number of observations, recorded in a total of 14 slices.

Depth of recording 100 µm.

†$p \leq 0.0001$ for comparison between baseline and peak for each column (paired t-test).

*$p \leq 0.0001$ for comparison of $\Delta[Cl^-]_o$ between SR and SP within a single type of treatment (unpaired t-test).

Table VIII. Effects of 5 mM GABA and anoxia on $[Na^+]_o$ in stratum pyramidale (SP) and stratum radiatum (SR).

	GABA		N_2	
	SP	SR	SP	SR
Baseline $[Na^+]_o$ (mM)	149.0 ± 8.5 (n = 8)	155.0 ± 7.0 (n = 8)	154.0 ± 7.4 (n = 9)	153.0 ± 4.0 (n = 9)
Peak $[Na^+]_o$ (mM)	132.0 ± 9.4† (n = 8)	132.2 ± 12.0† (n = 8)	129.0 ± 7.5† (n = 9)	121.0 ± 10.4† (n = 9)
$\Delta[Na^+]_o$ (mM)	–17.0 ± 0.7 (n = 8)	–23.5 ± 2.2* (n = 8)	–25.2 ± 0.5 (n = 9)	–32.3 ± 2.6* (n = 9)

Values are mean ± SE; n = number of observations, recorded in a total of 9 slices.

Depth of recording 100 µm.

†$p \leq 0.0001$ for comparison between baseline and peak for each column (paired t-test).

*$p \leq 0.05$, for comparison of $\Delta[Na^+]_o$ between SR and SP within a single type of treatment (unpaired t-test).

Table IX **Percentage change in extracellular space (ECS) evoked by 5 mM GABA and by anoxia at different depths of recording in stratum pyramidale (SP) and stratum radiatum (SR).**

		50 µm	100 µm	150 µm
SP	GABA	1.17 ± 0.13 (n = 4)	4.64 ± 0.23 (n = 7)	4.73 ± 0.40 (n = 3)
	N_2	1.50 ± 0.28 (n = 4)	5.28 ± 0.28 (n = 7)	5.50 ± 0.28 (n = 3)
SR	GABA	1.45 ± 0.25 (n = 2)	4.75 ± 0.25* (n = 6)	5.0 ± 0.11 (n = 3)
	N_2	1.83 ± 0.44 (n = 3)	6.16 ± 0.80* (n = 6)	6.6 ± 0.30 (n = 3)

Values are mean ± SE.

n = number of observations, measured at different depths in 3 groups of slices (n=4, 7 and 3 different slices for 50, 100 and 150 µm depth recordings, respectively.

*$p < 0.05$ for comparison between SR and SP (paired observations) within a single type of treatment.

Calculations from changes in $[TMA^+]_o$ which were recorded with an ISM containing the Corning 477317 ion exchanger in the presence of 3 mM TMA in the bath perfusate, using equation [4] (see text).

Table X. **Summary of uncorrected ion changes evoked by 5 mM GABA and anoxia in stratum pyramidale (SP) and stratum radiatum (SR).**

		Stratum Pyramidale	Stratum Radiatum
$\Delta[K^+]_o$ (mM)	GABA	+0.49 ± 0.08 (n = 10)	+0.65 ± 0.07 (n = 11)
	N_2	+1.01 ± 0.21 (n = 9)	+3.18 ± 0.17** (n = 12)
$\Delta[Cl^-]_o$ (mM)	GABA	+21.3 ± 2.0 (n = 14)	–14.7 ± 0.9** (n = 8)
	N_2	+25.1 ± 2.7 (n = 10)	–33.8 ± 5.0** (n = 9)
$\Delta[Na^+]_o$ (mM)	GABA	–17.0 ± 0.7 (n = 8)	–23.5 ± 2.2* (n = 8)
	N_2	–25.2 ± 0.5 (n = 9)	–32.3 ± 2.6* (n = 9)

Data from Tables VI, VII and VIII.

Values are mean ± SE; n = number of observations, recorded in a total of 35 slices.

Depth of recording 100 µm.

*$p \leq 0.05$; **$p \leq 0.001$ for comparison of $\Delta[ion]_o$ between SR and SP within a single type of treatment (unpaired t-test).

Note: paired t-test evaluation of data within each group shows significant change from baseline, $p \leq 0.0001$.

Table XI. Ion changes evoked by 5 mM GABA and anoxia and corrected for extracellular space shrinkage.

		Stratum Pyramidale	Stratum Radiatum
$\Delta[K^+]_o$ (mM)	GABA	+0.27 ± 0.11 (n = 10)	+0.45 ± 0.20 (n = 11)
	N_2	+0.77 ± 0.22 (n = 9)	+2.77 ± 0.35* (n = 12)
$\Delta[Cl^-]_o$ (mM)	GABA	+14.7 ± 4.0 (n = 14)	–20.3 ± 2.2* (n = 8)
	N_2	+16.8 ± 4.4 (n = 10)	–40.1 ± 13.0* (n = 9)
$\Delta[Na^+]_o$ (mM)	GABA	–23.2 ± 1.8 (n = 8)	–29.8 ± 5.9 (n = 8)
	N_2	–31.6 ± 2.6 (n = 9)	–39.7 ± 6.4 (n = 9)

Changes calculated using values from column 2 of Table IX to correct individual values for data summarized in Tables VI, VII and VIII and resummarized in Table X. Values are mean ± SE; n = number of observations, recorded in a total of 35 slices. Depth of recording 100 μm.

*$p \leq 0.001$ for comparison of $\Delta[ion]_o$ between SR and SP within a single type of treatment (unpaired t-test).

Note: paired t-test evaluation of data within each group shows significant change from baseline, $p \leq 0.0005 - 0.0001$.

Table XII. Effects of bicuculline methiodide (BMI) (100 μM) on $\Delta[K^+]_o$ evoked by GABA and anoxia in stratum pyramidale (SP) and stratum radiatum (SR).

	GABA		N_2	
	SP	SR	SP	SR
Baseline $[K^+]_o$ (mM)	3.4 ± 0.2 (n = 5)	3.5 ± 0.1 (n = 6)	3.6 ± 0.2 (n = 4)	3.5 ± 0.1 (n = 5)
$\Delta[K^+]_o$ – Control (mM)	0.48 ± 0.1 (n = 5)	0.53 ± 0.1 (n = 6)	0.81 ± 0.2 (n = 4)	3.10 ± 0.1†† (n = 5)
$\Delta[K^+]_o$ – with BMI (mM)	0.03 ± 0.1*** (n = 5)	0.0 ± 0.0** (n = 6)	0.23 ± 0.2* (n = 4)	0.95 ± 0.1***† (n = 5)

Values are mean ± SE; n = number of observations, recorded in a total of 6 slices.

Depth of recording 100 μm.

$^{*}p \leq 0.001$; $^{**}p \leq 0.0005$; $^{***}p \leq 0.0001$ for comparison of $\Delta[K^+]_o$ with and without BMI for each column (paired t-test).

$^{\dagger}p \leq 0.01$; $^{\dagger\dagger}p \leq 0.0001$ for comparison of $\Delta[K^+]_o$ in SP and SR for each treatment — GABA or N_2 (unpaired t-test).

Table XIII. Effects of bicuculline methiodide (BMI) (100 μM) on $\Delta[Cl^-]_o$ evoked by GABA and anoxia in stratum pyramidale (SP) and stratum radiatum (SR).

	GABA		N_2	
	SP	SR	SP	SR
Baseline $[Cl^-]_o$ (mM)	131.0 ± 3.1 (n = 6)	137.0 ± 1.3 (n = 3)	135.0 ± 2.8 (n = 4)	139.0 ± 7.8 (n = 4)
$\Delta[Cl^-]_o$ – Control (mM)	20.1 ± 6.5 (n = 6)	–15.5 ± 1.7 (n = 3)	32.1 ± 6.7 (n = 4)	–24.6 ± 3.7† (n = 4)
$\Delta[Cl^-]_o$ – with BMI (mM)	2.8 ± 1.3 (n = 6)	–7.3 ± 1.3 (n = 3)	14.6 ± 3.1 (n = 4)	–11.6 ± 3.5† (n = 4)

Values are mean ± SE; n = number of observations, recorded in a total of 6 slices.

Depth of recording 100 μm.

†$p \leq 0.0001$ for comparison of $\Delta[Cl^-]_o$ in SP and SR for each treatment — GABA or N_2 (unpaired t-test).

Table XIV. Effects of bicuculline methiodide (BMI) (100 μM) on $\Delta[Na^+]_o$ evoked by GABA and anoxia in stratum pyramidale (SP) and stratum radiatum (SR).

	GABA		N_2	
	SP	SR	SP	SR
Baseline $[Na^+]_o$ (mM)	146.0 ± 8.8 (n = 3)	150.0 ± 1.8 (n = 3)	143.0 ± 3.2 (n = 3)	150.0 ± 2.0 (n = 3)
$\Delta[Na^+]_o$ – Control (mM)	–17.3 ± 0.5 (n = 3)	–28.6 ± 6.3 (n = 3)	–25.6 ± 0.6 (n = 3)	–41.3 ± 1.5†† (n = 3)
$\Delta[Na^+]_o$ – with BMI (mM)	–11.2 ± 1.2 (n = 3)	–19.6 ± 3.7 (n = 3)	–20.2 ± 1.4 (n = 3)	–29.6 ± 3.2† (n = 3)

Values are mean ± SE; n = number of observations, recorded in a total of 3 slices.

Depth of recording 100 μm.

$^{†}p \leq 0.05$; $^{††}p \leq 0.001$ for comparison of $\Delta[Na^+]_o$ in SP and SR for each treatment — GABA or N_2 (unpaired t-test).

Table XV. Summary of effects of BMI (bicuculline methiodide, 100 µM) on ion changes evoked by 5 mM GABA and by anoxia in stratum pyramidale (SP) and stratum radiatum (SR).

		SP	SR
% of control $\Delta[K^+]_o$	GABA	6.6 ± 0.9 (n = 5)	0.0*** (n = 6)
	N_2	42.5 ± 2.1 (n = 4)	28.2 ± 1.6** (n = 5)
% of control $\Delta[Cl^-]_o$	GABA	12.5 ± 6.0 (n = 6)	45.3 ± 6.0 (n = 3)
	N_2	47.6 ± 2.4 (n = 4)	44.7 ± 3.9 (n = 4)
% of control $\Delta[Na^+]_o$	GABA	67.5 ± 6.2 (n = 3)	68.8 ± 1.6 (n = 3)
	N_2	79.2 ± 2.7 (n = 3)	72.5 ± 2.5 (n = 3)

Calculations from data of Tables XII, XIII and XIV.

Values are mean ± SE; n = number of observations, recorded in a total of 15 slices.

*$p \leq 0.01$; **$p \leq 0.001$; ***$p \leq 0.0001$ for comparison of % change in SP and SR for each row.

Table XVI. Concentration-dependent effects of baclofen on $[K^+]_o$ in stratum pyramidale (SP) and stratum radiatum (SR).

[baclofen]	$\Delta[K^+]_o$ (mM)	
	SP	SR
1 µM	0.0 ± 0.0 (n=5)	0.0 ± 0.0 (n=4)
10 µM	0.09 ± 0.04 (n=6)	0.11 ± 0.01 (n=5)
20 µM	0.19 ± 0.05 (n=8)	0.23 ± 0.02 (n=6)
50 µM	0.27 ± 0.07 (n=6)	0.30 ± 0.05 (n=6)
100 µM	0.47 ± 0.09 (n=10)	0.51 ±0.12 (n=9)
1 mM	0.58 ± 0.09 (n=3)	0.60 ± 0.11 (n=3)
3 mM	0.59 ± 0.10 (n=3)	0.65 ± 0.08 (n=3)

Values are mean ± SE; n = number of observations, recorded in a total of 10 slices.

Table XVII. Effects of anoxia on membrane parameters recorded intracellularly from neurons in CA1 (n = 12 cells, from 10 slices/animals).

V_M (mV)	$\Delta V_{H(N_2)}$ (mV)	$\Delta V_{D(N_2)}$ (mV)	ΔV_{PAH} (mV)	R_M (MΩ)	R_{N_2} (MΩ)
–64.9 ± 1.7 (n=16)	–5.1 ± 4.5 (n=15)	+5.0 ± 1.1 (n=10)	–12.3 ± 2.2 (n=16)	44.9 ± 2.6 (n=16)	40.3 ± 3.2 (n=16)

V_M — resting membrane potential or control potential with hyperpolarizing current.

$V_{H(N_2)}$ — N_2-evoked hyperpolarization.

$V_{D(N_2)}$ — N_2-evoked depolarization.

V_{PAH} — post-anoxic hyperpolarization.

R_M — resting/control membrane resistance.

R_N — membrane resistance during N_2 exposure.

n — number of observations.

Figure 1. **Hippocampal slice.** Immunohistochemical staining for calbindin-D_{28K} shows immunopositive pyramidal cell bodies and dendrites of CA1, CA2 (between CA1 and CA3), and dentate gyrus (DG) as well as the mossy fibres. SO – stratum oriens; SP – stratum pyramidale; SR – stratum radiatum; LM – lacunosum moleculare; PP – perforant pathway; GC – granule cell layer; H – hilus. Photomicrograph from rat brain, reproduced with permission of Dr. K.G. Baimbridge.

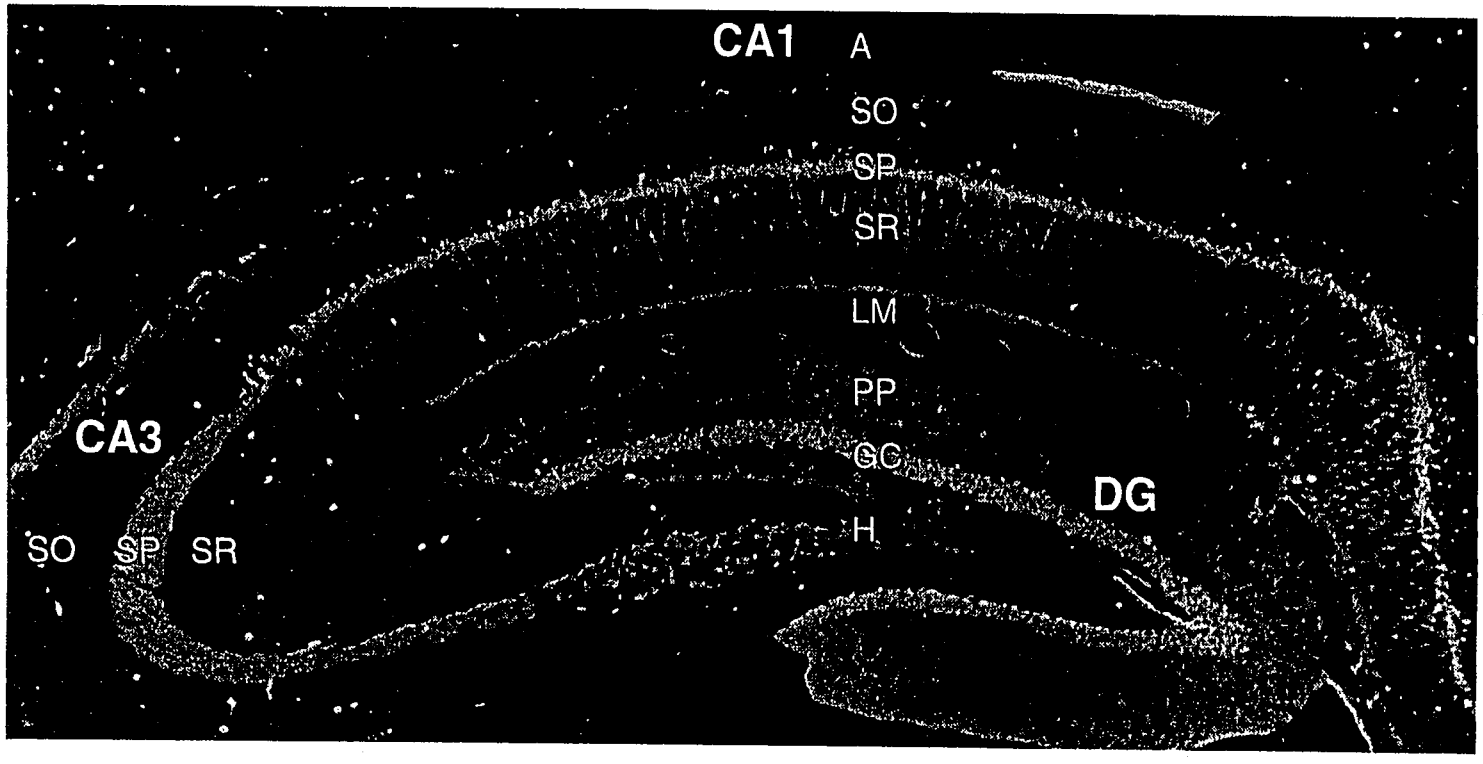
CA1
A
SO
SP
SR
LM
PP
GC
H
DG
CA3
SO
SP
SR

Figure 2. **Slice recording chamber (BSC–HT).** Haas-type interface chamber (Model BSC–HT, Medical Systems Inc.) is connected to inflow lines from ACSF reservoirs via eight-way valve (see diagram of Fig. 4) and secured above lower chamber (which contains distilled water and heater element for warming of perfusant and humidification of gas supply — see Fig. 3). Modified and reproduced with permission from instruction manual of manufacturer, Medical Systems Inc.

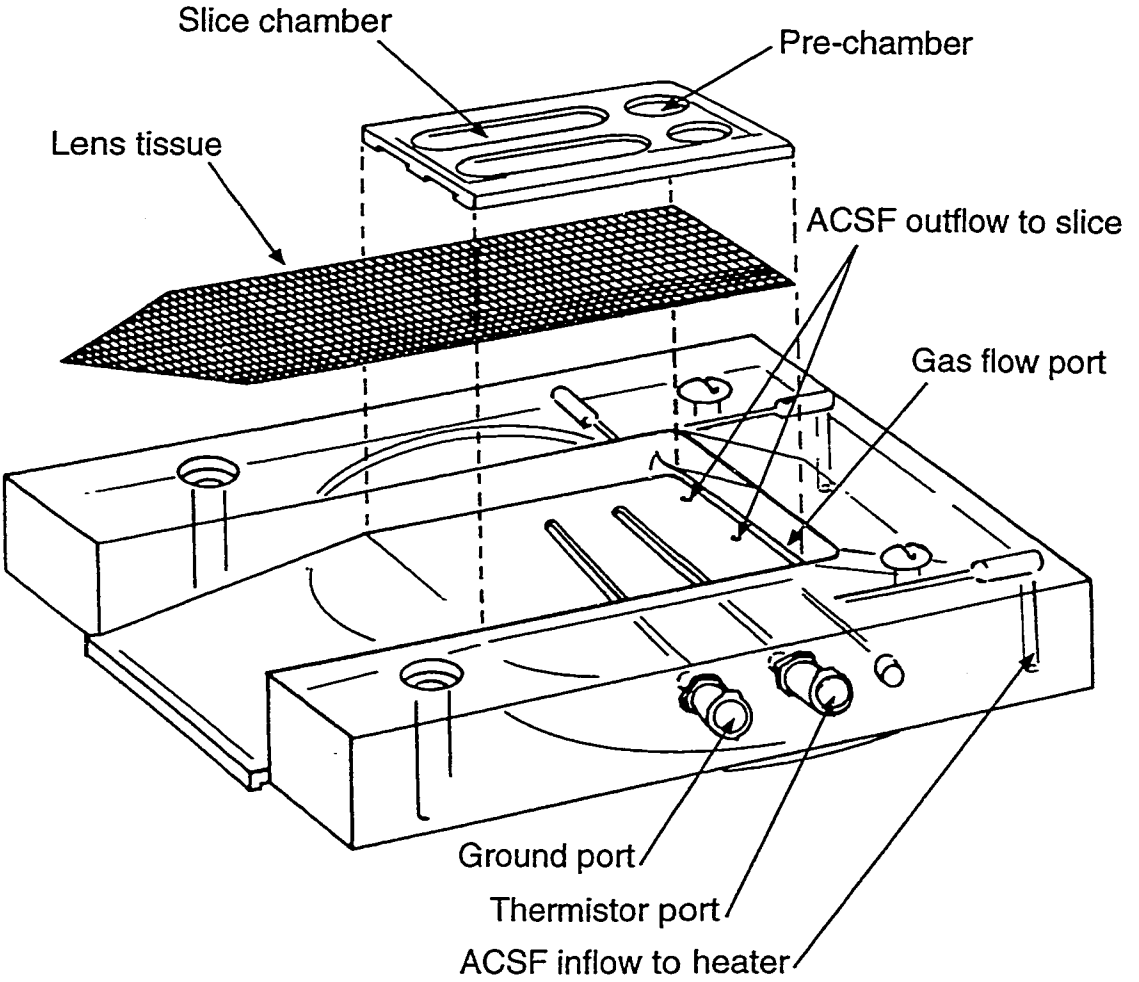
Slice chamber
Pre-chamber
Lens tissue
ACSF outflow to slice
Gas flow port
Ground port
Thermistor port
ACSF inflow to heater

Figure 3. **Base unit (BSC–BU) for heating and humidification.** Photograph of complete assembly (see legend of Fig. 2) reproduced with permission from instruction manual for Model BSC-HT, Medical Systems Inc.

