

MECHANISMS OF ANAESTHETIC DEPRESSION OF NEOCORTICAL AROUSAL

by

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ABSTRACT

The most widely accepted hypotheses suggest that general anaesthetics interrupt conscious processes in the brain by decreasing synaptic excitation or by potentiating synaptic inhibition, especially in the neocortex. The putative transmitters in the neurological systems that generate neocortical arousal include acetylcholine, glutamate and γ -aminobutyrate (GABA). The primary objective here was to determine the neuronal mechanisms by which anaesthetics may obtund this arousal.

The majority of the investigations were carried out on pyramidal neurons in layers IV and V of guinea pig neocortex (in vitro slices), using intracellular recording and pharmacological, including microiontophoretic, techniques. Bath applications of structurally dissimilar anaesthetics, isoflurane - a halogenated ether, and Althesin - a steroidal preparation, in concentrations of 0.5-2.5 minimum alveolar concentration (MAC) and 10-1300 μ M, respectively, produced a small hyperpolarization (3-5 mV) which was associated with an increase in input conductance (10-30%). The lower concentrations (0.5-1.5 MAC and 10-200 μ M) of these agents which are most relevant to the production of unconsciousness did not significantly affect the passive membrane properties. However, they produced striking decreases in spontaneous activities and the repetitive spike firing evoked by orthodromic (electrical) stimulation or intracellular current injections.

Because the observed changes in membrane properties could not explain the reduction in neuronal excitability, the effects of anaesthetics were investigated extensively on excitatory and inhibitory postsynaptic potentials (EPSPs and IPSPs). The application of isoflurane or Althesin induced a dose-dependent, reversible depression in the amplitude of EPSPs,

with EC_{50} s of 1 MAC and $\sim 50 \mu M$, respectively. The IPSPs also were reduced in a dose-dependent manner. In order to eliminate possible shunting of the EPSPs by the GABA-activated Cl^- -conductance that produces the IPSP in the observed EPSP-IPSP sequence, a GABA_A-antagonist, bicuculline, was additionally applied. Despite this IPSP-blockade, the anaesthetics strongly depressed the EPSPs as well as epileptiform activities evoked by subpial electrical stimulation.

In cognizance of the possibility that a postsynaptic attenuation of responsiveness to transmitter substances may be involved in the EPSP depression, the neuronal sensitivities to acetylcholine, glutamate and GABA were determined. Anaesthetic administration markedly reduced the depolarizations and associated conductance changes evoked by dendritic applications of acetylcholine, glutamate and N-methyl-D-aspartate (NMDA). The hyperpolarizing responses to somatic applications of GABA were not affected significantly whereas the depolarizing effects observed with its dendritic application were slightly depressed. Some degree of selectivity also was evident from the lower EC_{50} s for the isoflurane- and Althesin-induced depressions of responses to acetylcholine compared with glutamate. Under in vitro conditions of hypomagnesemia the responses to acetylcholine were totally blocked and the order of depression in the responses to GABA and glutamate was reversed; this may be of importance in the mechanism for the known increase in anaesthetic requirements in clinical syndromes associated with hypomagnesaemia.

Because the genesis of synaptic transients is affected by Ca^{2+} influx or disposition, the interactions of anaesthetics were investigated on spike afterhyperpolarizations (AHPs). The AHPs which are produced specifically by a Ca^{2+} -activated K^+ -conductance were suppressed by the anaesthetics in a

dose-dependent manner under conditions where contaminating IPSPs had been blocked by bicuculline. Since the passive membrane properties were unaffected, an interference with a transmembrane Ca^{2+} -influx may be involved in the anaesthetic actions.

The effects of anaesthetics on glutamate-induced and voltage-dependent increases in intraneuronal Ca^{2+} ($[\text{Ca}^{2+}]_i$) were determined in cultured hippocampal neurons with a Ca-sensitive probe (Fura-2) and microspectrofluorometric techniques. Isoflurane application depressed the increases in $[\text{Ca}^{2+}]_i$ produced by application of glutamate under conditions where its actions would be favoured at NMDA- and quisqualate-subtypes of receptors. K^{+} -induced increases in $[\text{Ca}^{2+}]_i$ also were reduced by application of isoflurane, probably due to actions on voltage-dependent Ca-channels in the membrane.

These investigations have provided evidence for the first time that excitatory transmitter actions in neocortex are selectively depressed by anaesthesia. A plausible mechanism would include suppression of the postsynaptic Ca-conductances associated with the AHPs and glutamatergic, as well as cholinergic interactions at pre- and post-synaptic sites on neurons involved in neocortical arousal.

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LIST OF ABBREVIATIONS

Acetylcholine	ACh
Afterdepolarization	ADP
Afterhyperpolarization	AHP
2-amino-5-phosphonovalerate	2-APV
4-aminopyridine	4-AP
γ -aminobutyrate	GABA
Ampere(s)	A
Artificial cerebrospinal fluid	ACSF
Cyclic guanosine monophosphate	cGMP
2,4-dinitrophenol	DNP
Effective concentration	EC
Effective dose	ED
Excitatory postsynaptic potential	EPSP
Extracellular calcium concentration	$[Ca^{2+}]_o$
Extracellular magnesium concentration	$[Mg^{2+}]_o$
Glutamate	Glu
Inhibitory postsynaptic potential	IPSP
Input resistance	R_i
Intracellular free calcium concentration	$[Ca^{2+}]_i$
Intracellular free magnesium concentration	$[Mg^{2+}]_i$
Kainate	Ka
N-methyl-D-aspartate	NMDA
Minimum alveolar concentration	MAC
Minute(s)	min
Ohm(s)	Ω
Protein kinase C	PKC
Quisqualate	Quis
Resting membrane potential	V_m
Reticular activating system	RAS
Second(s)	s
Standard deviation	SD
Standard error of mean	SEM
Tetraethylammonium	TEA
Tetrodotoxin	TTX
Volts	V

In the name of God, Most Gracious, Most Merciful

"Say: He is God, the One and Only. God the Eternal, Absolute.

He begetteth not, nor is He begotten. And there is none Like unto Him."

(Translation from the Holy Qur'an)

I wish to dedicate this thesis to my parents,

Nabila and Mostafa El-Beheiry,

who have struggled continuously for my well-being.

1 INTRODUCTION

1.1 General scope of topic

During the past two decades, virtually hundreds of actions of general anaesthetics on vertebrate and invertebrate neurons have been described. However, there are no hypotheses that adequately explain the neuronal mechanisms by which these agents produce the unconscious state. There are two main reasons for this. First, anaesthetic actions have not been thoroughly studied on relevant cell types in the central nervous system (CNS). Neurophysiological and biochemical (metabolic) evidence indicates that neocortical neurons are the likely target sites for anaesthetic agents. Certain technical difficulties and a modest understanding of the physiological mechanisms of arousal have limited the previous investigations to the more readily studied neurons of subcortical structures or tissue cultured neurons. Secondly, there is an absence of a common specificity in the chemical structure of hydrocarbons, ethers, barbiturates, pyrimidines, steroids and inorganic compounds that possess the anaesthetic property. Comprehensive reports that have compared the actions of structurally dissimilar anaesthetics on the same physiological type of neuron are rare, despite a perceived importance of such experimentation.

In the present research, the effects of two chemically distinct anaesthetics, isoflurane -- a relatively recent, volatile agent, and Althesin -- a steroidal preparation, were studied on neocortical neurons of the brain in order to address four fundamental questions:

- (a) do structurally different anaesthetics have similar (depressant) effects on the same neuronal type?

- (b) is anaesthetic-induced depression of neuronal activity in neocortex due to an attenuation of excitation or an enhancement of prevailing inhibition at synapses (or both)?
- (c) by what neuronal mechanisms do anaesthetics obtund neocortical arousal?
- (d) what are the plausible ionic mechanisms for the anaesthetic interference with excitatory or inhibitory processes?

A major hypothesis evolved during the course of these investigations, i.e., general anaesthetics act with varying degrees of efficacy on neuronal activities in the neocortex by selectively depressing certain electrical processes. Neuronal excitabilities are decreased in a specific manner and a cortical awareness of the external and internal environments are consequently obtunded, resulting in the unconscious state. In order to more fully understand the basis for this, a synopsis of current evidence as to how consciousness is brought about in unanaesthetized brain is presented below.

1.2 The Conscious System In The Brain

1.2.1 Definitions. Among all aspects of human behaviour consciousness is probably the most difficult to define. This is partly because consciousness is phenomenological and is regarded as an attribute of the CNS; this is somewhat analogous to the property of "luminescence" which is considered an attribute of cultured luminous bacteria (White 1987).

Recent attempts to visualize consciousness as a multicomponent system have met with some success (Kissin 1986). The various subsystems involved in the processes producing consciousness of the brain are closely integrated in function and electrical activity. As a consequence of the interdependency of the modular organization, consciousness is usually experienced as a

single, though integrated entity. In the multicompartmental conceptualization, consciousness has objective (alertness) and subjective (awareness) components. Awareness in turn may be vague, i.e., general in content or specific in discrimination (self-awareness). In addition, consciousness is characterized by a "level of energy" that has been referred to as "arousal" or "activation" and qualitatively, as "affect". Thus a beam of awareness may be focused in one direction or another under the paradigm of "attention". These components -- activation, alertness, arousal, affect, the dual facets of awareness and attention -- represent different aspects of consciousness. The above terms describe different dimensions of consciousness, each of which constitutes a separate and discrete process under the control of a defined anatomical and physiological system in the brain.

1.2.2 Integration of function and activity of the conscious subsystems. As mentioned above, the various subsystems of consciousness are closely integrated in function and activity. For example, an increase in arousal brought about by an increase in motivational excitation is usually associated, on an almost one-to-one basis with comparable increases in activation, alertness, awareness and attention. Therefore, motivational-emotional arousal produces an electrophysiological activation of the brain, which is translated epiphenomenally into alertness and awareness. Awareness is focused through attention onto the cognitively and motivationally significant events in the internal and external environments such that the final sequence of drive-oriented behavioural responses can be produced or repeated.

1.2.3 Functional neuroanatomy of the consciousness system (a) Activating systems of the brain. These are generated by the ascending reticular activating pathways that project from the medial portion of the

brainstem into the dorsal medial thalamus whereby impulses radiate to all portions of the basal ganglia, limbic system and most significantly, to the neocortex (Moruzzi and Magoun 1948; Morison and Dempsey 1962). The reticular activating system (RAS) controls the 'energy levels' in the brain. Hence, a state of diffuse background activation of the cerebral cortex can be maintained for the conscious state (Webb 1983). The 'pacemaker' of the RAS is the locus coeruleus of the pons. Electrical activity in the locus coeruleus is reflected by the presence of β - (high activity) or γ - (very high activity) waves in the cortically monitored EEG (Snyder and Scott 1972).

(b) Alertness subsystem. Alertness is the behavioural level of consciousness when the RAS is mildly active as in the so-called resting, relaxed state (Kissin 1986). An individual may be conscious but generally unattentive during which the thalamus induces α -waves in the EEG. This is different from the behavioural level of awareness which reflects the activation of the awareness system by the RAS of the brain (see below).

(c) Awareness systems of the brain. These consist of general and self-awareness subsystems of the brainstem. Both are connected to the activating systems and to each other. The general awareness system consists of the posterior hypothalamus, the dorsal median and anterior nuclei of the thalamus, the globus pallidus and the putamen of the basal ganglia and the nucleus basalis of Meynert in the substantia innominata of the paleocortex. The individual anatomical units of this system, though anatomically disperse, operate in an integrated fashion to provide all the primitive elements of consciousness and attentional activities. Lesions in this system can result in a syndrome known as akinetic mutism, in which there is no reaction to external stimuli (Girvin 1975). The self-awareness system is responsible

for the awareness of the internal environment i.e., "one's self". The parts of the brain that are involved in the phenomenology of self-awareness according to Mesulam and Geschwind (1978) are the nucleus accumbens septi of the diencephalon, substantia innominata and the posterior inferior parietal lobes. The latter receive cholinergic projections from the diencephalic and paleocortical centres. Heilman and Watson (1977) and Mesulam and Geschwind (1978) have described a characteristic behaviour in patients with unilateral lesions in the inferior parietal lobe secondary to either a stroke or tumor. The individual often behaves as though one-half of the world did not exist at all, i.e., a patient may shave only one side of his face or another patient may dress only half of his body.

(d) The attention apparatus. Activation of the thalamo-basal ganglia complex, brainstem general awareness system and the nucleus accumbens septi, substantia innominata and posterior inferior lobe self-awareness system produces a generalized state of alertness and awareness. Such activation would be meaningless unless it were focused on critical events in the environment. The sequence of events can be reconstructed as follows: The attention apparatus (1) assesses environmental events that are deemed biologically significant, (2) directs more specific aspects of consciousness to these events, (3) ties together the events directly into the activated self-concept system and (4) mobilizes all reserves to deal with them most effectively .

The neocortex is the site of these complex consciousness-directing mechanisms (cf. Kissin 1986). The sensory cortex and the inferior temporal lobe receive primary sensory (processed) information from the thalamus and contiguous hypothalamus as well as the basal ganglia, for cognitive processing and motivational emotional/evaluation, respectively. Integrated

information from these control centres flows to the prefrontal area which in turn controls the flow through the amygdaloid-hippocampal complex, i.e., for the attention-directing activities. In addition, there exists a prefrontal-thalamic feedback arrangement which inhibits electrical activity in the unattended sensory channels. Thus attention can be concentrated towards a given sensory modality.

1.2.4 Neocortical activation. A functioning consciousness system is characterized by a steady state level of cortical excitation (Matsumura et al. 1988) which is prerequisite to the fast, 'phasic' transfer of information bidirectionally in the attention system. Neocortical activation results from subcortical as well as intracortical (horizontal or vertical) excitatory transmission (Enzure and Oshima 1981a; Giuffrida and Rustioni 1989). Cholinergic ascending reticular pathways project to the deep cortical layers (Pauly et al 1989) originating from the midbrain, pons, thalamus and magnocellular neurons in the basal forebrain (Shute and Lewis 1967; Divac 1975; Hoover and Jacobowitz 1979; Lehmann et al. 1980; Kimura et al. 1981; Spencer et al 1986). Adrenergic and histaminergic influences in neocortical activation may be significant (Rothballer 1956; Ling and Foulks 1959; Wanatabe et al. 1984; Reiner and McGeer 1987) as indicated by demonstrations of specialized groups of biogenic amine-containing neurons in the midbrain and pons that project diffusely to the cortex (Reader et al. 1988). Adrenergic and serotonergic ascending systems to the neocortex have been described (Levitt and Moore 1978; Lidov et al. 1980) although these are more related to the sleep-waking cycle (Aston-Jones and Bloom 1981) than to the maintenance of consciousness. More recently, direct morphological evidence has been interpreted for the coexistence of the neuropeptide substance P with acetylcholine in RAS neurons (Vincent et al. 1983).

1.2.5 Activating neurotransmitters in the neocortex. Acetylcholine (ACh) is considered a major cortical 'activating transmitter' because neocortical activation can be elicited with cholinomimetics and blocked by atropine (Funderburk and Case 1951). A role for ACh at neocortical synapses has been suggested in the generation of a state of enhanced responsiveness of neurons. Applications of ACh enhance the neuronal firing evoked by electrical stimulation of synaptic inputs (likely glutamatergic pathways) and prolongs afterdischarges such that the spike bursts are more numerous and prolonged in duration (Krnjević 1987, 1988). Also, exogenous ACh can induce repetitive firing which is believed to be prerequisite to the generation of mnemonic traces (Krnjević et al. 1971a).

Another likely major transmitter in the neocortex is glutamate (Glu) (Krnjević and Phillis 1963a; McLennan 1983; Puil and Benjamin 1988). In contrast to ACh, Glu has a rapid onset/offset action. Glu, acting as a transmitter, may be responsible for the fast transfer as well as processing of information at synapses in pathways for conscious processing (Krnjević 1987). However, ascending cholinergic excitation of the neocortex modulates the rate of transfer of information, intracortically, as well as between cortical and subcortical structures (Conti et al. 1987; Giuffrida and Rustioni 1989). For example, in a comatose patient, the tonic ACh excitation may be attenuated to a lower level than that present in an alert individual. Hence, the processing of sensory information is obtunded in the unconscious state.

A contribution of inhibitory transmission to neocortical activation has yet to be elucidated. However, some reports suggest a model of neuronal circuitry that may make up the elementary structure of cortical activation (Enzure and Oshima 1981b). In addition to excitatory neurons, inhibitory

GABAergic neurons have been postulated in the model; these have been identified during stimulation of the RAS in awake cats (Inubushi et al. 1978a, b; Enzure and Oshima 1981a). Consequently, GABA could be an important transmitter responsible for a negative feed-back loop in the neuronal organization of the consciousness system.

1.2.6 The 'activating transmitters' and their ionic mechanisms of action

1.2.6.1 Acetylcholine (ACh). Excitation mediated by ACh or by other transmitters is brought about through the following ionic membrane mechanisms: (a) a decrease in the resting K^+ conductance, (b) a slowing of repolarization due to reduction in the delayed K^+ rectifying conductance, (c) a reduction in the slow afterhyperpolarization (AHP) due to inhibition of the slow Ca^{+2} -activated K^+ conductance and (d) inhibition of the M-current.

(a) Decrease in the resting K^+ conductance. Krnjević and his associates (1971a) first demonstrated this mechanism in neocortical neurons of the lightly anaesthetized cat in in vivo experiments. Subsequent investigations by Woody et al. (1978) confirmed these unconventional actions of ACh in consciously freely moving cats. McCormick and Prince (1986) also demonstrated the actions of ACh on neurons in in vitro slices of anterior cingulate cortex of guinea pigs. In the early studies a tendency for the input resistance to increase during an application of ACh and also a reversible potential close to -100 mV for the depolarizing actions were observed. Since there was no convincing evidence of inward (anomalous) rectification in these neurons, the results could be best explained by a reduction in Cl^- or K^+ conductance (both ions have an equilibrium potential close to -100 mV). Intracellular injections of Cl^- which produced large positive shifts in the Cl^- equilibrium potential and the

IPSP reversal potential did not obviously change the character of the neuronal responses to ACh. Therefore, Krnjević et al. (1971a) concluded that ACh probably acted by reducing membrane conductance for K^+ . As supporting evidence, Ba^{2+} , which is known to interfere rather specifically with movements of K^+ in a variety of excitable tissues, was shown to excite cortical cells in a manner similar to ACh (Krnjević et al. 1971b).

(b) Reduction in the delayed K^+ rectifier. This effect (Krnjević 1971a; Woody et al. 1978; Bernardo and Prince 1982), which is mimicked by iontophoretic application of Ba^{2+} (Krnjević et al. 1971b) leads to a "slowing down" of repolarization, i.e., the falling phase of the action potentials, and enhances repetitive firing and afterdischarges.

(c) Depression of the Ca^{2+} activated K^+ conductance. The depression of this net outward current abolishes the AHP. McCormick and Prince (1986) showed that when ACh was applied by pressure ejection, the postspike AHP in neocortical neurons was substantially reduced and even replaced by an afterdepolarization (ADP). This finding is in agreement with the early observations (Krnjević et al. 1971a, 1978a,b). However, the exact mechanism of the AHP depression is not well understood; the effect could be mediated by an increased Ca^{2+} binding to calmodulin (a Ca^{2+} -binding protein), a reduction in Ca^{2+} -influx, diminished internal Ca-sequestration or it could be due to a more direct interaction between the activated ACh-receptor and the relevant K-ionophores (cf. Krnjević 1984). On the other hand, the replacement of the AHP by an ADP may be due to an unmasking of a slow depolarizing event mediated by Ca^{2+} or Na^+ which ordinarily would be obscured or blocked by the AHP (McCormick and Prince 1986).

(d) Suppression of the M-current. There is now good reason to believe that the muscarinic actions lead to selective suppression of a voltage- and time-dependent, non-inactivating K-current generated by relatively small depolarizations. As a consequence of the excitation of this current, the neuron tends to hyperpolarize and repetitive firing subsides. Suppression of this slow outward current greatly facilitates the firing and afterdischarges induced by concomitant actions of excitatory transmitters like Glu.

The M-current was first described in bullfrog sympathetic ganglia (Brown and Adams 1980), then in hippocampus (Halliwell and Adams 1982; Malenka et al. 1986; Moore et al. 1988), followed by guinea pig olfactory cortex (Constanti and Sim 1987) and human neocortex (Halliwell 1986). Because Gähwiler and Dreifuss (1982) reported that ACh was ineffective in the absence of Ca^{2+} in hippocampal neurons, at least part of the M-current may be a result of a Ca-dependent K-conductance as speculated earlier by Krnjević (1977). In more recent voltage-clamp investigations of rat sympathetic ganglia, Belluzzi et al. (1985) found that muscarine blocks only inward-Ca currents and is ineffective in the absence of extracellular Ca^{2+} . Hence, they concluded that the "M-channels" are really Ca-activated K-channels and that their apparent voltage- and time-dependencies reflect the membrane properties of the Ca-channels. The idea that ACh acts primarily on Ca-channels is attractive because it provides a unitary explanation for all muscarinic actions discussed thus far for CNS neurons.

Cholinergic inhibition has been reported for all levels of the nervous system, including the cerebral cortex, neostriatum, thalamus, hypothalamus, brainstem, spinal cord and sympathetic and parasympathetic ganglia (Krnjević 1974a; Libet 1970; Zieglgänsberger and Reiter 1974; Hartzell et al. 1977; Dodd et al. 1981; Bernardo and Prince 1982; French-Mullen et al.

1983). Inhibition has been observed just prior to the depolarizing ACh response, in many cases. Cholinergic inhibition had been observed mostly within the superficial layers (II and III) of the cerebral cortex (Krnjević 1974a). It is also present in layer V, although the inhibitory responses in neurons in the superficial layer are considerably larger (McCormick and Prince 1986). In the neocortex, cholinergic inhibition may be a consequence of GABA-ergic interneurons activated by the extracellularly applied ACh, which impinge on the impaled neuron thereby producing IPSPs (Haas 1982; McCormick and Prince 1986).

The slowness in ACh action on cortical neurons suggests an involvement of a secondary messenger system in the transduction of the response. Cyclic GMP (cGMP) does not mimic the muscarinic actions of ACh in subcortical neurons (Krnjević et al. 1976; Bernardo and Prince 1982). However, the actions of ACh could be mediated intracellularly by cGMP and by a cGMP-dependent protein kinase in neocortical neurons (Woody and Gruen 1988).

1.2.6.2 S-Glutamate (Glu). The excitant effect of Glu is a result of the depolarization of the neuron to the threshold for voltage-dependent spike activation. An involvement of another voltage-dependent process associated with the receptor-coupled ionophore complex contributes to the conductance change measured during glutamate excitation after the blockade of spike genesis. The net change represents a combination of both voltage-dependent and voltage-independent behaviour of the ionic channels activated by the respective interactions of Glu at presumed receptors for N-methyl-D-aspartate (NMDA) and at non-NMDA receptors for quisqualate (Quis) and kainate (ka). The differentiation between NMDA and non-NMDA responses has been made mostly on the basis of blockade at NMDA receptors by specific antagonists such as 2-amino-5-phosphonovalerate (2-APV) or recently

MK-801 and by Mg^{2+} (Hwa and Avoli 1984; Nowak et al. 1984; Thomson 1986; Coan et al. 1987; Mayer and Westbrook 1987; Puil and Benjamin 1988; Collingridge and Lester 1989).

The voltage-sensitivity of the cationic channels activated by Glu-interactions at NMDA receptors is a direct consequence of a voltage-dependent blockade by Mg^{2+} (Nowak et al. 1984). This blockade occurs when Mg^{2+} enters the activated channels and decreases in magnitude on depolarization, thereby conferring a bistable state of the membrane potential (MacDonald et al. 1982; Thomson 1986). NMDA induces an inwardly rectifying current and a bistable state change in the input/output behaviour of the neuron; in neocortical neurons this can lead to a greater tendency to fire action potentials repetitively and to develop pacemaker-like oscillations in membrane potentials (Flatman et al. 1986).