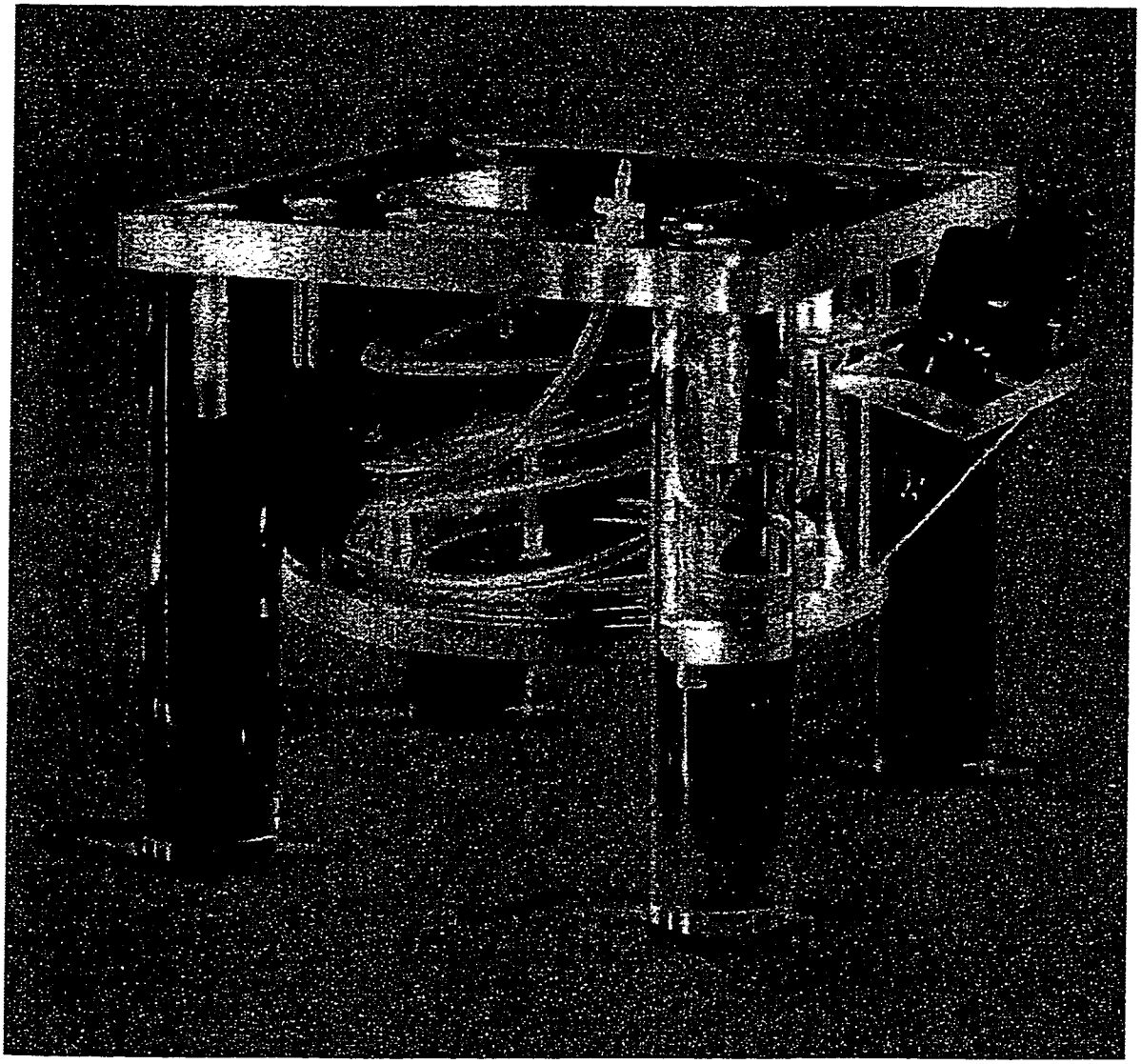

Figure 4. **Diagram of bath perfusion system.** A1–A6: reservoirs for ACSF solutions. S1–S6: switches for 12 V activation of V1–V6 solenoid valves to allow selection of flow through eight-way connector to measuring chamber. Diagram modified from original in Fig. 2 of Barolet et al. 1989, and reproduced with permission.

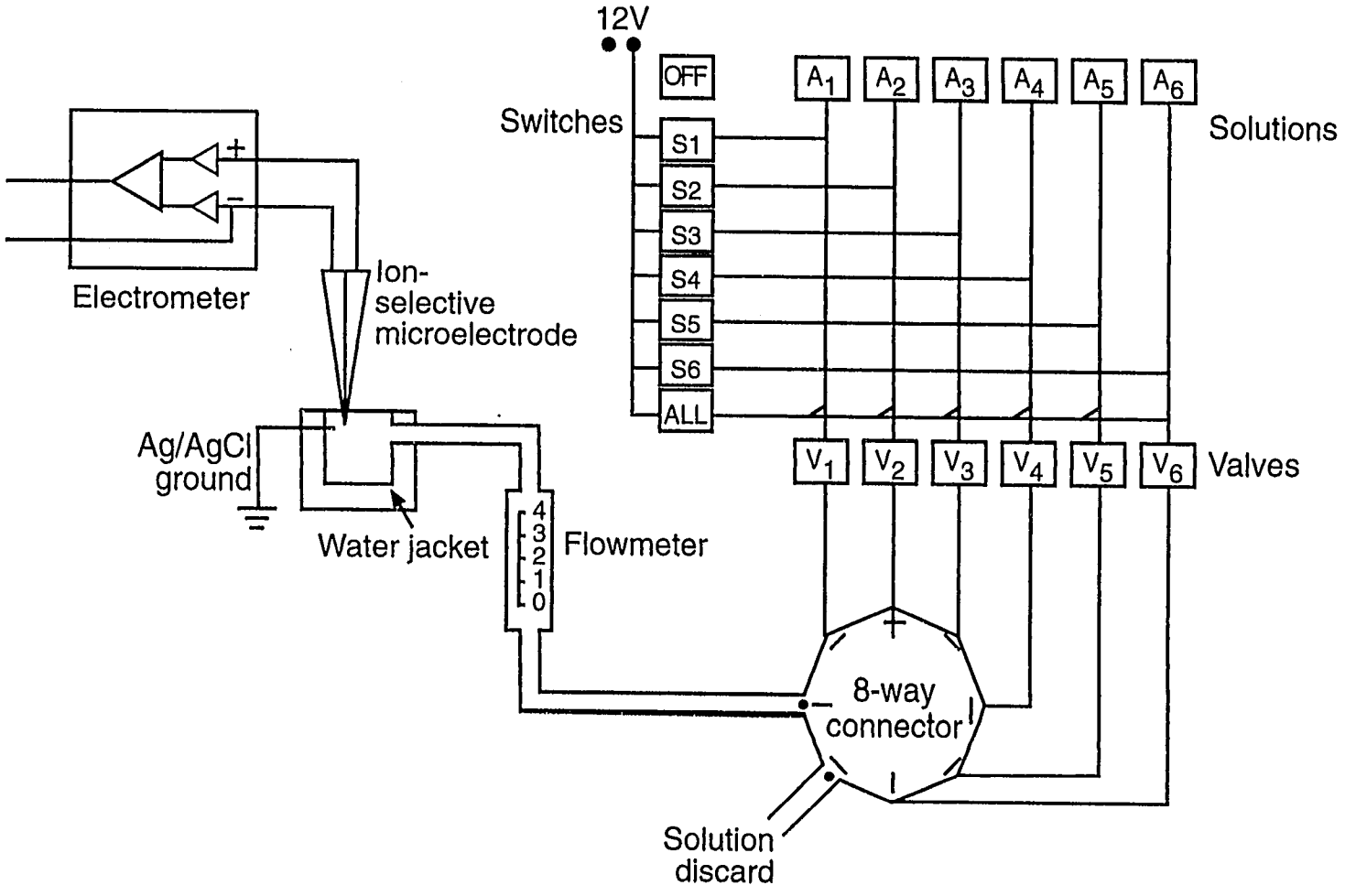
12V
OFF
Switches
S1
S2
S3
S4
S5
S6
ALL
A1
A2
A3
A4
A5
A6
Solutions
V1
V2
V3
V4
V5
V6
Valves
+
−
Electrometer
Ion-
selective
microelectrode
Ag/AgCl
ground
Water jacket
4
3
2
1
0
Flowmeter
8-way
connector
Solution
discard

Figure 5. **Diagram of hippocampal slice.** Recording with double-barrel ion-selective microelectrode (ISM) in CA1 region. Placement of stimulation electrode in CA2/CA3 region allows activation of Schaffer collateral input to CA1 neurons.

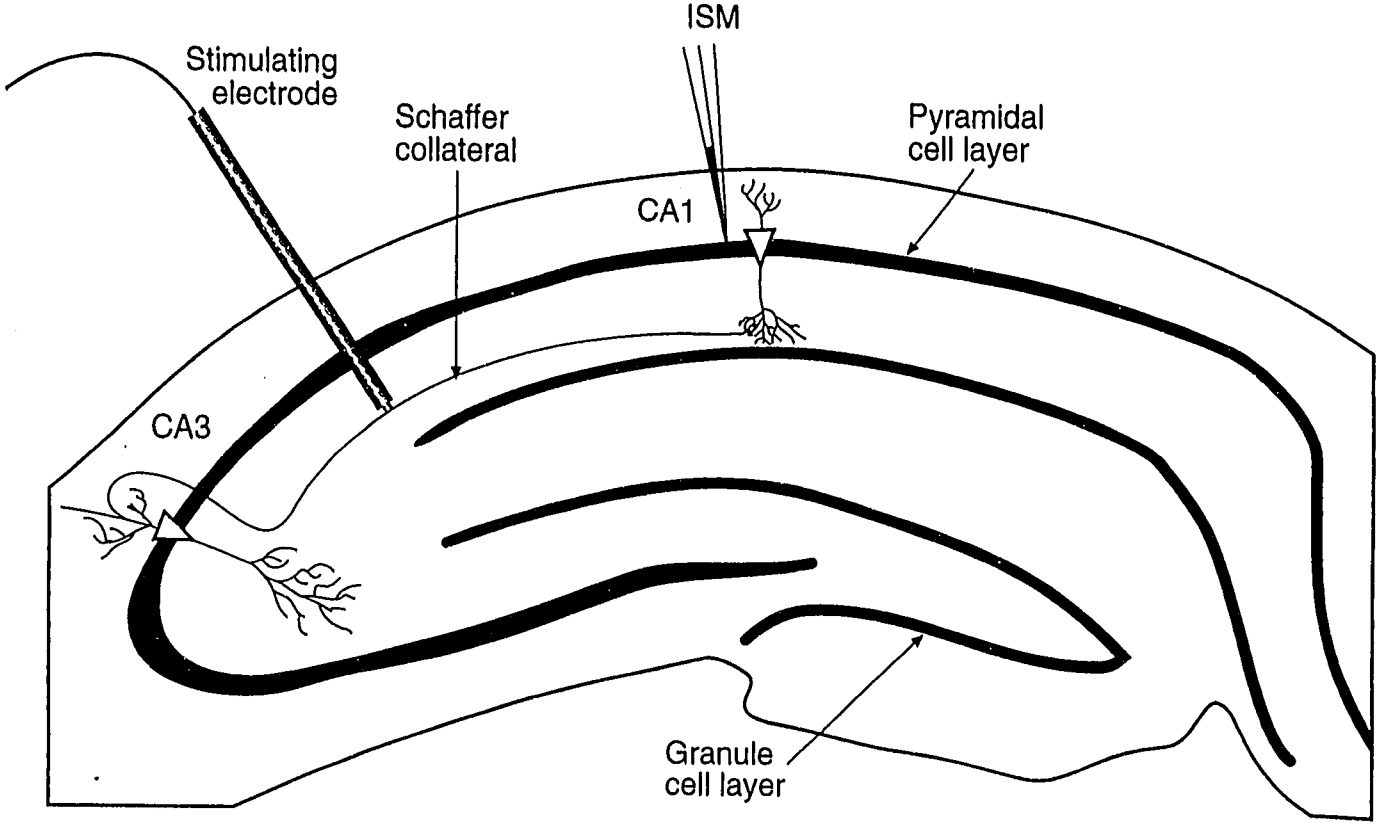
ISM
Stimulating electrode
Schaffer collateral
Pyramidal cell layer
CA1
CA3
Granule cell layer

Figure 6. **Stimulation and recording set-up** for ion-selective microelectrode measurements.

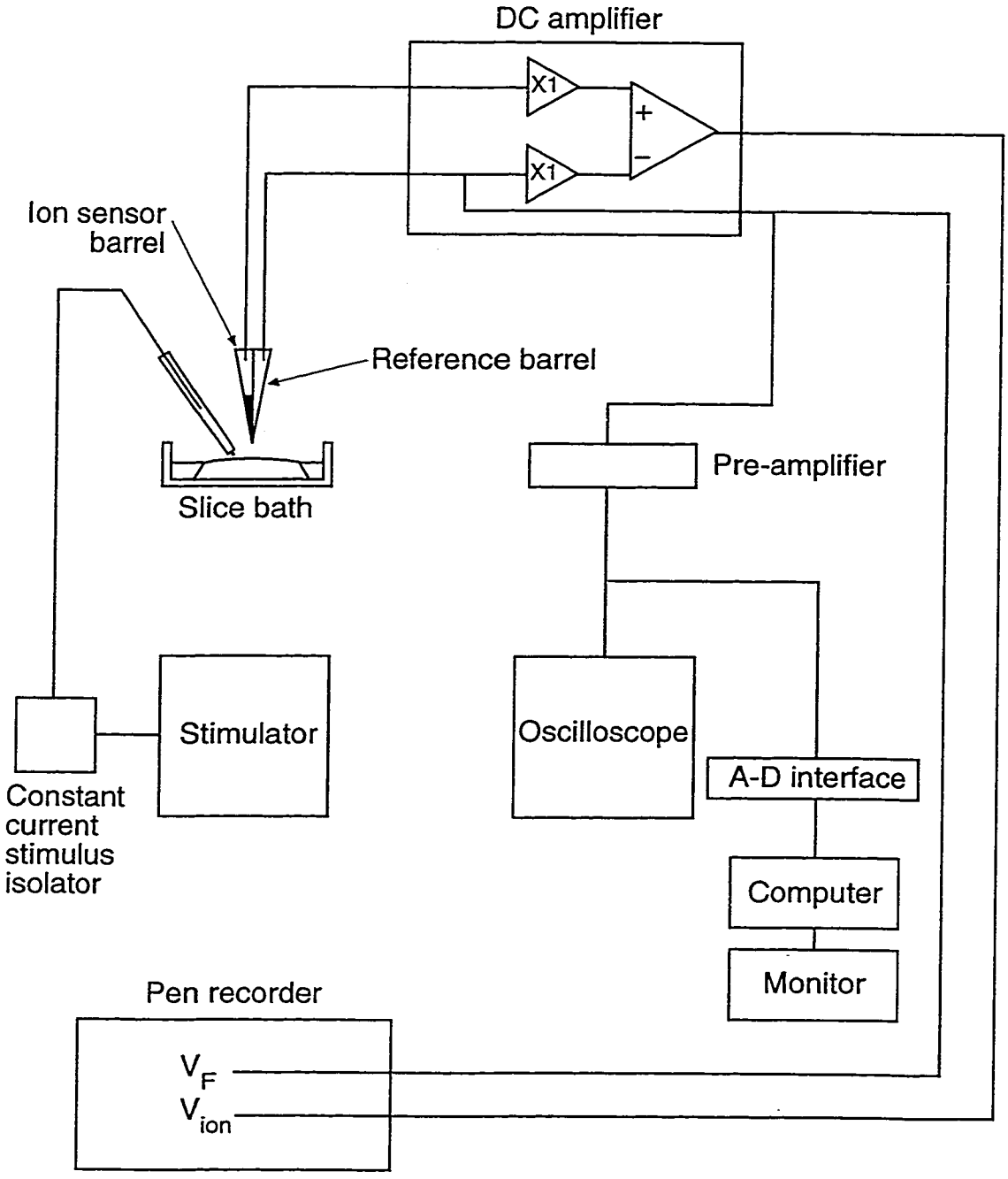
DC amplifier
X1
X1
+
−
Ion sensor barrel
Reference barrel
Slice bath
Pre-amplifier
Stimulator
Constant current stimulus isolator
Oscilloscope
A-D interface
Computer
Monitor
Pen recorder
V_F
V_{ion}

Figure 7. **Calibration curve for K^+-sensitive microelectrode.** ISM was prepared with the FLUKA 60398 valinomycin-based Potassium Ionophore I — Cocktail B. Graph shows voltage responses to six different concentrations of KCl in ACSF. Total ionic activity was maintained constant by varying NaCl. Curve fitted with extended Nicolsky-Eisenman equation by non-linear least-squares regression (Barolet et al. 1989).

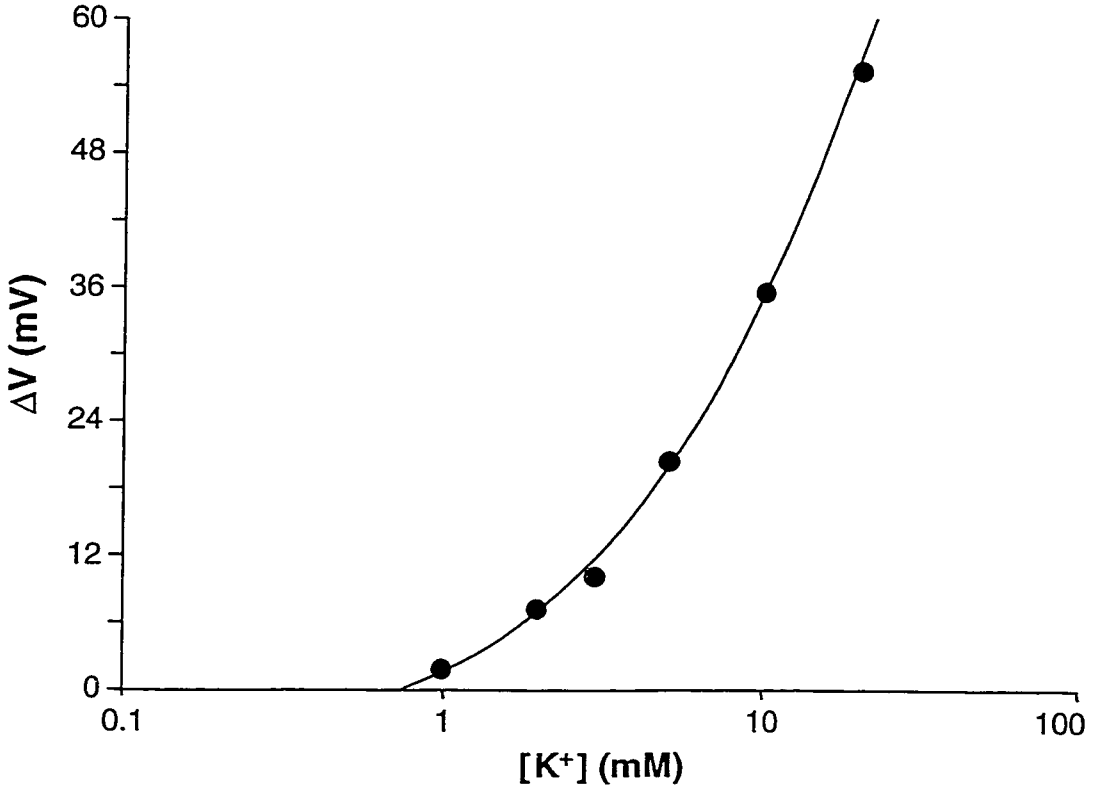
60
48
36
24
12
0
ΔV (mV)
0.1
1
10
100
[K+] (mM)

Figure 8. **Calibration curve for Cl^--sensitive microelectrode.** ISM was prepared with the FLUKA 24902 Chloride Ionophore I — Cocktail A. Graph shows voltage response to five different concentrations of NaCl. Total ionic activity of solutions was maintained constant by substitution with Na glucuronate. Curve fitted with extended Nicolsky-Eisenman equation by non-linear least-squares regression (Barolet et al. 1989).

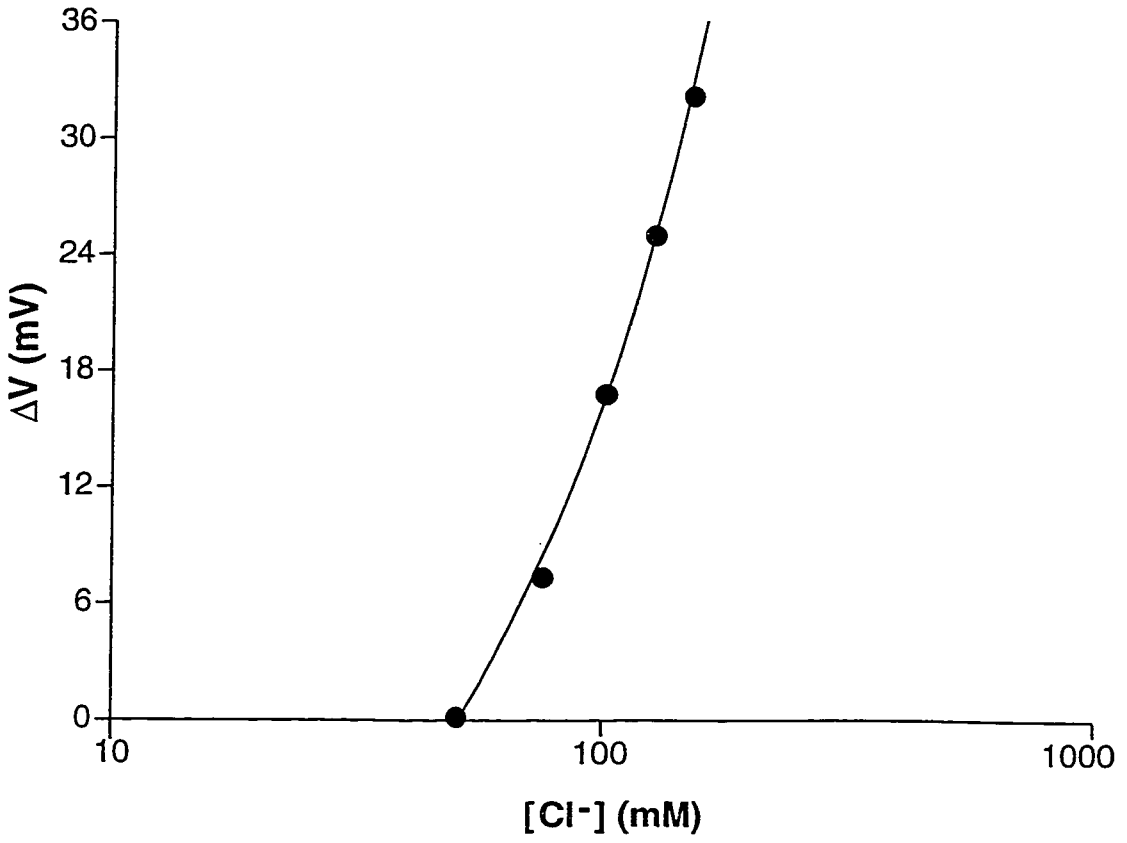
36
30
24
18
12
6
0
ΔV (mV)
10
100
1000
[Cl⁻] (mM)

Figure 9. **Calibration curve for Na^+-sensitive microelectrode.** ISM was prepared with the FLUKA 71178 Sodium Ionophore II — Cocktail A. Graph of voltage responses to five different concentrations of NaCl. Total ionic activity was maintained constant by substitutions with KCl. Curve fitted with extended Nicolsky-Eisenman equation by non-linear least-squares regression (Barolet et al. 1989).

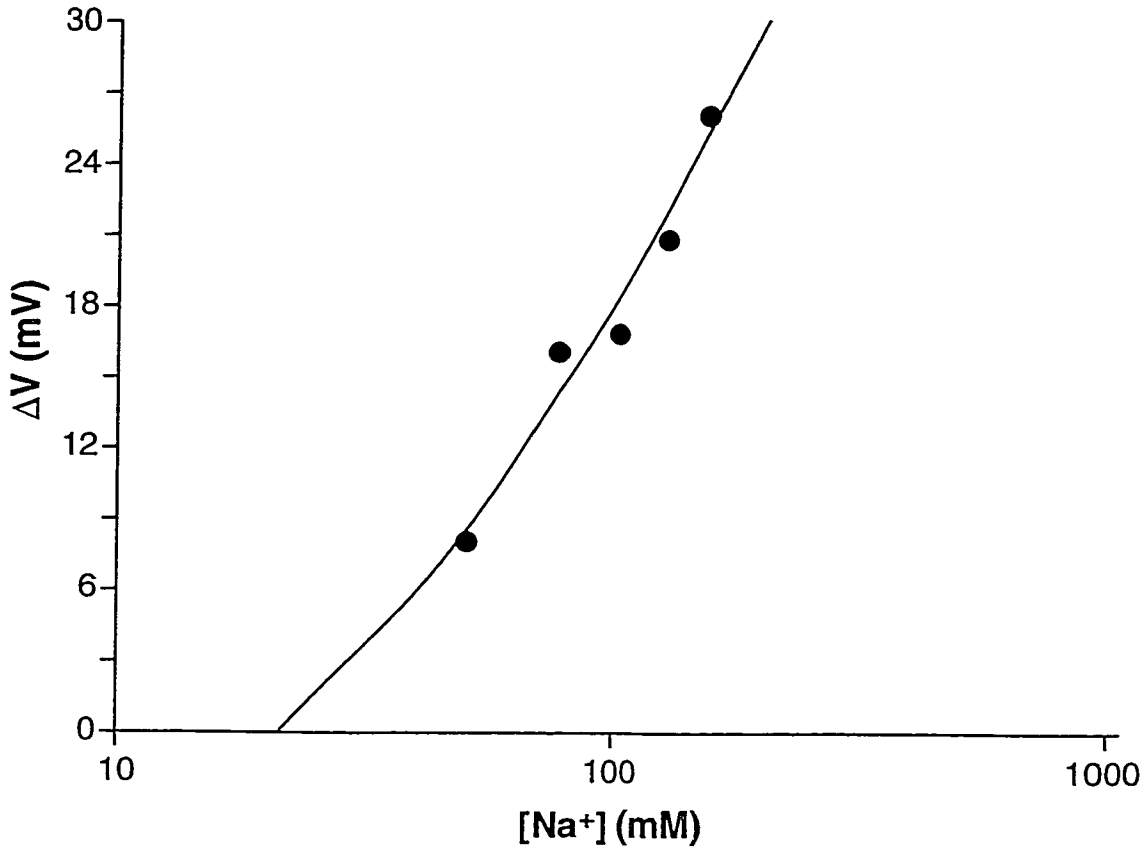
30
24
18
12
6
0
ΔV (mV)
10
100
1000
[Na+] (mM)

Figure 10. Responses of Corning 477317 ISM to changes in [K^+] and [TMA^+]. A — shows 27 mV shift in voltage recorded by an ISM when [K^+] concentration in ACSF was increased from 3 mM to 10 mM and back to 3 mM. B — shows voltage changes recorded by same electrode in response to 1, 2 and 3 mM [TMA^+] in ACSF containing 3 mM KCl. Note absence of response to 6 and 10 mM [K^+] in the presence of 3 mM [TMA^+]. Note different recording gains for A and B.

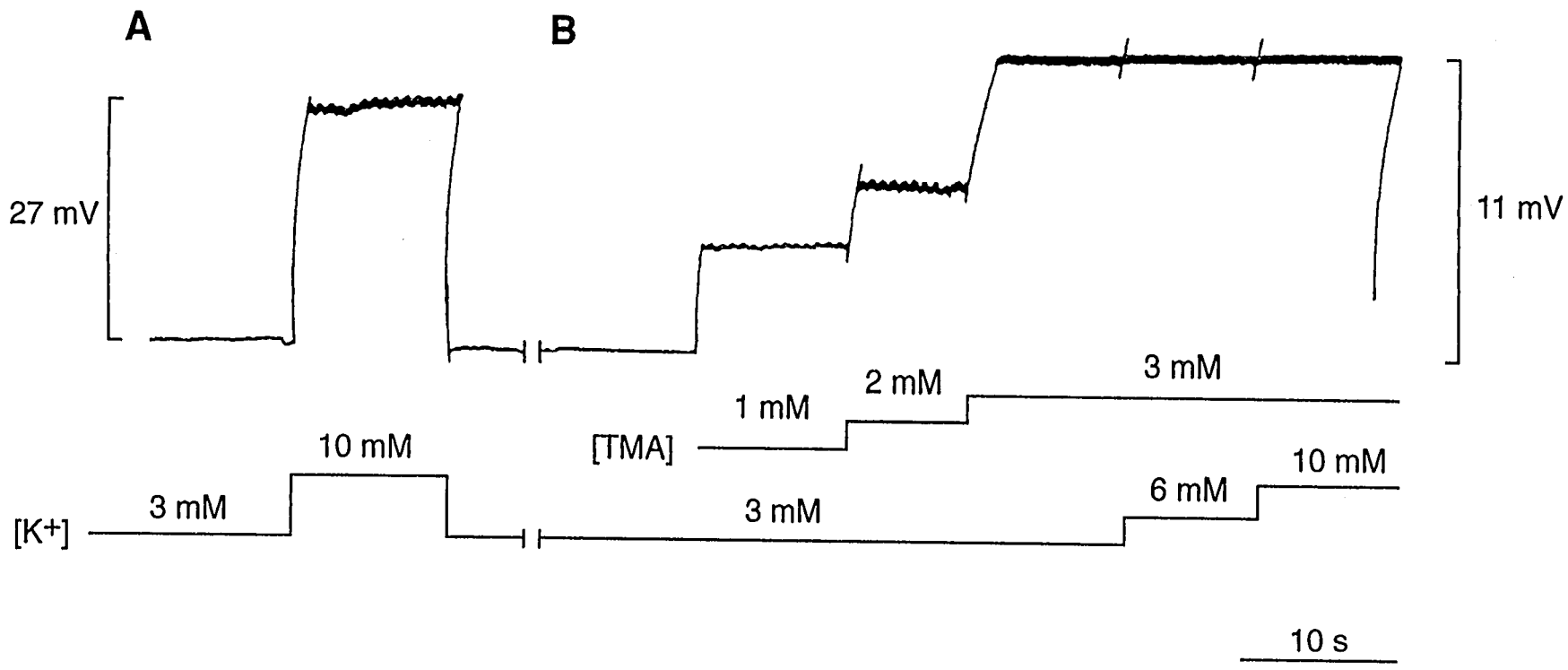
A
B
27 mV
11 mV
1 mM
2 mM
3 mM
[TMA]
10 mM
3 mM
[K+]
3 mM
6 mM
10 mM
10 s

Figure 11. Calibration curve for TMA^+-sensitive microelectrode. ISM was prepared with the classical Corning 477317 ion exchanger for K^+, which has greater selectivity for quaternary ammonium ions than for K^+ (see Fig. 10 and Table II). Graph shows voltage response to six different concentrations of TMA chloride in ACSF. Total ionic activity was maintained constant by varying NaCl. Curve fitted with extended Nicolsky-Eisenman equation by non-linear least squares regression (Barolet et al. 1989).

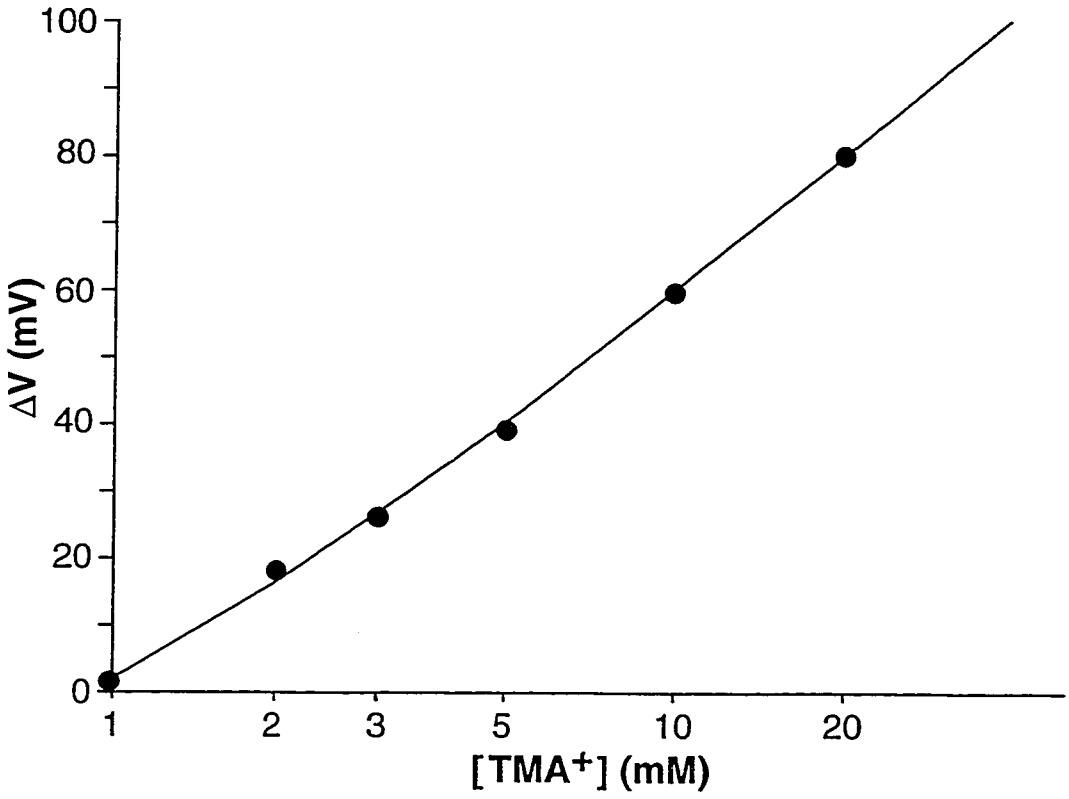
100
80
60
40
20
0
ΔV (mV)
1
2
3
5
10
20
[TMA+] (mM)

Figure 12. Stimulation-evoked changes in $[K^+]_o$. Responses were evoked by orthodromic stimulation of Schaffer collaterals and recorded in SP and SR of CA1. Graph shows peak $\Delta[K^+]_o$ when frequency of stimulation (0.1 ms, 20 s) was varied between 1 and 10 Hz. Data from 4 slices; n = 1–5 observations at each frequency; SEM values $\leq$ 0.1 not displayed. SP — open circles ($r = 0.998$, $p \leq 0.0001$, slope of regression = 0.923 ± 0.03, n = 6). SR — filled circles ($r = 0.998$, $p \leq 0.0001$, slope of regression = 1.18 ± 0.03, n = 6).

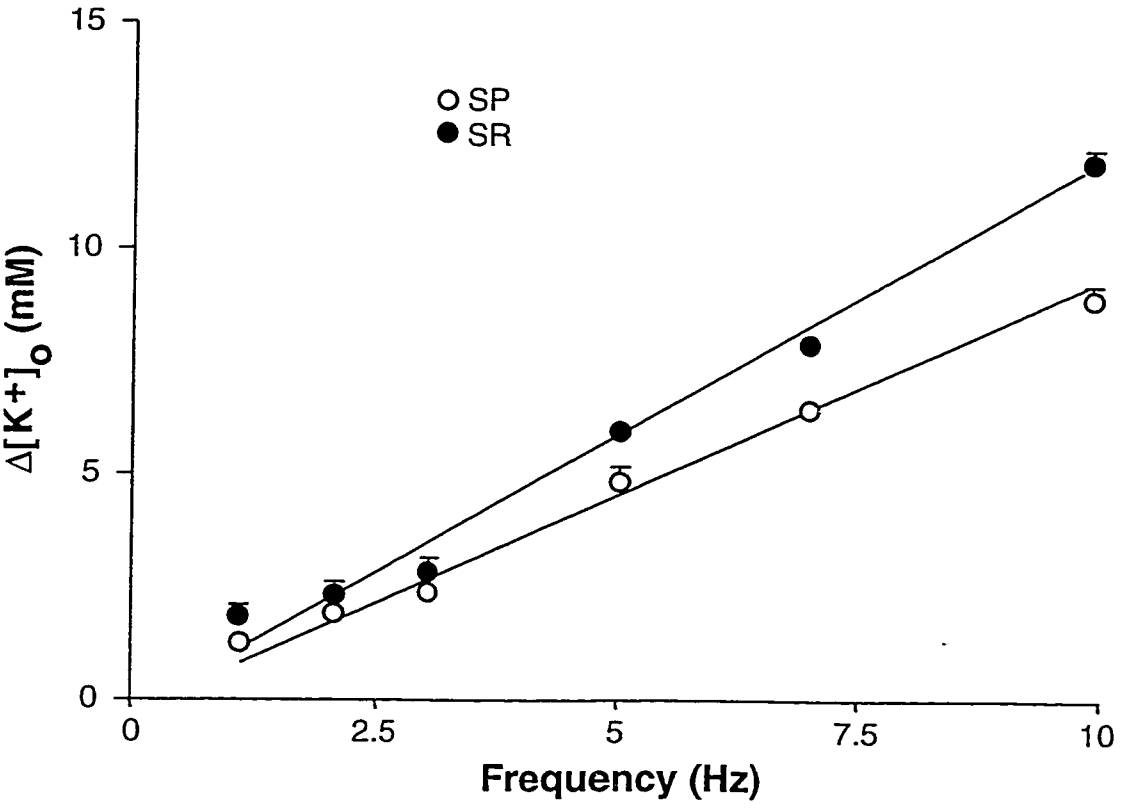
15
10
5
0
0
2.5
5
7.5
10
SP
SR
Δ[K+]o (mM)
Frequency (Hz)

Figure 13. Concentration-dependent $\Delta[K^+]_o$ evoked by GABA in SP and SR. Bath applications of 1–10 mM GABA were for 5 min. Bars of histogram (unfilled — SP; filled — SR) show mean values ± SE (3–13 observations at each concentration, n = 13 slices).

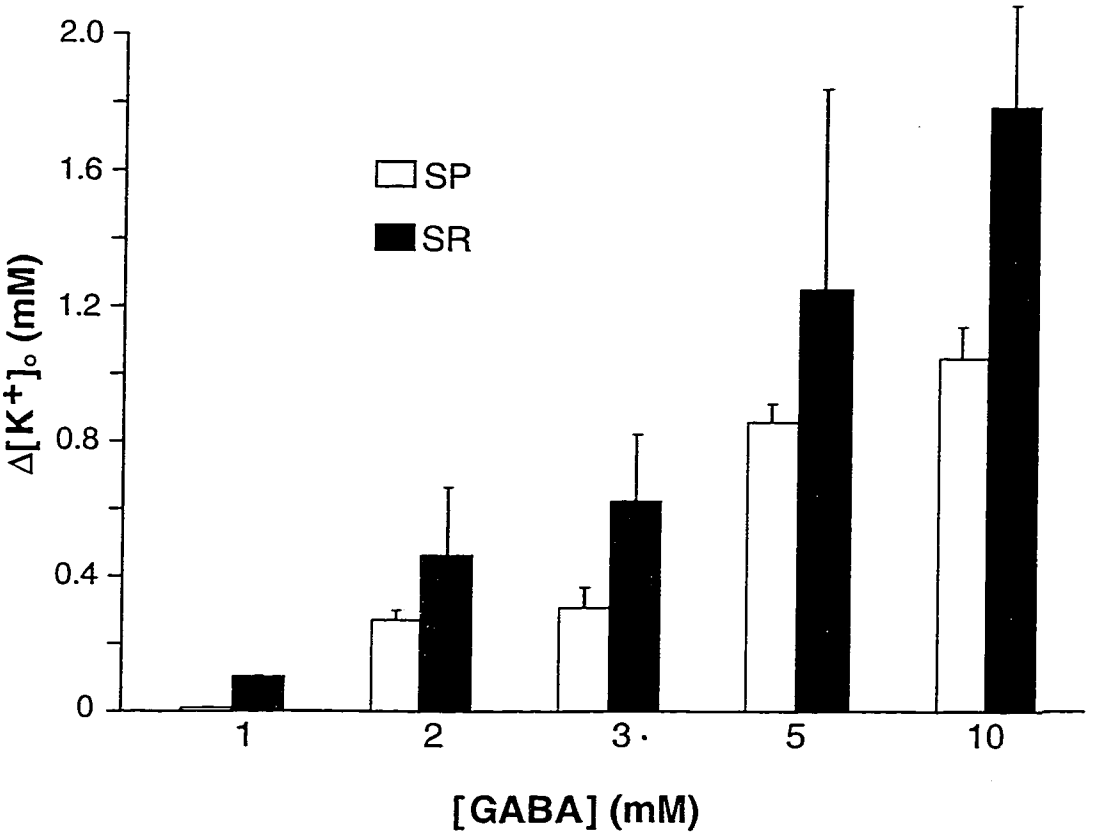
2.0
1.6
1.2
0.8
0.4
0
SP
SR
Δ[K+]o (mM)
1
2
3
5
10
[GABA] (mM)

Figure 14. Concentration-dependent depression of population spikes by GABA. Bath applications of 1–10 mM GABA for 5 min. Field potentials were evoked by 50 μA, 0.1 ms, 0.5 H_z stimulation of Schaffer collaterals and recorded in SP in 6 slices (n = 2-6 observations). Graph shows means ± SE and linear regression (slope = 10.19 ± 0.59, $r^2 = 0.987$, $p \leq 0.0001$, n = 6).

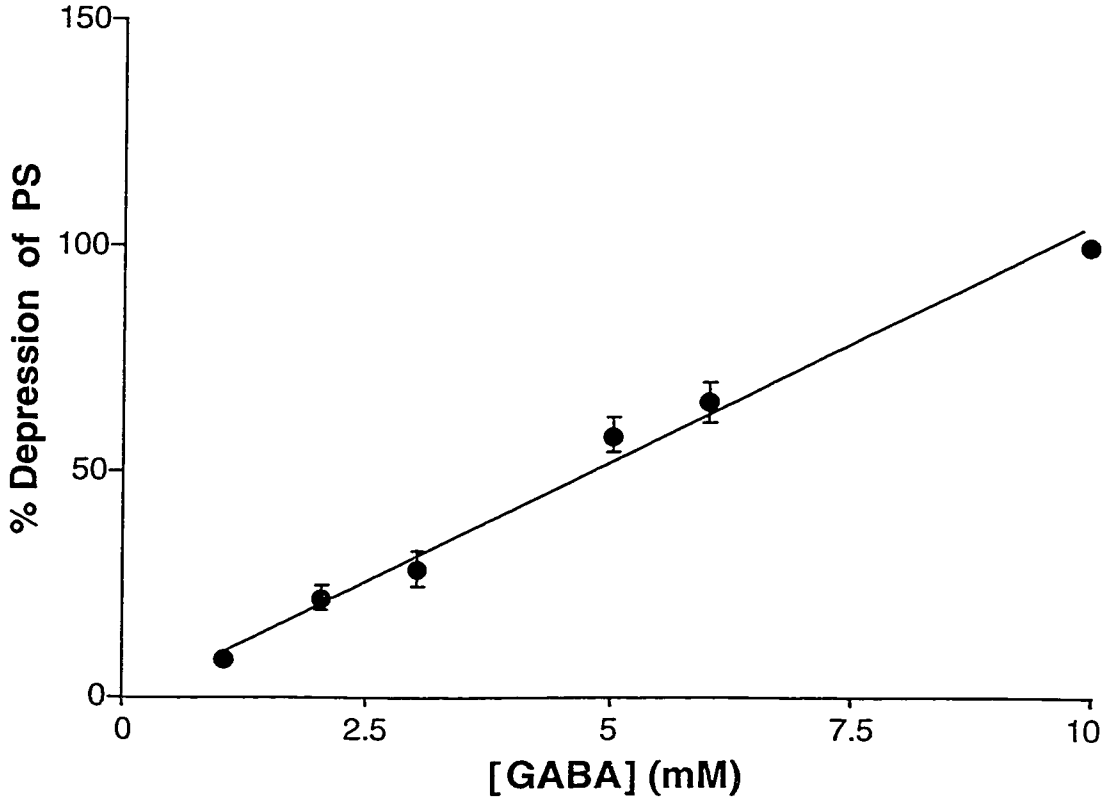
150
100
50
0
% Depression of PS
0
2.5
5
7.5
10
[GABA] (mM)

Figure 15. Effects of GABA and anoxia on field potentials recorded in SP. A and B illustrate examples of oscilloscope traces from two different slices of the depression and recovery of SP field potentials evoked by orthodromic stimulation of Schaffer collaterals following bath application of 5 mM GABA (5 min) and exposure to 95%N_2/5%CO_2 (5 min), respectively.