The depolarizations evoked by NMDA and non-NMDA agonists have reversal potentials near zero millivolts. The reversal potentials become more hyperpolarized with progressive removal of extracellular Na^+ ; this suggests that both types of agonists increase membrane permeability to Na^+ and K^+ . The reversal potential of non-NMDA responses is much more sensitive to changes in external $[Na^+]$ whereas the reversal potential for the NMDA responses is markedly affected by changing the external $[Ca^{2+}]$ (cf. Padjen and Smith 1981; Puil 1981). In cultured spinal cord neurons, applications of Glu that activate receptors of the NMDA-subtype increase the intracellular Ca^{2+} -activity measured with Ca^{2+} -sensitive fluorescent probes. Here, Mg^{2+} can be used to distinguish the NMDA-receptor-coupled Ca^{2+} -influx from the voltage-dependent source of Ca^{2+} -influx that also is initiated by the NMDA-actions (MacDermott et al. 1986). In neocortex, Glu application produces tetrodotoxin (TTX)-insensitive decreases in

extracellular $[Na^+]$, $[Ca^{2+}]$ and $[Mg^{2+}]$ measured with selective ion-sensitive microelectrodes (Pumain et al. 1988). Glu and the specific agonists -- NMDA, Quis and ka -- also produce responses that are dependent on external $[K^+]$ indicating that the activated channels include those that are permeable to K^+ and that the efflux of K^+ may be partly passive or due to active extrusion by a pumping process (Bührle and Sonnhof 1983; Padjen and Smith 1983; Pumain and Heinemann 1985; Pumain et al. 1987).

1.3 The Anaesthetic State

1.3.1 Historical introduction and definitions. Anaesthesia is considered an American contribution since the first successful public demonstration by W.T.G. Morton took place in Massachussetts General Hospital in 1846 (Bigelow 1846). However, anyone who has examined the history will realize that innovation of such significance could have hardly arisen spontaneously. For example, diethylether which was the agent used by Morton, had been discovered in Switzerland around 1540 by Valerius Cordus and named Aether by Frobenius. Its pharmacological and particularly anaesthetic properties in animals were described by a physician and alchemist named Paracelsus (cf. Smith 1986).

The word "anaesthesia" was first used as in its modern sense in the first century AD by the Greek philosopher, Dioscorides, who described the narcotic effects of the plant Mandragora. Its usage in the English language is usually ascribed to Oliver Wendell Holmes, though it was defined considerably earlier in 1751 in Bailey's English Dictionary as "a defect of sensation" (Beecher 1968).

In the clinical context, Rees and Gray (1950) defined anaesthesia as a state characterized by narcosis, analgesia and relaxation. Gray in 1960 renamed this triade as narcosis, reflex suppression and relaxation. More

recently, White (1987) attempted to define general anaesthesia as a state in which the CNS is reversibly depressed to such a degree that consciousness is lost and that on recovery, nothing is recalled relating to the period of anaesthesia. Atkinson and associates (1987) provided a mechanistic (i.e., operational) definition by suggesting that anaesthesia is a process of modification of the normal physiological reflex response to stimuli provided by surgery. Accordingly, they considered the triade of anaesthesia as inhibition of the afferent part of the reflex system, depression of the central synaptic mechanisms of coordination and blockade of the efferent part of the reflex arc. This definition, though uncommon, provides the anaesthesiologist with two main advantages. First, it avoids the different problems in defining unconsciousness (or "narcosis"). Secondly, it provides an objective definition applicable to humans as well as experimental animals.

1.3.2 Site of action of anaesthetics in the consciousness system.

Himwich (1951) was the first to suggest the concept of horizontal levels within the brain that were affected reversibly and sequentially by increasing depths of anaesthesia. The cerebral cortex is depressed before the subcortical diencephalon, and then as anaesthesia deepens, the mesencephalon and pons are gradually inhibited. Finally the deepest anaesthesia occurs when the vital centres of the medulla are depressed.

More recent developments in neurophysiology have given support for a vertical stratification of the brain. Livingstone (1954) visualized the CNS as composed of three highly interrelated, though vertical systems -- the specific sensory system, the nonspecific sensory and the motor system. The nonspecific sensory system has the responsibility of interrelating and modulating all sensorimotor interrelations. This nonspecific system is located essentially within the midline structures, extending from the

medulla to the diencephalon, and containing the ascending RAS. Because the reticular formation has been demonstrated to be a vital component for the control of consciousness, several investigators have attempted to relate anaesthesia to alterations in the function of the RAS (French et al. 1953; Arduini and Arduini 1954; King 1956).

Two lines of experimental evidence have altered this concept. Consciousness, as reflected in a desynchronized EEG, is possible in chronic preparations of experimental animals without the midbrain reticular formation (Genovesi et al. 1956). Also, changes can be noted with at least some anaesthetics, in the pattern of cortical activity, before there are changes in the activity of the reticular formation neurons (Winters et al. 1967, 1969; Darbinjan et al. 1971; Mori et al. 1971, 1972; Mori 1972). Shimoji and Bickford (1971) observed that anaesthetics such as halothane produced excitatory effects at the unitary level in the reticular core resulting in an increased firing of mesencephalic reticular neurons. Moreover, local cerebral glucose utilization was found to be specifically and consistently depressed in neocortex and not in brainstem of rats anaesthetized with pentobarbital or halothane (Savaki et al. 1983; Sakabe et al. 1985; Peschanski et al. 1986).

From the above discussion, it is apparent that any hypothesis which simply proposes brainstem depression as the sole site at which anaesthetics produce unconsciousness needs careful assessment. In summary, data on local cerebral glucose consumption as well as neurophysiological observations suggest that the neocortex is the likely site of action of anaesthetics. The exact reasons for such specificity are not exactly known. The role of the neocortex in the neurological mechanisms of the anaesthetic state requires comprehensive investigation.

1.3.3 Mechanisms of action of general anaesthetics. Though few investigators would debate the importance of the neocortex as a site of action of anaesthetics (Robson 1967; Mori 1972; Mori et al. 1971, 1972; Krnjević 1974; Peschanski et al. 1986), the exact target sites at the cellular and molecular levels remain a matter of considerable controversy (Richards 1980; Franks and Lieb 1982; Dluzewski et al. 1983; Haydon et al. 1984; Miller 1985; Bangham and Hill 1986). At the molecular level, suggestions about the effects of anaesthetic molecules on neurons have ranged widely from a very general depression of neuronal excitability by a uniform physiochemical change in membrane properties to highly specific mechanisms such as presynaptic reduction of transmitter release or a selective prolongation of inhibitory actions on the postsynaptic membrane (Krnjević 1972).

1.3.3.1 Non-specific (lipid) hypotheses. These imply that anaesthetics act by changing the physical properties of the macromolecular structure of the plasma membrane, i.e., no specific site or receptor is required. Here, the classical view is that anaesthetics act through dissolution in membrane lipids as viewed by Overton (1901) and Meyer (1899). Their observations were the excellent correlation between the potency of a variety of anaesthetics and their lipid solubilities (olive/oil partition coefficient). However, this correlation does not explain how the drugs produce anaesthesia.

The earliest attempt to overcome this difficulty was the critical volume hypothesis of Mullins (1954) which postulated that anaesthesia occurs when a critical volume fraction of anaesthetic molecules is achieved in neuronal membranes. Mullins suggested that the excess volume would cause lateral pressure on the ionic pores of the membrane which tends to occlude them. As

a result, impulse conduction and synaptic transmission would be impaired. After further modifications by Paton and Pertwee (1972), Seeman (1972, 1975), Seeman and Roth (1972), Haydon et al. (1977) and Halsey et al. (1978) the critical volume hypothesis provided a reasonable model for explaining the phenomenon of reversal of anaesthesia at high pressures (Miller et al. 1973). Its weakness was the implicit assumption that all lipid soluble substances (e.g., soaps) should be anaesthetics and that they should all work in the same way. Certain compounds which are very similar in chemical structure may have quite different effects; for example, hexafluoroisopropylmethyl ether is an anaesthetic, whereas hexafluorodiethylether is a powerful convulsant (Richards 1980). The pattern of uptake and distribution of these compounds in the CNS also should be nearly identical as both processes are determined only by the partition coefficient and the lipid solubility. The difficulties become further compounded when barbiturates and steroids are considered, since both of these groups show pronounced structure/activity relations which cannot be predicted from the simple critical volume hypothesis (Halsey 1974).

In view of these difficulties, attention was directed towards mechanisms by which anaesthetics could disrupt the structure of the lipid bilayer to cause indirect changes in the activity of functional membrane proteins. Three models have been proposed:

- (a) The phase-transition hypothesis suggests that lipids immediately surrounding an excitable membrane channel are exclusively in a gel phase, which tends to maintain the patency of the channel. The lipids become fluid on addition of an anaesthetic (i.e., there is a phase-transition), which allows the channel to close (Lee 1976). This hypothesis is

supported by the finding that anaesthetics decrease the temperature at which phase-transition occurs in purified phospholipid model membranes (Lee 1976; Hill 1978). In addition, high pressure antagonizes both the development of anaesthesia (pressure reversal), and the anaesthetic-induced decrease in phase-transition temperature (Kamaya et al. 1979).

(b) The lateral phase separation hypothesis postulates that under normal conditions membrane phospholipids co-exist in both fluid and gel forms (Trudell 1977). Conversion of one form to another permits the membrane to expand or contract with less expenditure of energy than would be required if the membrane were purely fluid or gel. An analogy is provided by the reduction in volume that results when ice melts. Less energy is required to decrease the volume than to compress ice or water without a phase change. Anaesthetics may "melt" the gel phase and thereby increase the energy required to displace portions of the membrane and decrease the membrane lateral compressibility. This decrease may prevent the opening of protein channels that permits translocation of ions across the postsynaptic membrane or conformational changes in proteins responsible for transmitter release. Indeed, general anaesthetics disrupt lateral-phase separations in model membranes composed of two types of phospholipids (Trudell et al. 1975). These effects are partially reversed by high pressures (Trudell et al. 1975).

c) The fluidized-lipid hypothesis states that anaesthetics increase the lateral and rotational motions of membrane components. As in the phase-transition hypothesis, this increase in the membrane disorder is thought to facilitate ionic-channel closure by reducing the stability of the channel proteins. The enhancement of the decay of the miniature

postsynaptic current by inhalational agents is consistent with the fluidized-lipid or phase-transition hypothesis (Gage and Hamill 1975).

The hypotheses related to phase transition, lateral phase separation and fluidized lipid suggest that anaesthetics act by increasing membrane fluidity. These suffer from some serious constraints and inconsistencies, such as the following:

- (1) fluidity changes in lipid bilayers cannot be detected unless the anaesthetic concentrations which are used are well in excess of those used clinically (Boggs et al. 1976; Franks and Lieb 1978; Turner and Oldfield 1979; Lieb et al. 1982);
- (2) an increase in temperature (less than 1°C) also increases membrane fluidity (Harris and Groh 1985) and therefore should enhance anaesthesia. However, an increase in body temperature consistently decreases anaesthetic potency (Eger et al 1965) and does not produce anaesthesia;
- (3) on the other hand, cooling should offset the anaesthetic action because it should oppose the fluidizing effect according to the above-mentioned models. In contrast, Richards and his associates (1978) demonstrated that cooling of a nerve did not render it less susceptible to blockade by anaesthetic agents;
- (4) the cis and trans isomers of 9,10-tetradecanol, which have identical anaesthetic potencies do not induce similar changes in membrane fluidity (Pringle and Miller 1978). One isomer lowers and the other elevates the phase transition temperature of a phospholipid membrane;
- (5) some chemical congeners or isomers, differing only trivially from an anaesthetic member in chemical and physical properties such as lipid solubility, show convulsant activity. Examples of molecular modifica-

tions that convert an anaesthetic to a convulsant molecule include addition of a methyl group to the aliphatic side chain of barbiturates, addition of a single double bond in the steroid nucleus of alphaxalone and replacement of one fluorine atom in halothane by a hydrogen atom (cf. Labella 1981, 1982).

1.3.3.2 Specific (protein) hypotheses. Because the actions of anaesthetics on membrane lipids do not appear to explain their physiological effects, it is natural to consider the possibility that anaesthetics may disrupt membrane function by direct interaction with the proteins of the membrane. Anaesthetic molecules may combine with a hydrophobic region of a protein molecule, induce a conformational change and thereby make it less active (Eyring et al. 1973). A more complex hypothesis suggests that different sets of hydrophobic sites exist on target protein molecules, with each of the hydrophobic sites accomodating anaesthetic molecules of different sizes (Richards et al. 1978). General anaesthetics have been shown to induce changes in conformation and properties for a wide variety of purified proteins and protein structures such as actomyosin (Strosberg et al. 1972), microtubular protein of an axopod (Allison et al. 1970), glutamic dehydrogenase (Hulands et al. 1975), myoglobin and haemoglobin (Schoenborn and Featherstone 1967), luciferase (Franks and Lieb 1984; 1985), albumin and lactoglobulin (Balasubramanian and Wetlaufer 1966), calcium-dependent ATPase (Price et al. 1974), and receptors for acetylcholine (Young et al. 1978; Aronstam et al. 1986), dopamine, ouabain, or opioids (Labella et al. 1979), as well as α -adrenergic ligands (Fairhurst and Liston 1979) and aequorine (Baker and Schapira 1980). The kinetics of interaction between the anaesthetic molecules and functional proteins often indicate competitive antagonism according to Labella's (1981) observations on anaesthetic-induced

perturbations of specific binding to brain membranes of ligands for the opioid, digitalis and dopamine receptors (Labella et al. 1979). This is supported by the observations of a halothane induced-depression of the purified cardiac myofibrillar ATPase and the competitive antagonism by Ca^{2+} of the maximal isometric force of isolated cardiac muscle (Price 1974; Price et al. 1975). However, there are other reports indicating non-competitive and mixed inhibition of enzyme activity by anaesthetics. For example, diethylether, methoxyflurane, trichloroethylene, chloroform and halothane show "mixed" kinetics in inhibiting brain and erythrocyte acetylcholinesterase activity (Braswell and Kitz 1977). This discordance with the results of other structural, functional and binding studies suggest that there are clearly distinguishable types of binding sites on proteins. Hydrophobic pockets can result from inefficient folding of the proteins and can bind anaesthetics with high selectivity or cause little or no conformational change, although at higher concentrations, anaesthetics can cause marked structural changes e.g., in hemoglobin (Harkey et al. 1979). Other hydrophobic pockets occur where proteins may fold around a substrate or cofactor (e.g., adenylate kinase; Sachsenheimer et al. 1977 and luciferase; Franks and Lieb 1984) and there appears to be considerably more flexibility which allows a wider range of chemical structures to bind. There are also hydrophobic regions (or clefts of certain sizes), perhaps intermediate between the above two, where one or more anaesthetics may bind (e.g. β -lactoglobulin; Wishina and Pinder 1966). The hydrophobic surfaces or grooves where anaesthetics bind in a nonhyperbolic pattern are less discrete and have poorly defined stoichiometry (e.g., bovine serum albumin; Wishina and Pinder 1964). Whether such binding reflects a single large patch or many sites of low affinity has yet to be established.

The consequences of anaesthetic binding to protein function are less well understood. Nonetheless, three basic mechanisms have been proposed (cf. Miller 1985):

- (a) competition for cofactors needed for activation,
- (b) minor sterically induced rearrangements in the target protein resulting from binding to hydrophobic sites near the active center and,
- (c) allosteric effects induced by anaesthetic protein interaction that require anaesthetics to bind to different conformations of a protein with different affinities, although this has not been established.

Two main criticisms have been launched against the "protein perturbation hypothesis" (Ueda and Kamaya 1984). The large number of proteins that show specific anaesthetic binding suggest that non-specificity is favoured. Second, there is virtually no evidence for the mechanism by which protein hypothesis can account for the pressure reversal phenomenon. It is true that most proteins studied thus far bear no relation to those involved in synaptic transmission; however, they can be considered as models for illustrating the possibility of specific binding of anaesthetics to protein macromolecules. This situation is similar to the use of membrane models and olive oil/water partition coefficients to demonstrate the feasibility of the lipid hypotheses. Recently, however, a specific binding of halothane to brain muscarinic receptors has been reported (Aronstam 1986). The impressive agreement between the predictions of the critical volume hypothesis (a non-specific theory) and the pressure reversal data could be more obtained precisely by assuming a single molecular model in which one anaesthetic molecule binds to, and inactivates a single site (Franks and Lieb 1982). In addition, pressure reversal could be explained by pressure causing a general increase in excitability, which counteracts a generalized decrease in

excitability caused by anaesthetic binding to specific protein sites (Kendig et al. 1975, 1978) or simply to anaesthetics being "squeezed away" from their target sites.

1.3.3.3 Anaesthetic molecular mechanisms and cellular excitability.

In order to accept a molecular hypothesis for anaesthetic actions, the question about how lipid or protein perturbations could produce depression in cellular excitability should be addressed. Because this is difficult to answer, few reports have attempted to explore the link between molecular mechanisms and neuronal depression.

Haydon and associates (1977) reported a decrease in capacitance and thickening of black lipid membranes induced by alkanes and anaesthetics in a dose-dependent manner. Later, the same group (Haydon and Urban 1983 a, b, c) using two-electrode voltage-clamp techniques in squid axon found that an increase in the steady state inactivation of Na-channels was associated with a decrease in input capacitance (i.e., an increase in membrane thickness) when an anaesthetic was applied in the bathing solution. Urban (1985), using the same techniques, published a Meyer-Overton type of correlation between anaesthetic concentrations needed to block evoked inward sodium currents by 50%, and their membrane/buffer partition coefficients. It may be suggested that this is the missing link between the lipid hypotheses and the inhibition of excitability. For example, anaesthetic molecules fluidize the lipid membrane bilayers thereby inducing membrane thickening and destabilize the open ionic channels activated during excitation.

Franks and Lieb (1978) using X-ray diffraction studies of multilamellar lipid bilayers showed no change in thickness following applications of several anaesthetics. Fernandez et al. (1982) found that in the squid axon, chloroform reduces the magnitude of the Na-gating currents but has no effect

on their kinetics. In contrast chloroform increases the rate of charge translocation of the lipophilic ion dipicrylamine. It is therefore unlikely that general anaesthetics act to modify the kinetics of opening and closing of ionic channels via modification of the structural parameters of the lipid matrix. More recent evidence showed that membrane capacitance of hippocampal neurons had been increased (not decreased) by ethanol applications (Carlen et al. 1986). Puil and Gimbarzevsky (1987) also reported an increase of the input membrane capacitance in trigeminal root ganglion neurons induced by halothane and isoflurane i.e., in neurons that are believed to be devoid of synaptic inputs (Lieberman 1976). Hence, if the theory of membrane thickening can survive the criticism of extrapolating observations from squid axon to mammalian central neurons, some difficulty will be encountered in reconciling the abovementioned observations that are contradictory to those discussed above (Haydon et al 1977; Haydon and Urban, 1983a, b, c).

Another attempt to bridge the gap between the molecular hypotheses and the depression of excitability was the discovery of a novel "anaesthetic K-current" specifically activated by halothane in molluscan neurons and insensitive to 4 amino-pyridine (4-AP), tetraethylammonium (TEA), Co^{2+} and Na^+ replacement (Franks and Lieb 1988). They suggested that anaesthetics bind directly to the relevant channel protein and stabilize it in the activated state or alternatively act at some point on the second messenger system that regulates the channel. However, this may not provide the appropriate link between the protein perturbation hypothesis and the attenuation of membrane excitability. A large variety of anaesthetics including volatile and intravenous agents and their abilities to activate such current should be investigated in cases where their EC_{50} 's are

strongly correlated with their minimum alveolar concentration (MAC) values. In addition, this novel anaesthetic activated K-conductance requires demonstration in neurons of the CNS in vertebrate animals.

In conclusion the data in the literature does not fill the gap between molecular mechanisms of anaesthesia and the anaesthetic-induced depression of excitability.

1.3.3.4 Anaesthetic actions on cytoplasmic membrane sites. In the absence of a plausible explanation of how anaesthetics depress neurons by interacting with plasma membrane lipids or proteins, Krnjević (1972, 1974b, 1975; 1986) hypothesized that anaesthetics could act indirectly at cytoplasmic membrane sites. For example, anaesthetics might depress the Ca^{2+} accumulating activity of mitochondria and other cytoplasmic Ca^{2+} -binding membranous proteins, thus increasing intraneuronal levels of free Ca^{2+} , which in turn could influence the conductance properties of excitable membranes as well as alter the presynaptic release of transmitters. Support for this hypothesis is suggested by both biochemical and electrophysiologic evidence:

(a) inhalational agents inhibit mitochondrial activity (Brunner et al. 1971; Biebuyck 1973; Rosenberg and Haugaard 1973; Hawkins and Biebuyck 1980). Large reductions in mitochondrial Ca^{2+} uptake by intravenous anaesthetics also have been observed in in vitro preparations (Lee et al. 1979; Sweetman et al. 1981) and in in vivo preparation of rat brain (Sweetman and Esmail 1975),

(b) barbiturates increase free intracellular Ca^{2+} activity in CNS neurons of cats in vivo (Morris and Krnjević 1985; Morris et al. 1986). In brain synaptosomes several inhalational anaesthetics, in higher doses, increase the intracellular free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$; Daniell and Harris 1988),

(c) 2,4-dinitrophenol (DNP), a metabolic inhibitor, produces electrophysiological effects on central neurons similar to those of anaesthetics, particularly the altered responsiveness to excitatory amino acids and peptides (Godfraind et al. 1970; 1971; Catchlove et al. 1972; Krnjević et al. 1978a; Sastry 1978),

d) hypoxia in in vivo or in vitro preparations induces excitability changes similar to anaesthetic action (Glotzner 1967; LeBlond and Krnjević 1989; Krnjević and LeBlond 1989) and

(e) more recently, Krnjević and Puil (1988) showed that halothane in clinically relevant concentrations depressed inward Ca^{2+} currents in hippocampal CA 1 neurons in a dose-dependent manner without any effect on the leak conductances.

Although it seems that this elegant hypothesis might explain the cellular basis of anaesthetic actions, there are several critical points that remain unanswered. For example, halothane-induced depression of mitochondrial respiration is not reversed by high pressure (Cohen 1973). The question of how anaesthetics act on mitochondrial membranes (or other Ca-binding cytosolic proteins) has not been resolved by experimental evidence, i.e., anaesthetic molecules alter these membrane structures by unknown biophysical mechanisms (Krnjević 1974b). The elevated $[\text{Ca}^{2+}]_i$ was suggested to substantially increase the input conductances of the neurons inducing hyperpolarization secondary to an enhanced K^+ -efflux. Anaesthetics do not consistently evoke hyperpolarization even in the same neuronal type (e.g., hippocampal CA1 neurons; Berg-Johnsen and Langmoen 1987; Fujiwara et al. 1988; Miu and Puil 1989).

Acceptance of this hypothesis will depend on further electrophysiological investigations of a single cell type using a variety of intravenous and inhalational anaesthetics, as well as a systematic biochemical approach to

elucidate the effects of the spectrum of anaesthetics on the respiratory chain enzyme system combined with $[Ca^{2+}]_i$ measurements in both in vivo and in vitro mammalian preparations. Such investigations should be carried out in nervous tissue relevant to the neurological mechanisms of consciousness discussed above.

1.4 Hypotheses and objectives.

The primary objective of the present investigations was to study the neuronal mechanisms by which anaesthetics depress neocortical arousal. However, this objective could not have been attained unless three basic questions were addressed:

1. What are the effects of anaesthetic agents on the neuronal excitability of neocortical neurons?
2. Is the anaesthetic depression of neocortex due to potentiated inhibition or depressed excitation?
3. What are the plausible ionic mechanisms of the anaesthetic actions?

During the course of the investigations, a major hypothesis developed suggesting that cholinergic excitation in the neocortex is more vulnerable to depression by anaesthetics than glutamatergic stimulatory effects. Accordingly, an extensive study was designed to determine the effects of anaesthetics on the chemical sensitivities of neocortical neurons to applied transmitter substances.

2 METHODS

2.1 Animals

Duncan Hartley guinea pigs (males or females) were obtained from the Animal Care Centre of the University of British Columbia. The guinea pigs were weaned at this Centre, from lactating females after 14 days, and fed on

a vitamin C-supplemented chow, with free access to water. Once a week, 5-6 guinea pigs (150-250 g, or approximately 20 days old) were received from the Animal Unit and placed in a wire cage in the animal care room of the Department of Pharmacology & Therapeutics for less than one week before an experiment. The temperatures and humidity in these facilities were controlled at 22-23°C and 50-55%, respectively.