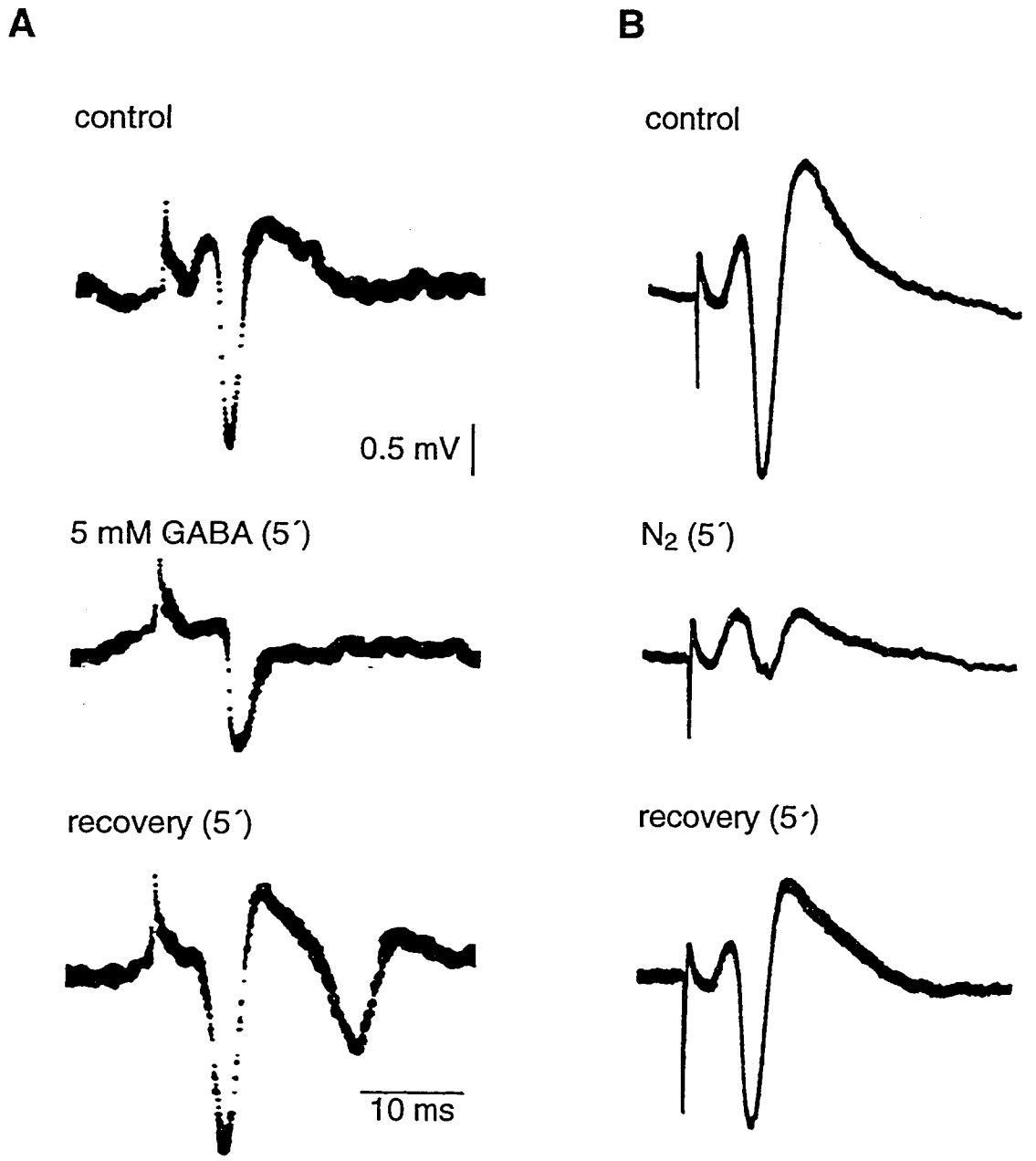
A
B
control
0.5 mV
control
5 mM GABA (5´)
N2 (5´)
recovery (5´)
recovery (5´)
10 ms

Figure 16. Effects of GABA and anoxia on $[K^+]_o$ in SP and SR. Polygraph records from 2 different slices of responses to bath application of 5 mM GABA and N_2, respectively. Horizontal bars mark duration of exposure. In each case recordings in SP and SR made at separate times; recovery not illustrated. Dotted lines indicate baseline level of $[K^+]_o$. Records of A and C from one slice; those of B and D from a second slice.

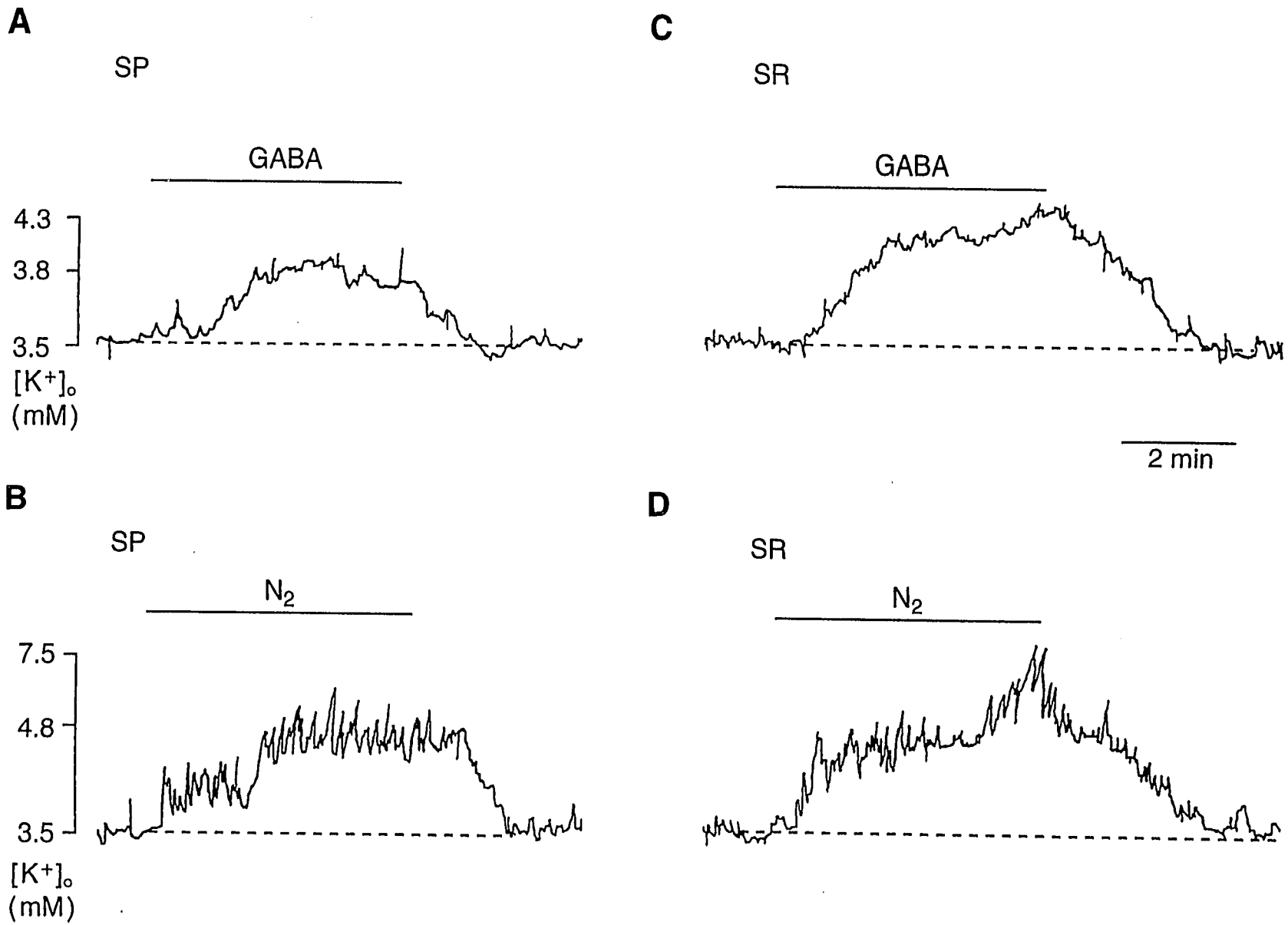
A
SP
GABA
4.3
3.8
3.5
[K+]o
(mM)
C
SR
GABA
2 min
B
SP
N2
7.5
4.8
3.5
[K+]o
(mM)
D
SR
N2

Figure 17. Mean $[K^+]_o$ changes evoked by GABA and anoxia in SP and SR. Bath application of 5 mM GABA and N_2 exposure for 5 min; recordings in SP and SR at separate times in 12 slices. Histogram shows means ± SE at left for GABA; at right for N_2 (n = 9 for SP, n = 12 for SR). Unfilled bars — SP; filled bars — SR.

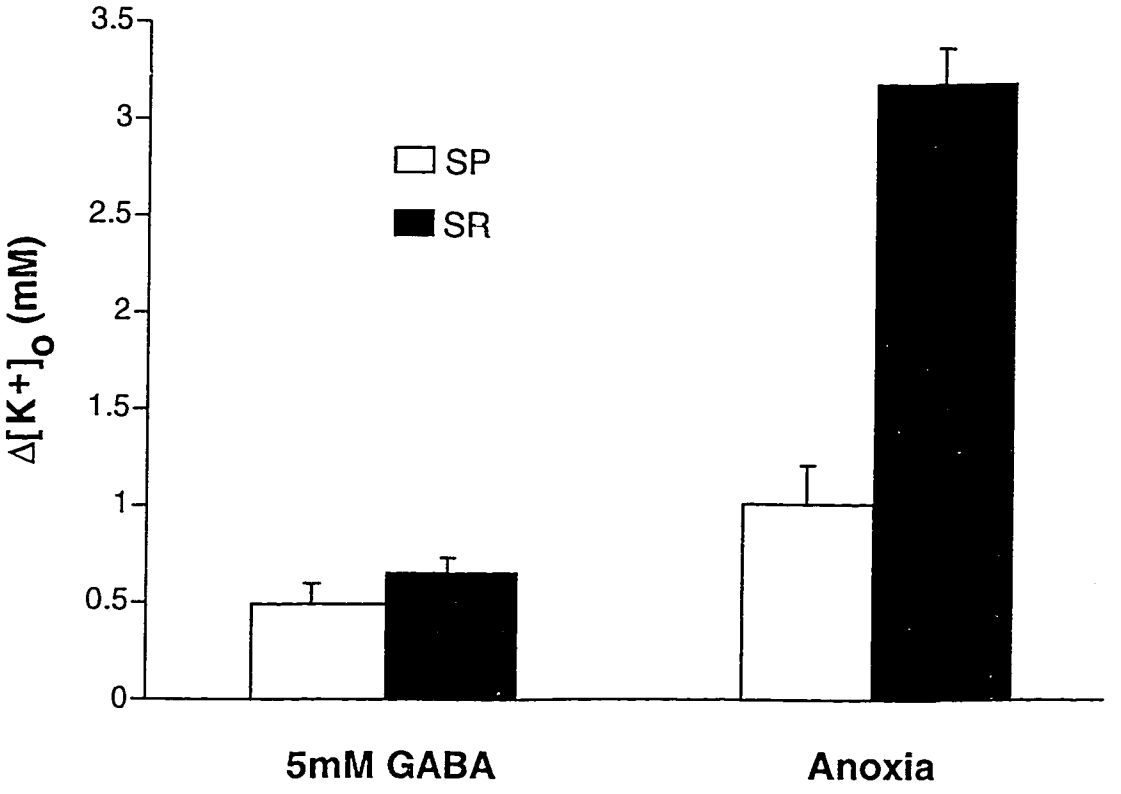
3.5
3
2.5
2
1.5
1
0.5
0
SP
SR
Δ[K+]o (mM)
5mM GABA
Anoxia

Figure 18. **Stimulation-evoked changes in $[Cl^-]_o$.** Responses were evoked by orthodromic activation of Schaffer collaterals (0.1 ms, 50 μA, 1–10 H_z for 20 s) and recorded in SP (open symbols) and SR (filled symbols). Graph shows mean ± SE (n = 5–7 observations at each frequency, n = 4 slices). Slopes of regression for SP and SR respectively are 9.94 ± 0.46 (r^2 = 0.989, n = 6, $p \leq 0.0001$) and –10.66 ± 0.74 (r^2 = 0.976, n = 6, $p \leq 0.0001$).

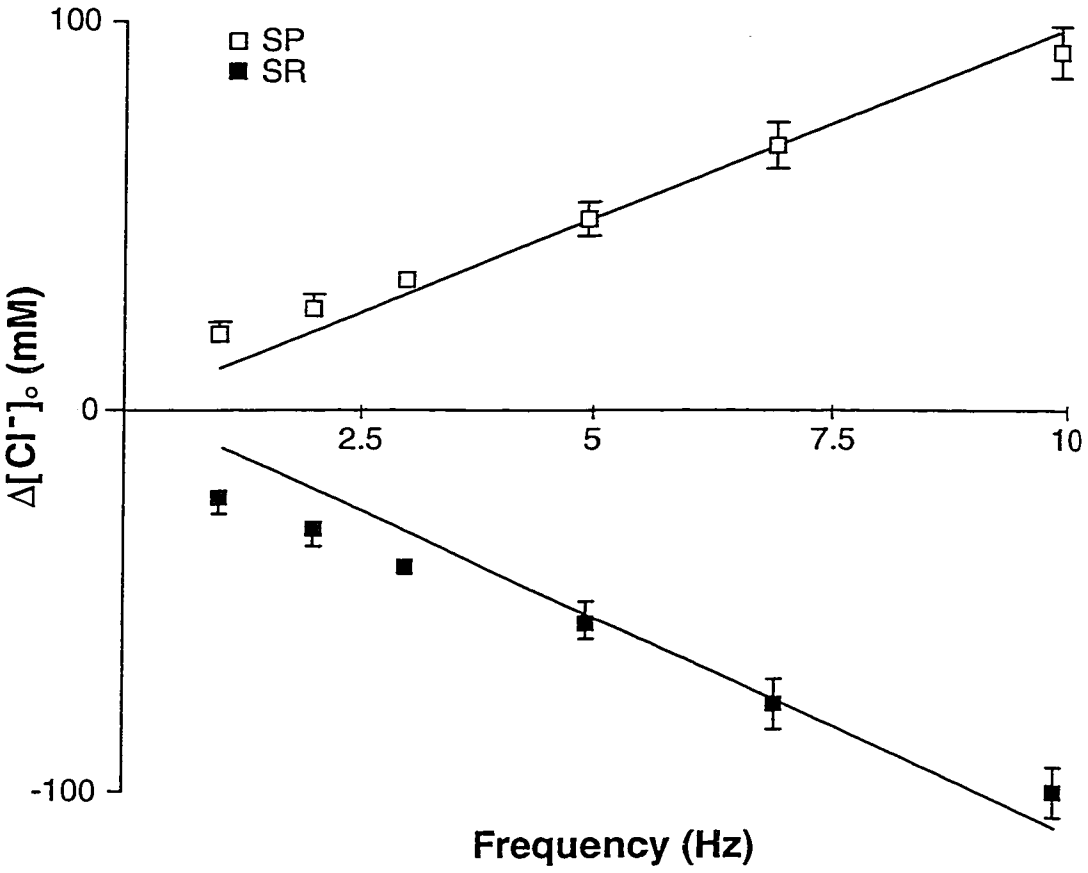
100
0
-100
SP
SR
2.5
5
7.5
10
Δ[Cl⁻]ₒ (mM)
Frequency (Hz)

Figure 19. Concentration-dependent $[Cl^-]_o$ changes evoked in SP and SR by GABA. Bath applications of 1–10 mM GABA for 5 min. Bars of histogram (unfilled — SP; filled — SR) show means ± SE from n = 6 slices (total number of observations = 30). EC_{50} values from non-linear regression curve fit of data are 4.1 and 5.6 mM for SP and SR, respectively.

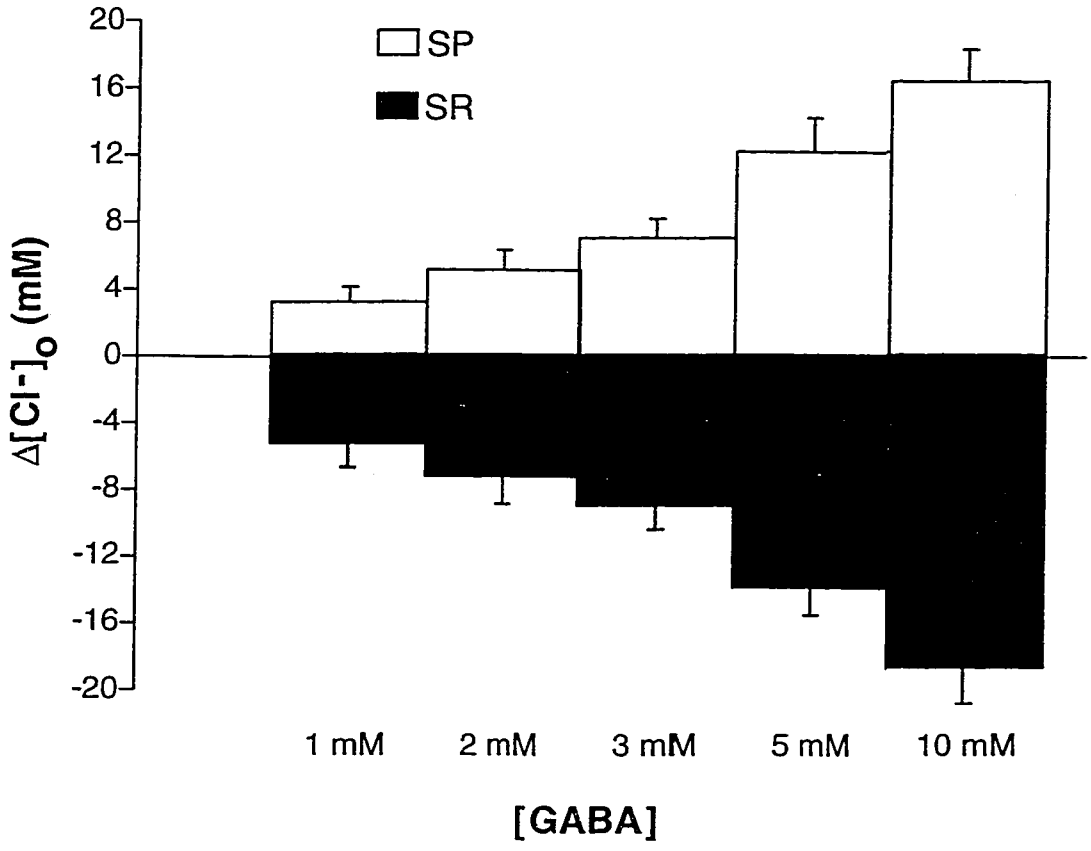
SP
SR
20
16
12
8
4
0
-4
-8
-12
-16
-20
Δ[Cl-]o (mM)
1 mM
2 mM
3 mM
5 mM
10 mM
[GABA]

Figure 20. Effects of GABA and anoxia on $[Cl^-]_o$ in SP and SR. Polygraph records from 2 different slices of responses to bath application of 5 mM GABA and N_2, respectively. Recordings in SP and SR at separate times; horizontal bars mark duration of exposure. Dotted lines indicate baseline level of $[Cl^-]_o$. Records of A and B from one slice; those of C and D from a second slice.

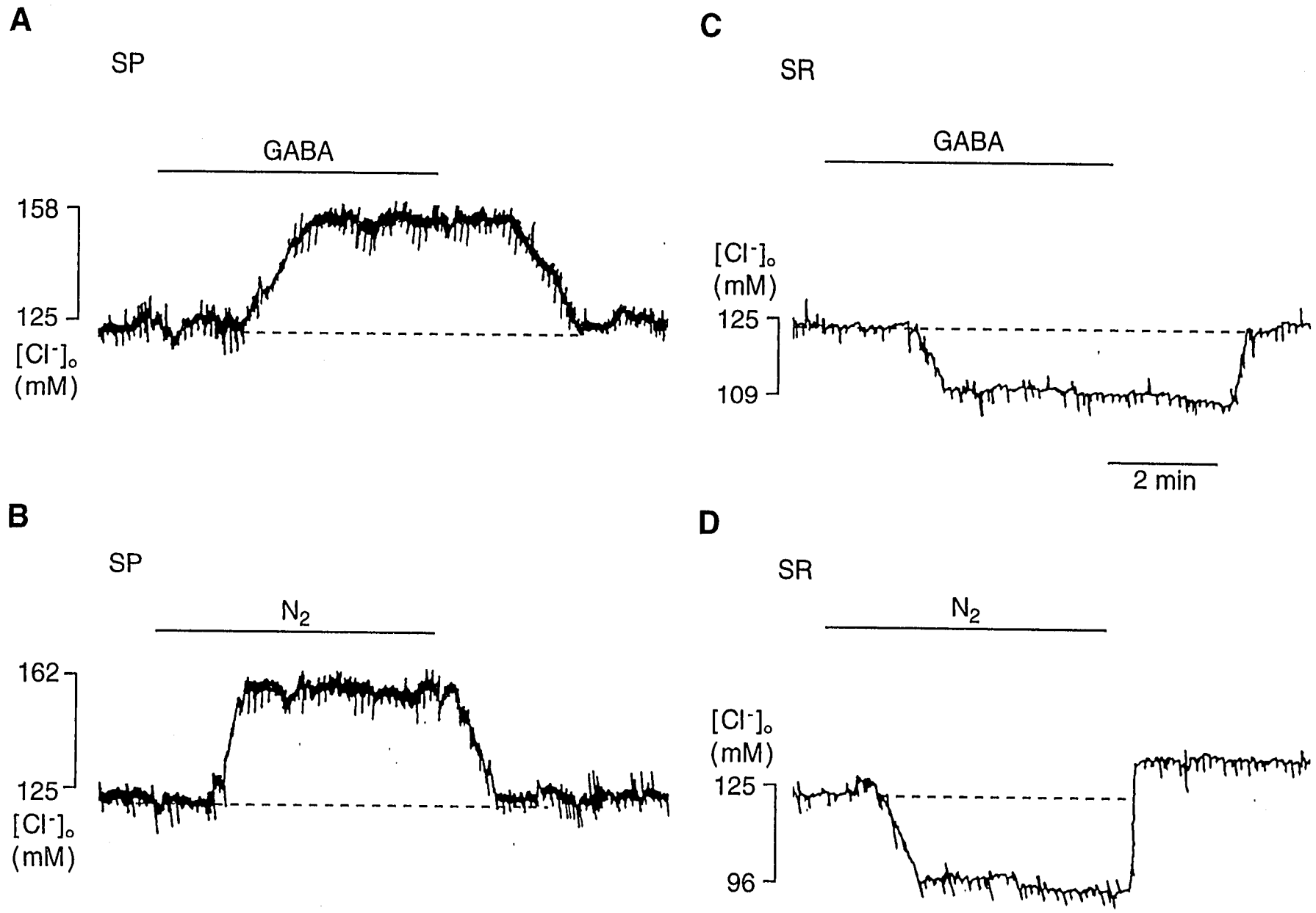
A
SP
GABA
158
125
[Cl⁻]o
(mM)
C
SR
GABA
[Cl⁻]o
(mM)
125
109
2 min
B
SP
N2
162
125
[Cl⁻]o
(mM)
D
SR
N2
[Cl⁻]o
(mM)
125
96

Figure 21. Mean $[Cl^-]_o$ changes evoked by GABA and anoxia in SP and SR. Bath application of 5 mM GABA and N_2 exposure for 5 min; recordings in SP and SR at separate times in 14 slices. Data are means ± SE at left for GABA (n = 14 for SP, n = 8 for SR) and at right for N_2 (n = 10 for SP, n = 9 for SR); unfilled bars — SP, filled bars — SR.

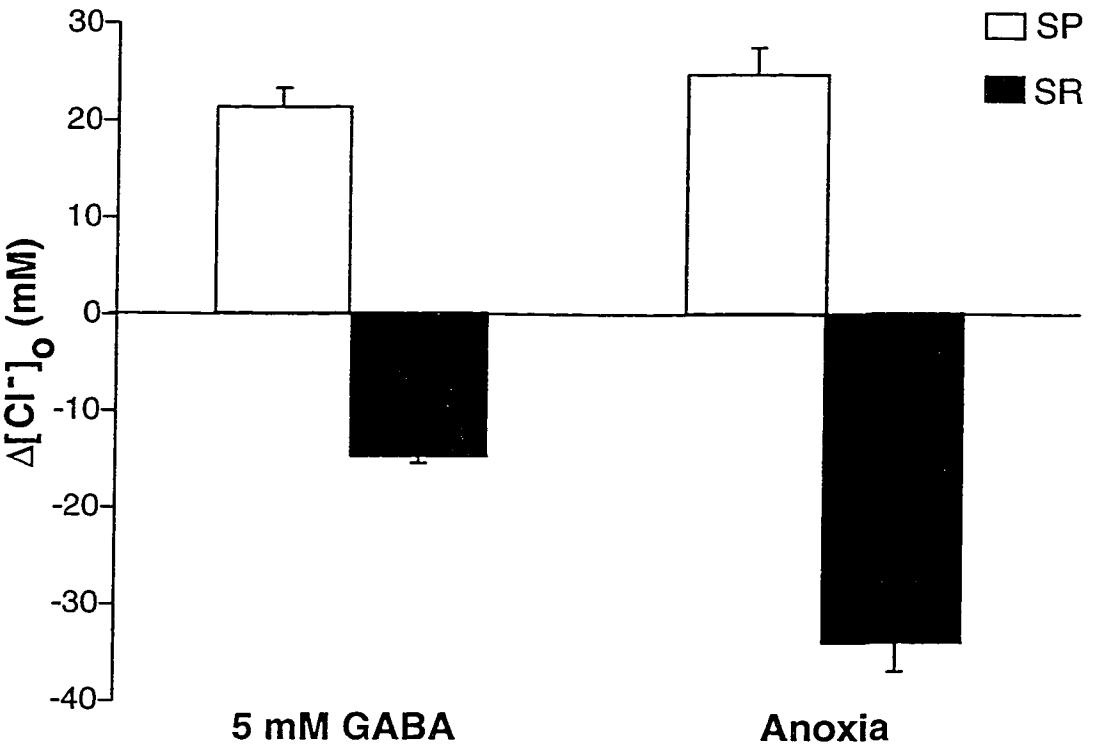
30
20
10
0
-10
-20
-30
-40
Δ[Cl⁻]o (mM)
SP
SR
5 mM GABA
Anoxia

Figure 22. **Effects of GABA and anoxia on $[Na^+]_o$ in SP and SR.** Polygraph records from two different slices illustrate responses to bath application of 5 mM GABA and exposure to N_2, respectively. Recordings in SP and SR at separate times; horizontal bars mark duration of exposure. Dotted lines indicate baseline level of $[Na^+]_o$. Records in A and B are from one slice; those in C and D are from a second slice.

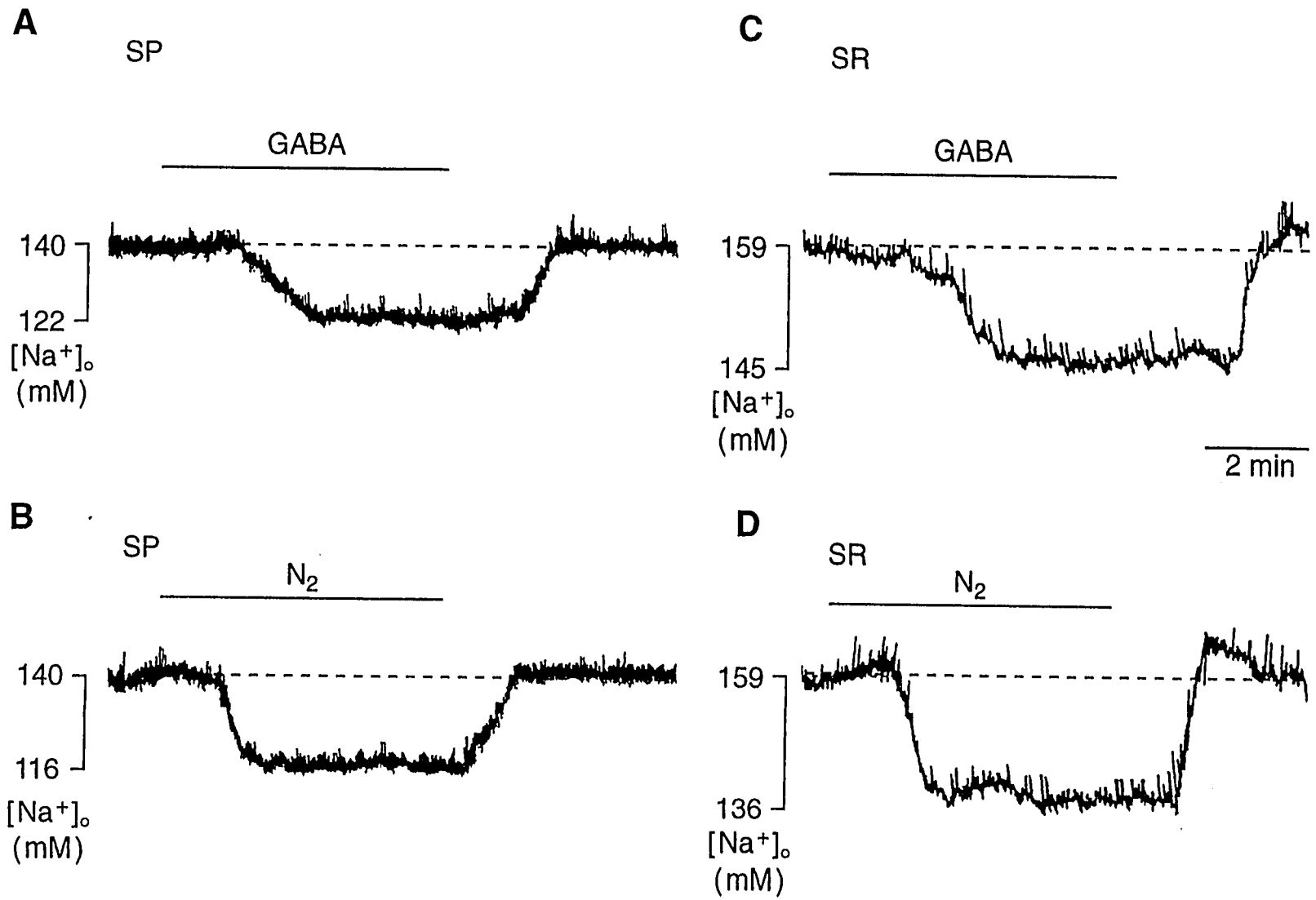
A
SP
GABA
140
122
[Na+]o
(mM)
C
SR
GABA
159
145
[Na+]o
(mM)
2 min
B
SP
N2
140
116
[Na+]o
(mM)
D
SR
N2
159
136
[Na+]o
(mM)

Figure 23. **Mean $[Na^+]_o$ changes evoked by GABA and anoxia in SP and SR.** Bath application of 5 mM GABA and N_2 exposure for 5 min; recordings in SP and SR at separate times in 9 slices. Data are means ± SE at left for GABA (n = 8 for SP and SR) and at right for N_2 (n = 9 for SP and SR); unfilled bars — SP; filled bars — SR.

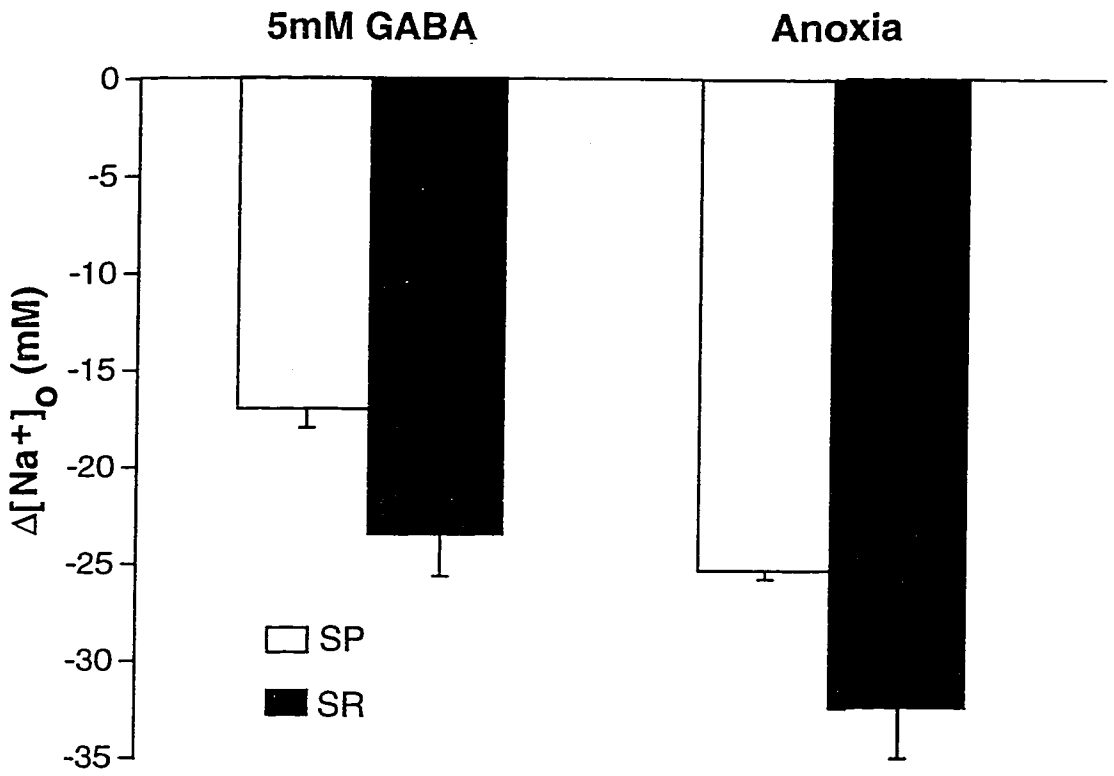
5mM GABA
Anoxia
0
-5
-10
-15
-20
-25
-30
-35
Δ[Na+]o (mM)
SP
SR

Figure 24. Effects of GABA and anoxia on $[TMA^+]_o$ in SP and SR. Responses recorded in two different experiments to bath application of 5 mM GABA and N_2 exposure, respectively. ACSF containing 5 mM TMA chloride was used for bath perfusion for 30 min prior to, during and following test treatments. Recordings in SP and SR at separate times; horizontal bars indicate duration of exposure to GABA or N_2. Records of A and B are from one slice; all four panels A, B, C and D have identical scales..

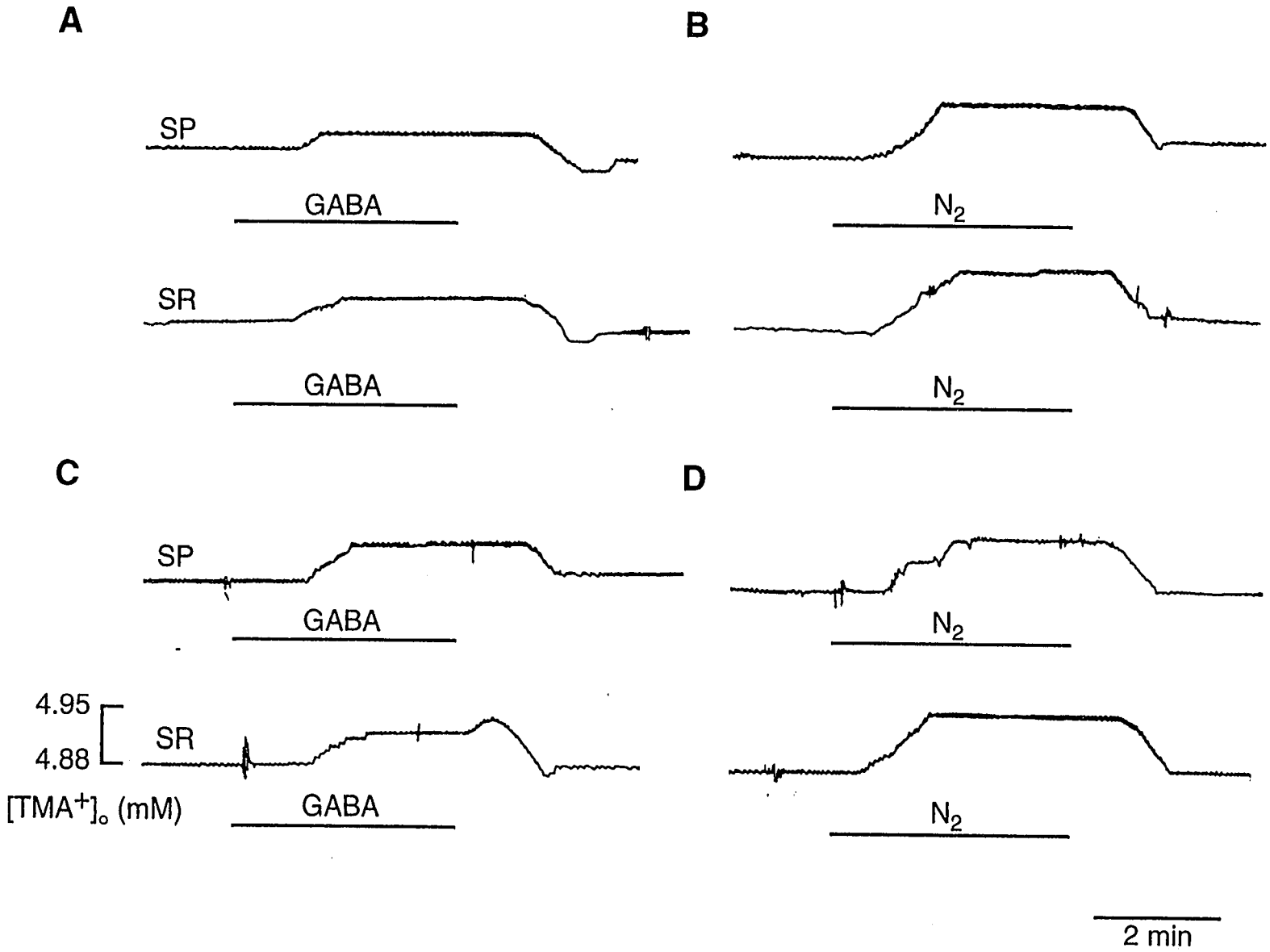
A
B
SP
GABA
SR
GABA
N_2
N_2
C
D
SP
GABA
4.95
4.88
SR
$[TMA^+]_o$ (mM)
GABA
N_2
N_2
2 min

Figure 25. Effects of BMI on $\Delta[K^+]_o$ evoked by GABA and anoxia in SP. Examples recorded in SP of reversible attenuation by the $GABA_A$ receptor antagonist, BMI (100 μM) (30 min pre-treatment and continuous perfusion) of $[K^+]_o$ responses evoked by 5 mM GABA (A–C) and exposure to N_2 (D–F), respectively, in two different slices. Horizontal bars mark duration of exposure. Records in C and F are at 30 min recovery.

Stratum Pyramidale

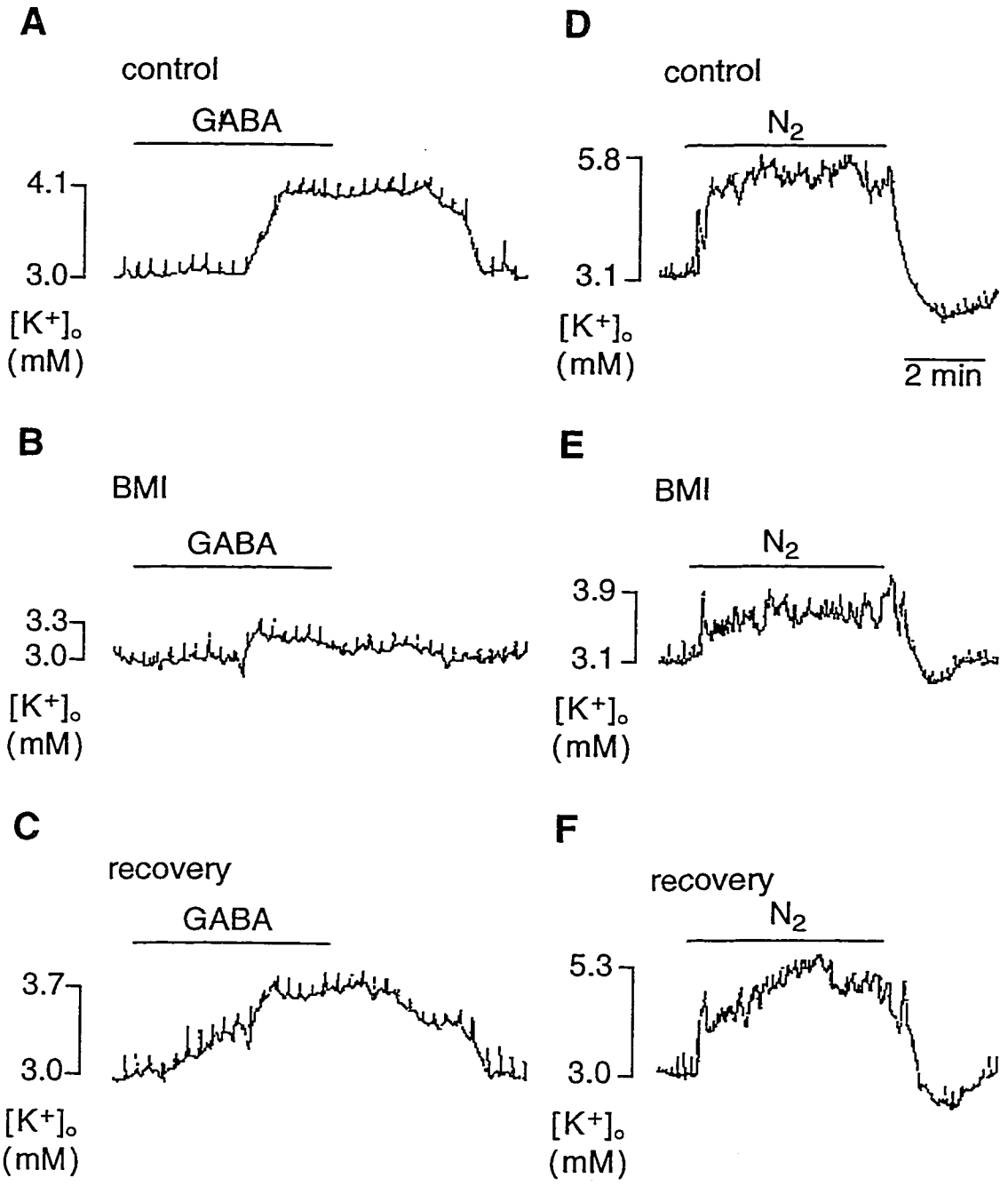

Figure 26. Effects of BMI on $\Delta[K^+]_o$ evoked by GABA and anoxia in SR. Examples recorded in SR of reversible attenuation by the $GABA_A$ receptor antagonist, BMI (100 μM) (30 min pre-treatment and continuous perfusion) of $[K^+]_o$ responses evoked by 5 mM GABA (A–C) and exposure to N_2 (D–F), respectively, in two different slices. Horizontal bars mark duration of exposure. Records of C and F are at 30 min recovery.