2.2 Slice preparations

Anaesthesia was induced with diethylether and maintained by endotracheal administration of 1-2% halothane. Attempts to lower intracranial tension were made with artificial ventilation by controlling the end-tidal CO₂ concentration at 30-35 mm Hg.

After frontotemporal craniotomy, the dura was reflected to expose the neocortex. In view of the vulnerability of neocortical neurons to hypoxia and other derangements of metabolism, the blood supply of the brain was uninterrupted up to the moment of the cortical excision. The sensorimotor and the anterior cingulate cortices were identified (Zeigler 1964; Verne 1974), excised with some contiguous subcortical tissue, and immersed in cold (2°C) artificial cerebrospinal fluid (ACSF) that was oxygenated with a 95/5% gaseous mixture of O₂/CO₂ (pH 7.4) within 10 s of excision. The constituents of the ACSF were (in mM): NaCl, 124; KCl, 3.75; KH₂PO₄, 1.25; MgSO₄.7H₂O, 2; CaCl₂.2H₂O 2; dextrose, 10; NaHCO₃, 26. The tissue was trimmed into ~5 mm square blocks, glued with α-cyanoacrylate to a Teflon disc and cut coronally with a Vibroslicer into 500-600 μm thick slices. During the slicing procedure which lasted a total of 15-20 min, the tissue was immersed in continuously oxygenated ACSF at ~4°C. The slices were transferred to a chamber containing oxygenated ACSF at room temperature (~22°C) and allowed to warm up gradually for at least 30 min. Each block of

tissue yielded ~5 neocortical slices together with some contiguous subcortical structures. A slice was transferred to the recording chamber (1 ml volume, Fig. 1) continuously superfused with ACSF in a humidified oxygenated atmosphere (Pandanaoboina and Sastry 1984). Here, the temperature was controlled at 32-34°C with a thermoregulator. A nylon mesh prevented floating movement of the slices during perfusion (rate 2-4 ml/min).

2.3 Solutions and drugs

Althesin (Glaxo), which is a 3:1 mixture of two steroids, alphaxalone and alphadolone in 20% v/v polyoxyethylated castor oil (cremophor EL), was diluted on the day of the experiment with the ACSF to obtain the desired concentrations. The concentrations of the vehicle in the solutions used in these investigations ranged between 2×10^{-5} and $16 \times 10^{-5}\%$ v/v. Previous investigations on the anaesthetic potencies of alphaxalone in the absence and presence of cremophor EL have ascertained that the observed activities of Althesin on neuronal membranes are attributable mainly to this steroid, and not to the vehicle (Pennefather et al. 1980; Cullen and Martin 1982, Minchin 1981). Before application by perfusion, isoflurane (Anaquest) was vaporized with the 95/5%, O_2/CO_2 gaseous mixture using five Mark 3 Fluotec vaporizers (Cyprane, Keighley, UK) that were recently calibrated, and bubbled into the ACSF reservoirs (inverted and covered 60 ml syringes) for a minimum of 20 min using a diluent gas flow rate of 1 L/min (Fig. 1). Isoflurane vapor also was introduced at the designated concentration into the chamber atmosphere immediately above the submersed slice. The concentration of isoflurane in the bath was determined using ^{19}F fluorine-nuclear magnetic resonance techniques (cf. Miu and Puil 1989). The molar values thereby obtained were converted to their corresponding MAC values for rodents

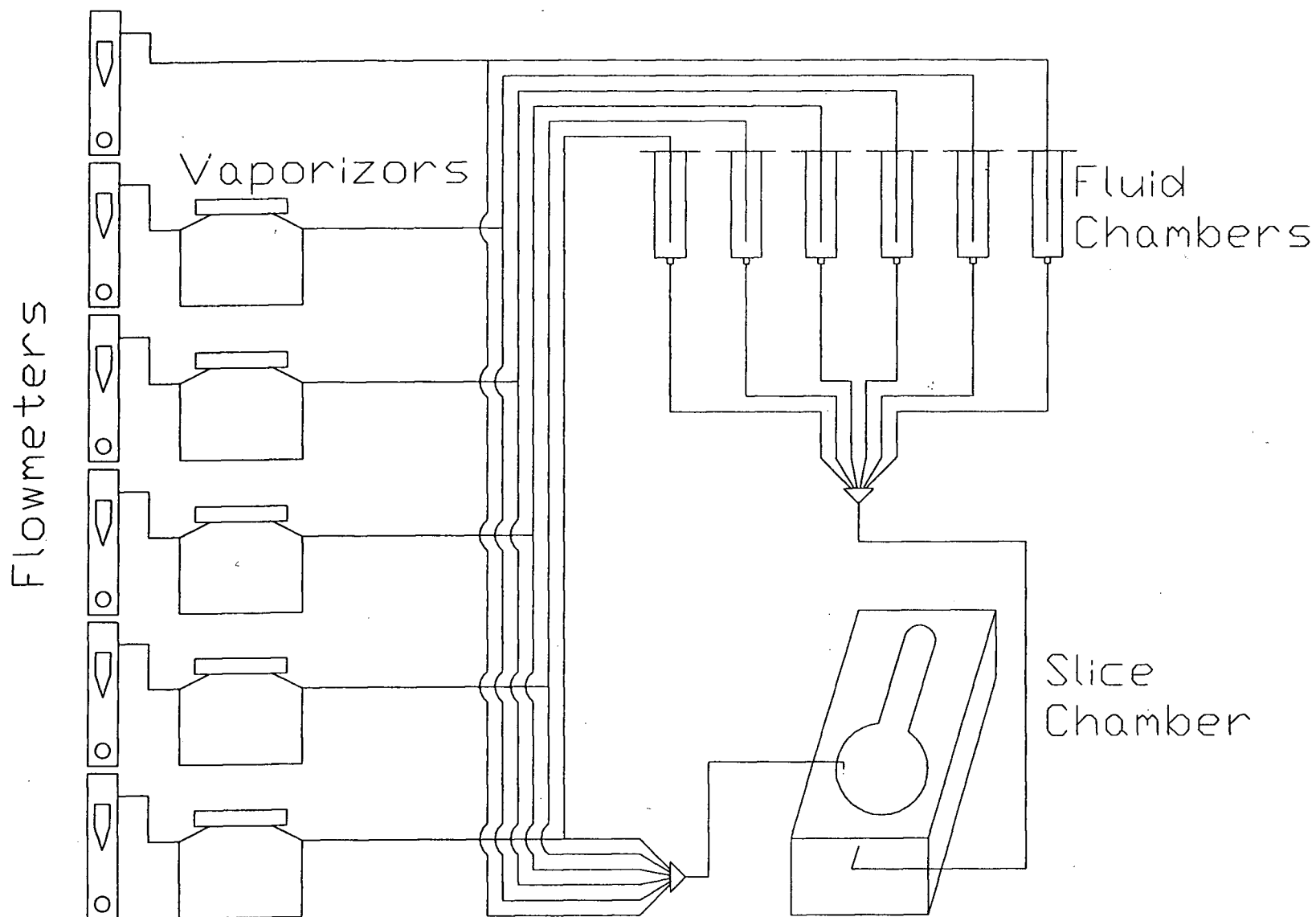


Fig. 1 Anaesthetic delivery system and recording chamber used in these investigations.

(Steward et al. 1973; Cullen 1986; Firestone et al. 1986). Aliquots of (-)bicuculline methiodide (Sigma) and (*)baclofen (Ciba-Geigy) were added to the ACSF from stock solutions (0.1 mM) that were kept frozen until required.

Ca^{2+} -deficient solutions were made without Ca^{2+} and either by adding 2 mM CoCl_2 or by increasing the $[\text{Mg}^{2+}]$ to 4 mM, according to the experimental procedure. In studies on the effects of extracellular $[\text{Mg}^{2+}]$ ($[\text{Mg}^{2+}]_0$), the total divalent cation concentration in the ACSF was kept constant by increasing or decreasing the $[\text{Ca}^{2+}]$ wherever the $[\text{Mg}^{2+}]$ was changed. The osmolality of the solutions was maintained approximately constant at 320 mosmol, by the addition of sucrose where necessary.

2.4 Intracellular recording arrangement

Intracellular recordings began approximately 30 min after incubating a slice in the recording chamber. A microelectrode mounted on a Narashige micromanipulator, was positioned under visual guidance using a dissecting microscope (40x), approximately at the middle and lower third portion of the slice (cf. Fig. 2a) .

Intracellular voltage recordings from neurons were obtained using microelectrodes filled with 3 M KCl, 3 M Cs_2SO_4 , 3 M K-acetate or 0.6 M K_2SO_4 . The tip resistances measured in the tissue were 50 to 90 M Ω . The electrodes were pulled from microfilament-incorporated glass blanks (1.0 and 1.2 mm external diameter; WPI Instruments) with a vertical puller (Narashige AP 81) using high heat and magnet-power settings. Each electrode was checked using high magnification light microscopy for its overall form and projected tip size. The electrodes then were vertically positioned on a retaining ring with the basal end immersed overnight in the concentrated electrolyte solution.

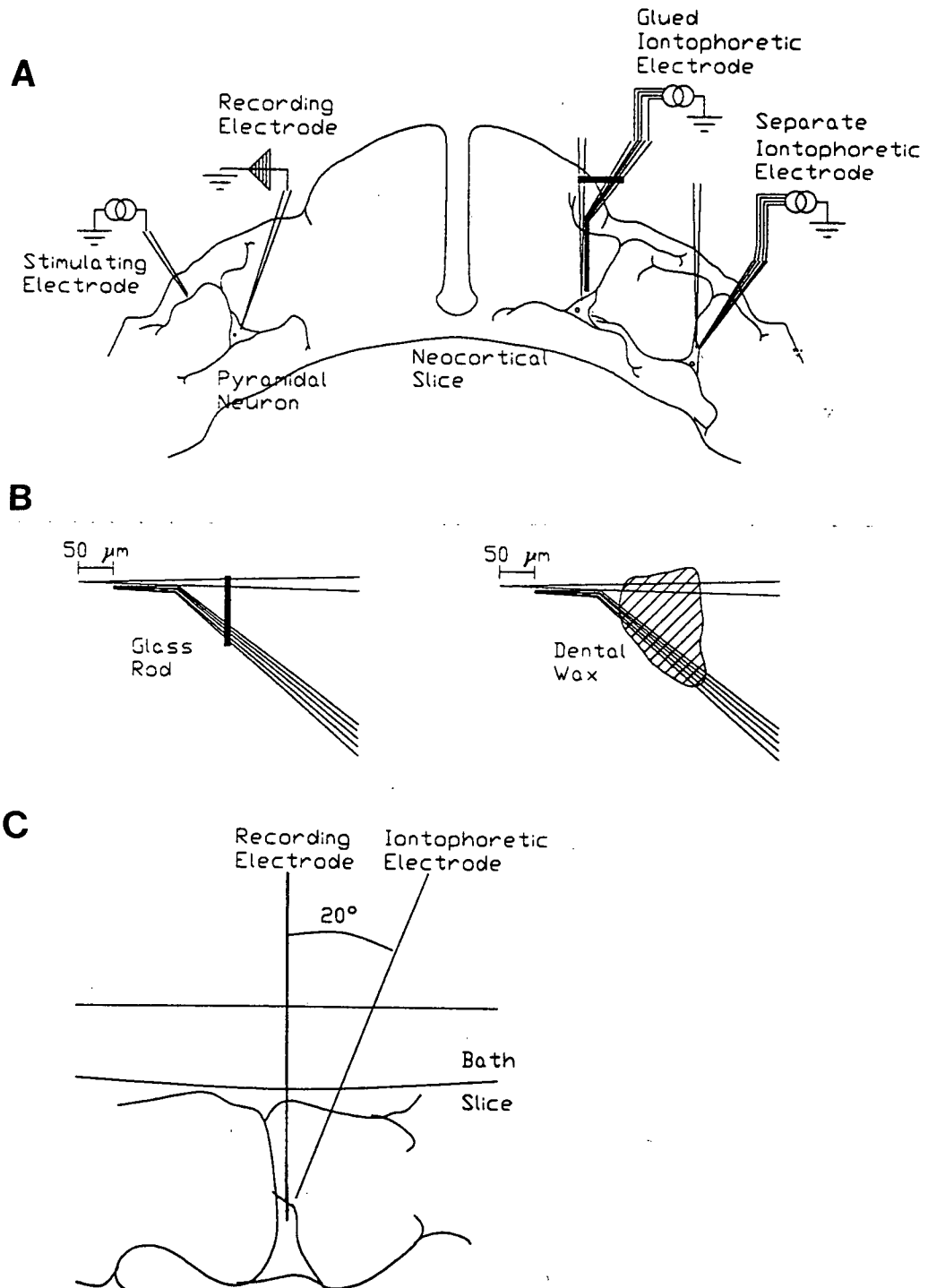


Fig. 2 Diagrams showing the neocortical slice and sites of recording and stimulation (a) and types of iontophoretic electrodes used (b and c).

The input resistances (R_i) of the neurons were measured using intracellular injections of specified step currents via the bridge-balance circuit of a WPI amplifier (model M701). A series of spikes and AHPs were elicited at 0.1 Hz by intracellular injections of current pulses before, during, and after an anaesthetic application; each of these pulses evoked 5 to 6 spikes. A total of 6 long-lasting AHPs were sampled consecutively for each experimental condition. These were quantified by averaging their peak amplitudes and durations. Because AHP parameters such as amplitude and duration can be affected by changes in resting membrane potential (V_m), continuous DC-injections were used to compensate for any anaesthetic induced changes in V_m . A tungsten bipolar stimulating electrode was placed in the subpial region of the neocortical slice for studying the effects of anaesthetics on synaptic potentials (Fig. 2a). Stimulation frequency was set to 0.2 Hz and the stimulus strength ranged from 5-10 V.

The amplified potentials were digitized with a PCM-1 instrumentation unit (VCR adaptor; Medical Systems Inc.) and stored with a super VHS video tape recorder (Canon model VR-HF 600) for off-line playback and analysis by computer. In some cases the intracellular signals (in analogue form) were analyzed with a waveform recorder (Biomation 805) which digitizes the input voltage sweep into 2048 points and thereby permits manual control of the digital-to-analogue conversion and storage for display on the oscilloscope, or reproduction on paper in an X-Y pen plotter (Hewlett-Packard 7015B; Fig. 3).

2.5 Computer analysis

Intracellular voltage signals, played back from the VHS video recorder, were fed into an interface panel of a data acquisition system (ERAT; laboratory-built) which converts the input analogue signals to digital form

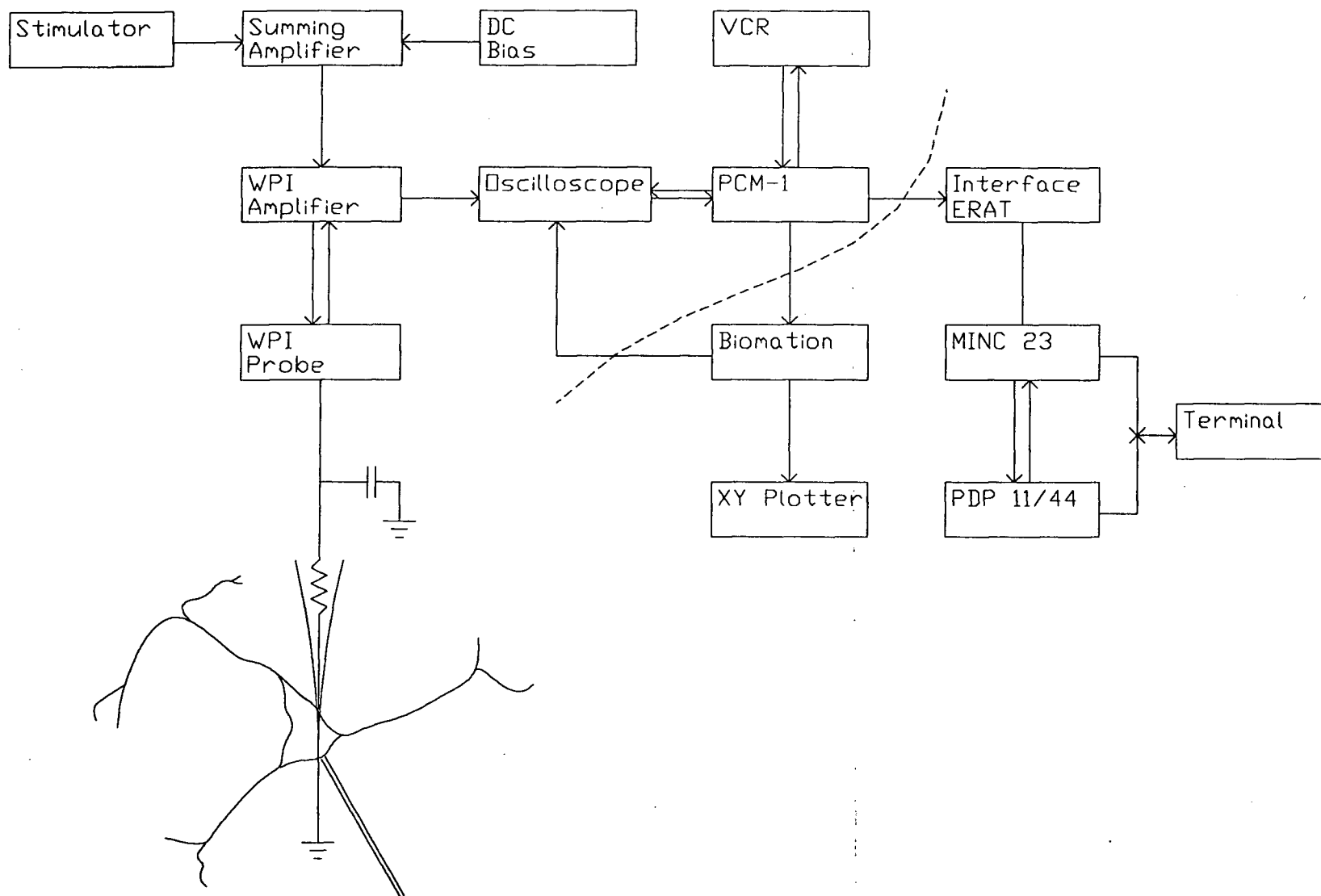


Fig. 3 Organization of the experimental set-up for data display, acquisition and retrieval.

(A/D conversion). The data were stored temporarily in the memory of a MINC-23 computer (DEC Inc.) during the data acquisition. After completion of this phase, the files were transferred and stored for the averaging and other procedures in another computer (PDP 11/44; DEC Inc.) which has a larger memory capacity (Fig. 3).

Fifteen to twenty individual traces of the synaptic transients in the raw data were selected from a MINC file for an averaging procedure. A new file was subsequently created for the averaged traces, and the amplitude, duration, rate of decay of the synaptic transients were measured from these averaged traces.

2.6 Iontophoretic techniques

2.6.1 Electrodes. In the initial experiments, 5 barrelled iontophoretic electrodes with a common tip diameter of 9-12 μm were glued with an ultraviolet (u.v.) sensitive fluid adhesive (Loctite glass bond or Loctite 358; Loctite U.K. Ltd.) to the recording electrodes (resistances, 70-90 $\text{M}\Omega$) at an angle of 20-30° and an intertip distance of 30-60 μm (Krnjević and Schwartz 1967; Zieglgänsberger and Puil 1973). The iontophoretic pipette was bent with a microforge at a point ~4 mm from the tip at an angle of 20-30°. The 'hockey-stick' shaped pipette then was positioned horizontally with two microscopes (one in the X-Y plane and the other in the X-Z plane) such that the 'blade' of the hockey stick was parallel to the recording microelectrode. The bonding material was applied and subjected to u.v. light for ~3 min with a Nuva Lite gun (Caulk Inc.). Secondary strengthening of the electrode assembly was achieved by gluing a glass tube across the two electrodes or by applying a rapidly setting dental wax (Fig. 2b) at about 0.5 - 1 cm from the tips. This assembly ensured that the transmitter substances would be applied in the vicinity of the soma of an impaled neuron.

In later experiments, separate recording and iontophoretic electrodes were used in combination with TTX application to eliminate the effects on nearby neurons activated by an ejected transmitter substance. In order to position the iontophoretic electrode tip as near as possible to an impaled neuron, it was initially assumed that the impaled neuron was located at a distance of $\sim 200\text{ }\mu\text{m}$ from the surface of the slice. Since the iontophoretic electrode was positioned at an angle of 20° with the horizontal plane, trigonometric functions were used to determine the amount of travel for the iontophoretic electrode into the slice (cf. Fig. 2C). This twin electrode placement permitted applications of the transmitters to the dendrites of the neurons. Once a neuron was impaled, Glu was ejected with a continuously applied current ($\sim 80\text{ nA}$); the "application electrode" then was advanced very slowly with an independent micromanipulator until a maximal depolarization was observed.

2.6.2 Solutions and equipment. Four barrels usually were filled using a 32 gauge needle with the following solutions of drugs (mostly from Sigma): acetylcholine Cl (0.5 M , pH 4), Na S-glutamate (1 M , pH 8.5), NMDA (200 mM , pH 9), GABA (0.5 M , pH 3.5) and (+)baclofen (Ciba-Geigy; 50 mM , pH 3). The fifth barrel was filled with a control solution (NaCl, 10 mM) which was used to compensate for the ejection currents; this balancing channel also served as a control for effects of currents on neurons.

The IDS 100 microiontophoresis unit used in these experiments possessed two main features. It allowed the automatic use of a 'push-pull' technique in which a current opposite in polarity and equal in magnitude and duration could be passed through the NaCl-containing barrel simultaneously with the "drug-current" and secondly, it had a built-in sequencer by which the transmitter substances could be applied sequentially in a pattern at desired (preset) currents for certain durations and inter-application intervals.

2.6.3 Experimental procedures. The effects of anaesthetics were assessed using the following procedures:

- (1) after stable recording conditions were achieved, the iontophoretic current was adjusted in the range of 30-350 nA (typically 80 nA) such that a submaximal (ED_{50}) or a just-maximal response could be elicited with a transmitter substance;
- (2) responses of relatively constant amplitude (usually 10-20 mV) were evoked at appropriate intervals (10-30s) for a period of at least 5-8 min;
- (3) equi-amplitude responses to at least two transmitter substances were evoked in the same neuron;
- (4) perfusion of the anaesthetic was commenced during the continuing, intermittent transmitter applications, and
- (5) at least 3 or 4 responses to each transmitter substance obtained just before anaesthetic application, after given intervals of anaesthesia, and during the recovery period, were averaged for the quantitative comparisons.

A slightly different procedure was used for investigation of NMDA responses because of the rapid development of desensitization or tachyphylaxis in the large pyramidal (layers IV-V) neurons (cf. layers II-III in Thomson, 1986) to a low dose of NMDA in the presence of $[Mg^{2+}]_0$. The just-maximal effect was determined using infrequent (~3 min) applications of short duration. Then, 3 or 4 equi-amplitude responses were obtained at 3 min intervals before commencing anaesthetic application for ~9 min.

2.7 Intracellular Ca^{2+} measurement

2.7.1 Neuronal cultures. Hippocampi were dissected out from 18-day-old Wistar rat embryos. Cells were dissociated, put onto 18mm coverslips and

incubated for 2 weeks in 5% CO₂ at 37°C in Dulbecco's modified medium which constituted 6 gm/L dextrose, 10 mM HEPES and 10% horse serum.

2.7.2 Storage and loading of Fura-2 AM. Fura-2 AM (1 mg; Molecular Probes Inc.) was dissolved in chloroform and 50 µL aliquots which were pipetted into small plastic ampules, placed in a dessicator and vacuum-dried for 3 hours. The dried aliquots were stored at -80°C. On the day of the experiment 50 µg of Fura-2 was dissolved in 50 µL dimethyl sulfoxide (DMSO) yielding stock solutions of ~1 mM. Stock Fura-2 (10 µL) was added to 2 ml preincubated Earl's balanced salt solution (EBSS) yielding a concentration of 10 µM Fura-2. EBSS constituted in mM: MgSO₄.H₂O, 0.8; K₂SO₄, 2.7; NaCl, 117; NaHCO₃, 26; NaH₂PO₄.H₂O, 1; dextrose, 5.6; HEPES, 10; CaCl₂.H₂O, 1.8 and 0.5% phenol red indicator (1 ml/L EBSS). One ml of EBSS-containing Fura-2 was immediately added to the well with 1 ml of standard EBSS; the hippocampal cell culture then was placed face-up in the well and incubated for 2 hours. Before recording, the Fura-2 incubated culture was rinsed by placing it into a separate dish of standard EBSS and incubated a further half-hour after which the cells were ready to use.