Stratum Radiatum

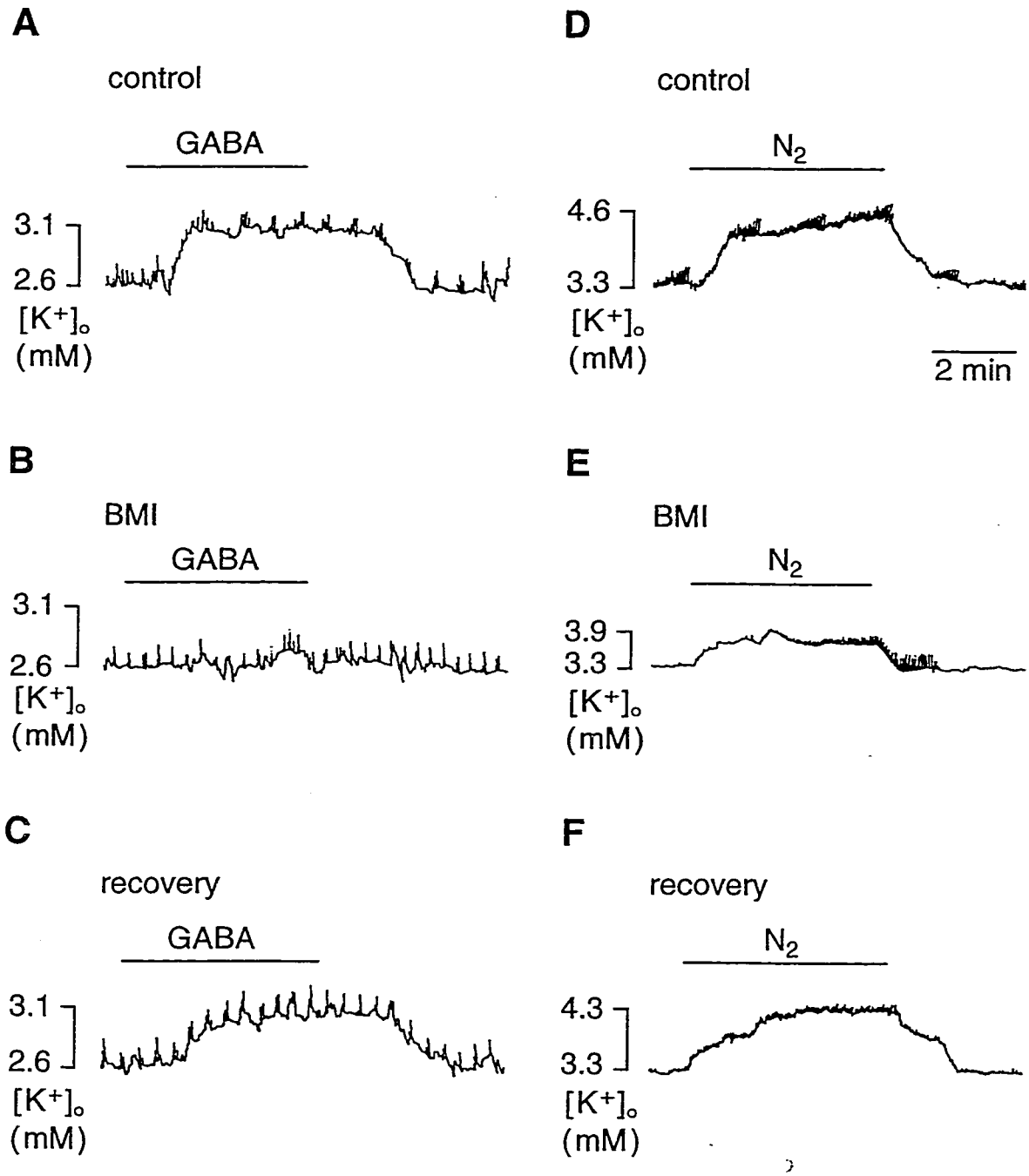

Figure 27. Concentration-dependent effects of BMI on anoxic-induced $[K^+]_o$ increases in SP and SR. Graph shows peak $\Delta[K^+]_o$ evoked by N_2 exposure (5 min) in absence of BMI (control at left) and following 30 min pre-treatment with 1–300 μM BMI. Bars are means ± SE (n = 3–10 observations for each, n = 10 slices); unfilled bars — SP, filled bars — SR. Values from curves fit by non-linear regression analysis are IC_{50} = 63 and 54 μM and r^2 = 0.956 and 0.986 for SP and SR, respectively.

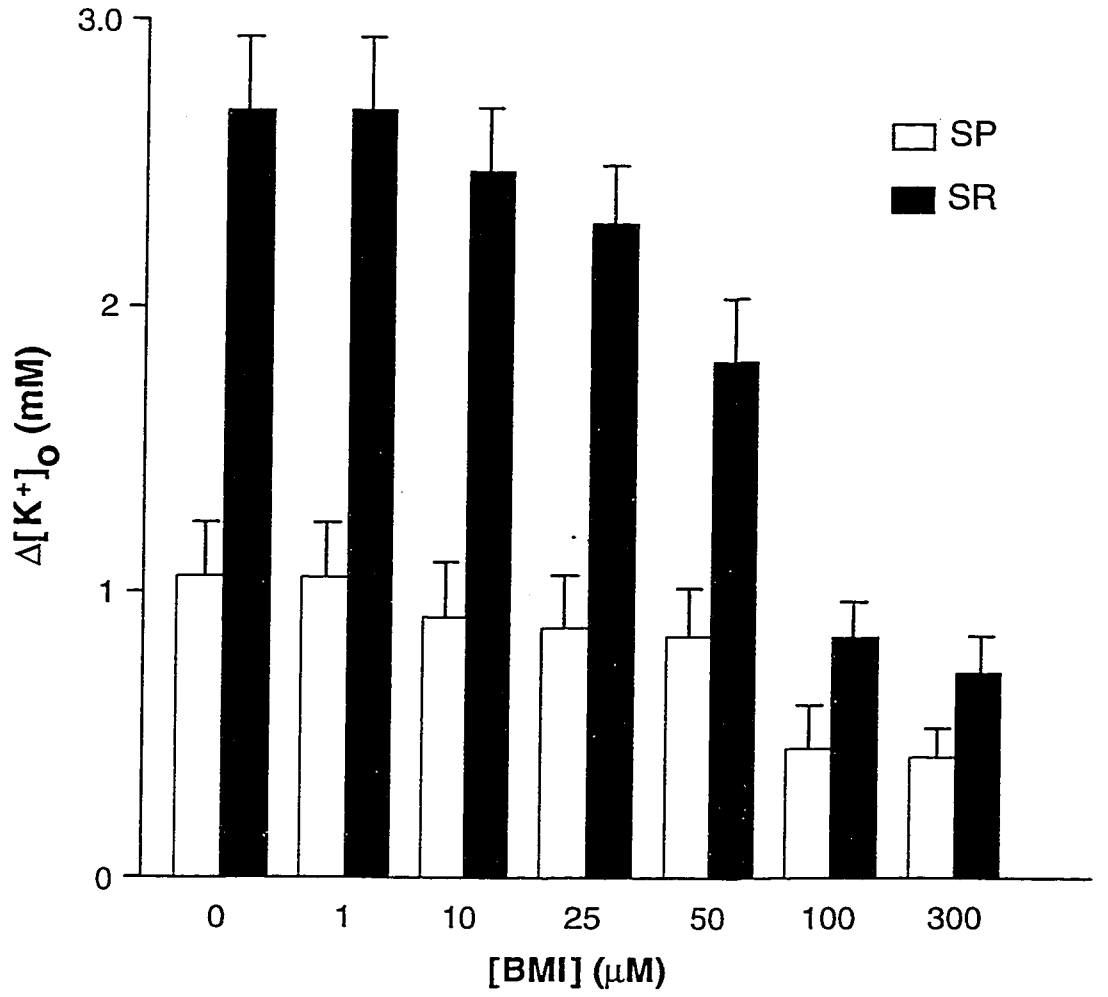
3.0
2
1
0
$\Delta[K^+]_o$ (mM)
SP
SR
0
1
10
25
50
100
300
[BMI] (μM)

Figure 28. Effects of BMI on $\Delta[Cl^-]_o$ evoked by GABA and anoxia in SP. Examples recorded in SP of reversible attenuation by the $GABA_A$ receptor antagonist, BMI (100 μM) (30 min pre-treatment and continuous perfusion) of $[Cl^-]_o$ responses evoked by 5 mM GABA (A–C) and exposure to N_2 (D–F), respectively, in two different slices. Horizontal bars mark duration of exposure. C and F were recorded at 30 min recovery.

Stratum Pyramidale

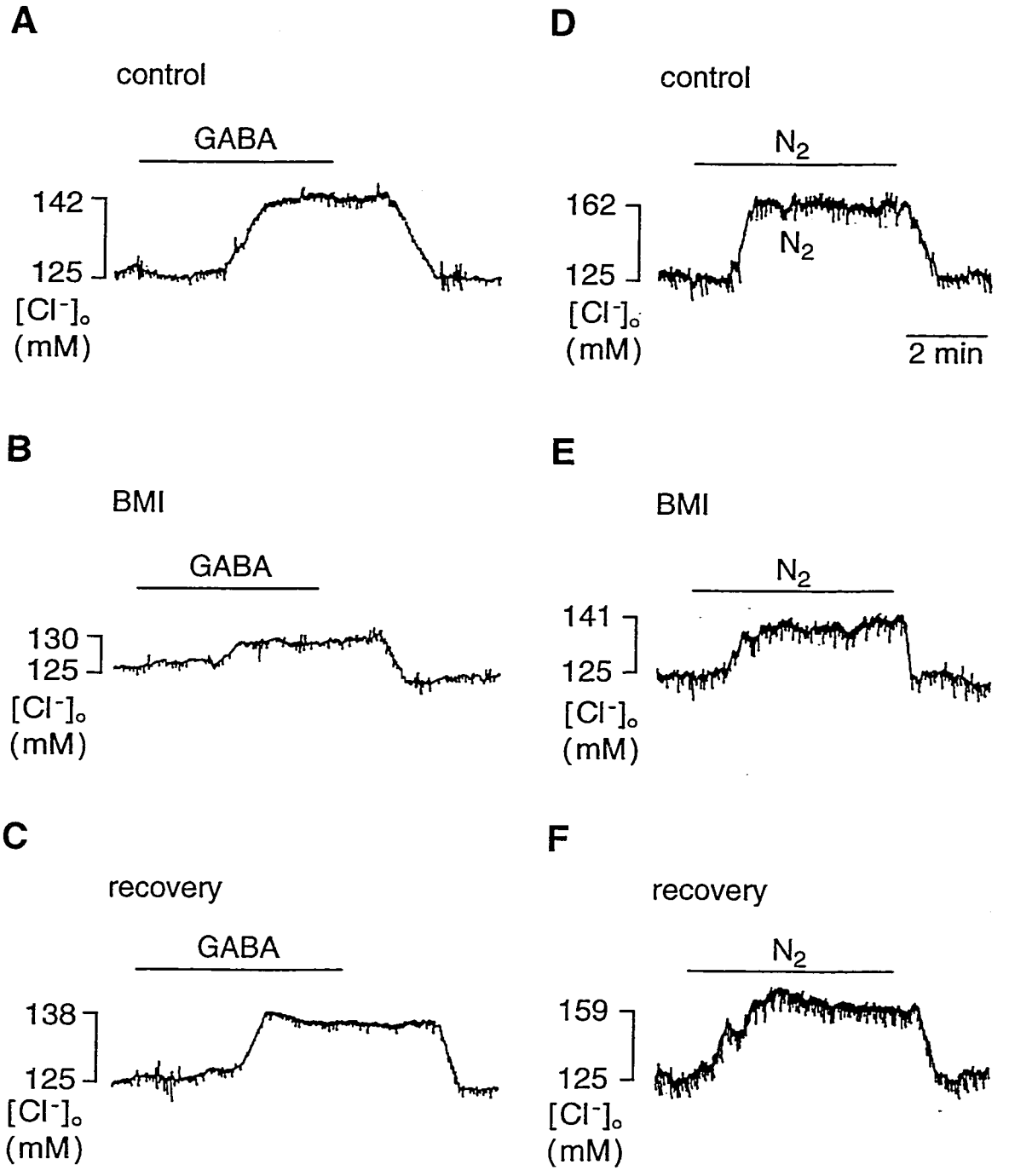

Figure 29. Effects of BMI on $\Delta[Cl^-]_o$ evoked by GABA and anoxia in SR. Examples recorded in SR of reversible attenuation by the $GABA_A$ receptor antagonist, BMI (100 μM) (30 min pre-treatment and continuous perfusion) of $[Cl^-]_o$ responses evoked by 5 mM GABA (A–C) and exposure to N_2 (D–F), respectively, in two different slices. Horizontal bars mark duration of exposure. Records of C and F are at 30 min recovery.

Stratum Radiatum

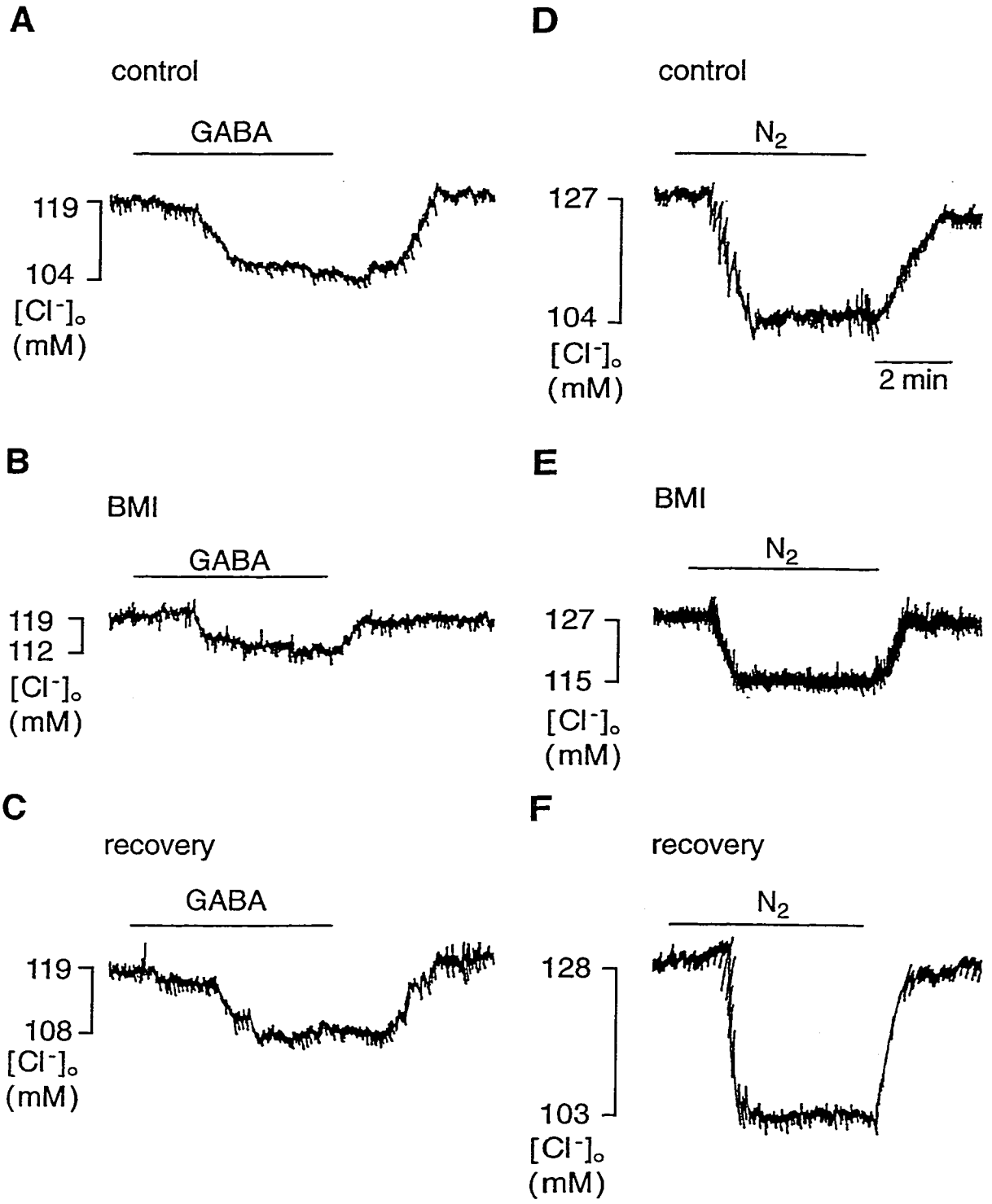

Figure 30. Effects of BMI on $[Na^+]_o$ changes evoked by GABA and anoxia. A and B show examples from one experiment of recordings in SP of reversible attenuation by the $GABA_A$ receptor antagonist, BMI (100 μM) (30 min pre-treatment and continuous perfusion) of $[Na^+]_o$ changes evoked by 5 mM GABA (A–C) and exposure to N_2 (D–F), respectively. Horizontal bars mark duration of exposure. Recovery observations in C and F are at 30 min.

Stratum Pyramidale

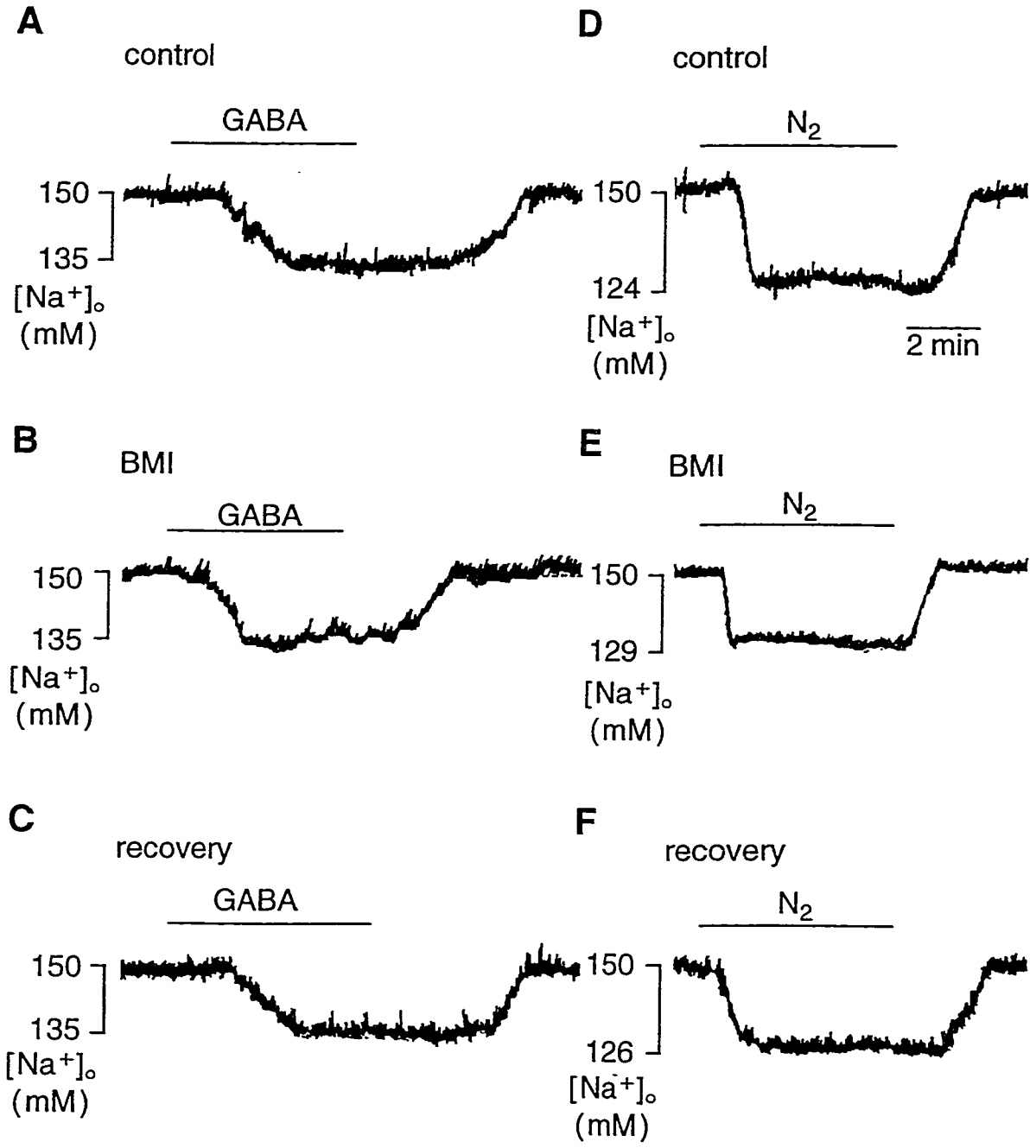

Figure 31. Effects of BMI on $\Delta[Na^+]_o$ evoked by GABA and anoxia in SR. Examples from one experiment of recordings in SR of reversible attenuation by the $GABA_A$ receptor antagonist, BMI (100 μM) (30 min pre-treatment and continuous perfusion) of responses to 5 mM GABA and exposure to N_2, respectively. Horizontal bars mark duration of exposure. Records of C and F are after 30 min recovery.

Stratum Radiatum

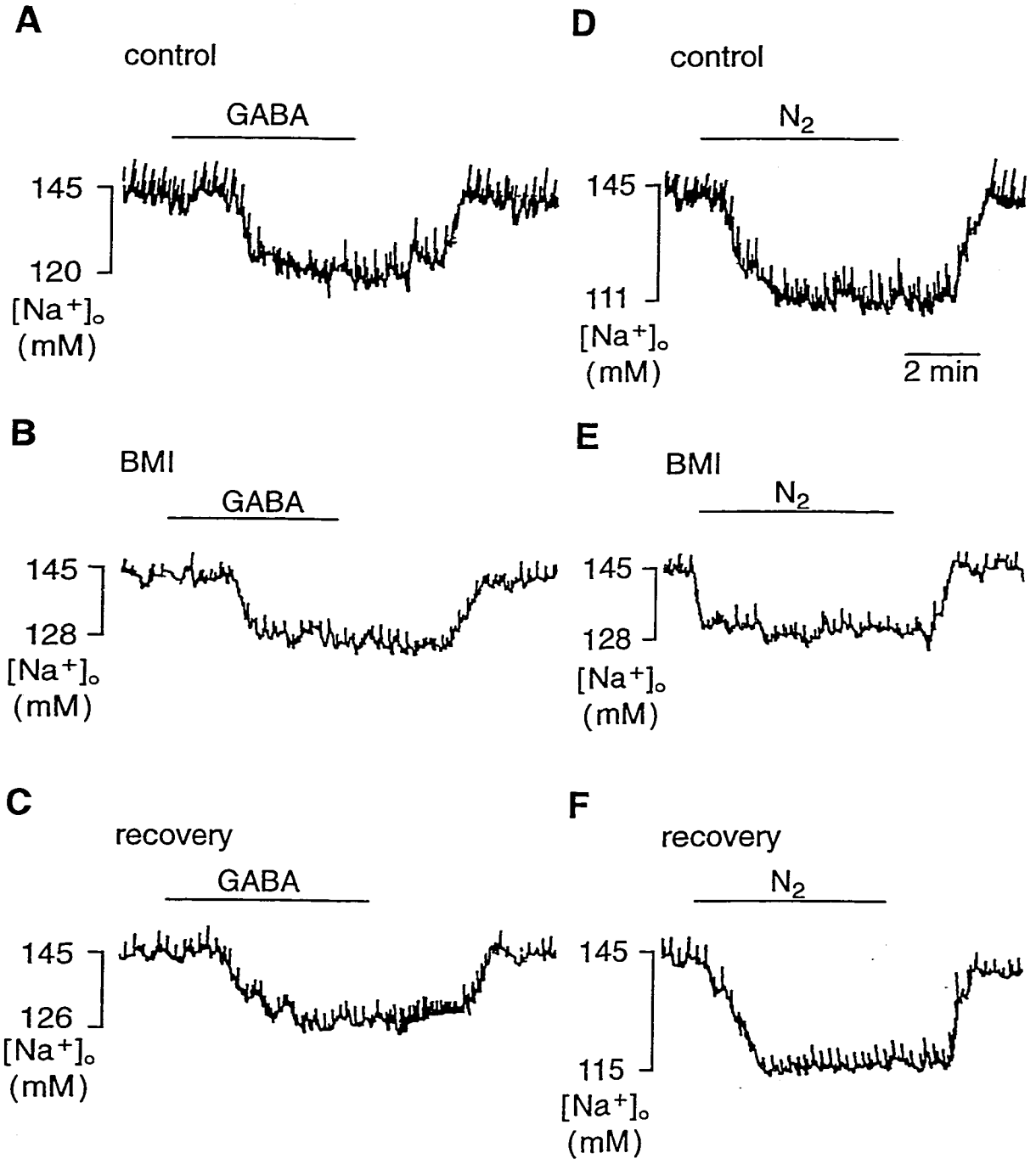

Figure 32. Effects of baclofen on $[K^+]_o$ and field potentials. Responses were evoked by applications of 100 μM baclofen for ≈ 5 min. A and B show examples of recordings made at separate times in stratum pyramidale (SP) and stratum radiatum (SR) from one experiment. In each group of traces $[K^+]_o$ changes are above the simultaneously recorded field potential (V_F). Duration of drug application is shown by horizontal bar; panels A and B have identical scales.

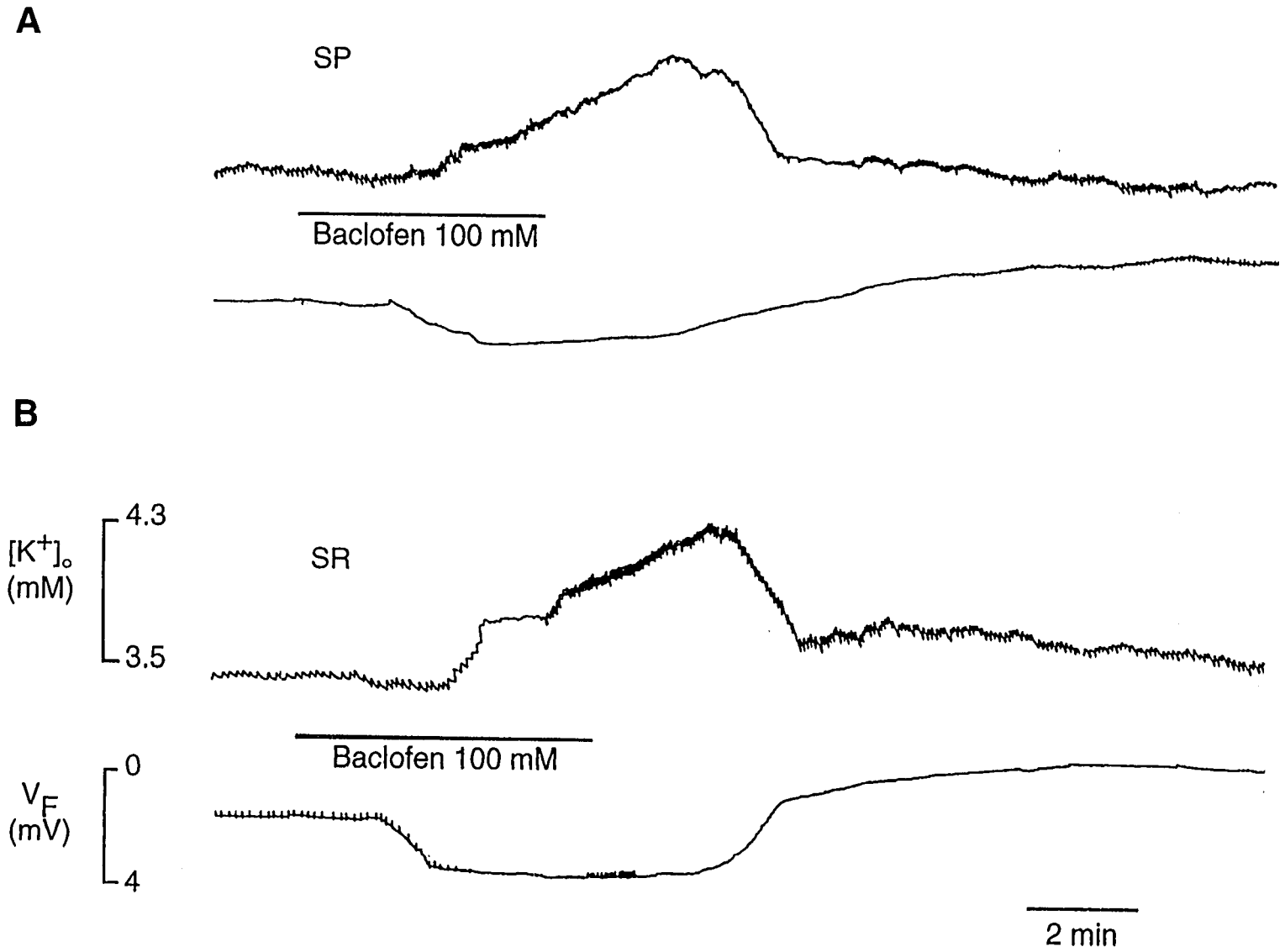
A
SP
Baclofen 100 mM
B
[K+]o
(mM)
4.3
3.5
SR
Baclofen 100 mM
VF
(mV)
0
4
2 min

Figure 33. Concentration-dependent changes in $[K^+]_o$ evoked by baclofen. Bath applications of seven different concentrations of baclofen (10^0 –3 x 10^3 μM) for 5 min. A — stratum pyramidale (SP) — open circles; B — stratum radiatum (SR) — closed circles. Data points are means ± SE for 3–10 measurements at each concentration, made in a total of 10 slices; n = 41 and 36 observations for SP and SR, respectively. Curves were fit by non-linear regression analysis to give values of R_{max} = 0.585 ± 0.03, and EC_{50} = 39.7 μM, r^2 = 0.978 for SP; and R_{max} = 0.648 ± 0.03, EC_{50} = 39.4 μM, r^2 = 0.982 for SR.

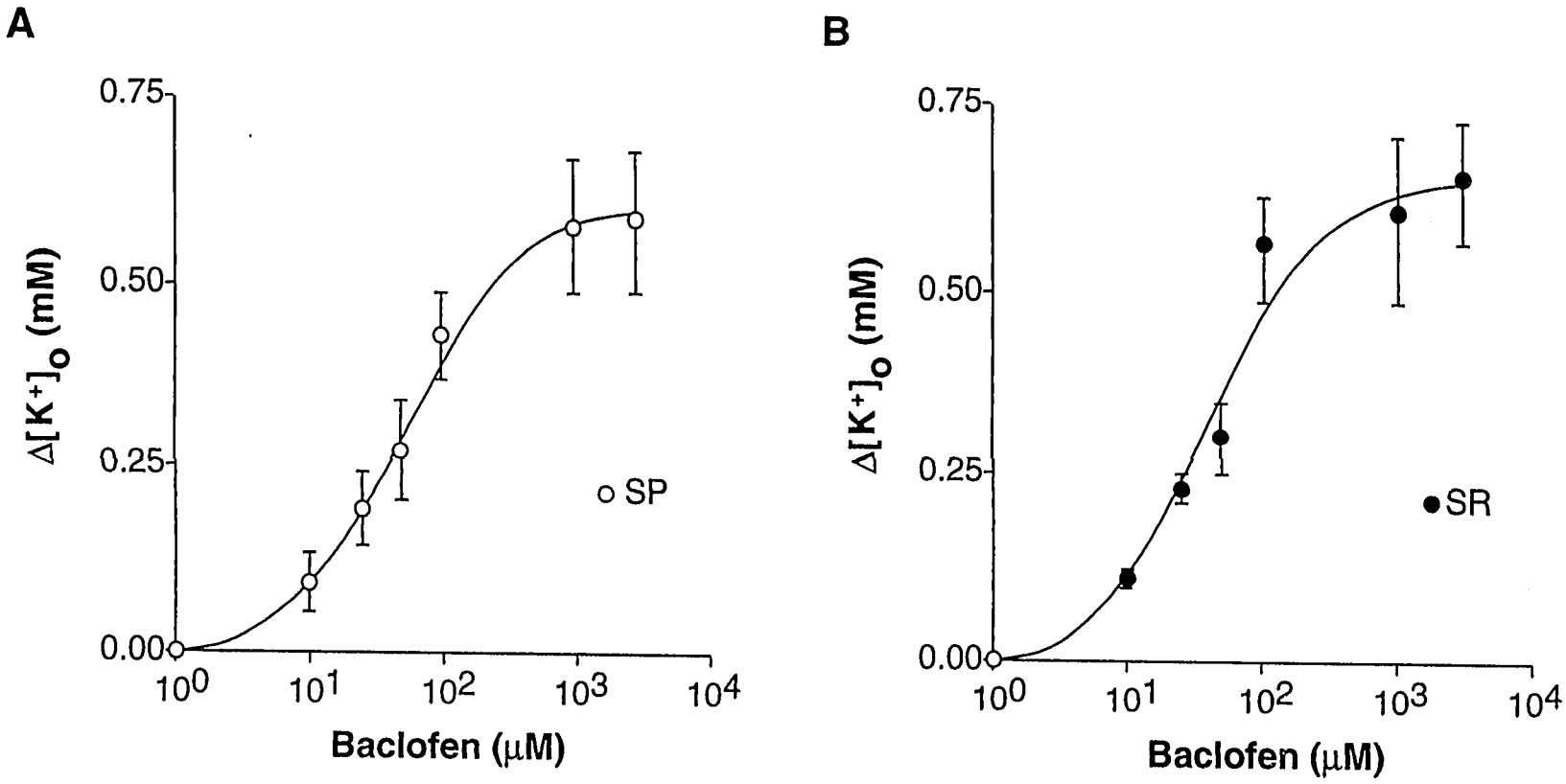
A
0.75
0.50
0.25
0.00
Δ[K+]o (mM)
o SP
10^0
10^1
10^2
10^3
10^4
Baclofen (μM)
B
0.75
0.50
0.25
0.00
Δ[K+]o (mM)
● SR
10^0
10^1
10^2
10^3
10^4
Baclofen (μM)

Figure 34. Correlation of changes in potassium (ΔV_K) and field (ΔV_F) potentials evoked by baclofen. Bath applications of 10 – 3 x 10^3 μM baclofen for 5 min (n = 39 observations, 18 slices). A — stratum pyramidale (SP) — open circles (r = 0.538, $p \leq 0.02$; slope of regression = 0.736 ± 0.519, n = 19). B — stratum radiatum (SR) — filled circles (r = 0.471, $p \leq 0.04$; slope of regression = 0.750 ± 0.332, n = 20).

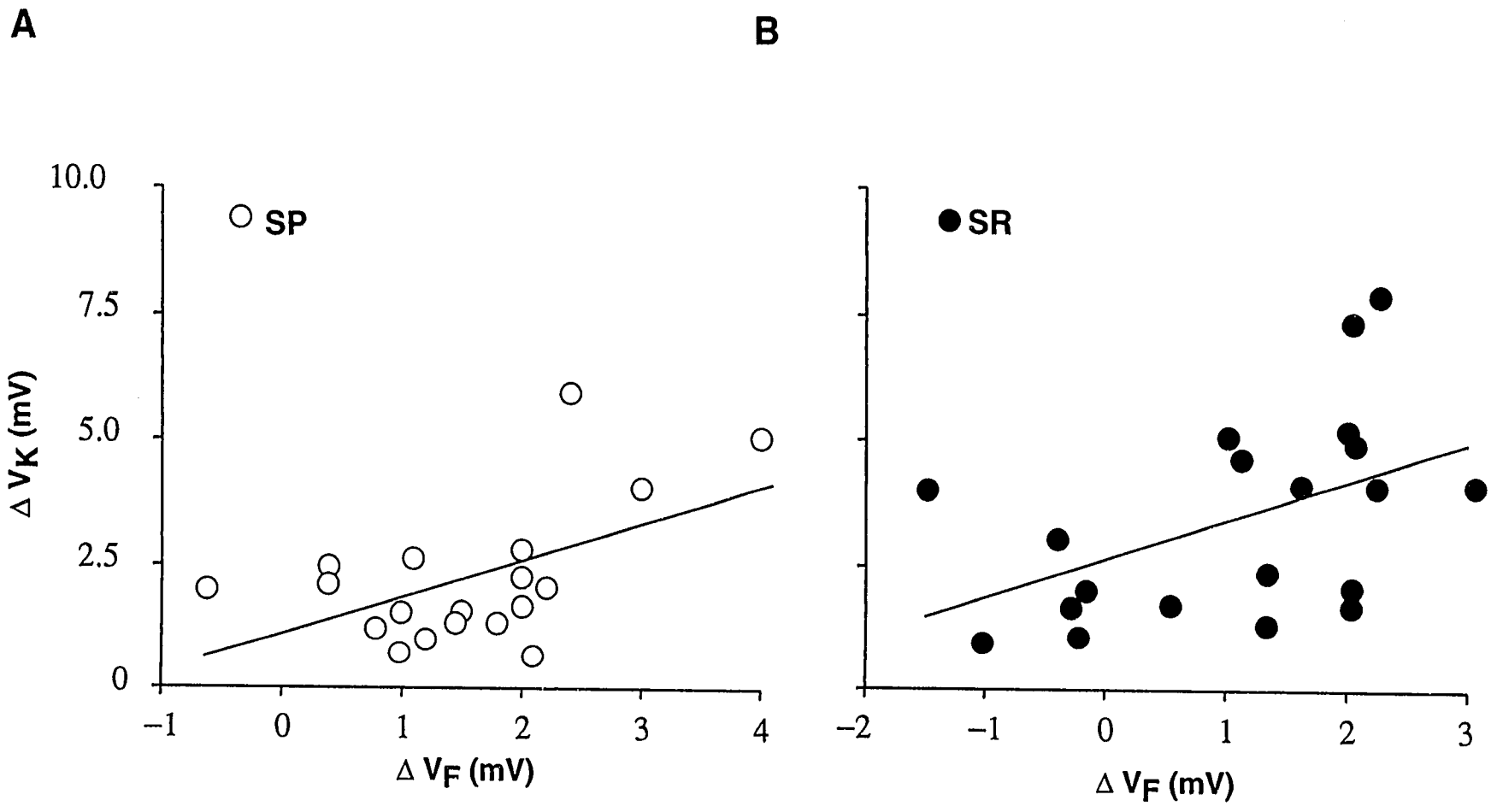
A
B
SP
SR
10.0
7.5
5.0
2.5
0
−1
0
1
2
3
4
−2
−1
0
1
2
3
Δ V_K (mV)
Δ V_F (mV)
Δ V_F (mV)

Figure 35. Recurrent potassium and field potentials evoked by baclofen. A — single example from a series of repetitive changes recorded in SR in one slice 2 min following application of 10 μM baclofen for 2 min. B — Changes recorded from a second slice (same animal) in SP 8 min after an application of 20 μM baclofen for 8½ min. Note different time-bases in A and B; in each group of traces $[K^+]_o$ changes are above the simultaneously recorded field potential (V_F).

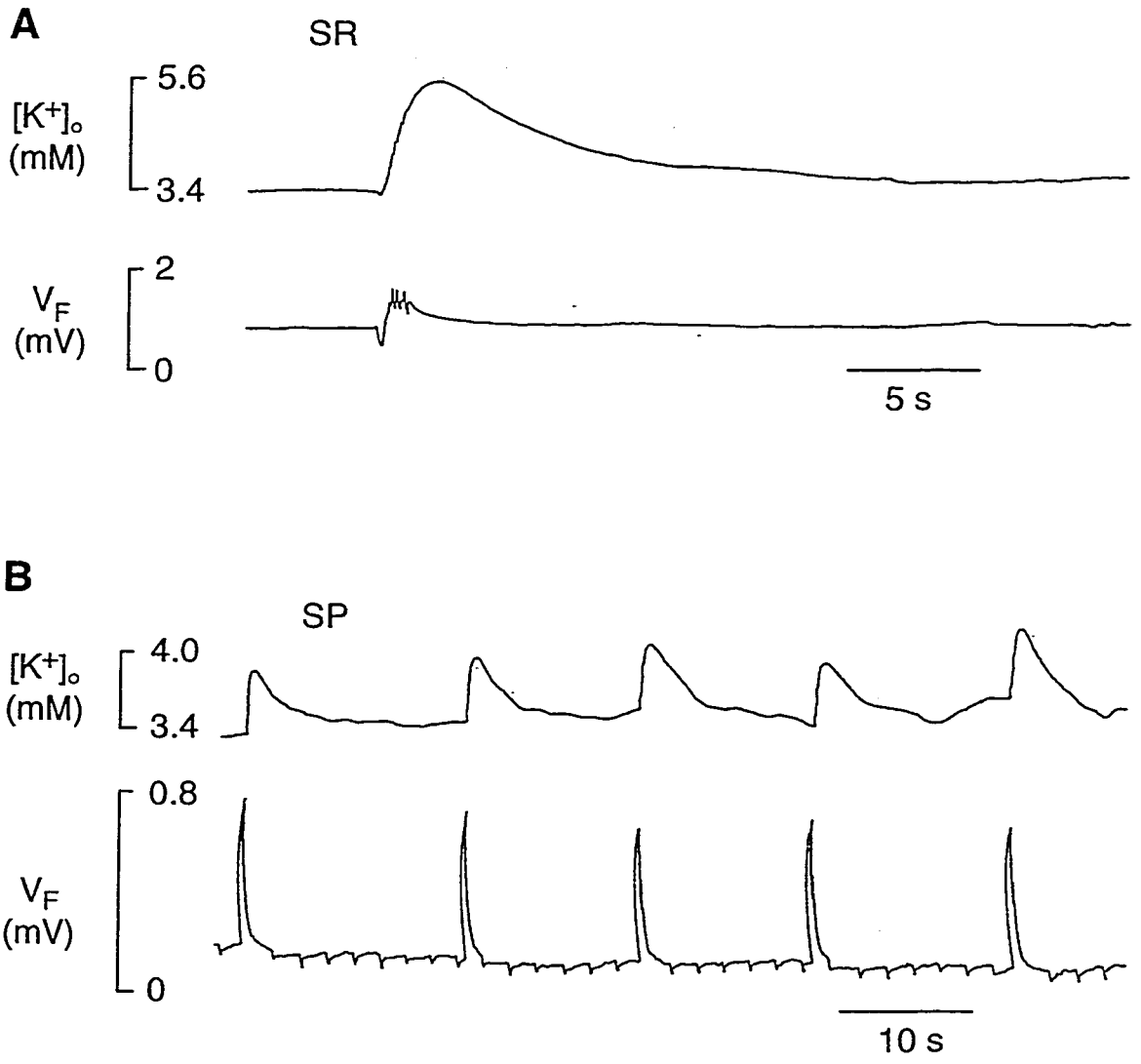
A
SR
5.6
[K+]o
(mM)
3.4
2
VF
(mV)
0
5 s
B
SP
4.0
[K+]o
(mM)
3.4
0.8
VF
(mV)
0
10 s

Figure 36. $[K^+]_o$ changes evoked by local application of baclofen. $[K^+]_o$ increases recorded in stratum pyramidale (SP) — (A), and stratum radiatum (SR) — (B), in response to pressure ejection of 100 μM baclofen for 300 ms (50 psi) from capillary electrode with tip located near recording site. Arrows mark times of application; identical scales for panels A and B.

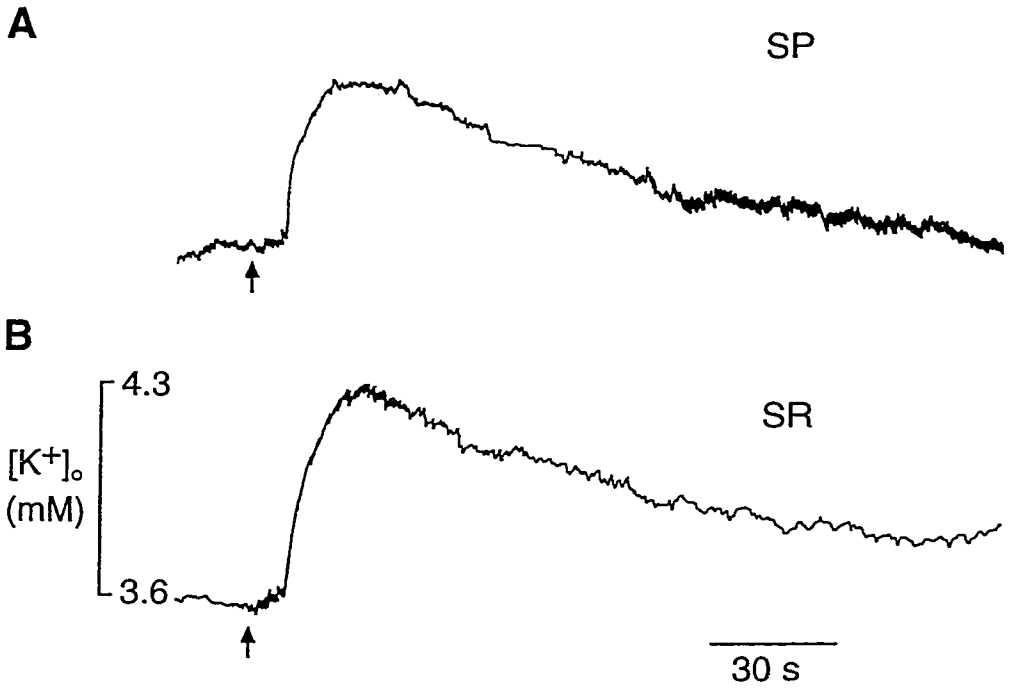
A
SP
B
4.3
[K+]o
(mM)
3.6
SR
30 s

Figure 37. Effects of pulse duration on $\Delta[K^+]_o$ evoked by local application of baclofen. Pressure ejection of 100 μM baclofen with 50 psi pulses of duration 100–900 ms in stratum pyramidale (SP) (unfilled columns) and stratum radiatum (SR) (filled columns). $[K^+]_o$ increases recorded near ejection sites. Data from 4 slices (each from a different animal); paired but separate recordings made in SP and SR for each observation (n = 3–5 for each pulse duration; total number of observations = 28; bars represent SE).