2.7.3 Intracellular Ca²⁺ measurement. Cover-slips containing dye-loaded cells were inverted and sealed into a specifically designed recording chamber allowing 2 perfusion inputs -- one for the control solution, low chloride EBSS in which NaCl was substituted for 117 mM Na isethionate, and the other for administration of anaesthetics. A third port was used for the injection of µl-volumes of the transmitter substances and agonists. Stimulation of the NMDA-receptor subtype by glutamate was favoured by adding glycine (1-2 µM) and exclusion of Mg during the preparation of the low chloride EBSS. Activation of quisqualate (Quis)-subtype of receptor was achieved by using low chloride EBSS without

the exclusion of Mg. Continuous suction was applied to an opposite opening and the temperature was controlled by a regulated heating device beneath the chamber.

The cells were viewed under oil immersion at 100x magnification on a Jenalumar Zeiss epifluorescent microscope which had an adjustable aperture for viewing single neurons. Recording of fluorescence from perikarya of single neurons was carried out at alternating excitation wavelengths of 350 and 380 nm u.v. light. Fluorescence signals (emitted from a neuron) was passed through a G125 glass barrier filter to reduce any background fluorescence and then to a photomultiplier which converted the fluorescence into DC-voltage. These signals were converted to digital form by an A/D converter and stored on floppy discs with a microcomputer. The results were presented as two values representing the intensity of emissions of both Ca-free-Fura-2 and Ca-bound-Fura-2 at wavelengths of 380 and 350 nm, respectively. The actual $[Ca^{2+}]_i$ then was calculated using the following formula:

$$[Ca^{2+}]_i = K_d 2.8 \beta (R - R_{min}) / (R_{max} - R) \dots\dots\dots(1)$$

where

K_d is the rate constant of association of the Fura-2 with the cytosolic free Ca^{2+} ,

β is the ratio of fluorescent values at 380 nm with zero and infinite extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$),

R is the experimentally determined ratio of the values of emission from the cell at 350 and 380 nm,

R_{min} is the ratio of values at 350 and 380 nm at zero $[Ca^{2+}]_o$,

R_{max} is the ratio of values at 350 and 380 nm at infinite $[Ca^{2+}]_o$,
and

β , R_{\min} and R_{\max} are determined from calibration of the system.

2.7.4 Calibration of the system. The following calibration procedure was employed:

Two hippocampal Fura-2 loaded cell cultures were equilibrated with the Ca^{2+} -ionophore A 23187 (10 μM), the first in the presence of 10 mM EGTA (calcium chelator) and zero $[\text{Ca}^{2+}]_o$, and the second in the presence of normal $[\text{Ca}^{2+}]_o$ and without EGTA. $[\text{Ca}^{2+}]_i$ measurements were obtained from both cultures for several cells until a 'plateau' of consistent values was reached. The β , R_{\min} and R_{\max} constants then were calculated from the averaged values obtained in these experiments.

2.8 Statistical analysis

The electrophysiological results were subjected to tests with ANOVA for statistical significance. Multiple comparisons between two groups were made using the Mann Whitney U-test (Zar 1984). Sample size in all experiments was usually 5 unless otherwise stated. The accepted level of significance was $p < 0.05$. Regression analysis was used to fit the data.

3 SPONTANEOUS ACTIVITY, REPETITIVE FIRING AND PASSIVE MEMBRANE PROPERTIES

3.1 Results

The 64 neurons selected for the study of anaesthetic applications on parameters of neuronal excitability had resting potentials more negative than -60 mV, spike amplitudes usually of 70-75 mV, and an ability to fire 5 such spikes repetitively during intracellular current-pulse injection. The depths of successful impalements corresponded to layers IV and V of typical cortex. The input resistances (mean \pm SD; $65.7 \pm 30.7 \text{ M}\Omega$) and resting potentials (V_m ; $-71.6 \pm 9.8 \text{ mV}$) remained stable for periods of 30 to

240 min. Forty-five applications of isoflurane in doses that ranged from 0.5 to 2.5 MAC were made to 34 neurons. Althesin was applied 48 times to 30 neurons, in concentrations from 15 μ M to 1500 μ M (alphaxalone content). The vehicle, cremophor EL did not have any effect when tested on the passive and active membrane properties as well as the spontaneous activities of neurons. In the initial investigations on 8 of the neurons, 1 MAC isoflurane or 100 μ M Althesin was applied continuously for 18 min. The maximal effects were evident at 6-9 min with isoflurane and at 4-6 min with Althesin, depending on the rate of perfusion. Therefore, the rate was adjusted to permit sampling of the maximal changes in parameters induced by either agent at 6 min. Halothane was applied 18 times to 10 neurons. No difference in anaesthetic effects was observed between the different types of sensorimotor and anterior cingulate neurons that have been classified into three categories (McCormick and Prince 1986).

3.1.1 Effects on spontaneous activity and evoked repetitive firing.

Twenty-five percent (i.e., 16/64) of the neurons were spontaneously active (synaptic transients and spikes) and arrhythmically fired spikes. Applications of an anaesthetic (Fig. 4) either had no effect or depressed spontaneous firing (isoflurane, 10/12 and Althesin, 5/8 neurons). An initial potentiation of firing followed by inhibition of spontaneous activity was not evident, as also found in hippocampal neurons in vitro (Berg-Johnsen and Langmoen 1987). Full recovery usually was observed within 6-10 min after an application. Occasionally, recovery was preceded by a rebound increase in firing. Complete recovery was not observed in some cases even after about 30 min (Fig. 4 A). However, most other anaesthetic induced changes were fully reversible.

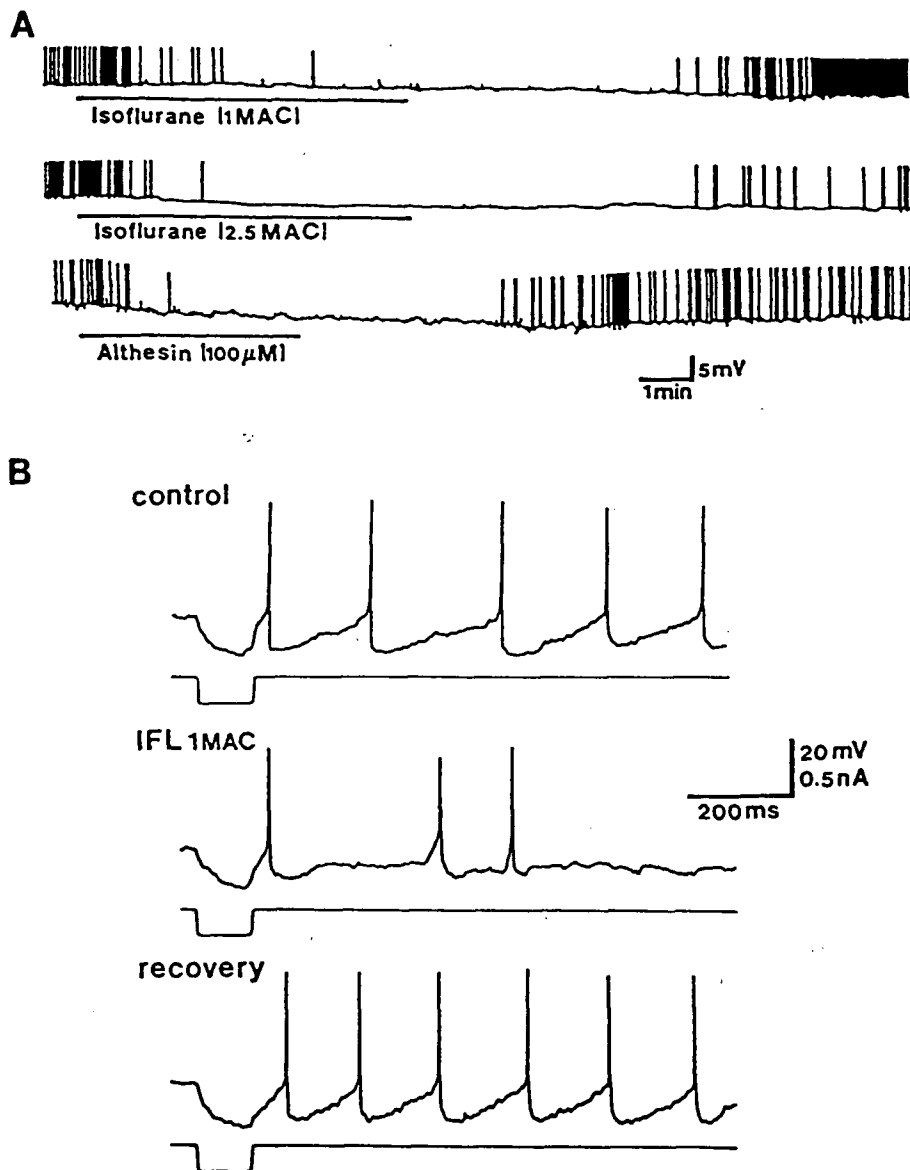


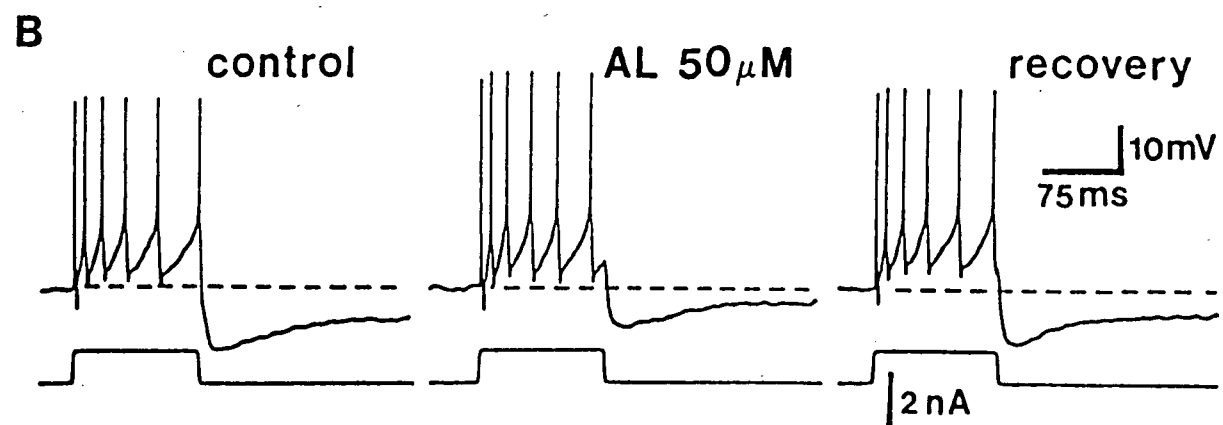
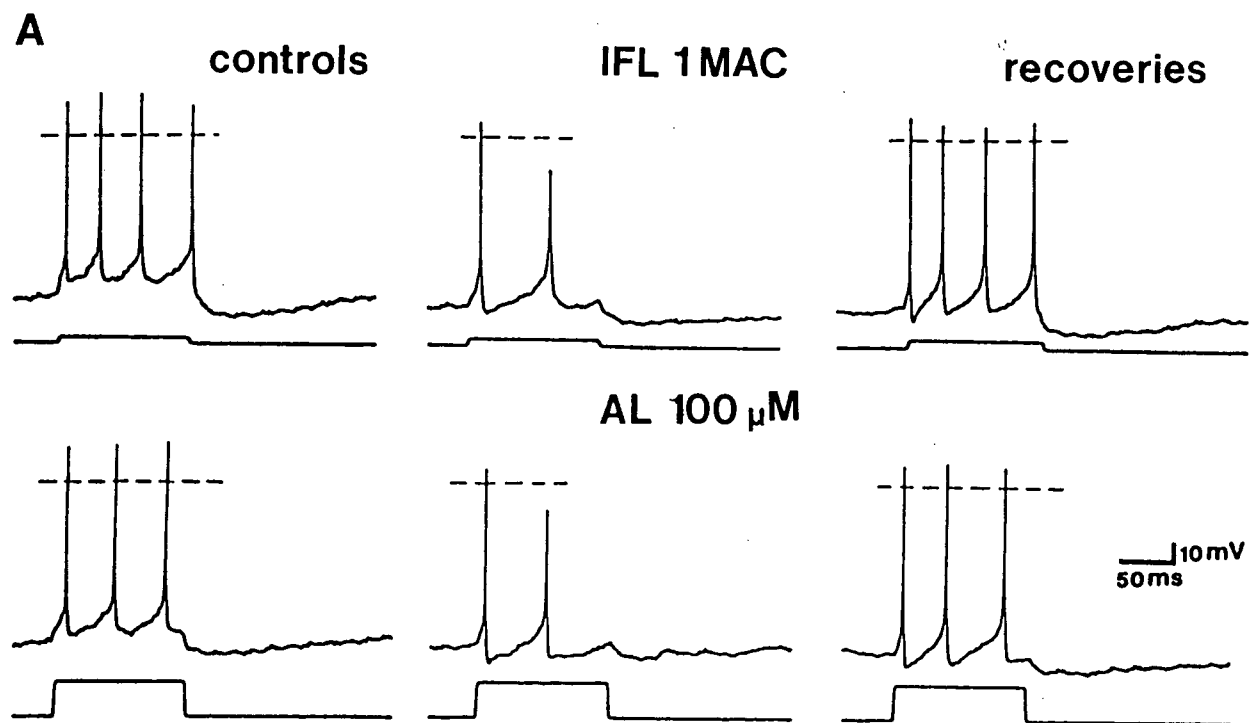
Fig. 4 Isoflurane and Althesin induced depression of spontaneous spike activities. (A) isoflurane application (1 MAC; upper trace) produced inhibition of the spontaneous firing and a rebound increase in firing after the application. Isoflurane (2.5 MAC; middle trace) in the same neuron produced a slight hyperpolarization (~ 3 mV) and a depression of spontaneous activity that was partly reversible. Althesin ($100 \mu\text{M}$; lower trace) induced ~ 5 mV hyperpolarization associated with complete attenuation of spontaneous activity in a second neuron. (B) on a faster time base, isoflurane depressed spontaneous spikes and blocked the AHPs. Voltage responses to hyperpolarizing current pulses may be seen at the left of the anodal break spikes. Complete recovery was observed after 10 min. There was no change in the V_m of this neuron.

The single spike threshold tested in 62 neurons by current injection was not altered significantly in (~70%) of cases. However, the amount of current required to evoke 3 to 5 spikes was only effective in producing <2 spikes during applications of isoflurane (23/32 neurons) in doses of 1 to 2.5 MAC or Althesin (17/30 neurons) in doses of 100-1500 μ M (Fig. 5A). Althesin administration at the 15 or 50 μ M doses did not produce inhibition of evoked repetitive firing (Fig. 5B). Hence, isoflurane and Althesin applications (9/32 and 8/30 neurons) compromised the ability of neurons to fire repetitively in response to direct current pulse injection through the recording electrode (Fig. 6A-B).

3.1.2 Effects on V_m and membrane electrical properties. Applications of isoflurane (1-2.5 MAC) hyperpolarized 24 of 34 cells by 3-8 mV. This effect was statistically significant only at 2 and 2.5 MAC (Fig. 7A). Isoflurane administrations at the highest dose evoked a mean hyperpolarization (from control) of $8.8 \pm 2.5\%$ in 5 neurons. The hyperpolarization elicited with Althesin applications in doses of more than 150 μ M was statistically significant (Fig. 7B).

Significant reductions in input resistance (R_i) were observed with isoflurane applications at 1.5, 2 and 2.5 MAC (Fig. 7B). The mean decreases in R_i at these doses were 15.34%, 20.71% and 28.53%, respectively. The effect of 1.5 MAC isoflurane on R_i is shown in Fig. 8. A maximal reduction of ~46% from control values was observed with Althesin applications at 1500 μ M (cf. Fig. 7D). Concomitant application of TTX (1.5 μ M) and isoflurane (1.5 MAC) or Althesin (100 μ M) produced the same effects on the V_m and R_i in 9 neurons as observed previously in the absence of TTX-blockade of Na^+ -spike genesis.

Fig. 5 Anaesthetic-induced increases in the threshold for spike genesis. (A) using same current intensities, isoflurane (IFL 1 MAC) and Althesin (AL 100 μ M) decreased the number of spikes evoked in two sensorimotor neurons. Note the depression of the amplitude of the second spike and the postspike AHP during anaesthetic application. V_m was held constant throughout the experiment. (B) no decrease in repetitive firing occurred but a reduction in the AHPs was evident during Althesin application (50 μ M). Complete recovery was observed after 9 min. Spikes are truncated due to frequency response of penwriter recorder. V_m did not change during the experiment.



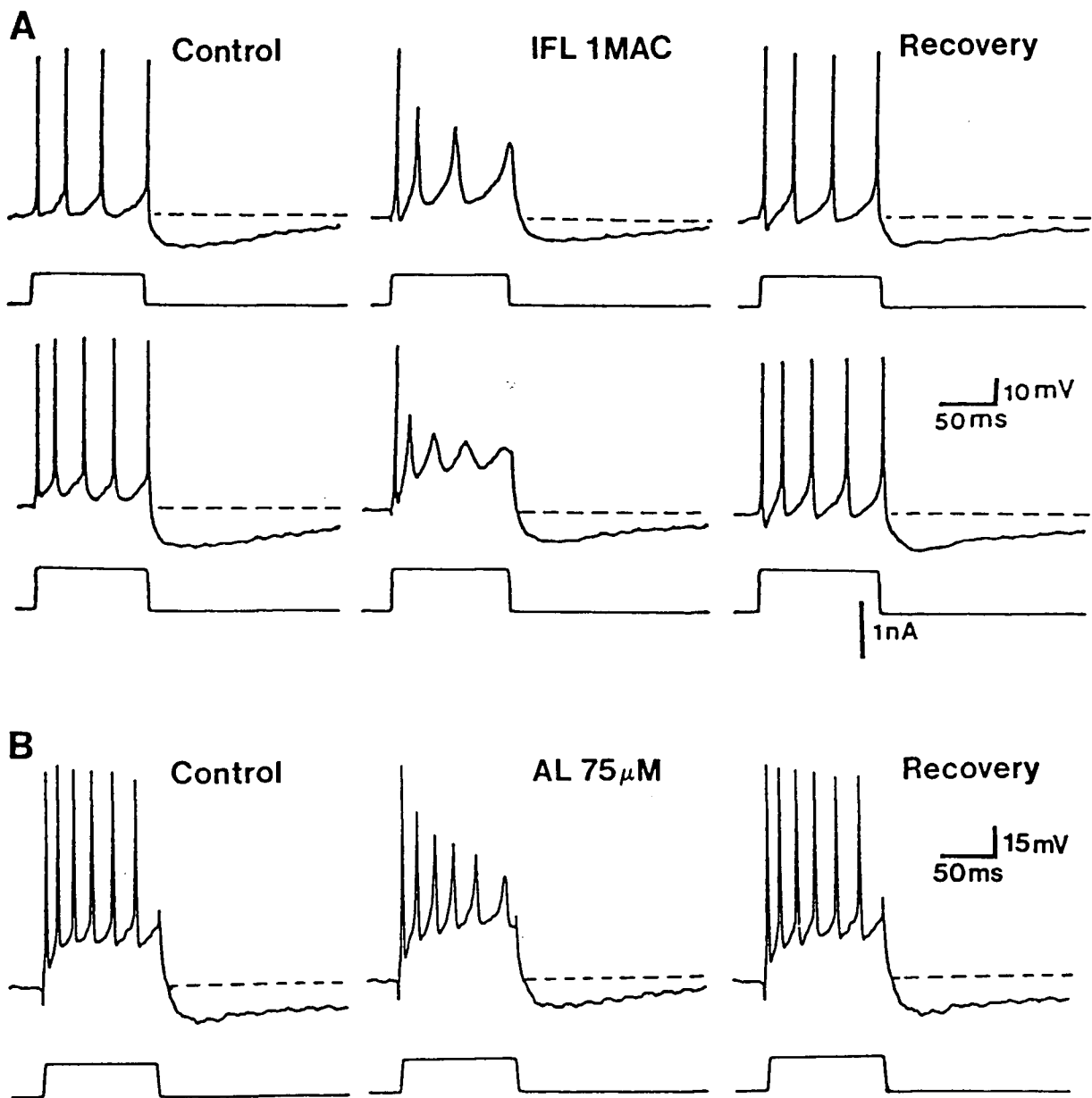


Fig. 6 Anaesthetic interference with the repetitive firing abilities of neocortical neurons. (A) isoflurane (IFL 1 MAC) interfered with repetitive firing elicited by current pulse injections. When the injected current was increased (lower traces), the ability of the neuron to fire more than 1 large amplitude spike still was severely compromised. The AHPs also were reduced. (B) Althesin (AL; 75 μ M) depressed the repetitive firing abilities of an anterior cingulate neuron. V_m s in A and B were controlled at the resting levels (~ -74 mV). Spikes are truncated due to frequency response of penwriter recorder.

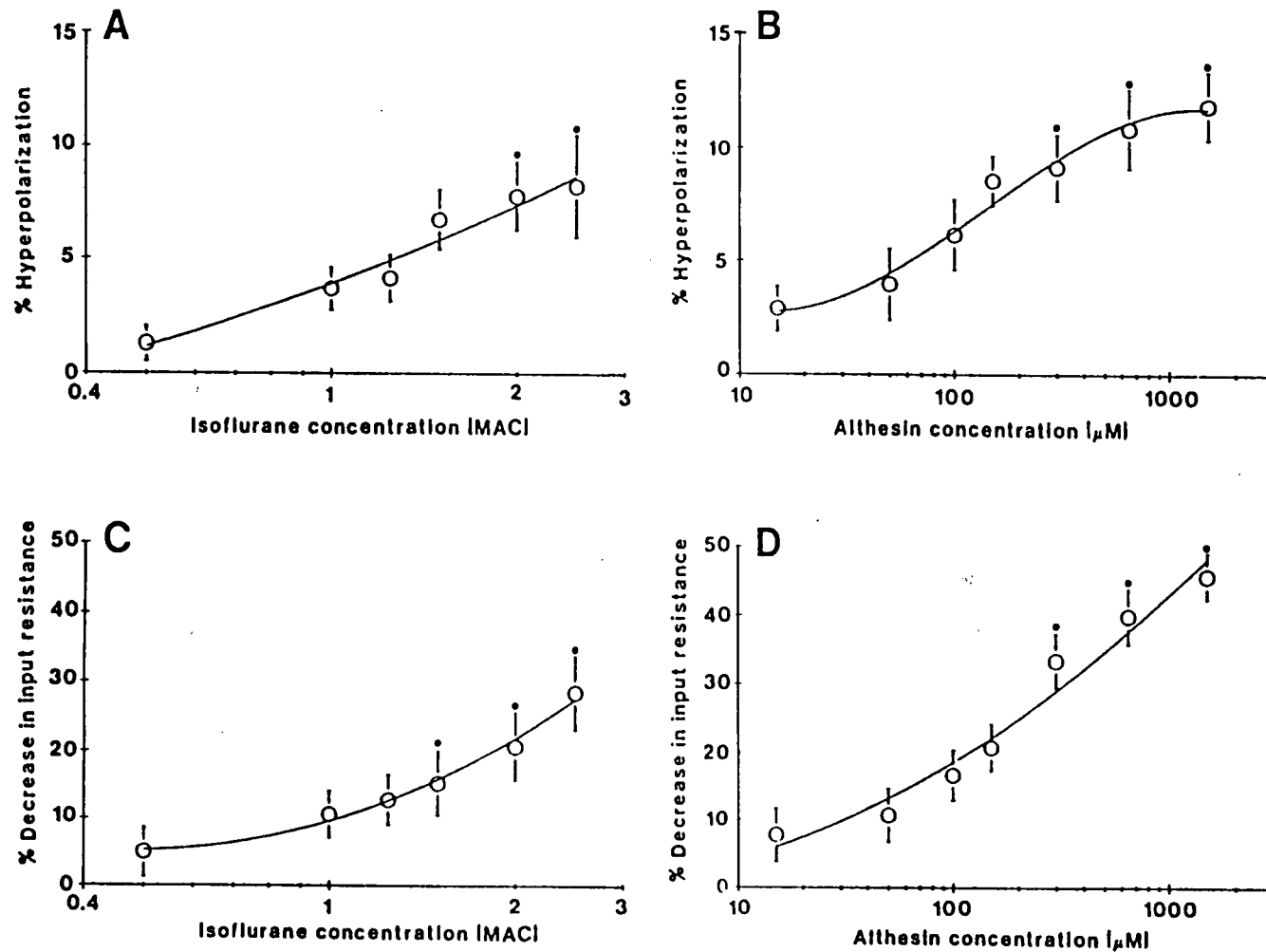


Fig. 7 Alterations in the passive membrane properties by applications of isoflurane (A,B) and Althesin (C,D). Symbols (●) indicate statistically significant difference from mean control values at $p < 0.05$.