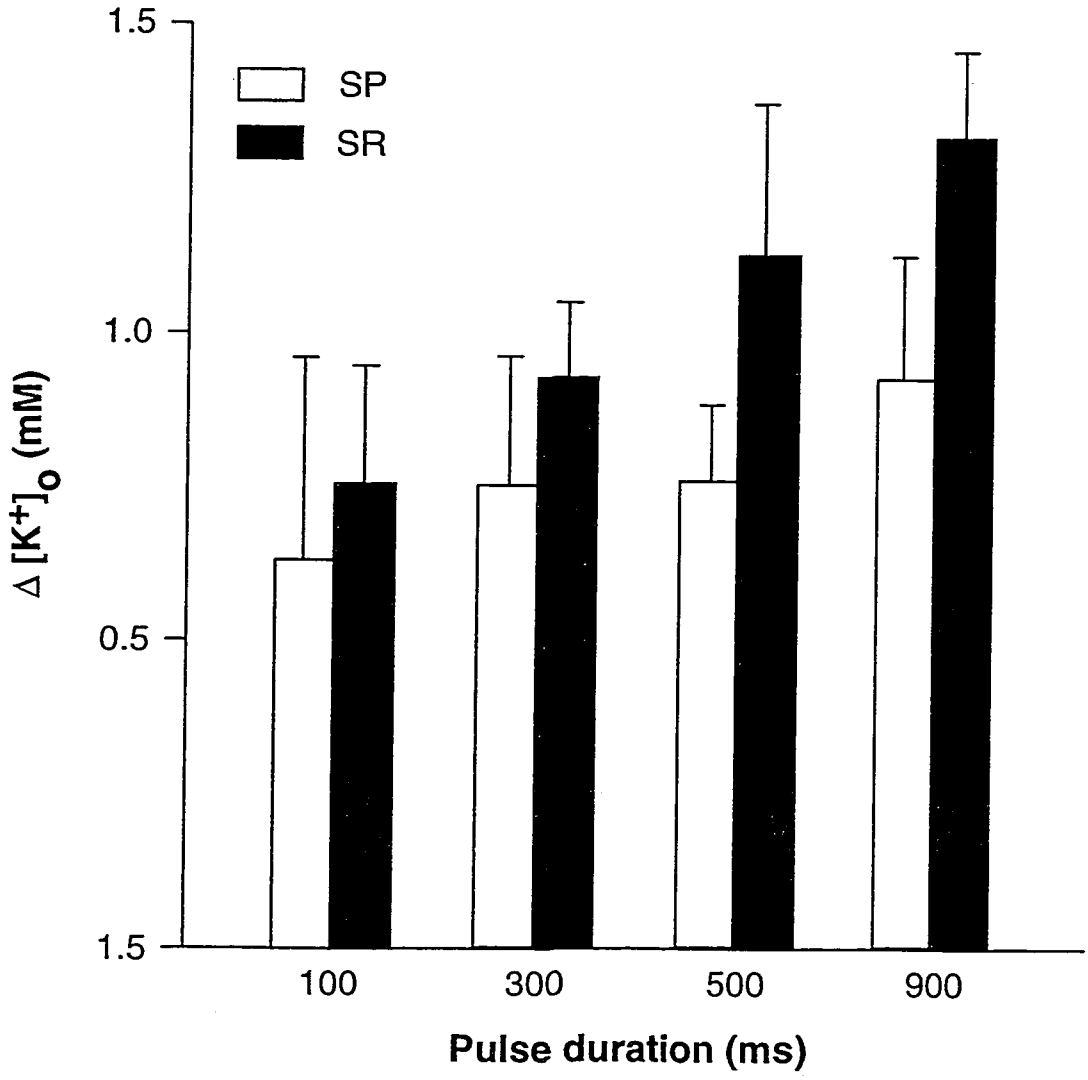
1.5
1.0
0.5
1.5
SP
SR
Δ [K+]o (mM)
100
300
500
900
Pulse duration (ms)

Figure 38. Effect of the $GABA_B$ receptor antagonist, CGP 35348 on baclofen-evoked $\Delta[K^+]_o$. A — control recording of $[K^+]_o$ increase induced by application of baclofen 100 μM for 5 min. B — shows attenuation of response by co-application of CGP 35348 50 μM with baclofen 100 μM. C — shows complete block of response to baclofen after presence of CGP 35348 for 25 min. D — recovery of baclofen-induced $[K^+]_o$ increase after 60 min washout of antagonist. Horizontal lines mark duration of applications of baclofen; all panels have identical scales.

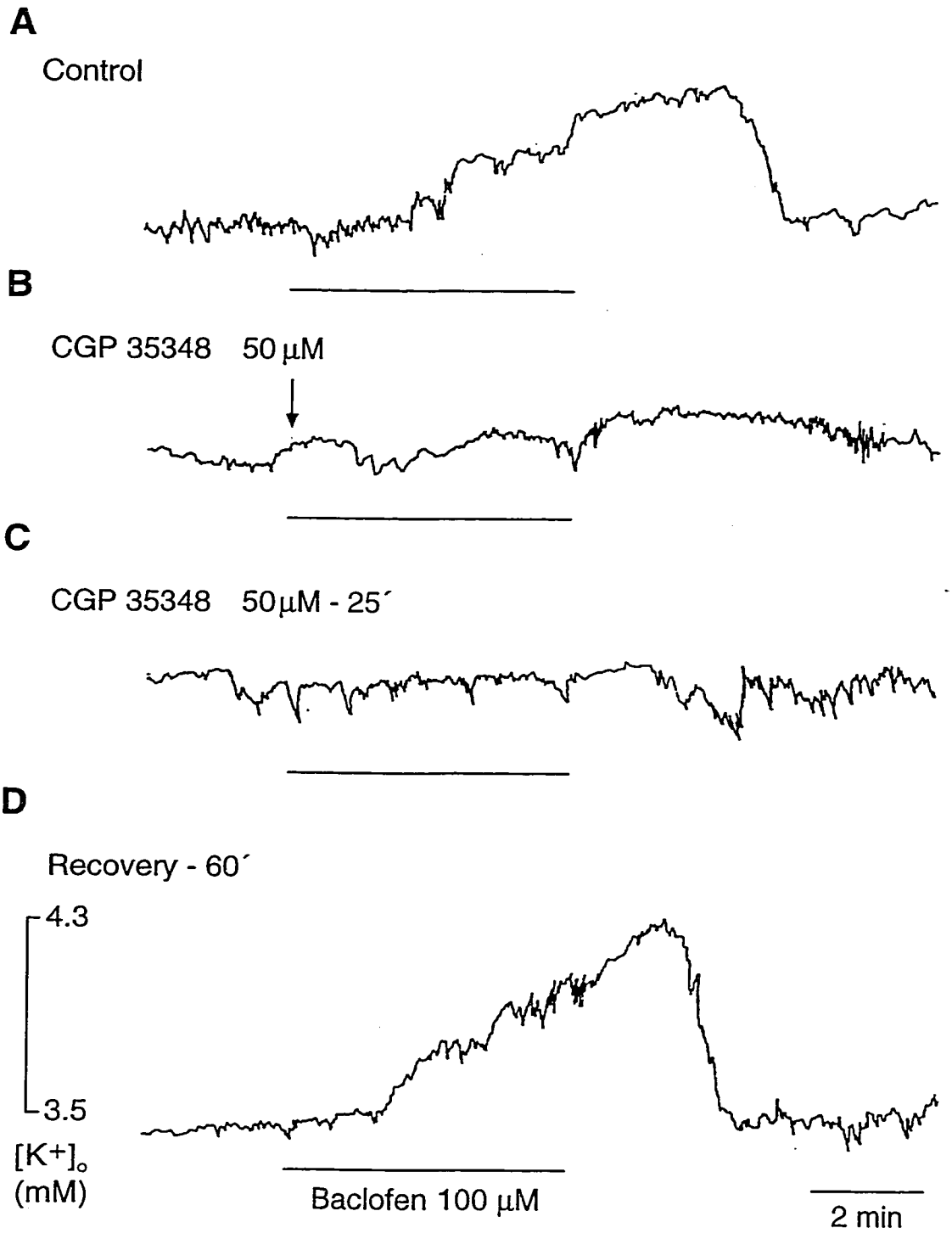
A
Control
B
CGP 35348 50 μM
C
CGP 35348 50 μM - 25´
D
Recovery - 60´
4.3
3.5
[K+]o
(mM)
Baclofen 100 μM
2 min

Figure 39. Hyperpolarization evoked by anoxia. A and B — hyperpolarizing responses evoked by brief N_2 exposure and recorded in two CA1 pyramidal neurons. R_M was monitored continuously by injection of hyperpolarizing pulses (0.2–0.3 nA); current monitor records (I_M) are below in each group. Numbers at left indicate membrane potential in mV; in lower trace of A hyperpolarizing current (I_{hp}) held membrane potential at –71 mV. Horizontal bars mark duration of N_2 exposure. Arrows indicate PAH (post-anoxic hyperpolarization). Dotted lines indicate pre-anoxic baseline resting potential.

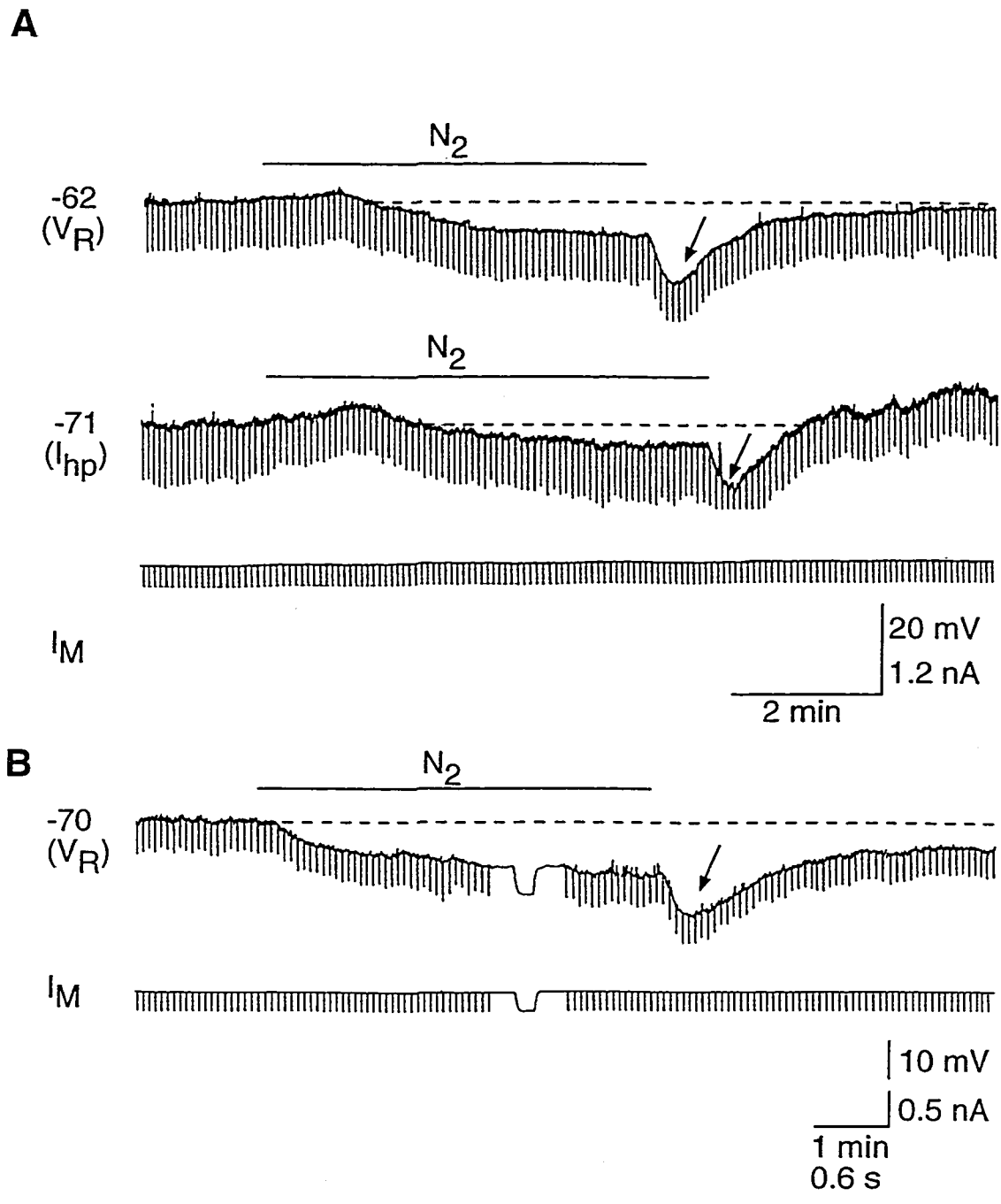
A
N2
-62
(VR)
N2
-71
(Ihp)
IM
20 mV
1.2 nA
2 min
B
N2
-70
(VR)
IM
10 mV
0.5 nA
1 min
0.6 s

Figure 40. **Depolarization evoked by anoxia.** A and B — depolarizing responses recorded in a CA1 pyramidal neuron during N_2 exposure. In A hyperpolarizing pulses (0.2 nA) show increase in R_M during anoxia. In B the anoxic depolarization is accompanied by depression of synaptic transmission — with earlier loss and delayed recovery of the IPSP. Inset below shows time course of EPSP-IPSP complex evoked by 1/30 s stimulation of Schaffer collaterals. Note burst of action potentials at peak of depolarization. Horizontal bars mark duration of N_2 application.

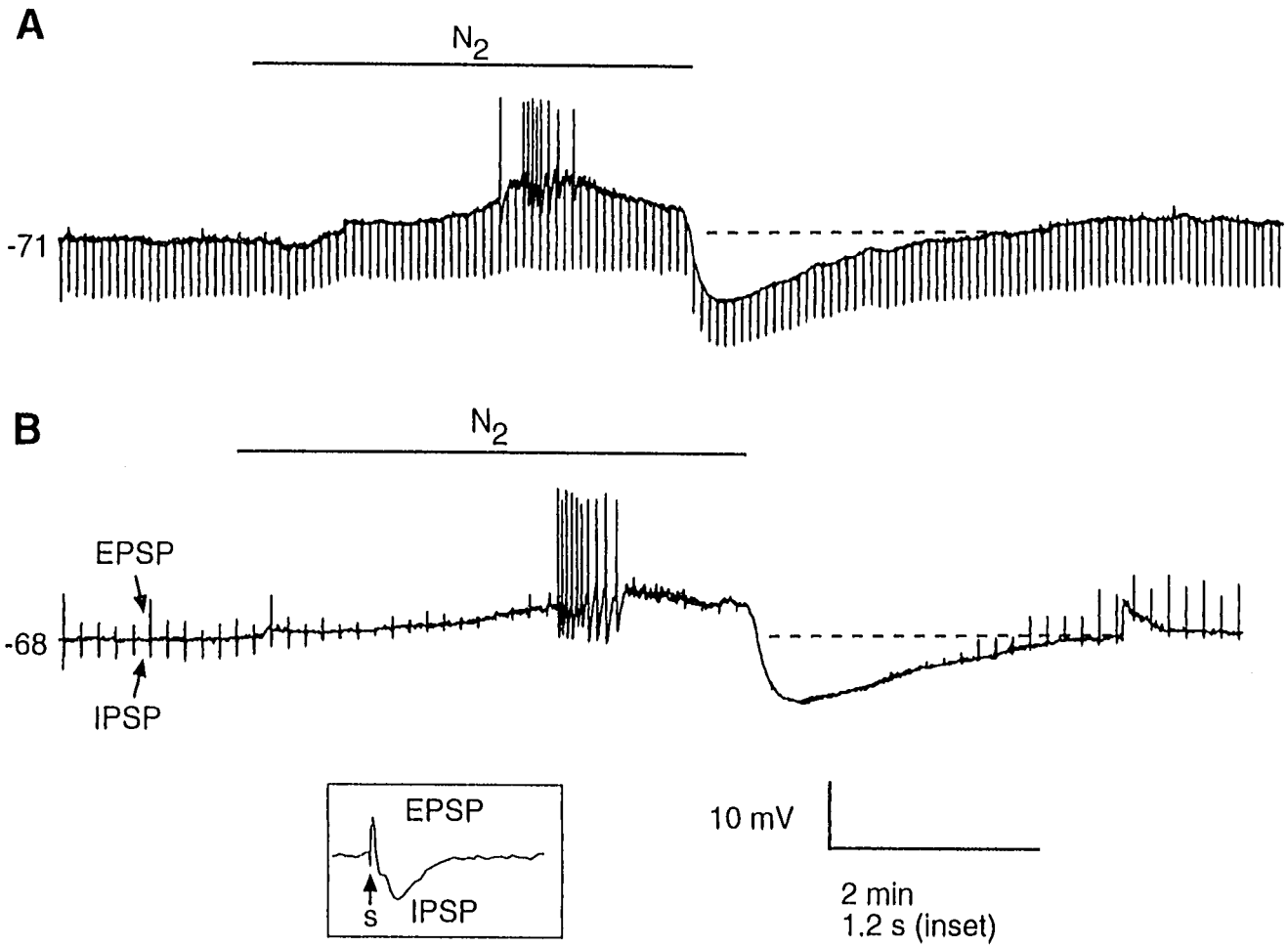
A
N_2
-71
B
N_2
EPSP
-68
IPSP
EPSP
s
IPSP
10 mV
2 min
1.2 s (inset)

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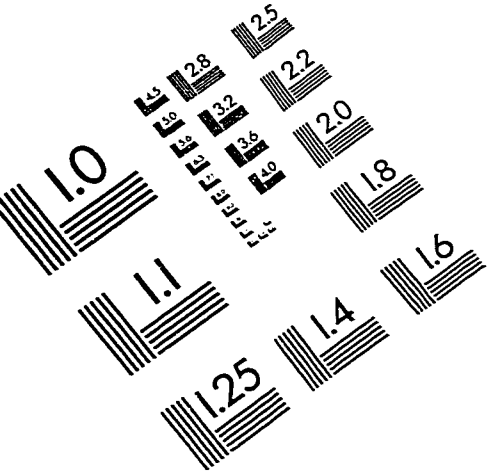
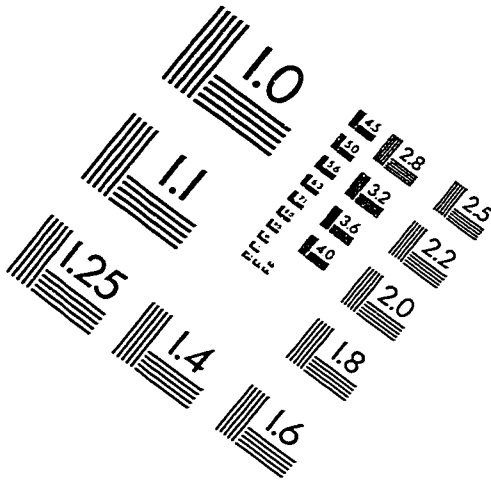
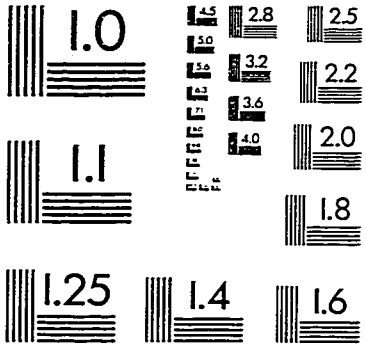
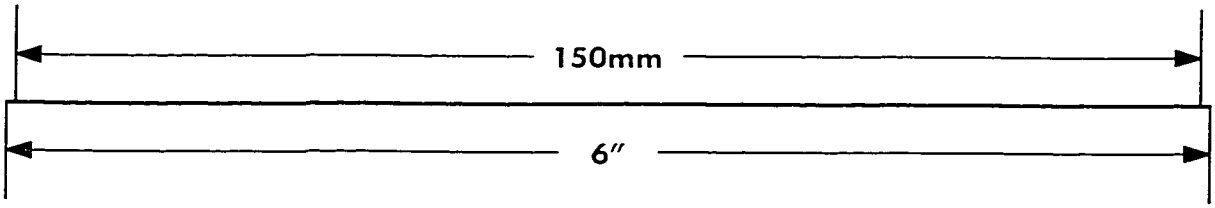
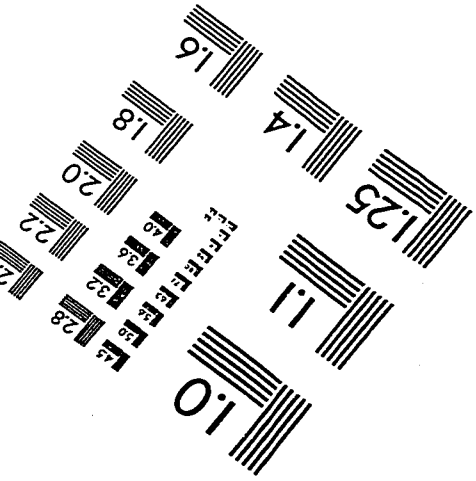
IMAGE EVALUATION TEST TARGET (QA-3)

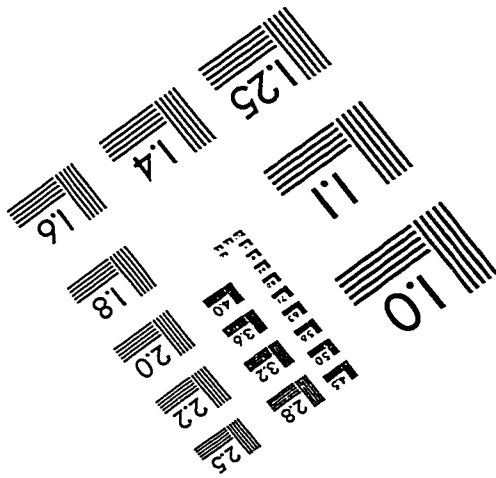












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