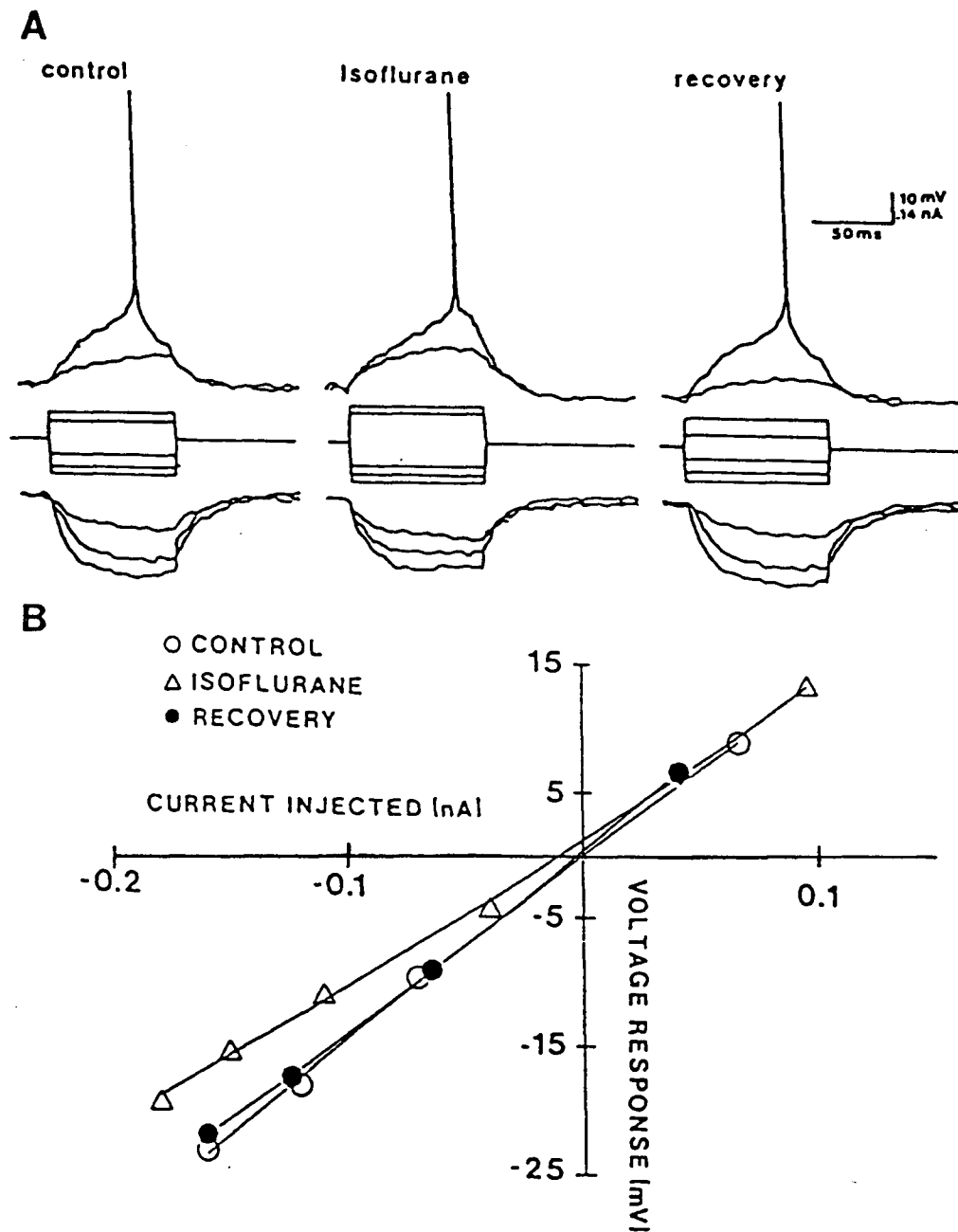


Fig. 8 Effects of isoflurane (1.5 MAC for 6 min) on the input resistance of an anterior cingulate neuron. (A) responses to injections of current pulses in control, isoflurane and recovery conditions. Note that isoflurane application produced a slight decrease in input resistance. The threshold amount of current for eliciting one spike was increased. (B) the slope resistance was decreased by ~28% during isoflurane application. Complete recovery was observed.

3.2 Discussion

The spontaneous activity of neocortical neurons has been correlated to the level of activation in the brain (Webb 1983). This level is optimally required for receiving, analyzing and reacting to internal and external stimuli (Krnjević 1974b; Richards 1983). The observed depression of spontaneous activity in the slice preparation that occurred without significant, concomitant changes in V_m and R_i , suggests that at least some of the observed actions of isoflurane and Althesin are at presynaptic sites. However, postsynaptic actions are indicated because significant increases in input conductance were observed with the anaesthetic applications in the higher dose range. When low doses of the anaesthetics were applied, the full extent of the changes in membrane conductance could have been partly masked by anaesthetic actions that decrease transmitter release. Addition of TTX, however, did not change the effects of anaesthetics on V_m or R_i . Consequently, another possibility may be considered where the failure to observe a change in conductance would be attributable to anaesthetic actions (in low doses) at dendritic sites distal to the recording electrode which was likely somatic in location. This can explain certain inconsistencies in the literature about the effects of anaesthetics on passive membrane properties. For example, some investigations have not revealed changes in neuronal excitabilities of CNS neurons (Zorychta and Čapek 1978; Scholfield 1980; Bosnjak et al. 1982 and Fujiwara et al. 1988). Nonetheless, profound reductions in the excitability of the postsynaptic membrane have been demonstrated during anaesthesia in some central neurons (Nicol and Madison 1982; Whitney and Glenn 1986; Berg-Johnson and Langmoen 1987; Takahashi and Takenoshita 1987) and in peripheral sensory neurons without known synaptic inputs (Puil and Gimbarzevsky 1987).

The repetitive firing evoked by current pulse injections was depressed by the anaesthetics, especially isoflurane. This effect could be due to change in Na^+ -inactivation in the neuron (Hodgkin and Huxley 1952). A local anaesthetic-like effect on the Na^+ -channels seems unlikely because diminutions in the amplitude and shape of the evoked single spikes were not observed in most neurons despite changes in the rate of current pulse injection. In addition, the first spike in an evoked burst was not affected by the anaesthetic applications (Fig. 6 A-B). The compromised repetitive firing ability can be attributed to an alteration in the level of Na^+ -inactivation as a result of anaesthetic attenuations of the AHPs (cf. Figs. 4B, 5 and 6 and Section VII) secondary to a depression of inward Ca^{2+} -currents (Krnjević and Puil 1988) which partly contribute to the ability of neocortical neurons to fire spikes repetitively (Stafstrom et al. 1985).

4 EFFECTS ON SYNAPTIC TRANSIENTS

In this series of investigations the effects of isoflurane (0.5-2.5 MAC) and Althesin (10 - 200 μM) applications were observed on 37 neurons. These neurons showed mean ($\pm\text{SEM}$) resting potentials of -74 ± 4 mV and a mean R_i of 65.9 ± 3.65 $\text{M}\Omega$. They fired repetitive spikes (<2 ms in duration) of >70 mV in amplitude with overshoots of ~ 20 mV. The average duration of recording was $125 (\pm 78)$ min. The electrodes were filled with K-acetate (2 M) and, on certain other occasions, KCl (3 M) for the excitatory postsynaptic potentials (EPSP) recordings or Cs_2SO_4 (3 M) for the dose/response studies on inhibitory postsynaptic potentials (IPSPs).

4.1 Results

4.1.1 Resting membrane properties. In this series, V_m was monitored continuously on a pen recorder whereas R_i was monitored by injection of

hyperpolarizing pulses in between two successive stimulations or by interrupting the rhythmic stimulation (0.2 Hz) just before, at the end, and after ~12 min of an anaesthetic application. An isoflurane-induced hyperpolarization was observed only at doses >1.5 MAC. Application at the highest dose (2.5 MAC) hyperpolarized 3 neurons by 3-5 mV. The hyperpolarizations elicited by Althesin were not statistically significant even at the 200 μ M dose where 4 neurons were hyperpolarized by ~4 mV. Statistically significant changes in R_i were observed during isoflurane applications at 1.5, 2 and 2.5 MAC. In 3 neurons, the maximum mean decrease from control values was 25% after 8 min of application (2.5 MAC). No significant changes in R_i were observed during Althesin applications at all doses (10-200 μ M).

4.1.2 Dose-response depression of excitatory postsynaptic potentials (EPSPs). Focal stimulation in the immediate subpial region of the neocortical slice produced EPSPs in the pyramidal cells located at the junction of the middle and lower third portions of the coronally sectioned slice. By using constant just-threshold stimuli (4-15 V; 0.1 ms), EPSPs without action potentials were initiated in ~50% of these stimulation experiments (at 0.2 Hz). When necessary (i.e., when an anaesthetic induced a change in V_m) the resting potential was held constant at the initial resting level by continuous DC-current injection throughout the experimental period. The mean amplitude (\pm SEM) of the control EPSPs was 18.5 (\pm 0.55) mV with an average duration of 210 (\pm 30) ms (cf. Avoli and Olivier 1989). The mean rate of rise was 0.25 (\pm 0.02) mV/ms whereas the rate of decay was 0.07 (\pm 0.01) mV/ms. Application of isoflurane (1-2.5 MAC) produced a dose-dependent reversible depression in the amplitude of the EPSPs (16/16 neurons; Fig. 9A). At 1 and 2 MAC the mean depressions from the control values were 52.56 and 80.25% respectively (Fig. 10). The rates of rise of

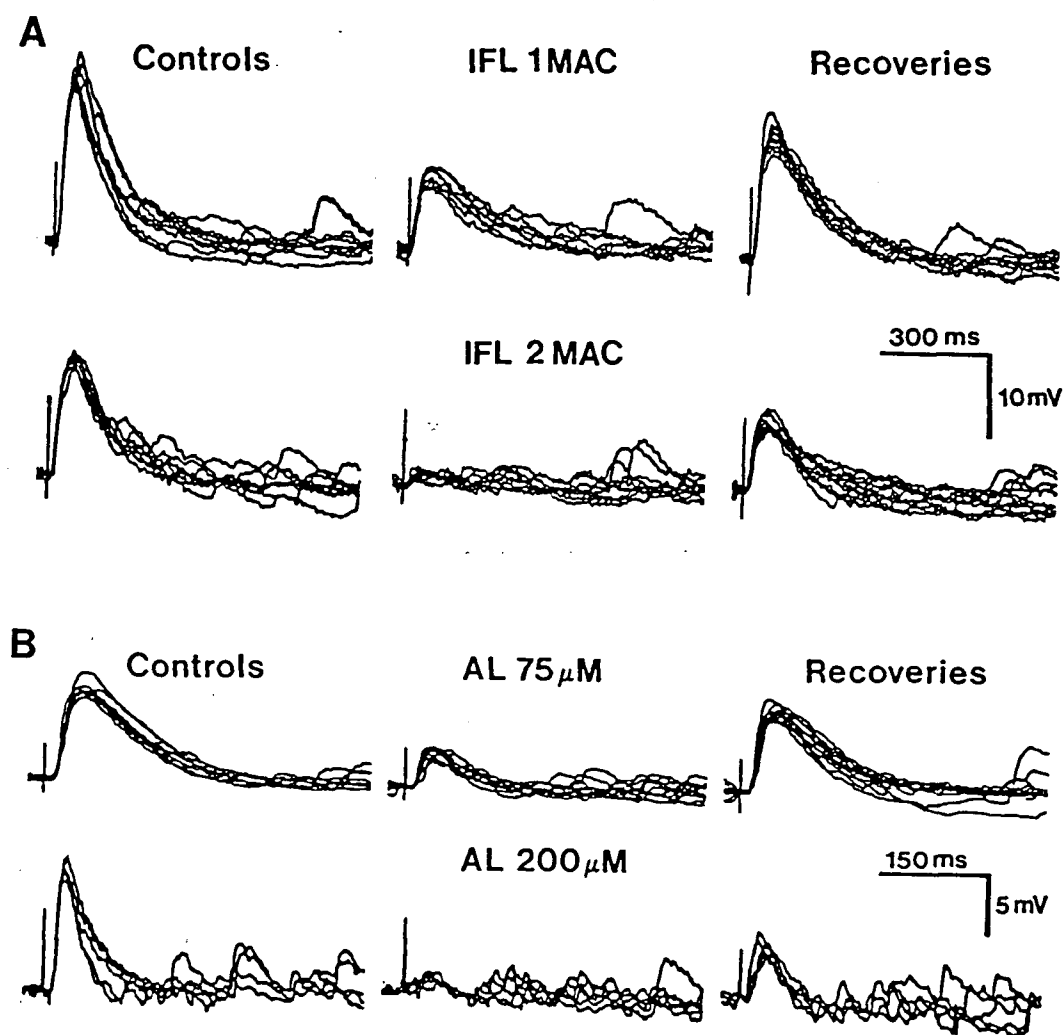


Fig. 9 EPSP-depression induced by isoflurane and Althesin. (A) isoflurane (IFL) was applied to two sensorimotor neurons for 10 min in doses of 1 and 2 MAC. The superimposed traces show almost complete depression at 2 MAC. Recovery was evident after 12 min. (B) Althesin 75 and 200 μ M was applied to two anterior cingulate neurons for 8 min. Partial recovery was obtained after 15 min in the second neuron (lower trace).

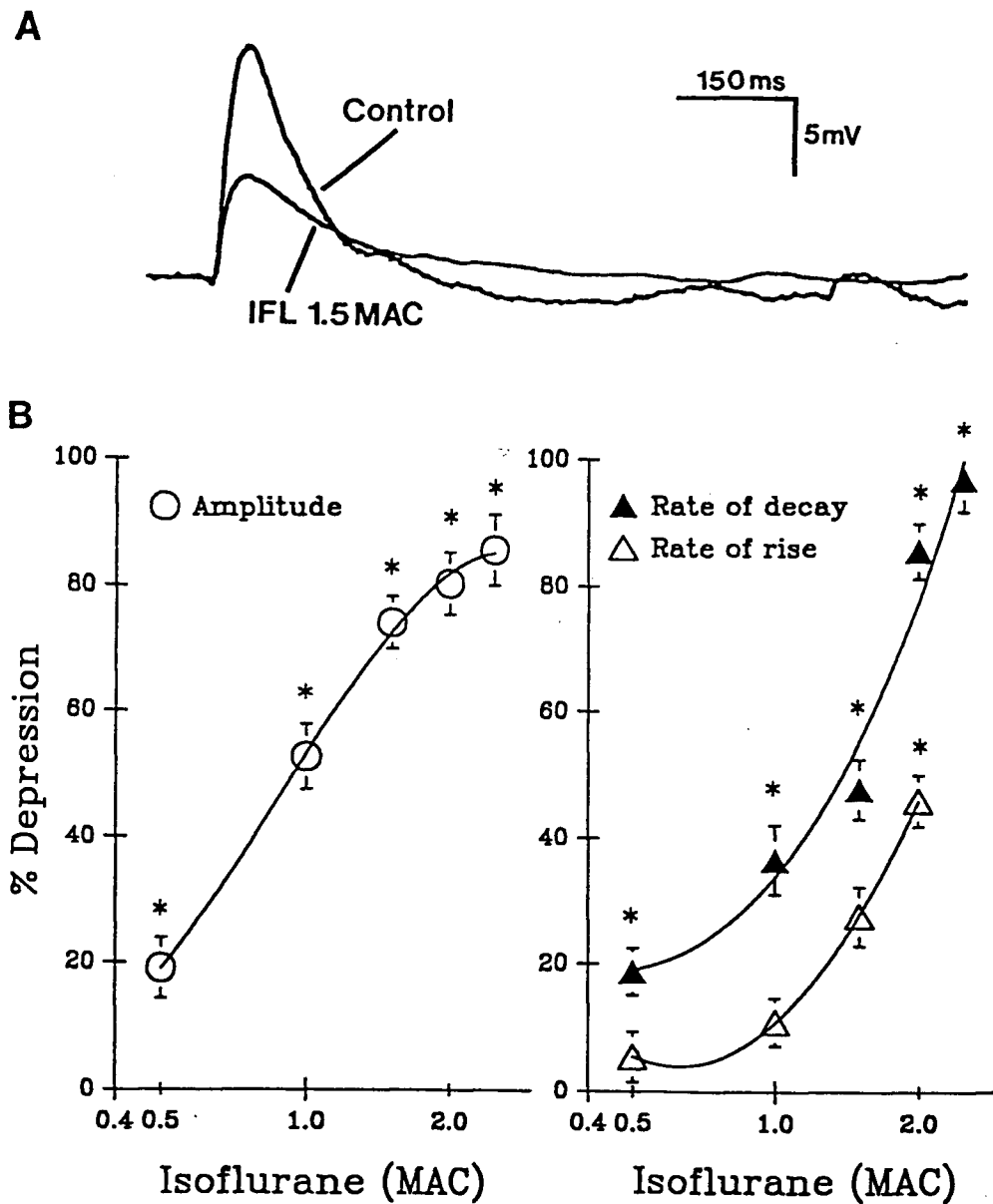


Fig. 10 Dose-dependent suppression of the amplitude and rate of decay by isoflurane (0.5-2.5 MAC). (A) computer-averaged traces of EPSPs evoked in a sensorimotor neuron during control and isoflurane (IFL; 1.5 MAC) perfusion using the same stimulus strength and duration. Traces averaged were obtained over a period of 2 min before, and at the end of isoflurane application (frequency of stimulation was 0.2 Hz). (B) dose-response relationships for the percent depression of amplitudes, rates of rise and rates of decay of the averaged EPSPs. The sample size for each dose was >4 . Statistical significance ($p < 0.05$) from control values is indicated by (*). Note that depression of the rate of rise was significant at 2.5 MAC. Error bars represent standard deviations.

the EPSP were not significantly affected except at concentrations >2 MAC whereas the rates of decay were reduced in a dose-dependent manner. At 0.5 and 1.5 MAC isoflurane, for example, the mean percent depressions from control conditions were 18.8 (± 3.67 , S.E.M.) mV/ms and 47.74 (± 1.74) mV/ms respectively (Fig. 10). The dose-dependent effects of Althesin occurred with a time course similar to that of the isoflurane effects in all the neurons ($n = 13$). Perfusion of the vehicle, cremophor EL (0.002 - 0.016% v/v), had no effect on EPSPs, or on the membrane conductance ($n = 4$ neurons). The EC_{50} for the depression of the EPSP amplitude by Althesin was slightly above 50 μ M (Fig. 11) and complete suppression was observed at 200 μ M. However, Althesin was less potent than isoflurane in depressing the rates of decay; the mean maximum percent depression was 77.88 (± 2.9) with concentrations as high as 200 μ M (Fig. 11).

4.1.3 EPSP attenuation in the presence of IPSP-blockade. Because the observed prolongation of allocortical IPSPs are presumed to contribute to the depression of the EPSPs produced by anaesthetic agents (Scholfield 1980), bicuculline (50 μ M) was used to pharmacologically isolate the EPSPs for a more exact isolation of the effects of isoflurane and Althesin. Using K-acetate or Cs_2SO_4 electrodes (cf. Fig. 14C), blockade of the IPSPs was observed within 5 min of bicuculline perfusion at a flow rate of 2 ml/min. No depolarization or increase in R_i was observed during application of the convulsant, possibly due to the preponderance of tonic excitatory inputs impinging on these neurons (cf. Matsumura et al. 1988; Buzsaki 1984). Under such conditions, the stimulus strength that was required to elicit the control, just-threshold EPSPs initiated only epileptiform bursts that usually were associated with 2-3 action potentials (Fig. 12A). Isoflurane (1.5 MAC) and Althesin (75 μ M) applied concomitantly with bicuculline

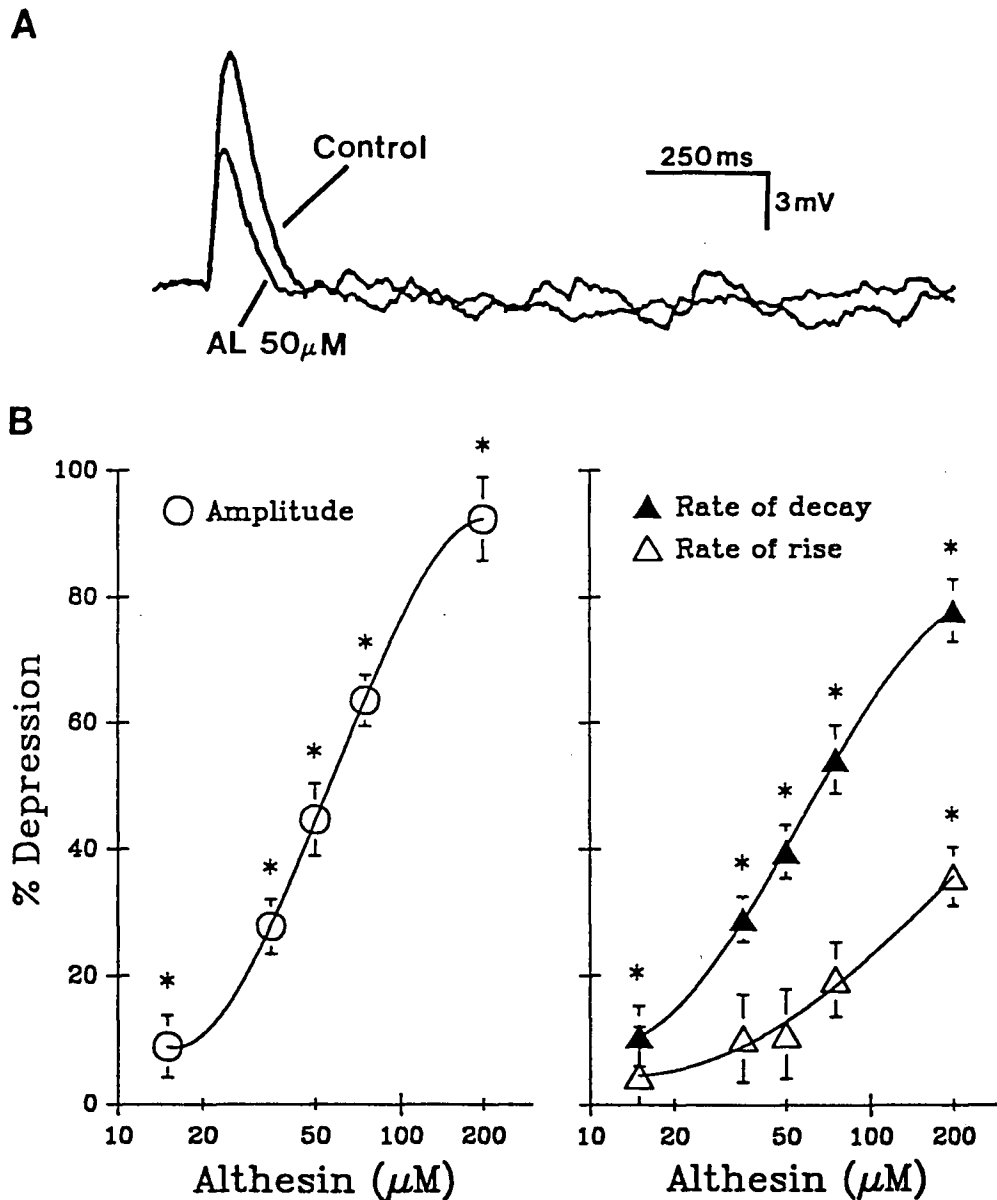
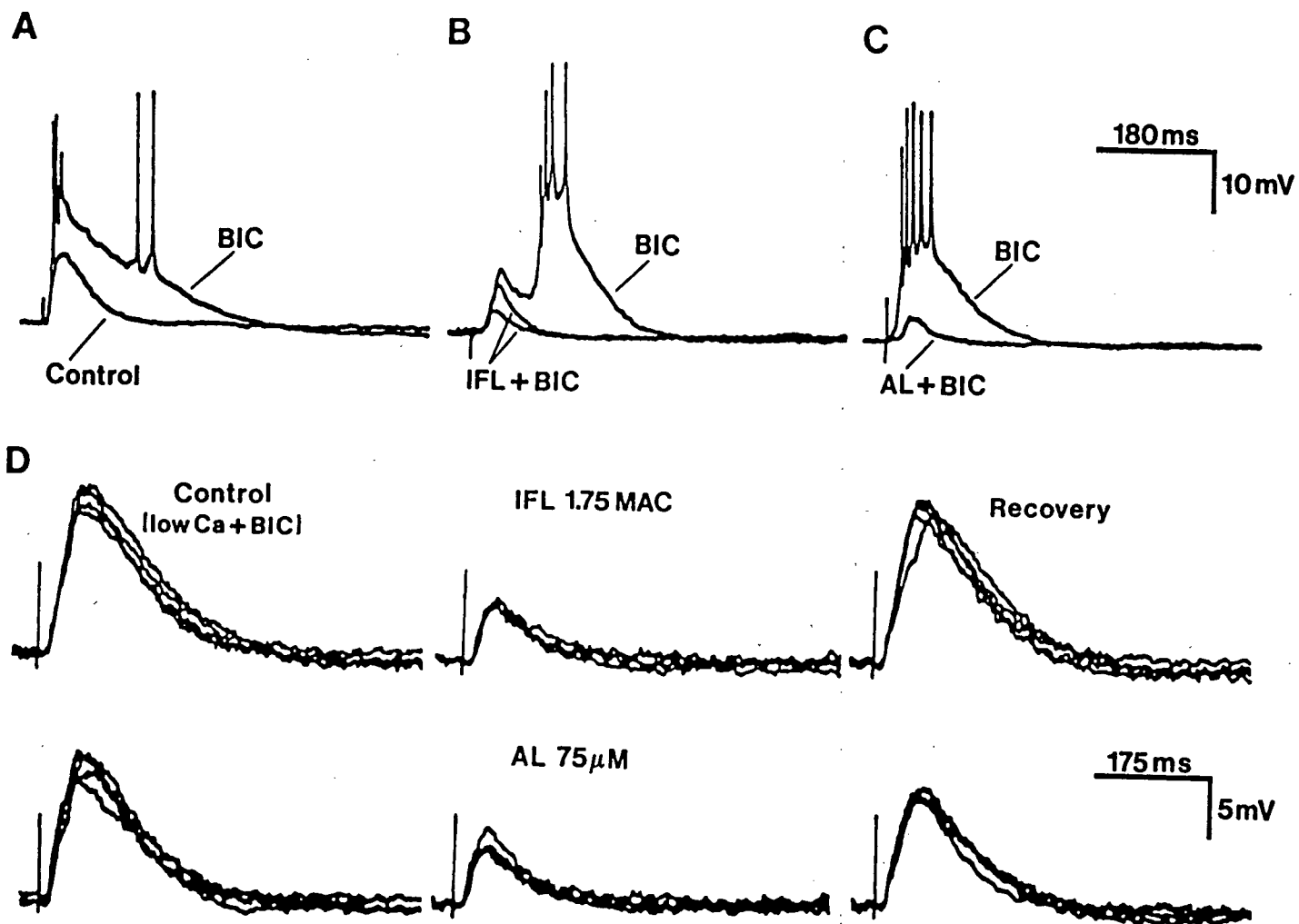


Fig. 11 Althesin-induced dose-dependent depression of the amplitudes and rates of decay of neocortical evoked EPSPs. (A) computer-averaged traces evoked in an anterior cingulate neuron are shown before and during Althesin application (AL; 50 μ M). Each averaged-trace represents at least 15 individual traces. (B) amplitudes and rates of decay were significantly depressed in a dose-dependent manner, whereas the rates of rise showed statistically significant suppression only at the highest dose (200 μ M). Symbols (*) indicate statistical significance ($p < 0.05$) from control. Sample sizes were ≥ 4 except at 15 and 200 μ M ($n = 3$ in each).

Fig. 12 Epileptogenic activity and EPSP suppression by anaesthetic applications in the presence of GABAergic blockade in neocortical neurons. (A) Application of bicuculline (BIC; 50 μ M) initiated epileptic activity in a neuron that was responding to the same stimulus with an EPSP. (B-C) epileptic bursts induced by bicuculline in two neurons were suppressed by the application of isoflurane and Althesin (IFL, 1.5 MAC, 15 min and AL, 75 μ M, 12 min respectively). In (B) the time course of burst suppression is shown (middle trace, 5 min and lower trace, 12 min). (D) in low Ca^{2+} media an EPSP could be evoked during the concomitant application of bicuculline. Under such conditions, a concomitant anaesthetic application (~10 min) attenuated the EPSPs in a similar manner to that observed in the absence of bicuculline (cf. Fig. 9).



depressed the amplitudes of the epileptiform bursts, unmasking small EPSPs (Fig. 12B-C). In order to eliminate the action potentials and epileptiform activity, the extracellular Ca^{2+} concentration was decreased to 1 mM and the $[\text{Mg}^{2+}]$ was increased by an equivalent amount. Because of this procedure, EPSPs subthreshold to spike genesis could be evoked. After the application of isoflurane and Althesin (1.75 MAC in 4 neurons and 75 μM in 4 additional neurons, respectively), the EPSPs were depressed in a manner similar to that observed in the absence of bicuculline (Fig. 12D).

4.1.4 Effects on orthodromically and intracellularly evoked spikes.

Supramaximal stimulation consistently evoked spikes that were blocked by anaesthetic perfusion (isoflurane at 1.5 MAC to 3 neurons and Althesin in 50 μM doses to additional 3 neurons) for 12 min at flow rate of 2 ml/min (Fig. 13, A1-B1). Recovery was evident after ~15 min on returning to the control solution. On interrupting the subpial stimulation, more than two spikes were evoked in these neurons with injections of depolarizing current pulses. The same current injection intensities used during the anaesthetic applications revealed the compromised abilities of the neurons to fire the same number of spikes as in control conditions (Fig. 13A2-B2).

4.1.5 Dose-response relationship for IPSP suppression during Cs-blockade of the K-conductances.

The use of intracellular Cs_2SO_4 electrodes was associated after about 15 min of impalement ($n = 17$ neurons), with an input resistance which increased from a mean control value of $55.75 \pm 3.25 \text{ M}\Omega$ to a mean of $74.57 (\pm 4.25) \text{ M}\Omega$, an average depolarization of ~11 mV and a markedly prolonged spike duration (Fig. 14A). After 20 min of Cs-application the postspike afterhyperpolarization was completely abolished (Fig. 14B). Application of bicuculline at this point blocked the IPSPs (4 neurons; Fig. 14C). Thus the use of the Cs_2SO_4 -containing electrodes

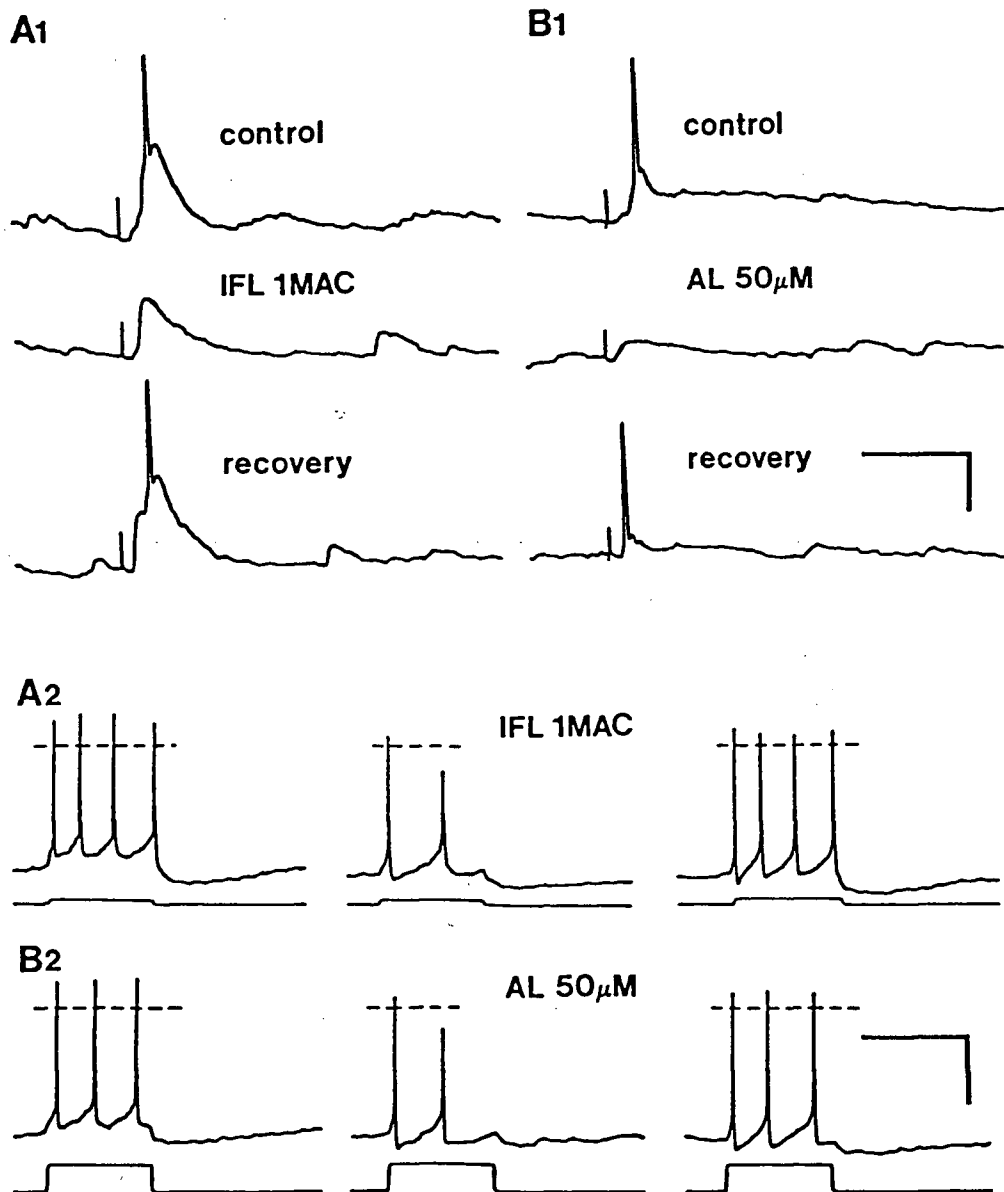


Fig. 13 Anaesthetic interference with the synaptically-induced spikes and directly-evoked action potentials by intracellular current pulse injections. Synaptically-induced (A1) and directly-evoked spikes (A2) were depressed by isoflurane (IFL; 1 MAC) in a sensorimotor neuron. (B1 and B2) the same effect was produced by Althesin (AL; 50 μ M) in another sensorimotor neuron. Calibration bars: 120 ms (horizontal) and 10 mV (vertical) in A1 and B1, 100 ms and 40 mV or 1 nA in A2 and B2.

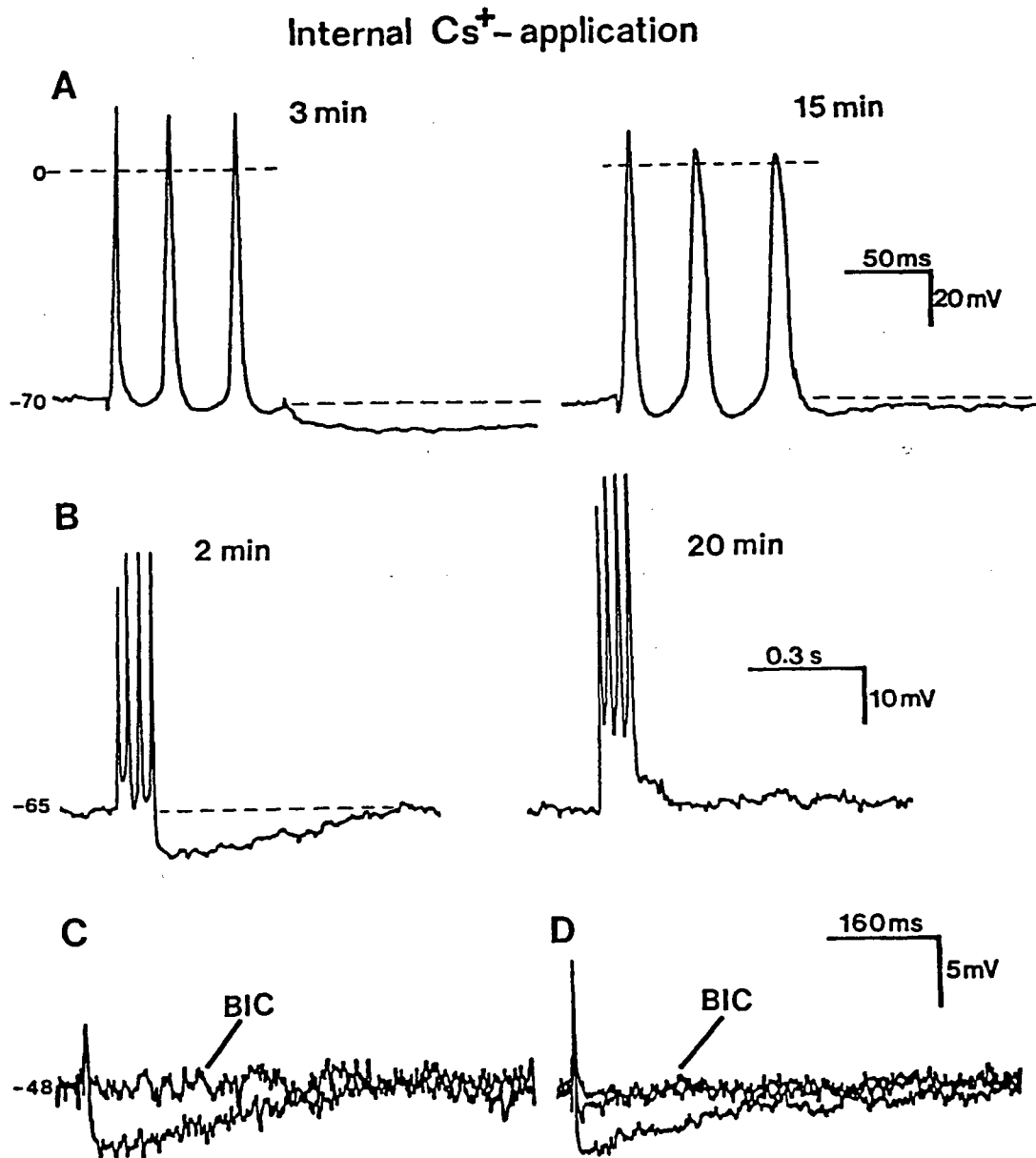


Fig. 14 Internal Cs⁺-application in 4 neocortical neurons. (A) Cs⁺ leakage from an intracellular micropipette caused prolongation of the action potentials, partial blockade of the AHP and a small local anaesthetic-like effect i.e., a reduction in the rates of rise and amplitudes of the spikes after 15 min of impalement. (B) in another anterior cingulate neuron complete blockade of the AHP was evident after 20 min of penetration. In (A) and (B), the membrane potential (V_m) was kept constant at the resting level by DC-current injection. In both cells V_m was depolarized by 13 and 15 mV respectively after ~25 min. (C) complete suppression of an IPSP after bicuculline application (BIC; 50 μ M; 5 min) in a neuron impaled by a Cs₂SO₄ electrode. (D) time course of blockade of the IPSPs in a sensorimotor neuron penetrated with a Cs₂SO₄ micropipette. Lower and middle traces were obtained after 1 and 3 min of bicuculline (50 μ M) perfusion. In (C) and (D), IPSPs were evoked after complete attenuation of the AHP and attainment of a steady state of V_m (-48 mV from a resting level -66 mV).

removed any "contamination" of the IPSPs by K-mediated late hyperpolarizing potentials. In addition, the Cs-induced depolarization increased the amplitude of the evoked IPSPs and rendered them more amenable for extensive examination of drug-actions. The mean amplitude of the IPSPs was 5.2 mV (± 0.35) whereas the mean duration and rate of decay were 410 (± 42) ms and 0.013 (± 0.007) mV/ms respectively (cf. Howe et al. 1987; Sutor and Hablitz 1989).

The application of isoflurane or Althesin consistently depressed the IPSPs in all neurons ($n = 17$); this effect was dose-dependent, reversible and reproducible on repeated applications to the same neuron. Isoflurane (1.5 MAC) and Althesin (75 μ M) depressed the amplitudes of IPSPs by ~54 and 55% respectively (Figs. 15 and 16). The effects were only evident after prolonged administration (12-15 min) in the case of both agents. Recovery usually was observed after 20 min. The EC_{50} for the attenuation of the IPSPs was greater than EC_{50} for depression of the EPSPs in the case of both agents. Although a dose-dependent slowing of the IPSP decay was evident, the dose-response curves for both agents had a lower slope values than that for the EPSPs. The maximum depressions of the rate of decay from the control values were ~54% for 2.5 MAC isoflurane and 45% for 100 μ M Althesin. Statistically significant differences were observed in the higher dose range (Fig. 16). Moreover, complete recovery of the rate of decay was observed in ~70% of neurons.

4.2 Discussion

The early studies by Eccles (1946), Brookes and Eccles (1947) and Larrabee and Posternak (1952) demonstrated that excitatory synaptic transmission was reduced by administration of various general anaesthetics including chloroform, diethylether and barbiturates. Later studies by Somjen

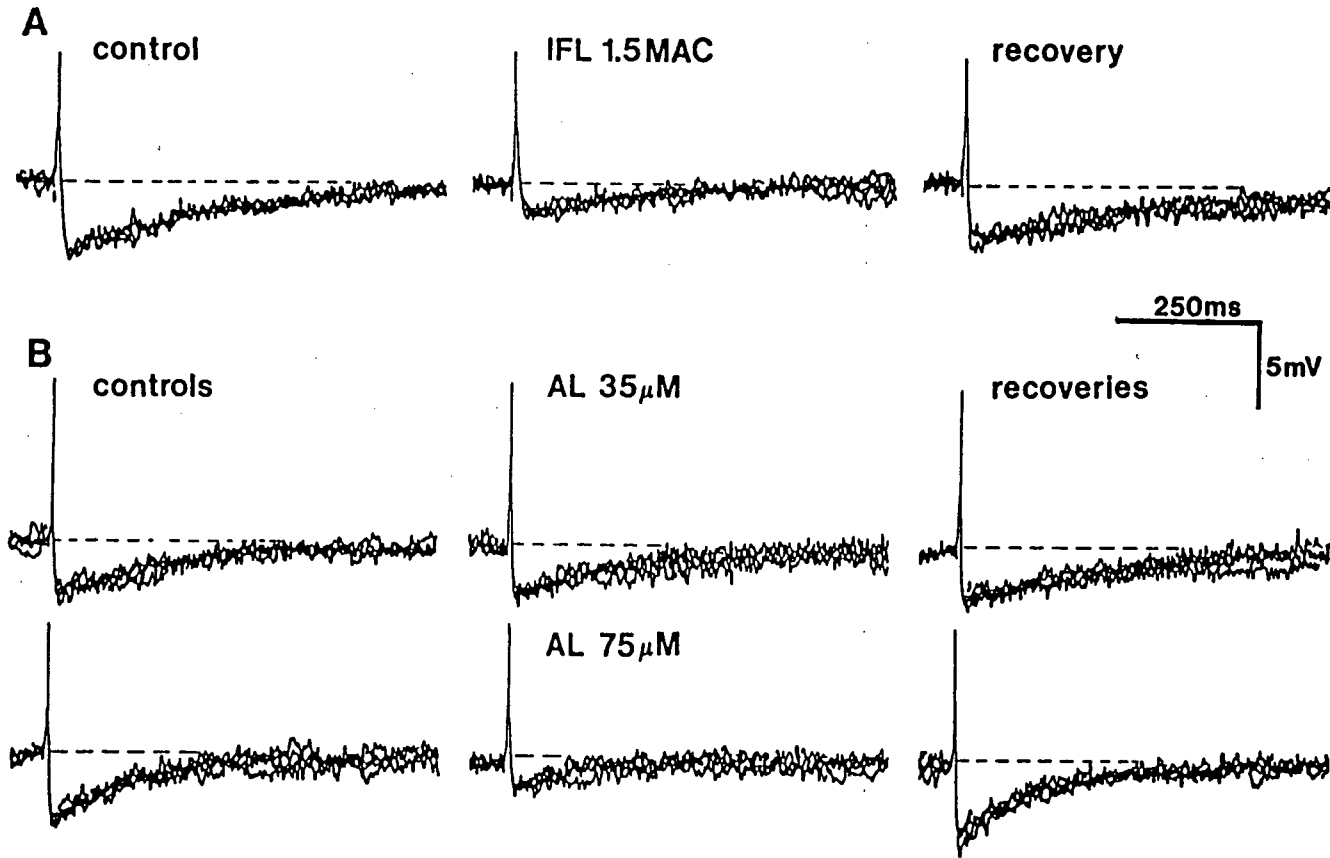


Fig. 15 IPSP-attenuation by isoflurane and Althesin after K^+ -conductance blockade by intracellular application of Cs^+ . (A) isoflurane (1.5 MAC; 12 min application) suppressed the IPSPs by ~60%. (B) Althesin (35 and 75 μ M; 12 min application) attenuated the IPSPs evoked in sensorimotor and anterior cingulate neurons respectively. In (A) and (B) the superimposed traces were at least 6 in each condition.

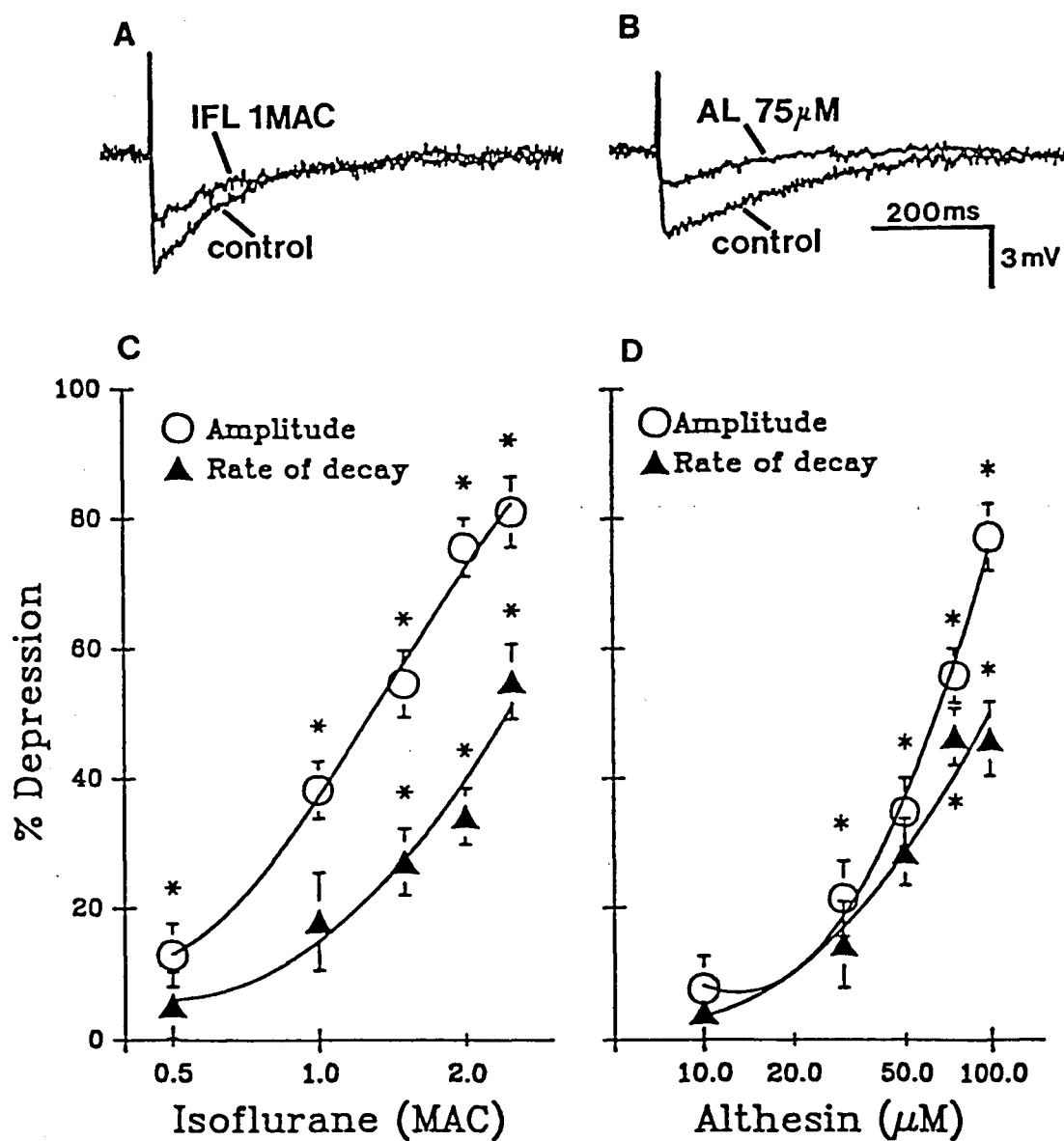


Fig. 16 Dose-dependent interference with the IPSPs in neocortical neurons with concomitant blockade of K^+ -conductances by intracellular Cs^+ . (A and B) computer-averaged traces in two neurons in which isoflurane and Althesin (IFL, 1 MAC; AL, 75 μ M) were applied. (C and D) dose-response curves for isoflurane and Althesin showing suppression of the amplitudes and rates of decay by at least 3 anaesthetic applications for each dose.

and Gill (1963) and Somjen (1963) confirmed that diethylether and barbiturates depressed the EPSPs in motoneurons. Hence clear evidence was obtained for direct actions of anaesthetics on central synaptic transmission. Subsequently, halothane and barbiturates were shown to depress excitatory synaptic transmission in the cuneate nucleus (Galindo 1969). At the supraspinal levels, a wide variety of anaesthetics has now been found to depress excitatory synaptic transmission particularly in the hippocampal formation (Berg-Johnson and Langmoen 1986a,b and 1987; MacIver and Roth 1988; Miu and Puil 1989) and olfactory cortex (Richards 1973; Richards et al., 1975). However, Nicoll (1972) has shown that the unusual dendrodendritic excitatory synapses of the olfactory bulb are relatively resistant to the depressive actions of general anaesthetics. Moreover, Fujiwara and his associates (1988) also found no effects of isoflurane, halothane or enflurane on evoked EPSPs in the hippocampal slice in vitro. There is no clear evidence at present time, that any general anaesthetic actually increases the efficacy of excitatory synapses. Such a mechanism might be expected to prevail during the early excitatory phase of the anaesthetic state or, given excitatory synaptic inputs to inhibitory interneurons, could contribute to the enhanced inhibitory processes during anaesthetic administration (see below; cf. Morris 1978).

The actions of general anaesthetics on inhibitory synaptic transmission are more complex than on those at excitatory synapses. Eccles and associates (1963) reported that presynaptic inhibition was enhanced by barbiturate or chloralose administration but depressed by diethylether and chloral hydrate application. Larson and Major (1970) reported that hexobarbitone application increased the duration of IPSPs in the spinal cord in in vivo feline preparations. Later, Nicoll and his associates (1975) also observed a

barbiturate-induced prolongation of inhibitory potentials in the hippocampus of in vivo feline preparations. More recently, increases in the spontaneous IPSCs (Gage and Robertson 1985) and the evoked IPSPs (Miu and Puil 1989) have been observed in hippocampal in vitro slice preparations. In in vitro studies of the olfactory bulb and cortex (Scholfield 1978, 1980) which were initiated by Nicoll (1972), administration of barbiturates, halothane, alphaxalone and ketamine increased the duration of postsynaptic inhibition. In the spinal cord, Weakly (1968) did not observe any specific increase in the direct inhibition of motoneurons in vivo whereas depression of IPSPs was evident in in vitro slice preparations of the newborn rat spinal cord (Takahashi and Takenoshita 1987). Synaptically evoked inhibitory potentials recorded in the CA1 pyramidal cells of the hippocampus are consistently attenuated by clinically used inhalational anaesthetics (Yoshimura et al. 1985; Fujiwara et al. 1988).

Modification of the synaptic transients during anaesthesia have not been investigated extensively in the neocortex which is an area that is very vulnerable to anaesthetic actions (cf. Introduction). In addition, experimental procedures to isolate the conductances associated with the EPSPs or IPSPs from other "contaminating" conductances have not been implemented in the previous investigations. This may have resulted in some of the apparent contradictions arising from the different experimental preparations used by previous investigators and also may have provided some difficulty in interpreting the "true effects" on these transients.

4.2.1 Anaesthetic depression of EPSPs. A striking feature of the anaesthetic effects on EPSPs is the dose-dependent depression in their amplitudes with EC_{50} s of 1 MAC and $\sim 50 \mu\text{M}$ for the respective depressions by isoflurane and Althesin; these concentrations are similar to those that

have been determined in the brain of anaesthetized animals (Smith et al 1974; Mills et al. 1987). An interesting effect which was observed in the present investigations was the dose-dependent slowing in the rate of decay of the synaptic transients. Quastel and Linder (1975) and Gage and associates (1975 and 1979) have reported similar effects of certain aliphatic alcohols on the miniature endplate currents at the neuromuscular junction. However, comparable findings have not been previously observed in neurons of the CNS (cf. Kullman et al 1989). The anaesthetic depression of EPSP amplitude in neocortex could be due to presynaptic or postsynaptic actions. Presynaptic mechanisms include depression of impulse conduction or nerve terminal excitability (Quastel and Saint 1986; Berg-Johnsen and Langmoen 1986b; Saint et al. 1986; Butterworth et al 1989) and, depression of transmitter release either by interfering with synthesis of transmitter and/or its mobilization, thereby inhibiting the number of released quanta per impulse or decreasing the quantal content (Cheng and Brunner 1978; Zorychta and Capek 1978; Lodge and Anis 1984; Johnson 1985; Bosnjak et al. 1988; Kullman et al 1989). Postsynaptically, an increase in membrane conductance (Nicol and Madison 1982) or a decrease in the receptor sensitivity to a transmitter (Anis et al. 1983; Sawada and Yamamoto 1985) can lead to attenuation of EPSPs.

The observed suppression of the EPSP amplitudes could be explained by a summation of pre- and postsynaptic depressant actions of the anaesthetic agents. The depression of the Ca-activated K-conductance that was "isolated" in neocortical neurons (cf. Section 6) provides indirect evidence that a Ca-conductance may be depressed in the presynaptic terminal. For example, an attenuated Ca^{2+} influx would decrease the number of quanta released per stimulus. This possibility is supported by the observations of

Krnjević and Puil (1988) who observed a depression of certain voltage-dependent Ca currents in voltage-clamped hippocampal CA1 neurons during halothane application (also cf. Morgan and Bryant 1977 and Nishi and Oyama 1983). Zorychta and collaborators (1975; Zorychta and Čapek 1978) reported that the decreased number of quanta released during halothane and diethyl-ether anaesthesia was the mechanism of suppression of monosynaptic EPSPs in the spinal cord of cats.

The prolongation of the decay with a concomitant depression of the amplitude of the EPSPs could be due to:

- (a) a proportional suppression of the uptake mechanisms (cf. Puil, 1981; Minchin 1981) with a reduction in the number of receptors activated;
- (b) a prolongation of the membrane time constant due to an increased input capacitance at membrane sites distant from the somatic recording electrode (cf. Puil and Gimbarzevsky 1987);
- (c) an increase in the mean open time of the receptor-operated channels mediating the EPSPs, concomitant with a decrease in the absolute number of receptors activated or
- d) the simultaneous depression of the IPSPs during the anaesthetic application.

The results of the present studies are at variance with the observations reported in olfactory cortex and hippocampal formation. Scholfield (1980) attributed the depression of the EPSPs in olfactory cortex to a shunting effect produced by the anaesthetic prolongation of the IPSPs. In the present investigations bicuculline (50 μ M) was applied to neocortical neurons before anaesthetic application and the IPSPs were additionally monitored in order to eliminate this possibility. Once blockade of IPSP was observed, isoflurane or Althesin was perfused simultaneously with bicuculline. Clinically

relevant doses of both agents not only depressed the EPSPs but also were effective in inhibiting the epileptiform neuronal discharges initiated by the GABA_A-antagonist (cf. Fig. 12). In the hippocampus, EPSPs recorded in the CA1 neurons were not affected by the applications of isoflurane, enflurane or halothane (Yoshimura et al. 1985; Fujiwara et al. 1988). According to the latter reports, a non-air-tight gas chamber was used and the anaesthetics were applied only by bath perfusion and were not introduced into the atmosphere above the slice. In addition, the volatile agents were not bubbled into the perfusate for a suitable equilibration period but were added and "shaken vigorously" in a stock solution before dilution and application in the chamber. Therefore, leakage of anaesthetic vapour could have occurred during such applications and could have lead to a decrease in the concentrations of the agents at the effector sites (cf. Bazil et al. 1987).

4.2.2 Anaesthetic actions on the IPSPs. The use of Cs₂SO₄ electrodes in these experiments provided two main advantages:

- (a) lower tip resistances than in the case of K-acetate filled micro-pipettes and
- (b) blockade of K⁺-conductances that could have contaminated endogenous activity mediated by GABA evoked Cl⁻ influx.

The dose-dependent depression of the IPSP amplitudes together with the decrease in the rate of decay suggests a common pre- and postsynaptic mechanism for the depression of the IPSPs and the EPSPs. Nonetheless, if inhibitory interneurons are activated by excitatory synapses, the anaesthetic-induced depression of EPSPs may lead to a concomitant attenuation of the IPSPs.

Takahashi and Takenoshita (1987) also observed an attenuation of the evoked EPSPs and IPSPs recorded from motoneurons in *in vitro* slice preparations. However, their results and those of the present investigations contradict the anaesthetic potentiation of the spontaneous and evoked IPSCs and IPSPs reported for olfactory cortex and hippocampal neurons (Scholfield 1980; Gage and Robertson 1985; Harrison et al. 1987). Despite the fundamental differences between the types of tonic synaptic inputs in the neocortical (Matsumura et al. 1988), hippocampal CA1 (Buzsaki 1984) and olfactory cortical neurons (Scholfield 1978), the discrepant findings could have arisen from the activation of K^+ -conductances that "contaminate" the IPSPs i.e., in the olfactory cortical and hippocampal neurons where internal Cs^+ had not been applied. Another possibility is that the low temperature (20-25°C) used during the experiments in the allocortex (i.e., olfactory cortex and hippocampus) would tend to slow down the kinetics of activated ionic channels and may increase their mean open-time. In the olfactory cortex, an anaesthetic depression of the EPSPs also was observed. Hence, the enhancement of the IPSPs could be attributed to a suppression of EPSPs which could have unmasked the full extent of the inhibitory ionic currents. In the case of the alphaxalone applications to cultured hippocampal neurons (Harrison et al. 1987), the doses which were used (0.1-10 μM) were six times lower than those that occur in the brain grey matter of anaesthetized rats (Smith et al. 1974). At such low concentrations in the *in vivo* situation alphaxalone may act as a sedative, e.g., by prolonging the IPSPs, but would likely not induce anaesthesia, particularly since previous studies with alphaxalone have revealed a depression of GABAergic responses in midbrain neurons of vertebrates (Cullen and Martin 1982).

5 ANAE STHETIC INDUCED ALTERATIONS IN NEURONAL RESPONSIVENESS

TO ACTIVATING TRANSMITTERS AND RELATED SUBSTANCES

The mean (\pm SD) resting potential and input resistance for the 65 neurons included in this series of experiments were -70 (± 2.25) mV and 51 (± 2.5) M Ω . These neurons fired spikes of >70 mV amplitude and were <2 ms in duration in response to depolarizing current pulses. The five-barrelled iontophoretic electrodes which were used here, were either glued to the recording pipettes or inserted separately at the surface of the slice (cf. Methods). The recording microelectrodes were filled with KCl (3 M) or K₂SO₄ (0.6 M).

5.1 Results

5.1.1 Control responses to iontophoretically applied agents

5.1.1.1 Acetylcholine. Applications of ACh (50-350 nA) evoked a mean (\pm SEM) depolarization of 12.3 (± 1.5) mV in 36 out of 50 neurons that were located in an area corresponding to layers IV and V of the neocortex. However, at more superficial layers (II and III), ACh application induced a long-lasting hyperpolarization in 3 neurons (cf. Wong and Gallagher 1989). The input resistances of the neurons were monitored by repetitive tests using constant hyperpolarizing pulses and were usually increased ($\sim 30\%$) by such applications; in 4 neurons the input resistances were decreased, probably due to membrane (delayed) rectification. The ACh-induced depolarizations were slow in onset as well as in decay (Fig. 17) and were reproducible on repeated application to the same neuron, without obvious signs of desensitization (Fig. 17A1). These actions could be blocked by the application of scopolamine (20 μ M; 3 neurons). Neurons that exhibited the largest responses to ACh application usually fired spikes in doublets when injected with depolarizing current pulses. These cells had input resistance

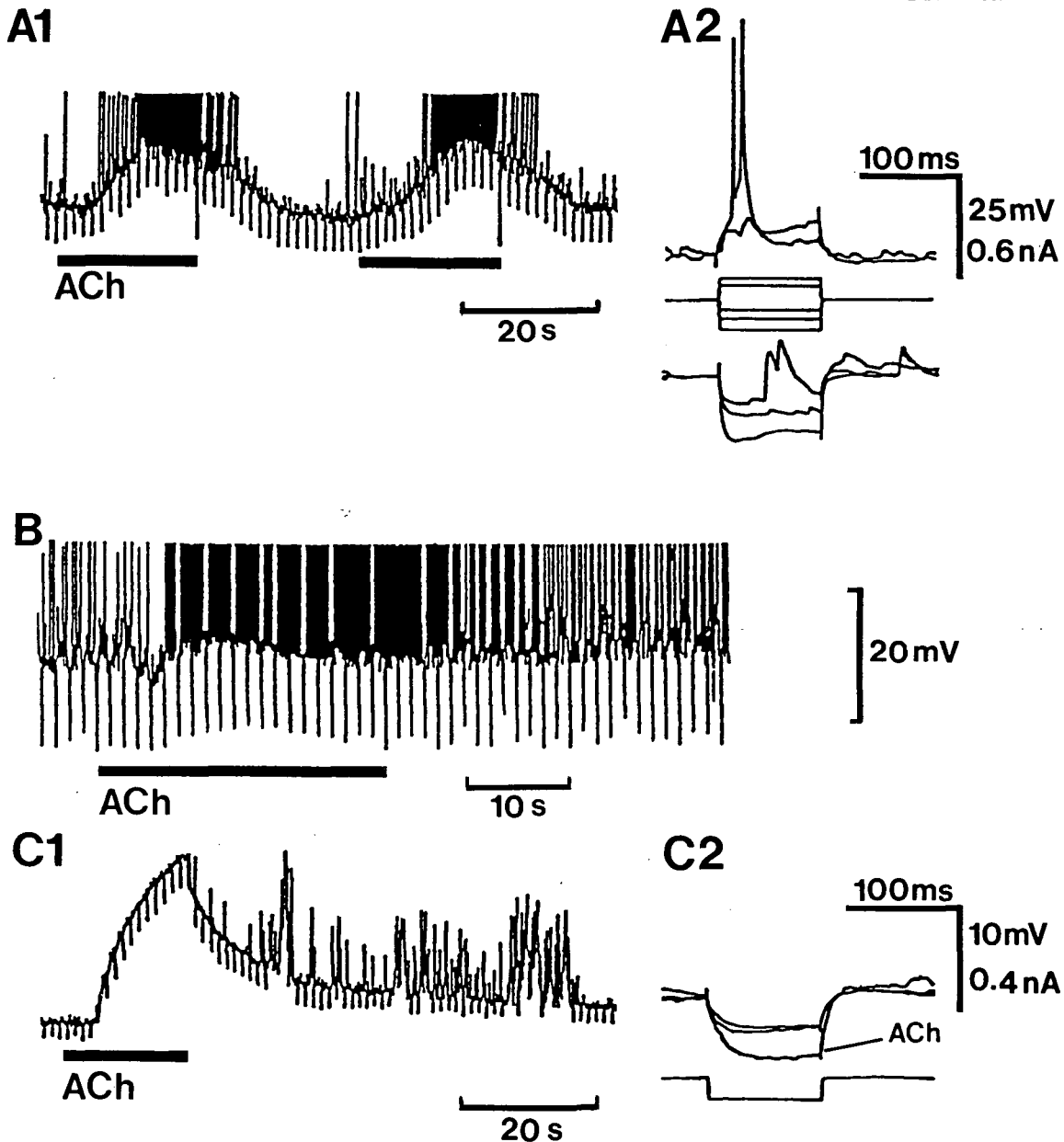


Fig. 17 Effects of iontophoretically applied acetylcholine (ACh) on neocortical neurons. (A) a typically slow onset response to ACh ejected from a compound electrode assembly is shown for a sensorimotor neuron. (A2) In the same neuron injections of just-threshold depolarizing current pulses evoked action potentials in doublets. Note the large amount of spontaneous synaptic activity as well as the sagging hyperpolarizing response to current pulse injection. (B) response of a sensorimotor neuron to ACh applied from a separate electrode consisted of a small depolarization and increased spiking activity which was preceded by a brief hyperpolarization. (C1) ACh evoked a depolarization with an afterburst of synaptic activity despite the presence of TTX. Hyperpolarizing voltage responses to intracellular current injections before, during, and after ACh application are shown in (C2).

of approximately 50 M Ω , and exhibited a large amount of spontaneous synaptic activity as well as 'sagging responses' to hyperpolarizing current pulse injections (Fig. 17A2). The responses to ACh could be grouped into two main categories:

- (1) depolarizing response with an afterburst of synaptic activity (Fig. 17C1) that was observed on several occasions in the presence of TTX and
- (2) depolarizing response that was preceded by a hyperpolarization (Fig. 17B); this hyperpolarizing potential which was obtained when the iontophoretic electrode was independent from the recording electrode, could be blocked with application of 1-1.5 μ M TTX.

5.1.1.2 Glutamate and N-methyl-D-aspartate. In contrast to the effects of ACh, the responses to Glu were abrupt in onset and had a rapid offset, i.e., were an "on/off" type of response (Figs. 18A-C). All 65 neurons were depolarized by Glu applications (40-300 nA) and did not exhibit desensitization. The mean (\pm SEM) depolarization was 20.25 (\pm 1.4) mV and was associated with a decreased input resistance of ~40-80% (Fig. 18A2). Bath application of TTX had no effect on the responsiveness of the neurons to Glu (Fig. 18B; cf. Zieglgänsberger and Puil 1972). In ~15% of the neurons, a brief period (2-3 s) of hyperpolarization (2-4 mV) was observed at the end of the depolarizing response (cf. Padjen and Smith 1983). It was not uncommon for the ratio of "ACh/Glu depolarizations" which varied between 0.7-1, to equal unity (Fig. 18C). There was a tendency towards a higher ratio when the electrode in the assemblies had intertip distances of 50 μ m. The Glu-evoked depolarizations produced by consistently spaced ejection pulses of equivalent current were remarkably reproducible despite the length of the experimental period (e.g., 3 hr.).

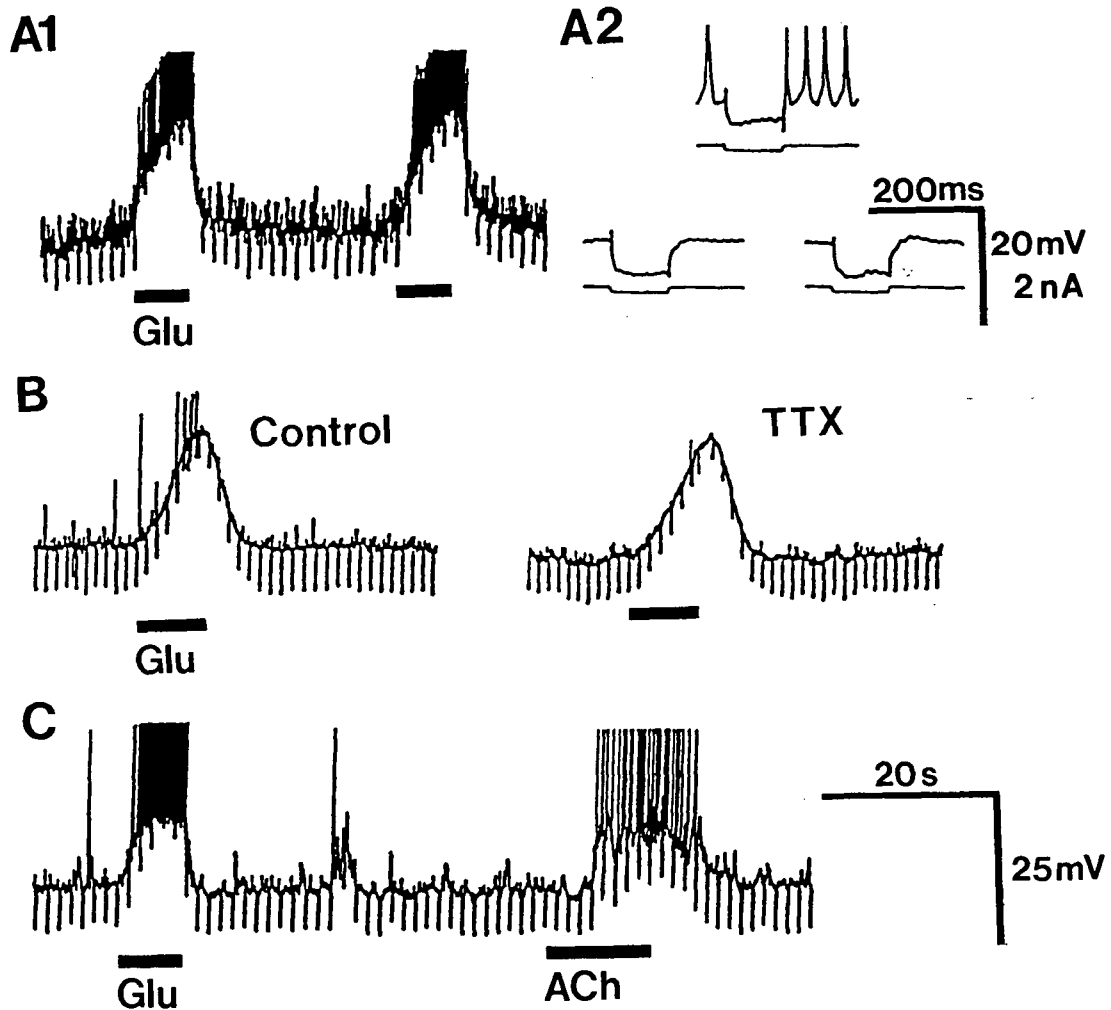


Fig. 18 Glutamate (Glu) evoked responses in neocortical neurons. (A1) Glu application induced a fast "on/off" type of depolarizing response which was associated with an increased conductance (A2). (B) the Glu actions were not affected by the perfusion of TTX ($1.5 \mu\text{M}$). (C) alternating applications of Glu and ACh on an anterior cingulate neuron produced depolarizing responses (ACh/Glu ratio = ~ 0.85) which are shown typically here.

Similarly brief NMDA applications depolarized 15/37 neurons despite the presence of 2 mM Mg^{+2} in the bath. NMDA induced slowly developing depolarizing responses that also had a slow decay (Fig. 19A); this response was apparent in conjunction with an increased input resistance that waned after the peak of the potential response (Fig. 19A). Two other types of responses also were observed:

- (1) depolarizing response that exhibited an acute form of desensitization during the application period (Fig. 19B; 5 neurons) and
- (2) a relatively fast Glu-like response that desensitized markedly on repeated applications (Fig. 19C; 4 neurons).

5.1.1.3 Gamma-aminobutyrate and baclofen. Iontophoretic application of GABA produced two types of responses (36 neurons):

- (1) a hyperpolarization which was observed only when the compound electrode assemblies were used (9/9 neurons) and
- (2) a depolarization which was evoked when the application pipette was separate from the recording electrode (29/29 neurons).

The hyperpolarizing responses (5-9 mV) to GABA were associated with decreased input resistance (40-60%). An acute desensitization (Fig. 20) was observed as well during the application period. Moreover, a long-lasting hyperpolarization was evident when the duration of GABA application was increased to >10 s (cf. Blaxter et al. 1986).

The depolarizing type of response (14-40 mV) was accompanied by an increase in input conductance and could be further subdivided into three forms:

- (1) without desensitization (Fig. 21A),
 - (2) with acute desensitization during the application period (Fig. 21B),
- and

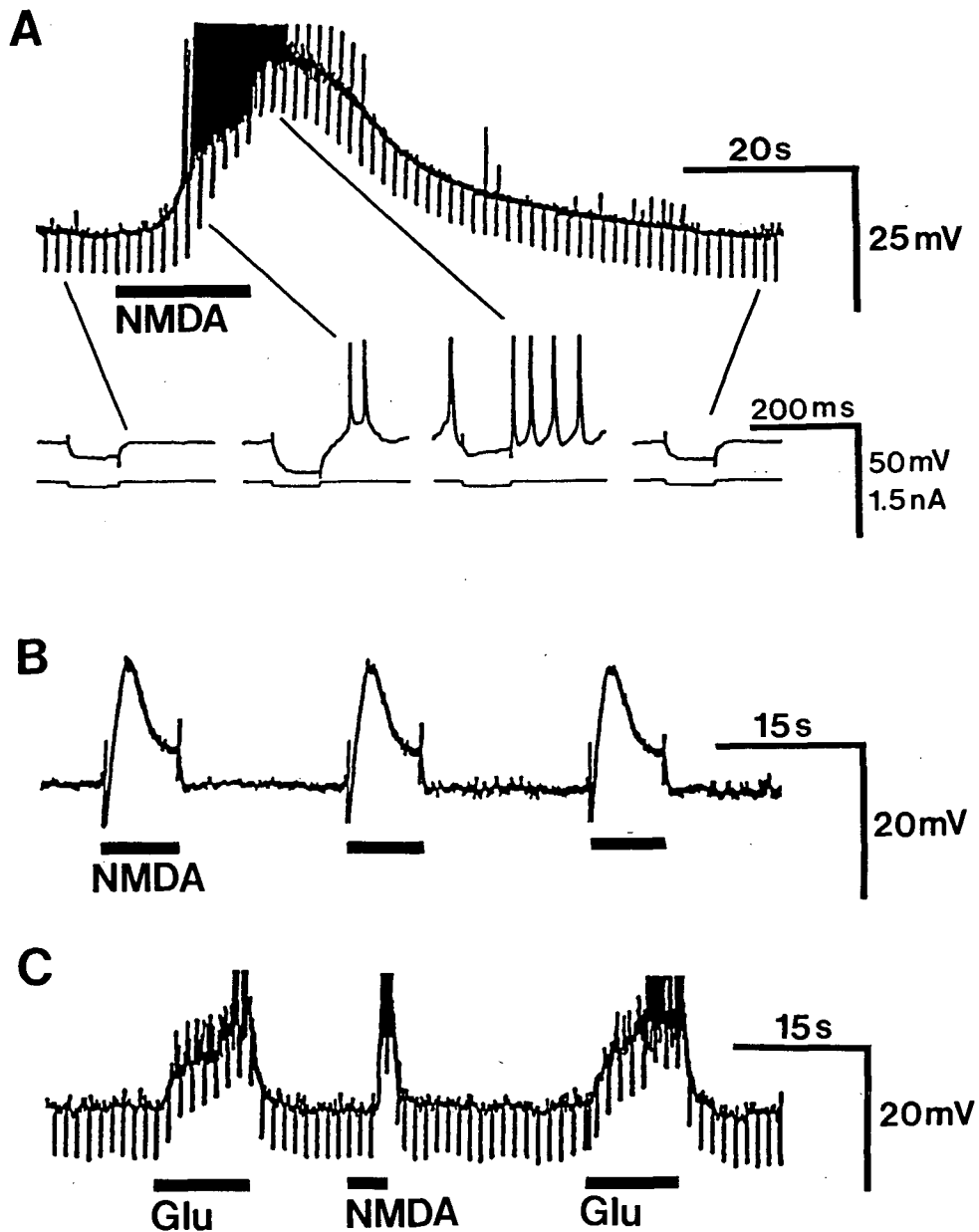


Fig. 19 NMDA-induced depolarizations in sensorimotor layer V neurons. (A) NMDA application evoked slow depolarizing response with initial increase in input resistance (lower traces). (B) acute desensitization exhibited by another neuron in response to NMDA ejection from a separate pipette. (C) a third type of NMDA action was a fast Glu-like response. This response desensitized markedly on repeated application.

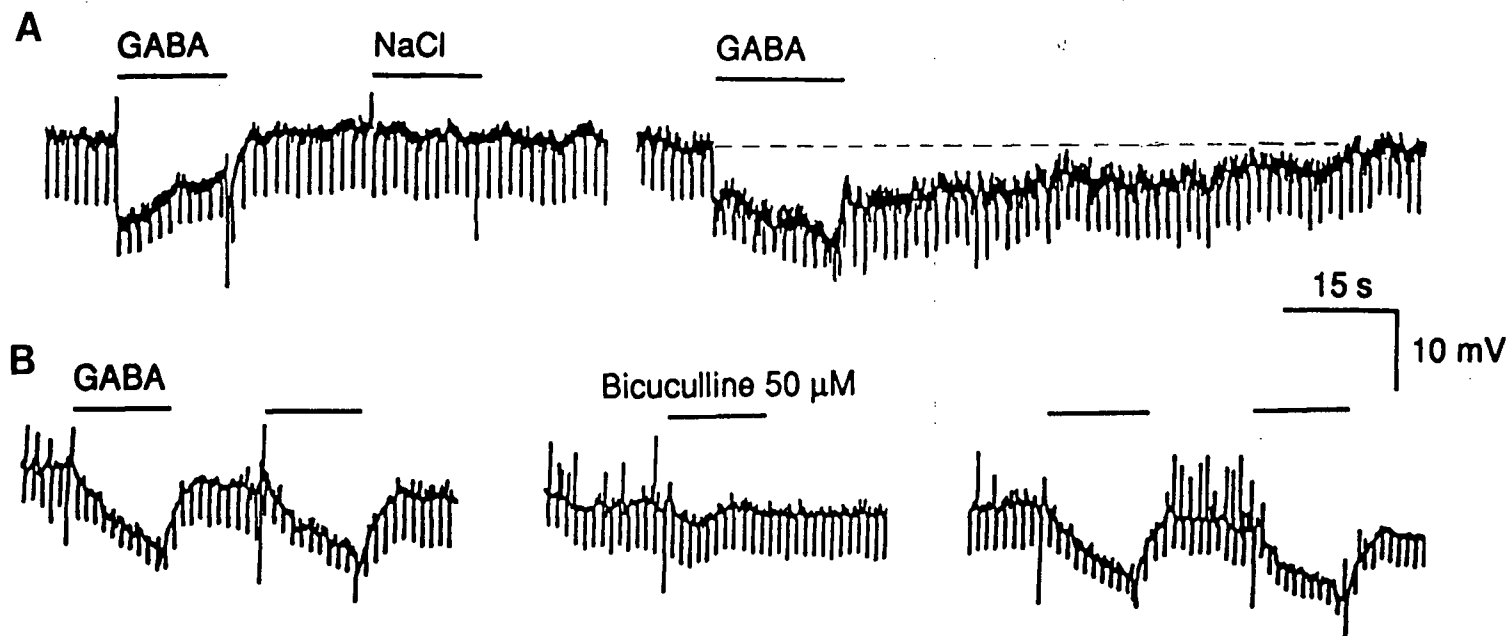


Fig. 20 A hyperpolarizing response to GABA applied from a compound electrode assembly. (A) acute desensitization of the GABA-evoked response is shown on the left and long-lasting hyperpolarization on the right. (B) The hyperpolarizing response in another neuron was blocked by bicuculline after 5 min and recovery was obtained after 20 min at a flowrate of 2 ml/min.

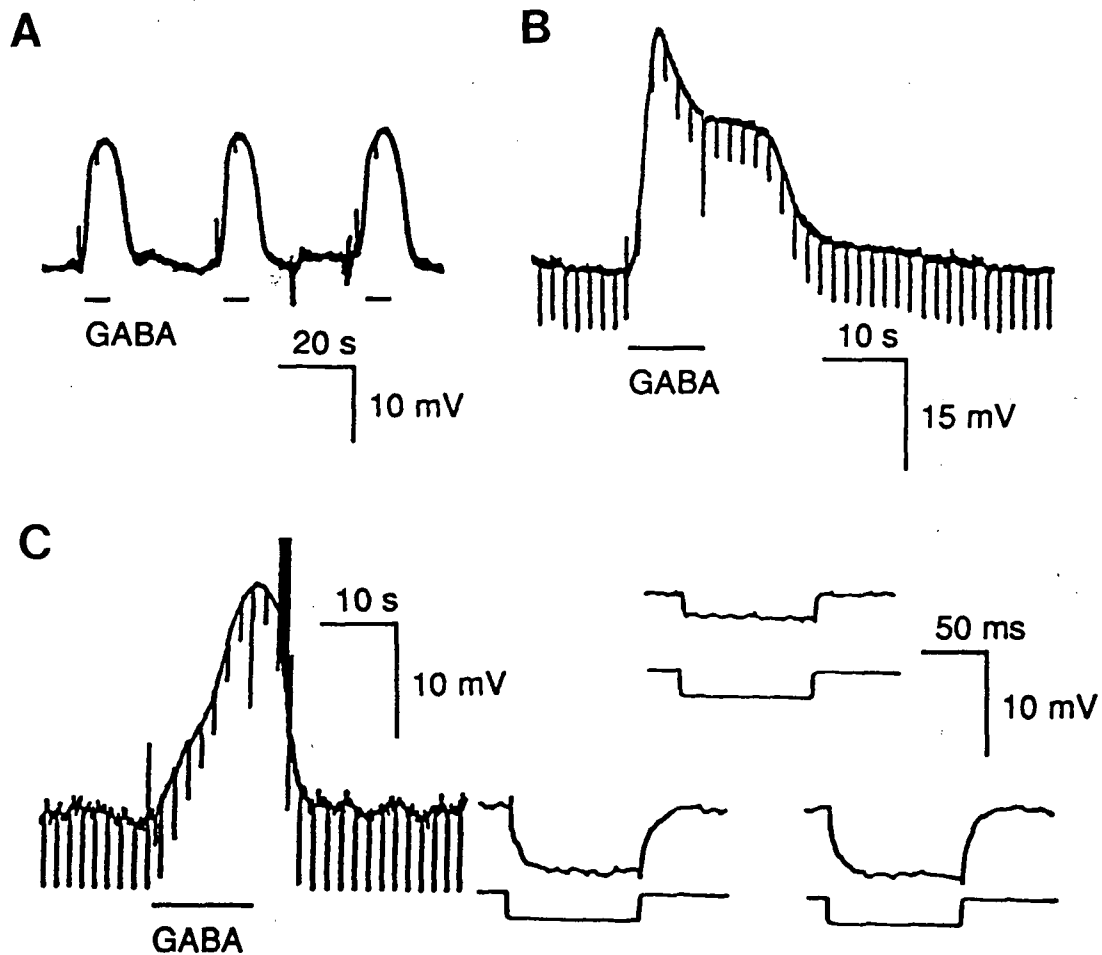


Fig. 21 Different types of depolarizing responses evoked by dendritic application of GABA. Non-desensitizing (A) and acutely desensitizing (B) responses are shown for two sensorimotor neurons. Conductance changes before, at the peak, and after a depolarizing effect of GABA are evident in (C). TTX (1.5 μ M) was applied in (A and B).

(3) with tachyphylaxis on frequent repeated applications (Fig. 22C). On several occasions the GABA depolarizations were associated with spikes with the rising and/or falling portions of each response (Figs. 21C and 22C).

In order to determine if the depolarizing response was indeed inhibitory, i.e., decreased membrane excitability, Glu was applied continuously to induce spike activity and this was followed by a concomitant pulse-like application of GABA (Fig. 22B). The depolarizing response induced by GABA suppressed the action potentials evoked by Glu application. In such cases, the inhibition of spike firing was not likely attributable to excessive depolarization because the combined depolarization was just suprathreshold.

GABA was applied to 4 neurons with resting potentials that were displaced by DC-current injection to levels between -100 and -30 mV in the presence of TTX to determine a reversal potential for the GABA depolarization (Fig. 22A). The depolarizations evoked by GABA increased in amplitude at more hyperpolarizing values of the resting membrane potential. However, at an imposed potential of ~ -30 mV the response was multiphasic (Fig. 22A).

After 4 min of bicuculline ($50 \mu\text{M}$) application (3 neurons) the hyperpolarizing response was blocked (flow rate 2 ml/min; Fig. 20). The GABA-evoked depolarization was blocked only by prolonged application of bicuculline in doses as high as $150 \mu\text{M}$ (5 neurons; Fig. 23A). A concomitant extracellular application of baclofen ($50 \mu\text{M}$) did not alter the response to GABA. Baclofen when applied iontophoretically from a separate pipette did not change the resting potential or input conductance (6 neurons). However, bath application of baclofen hyperpolarized 3 neurons by 2-3 mV with 10-15% increase in conductance and did not affect Glu depolarizations. Iontophoretically applied baclofen did not depress the number, amplitudes or shapes of Glu-evoked spikes (Fig. 23B).

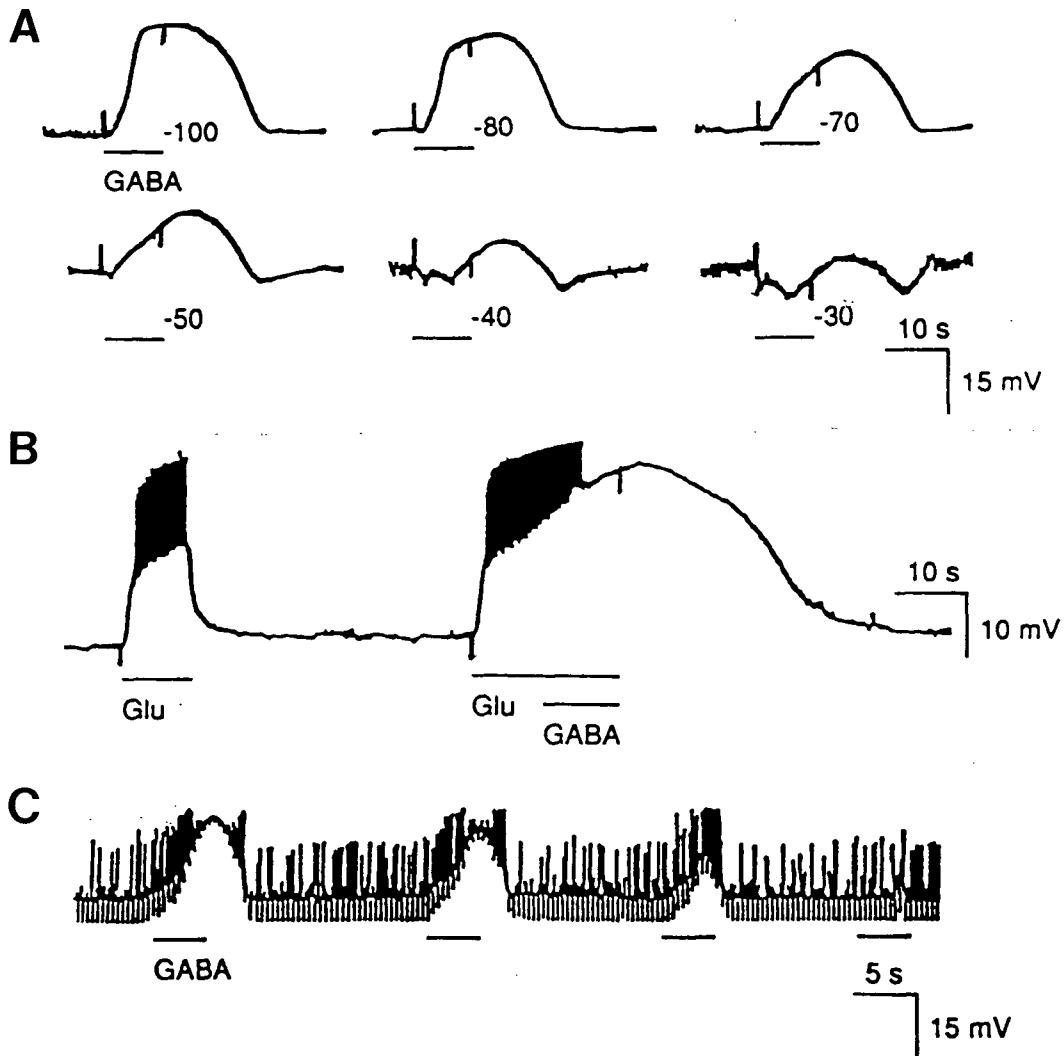


Fig. 22 Characteristics of the GABA-evoked depolarizations in neocortical neurons. (A) shifting the resting membrane potential by intracellular DC-injection revealed a biphasic response beginning at $V_m < -40$ mV. (B) concomitant application of GABA shunted the spikes evoked by Glu. (C) repeated application of GABA of < 10 s intervals desensitized the neuron completely to the subsequent GABA applications. The spikes on top of the GABA responses were probably due to excitatory actions of H^+ co-ejected with the agonist.

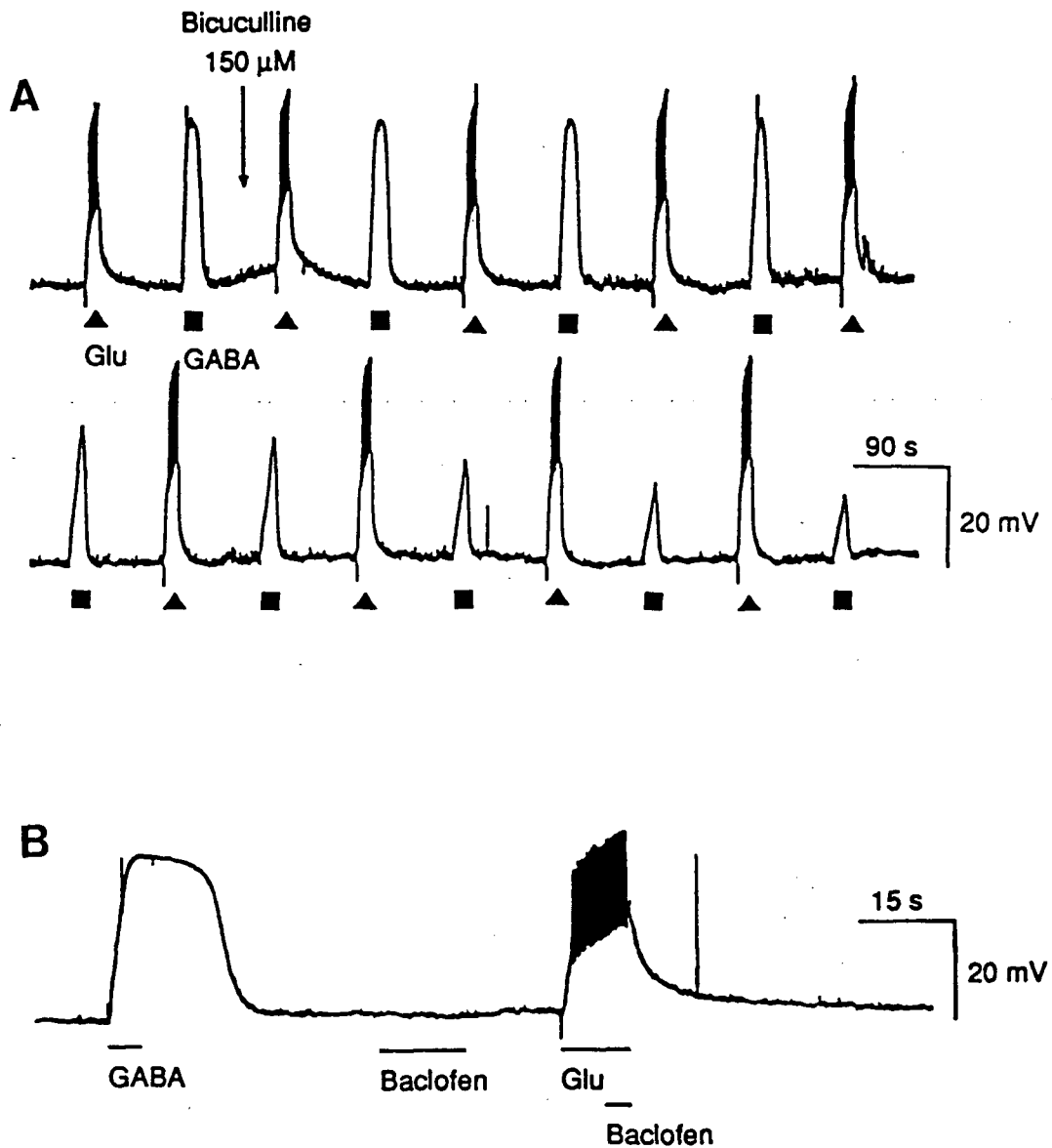


Fig. 23 Bicuculline, in high doses, blocked the GABA-induced depolarizations in neocortical neurons. (A) bicuculline (150 μ M) blocked the depolarizing responses to dendritic application of GABA whereas the Glu-responses were not affected. (B) baclofen, a GABA_B agonist, had no effect on V_m or on the Glu-evoked spikes in a sensorimotor neuron.

5.1.2 Anaesthetic-induced alterations in responses to excitatory and inhibitory transmitter substances. The observed changes in responses to the excitatory or inhibitory transmitter substances which are described below were not associated with marked changes in passive membrane properties. For example, only the highest doses of isoflurane and Althesin (2.5 MAC and 200 μ M respectively) hyperpolarized the neurons by 3-5 mV and decreased their input resistances by <20%.

5.1.2.1 Responses to ACh, Glu and NMDA. Isoflurane and Althesin applications (0.5-2.5 MAC; 25-200 μ M) depressed the ACh responses in a dose-dependent and reversible manner (Figs. 24 and 25). The EC_{50} s for the effects of isoflurane and Althesin were approximately 0.9 MAC and 75 μ M respectively. The suppression of the ACh responses was evident after 6-12 min of anaesthetic perfusion. Depending on the dose, full recovery was observed after 8-16 min (flow rate 1.5-3 ml/min). Usually, partial recovery and in some cases, full recovery was observed on high dose application of either agent (2-2.5 MAC isoflurane and 150-200 μ M Althesin; Fig. 25B).

Both anaesthetics depressed the Glu-induced depolarizations in a dose-dependent and reversible manner (Fig. 26 and 27A). The EC_{50} for the isoflurane-induced attenuation of the Glu-responses was ~1.9 MAC which is higher than that for the corresponding depression of ACh-induced depolarizations. In contrast, the Althesin concentration which suppressed the ACh response by 50% depressed the Glu-evoked depolarizations by about 40%. The Glu-induced responses were potentiated in 4 neurons (Fig. 28) and were not affected in 10 neurons, by either agent. Note that in Fig. 28, isoflurane application enhanced the Glu responses and almost totally blocked ACh actions.

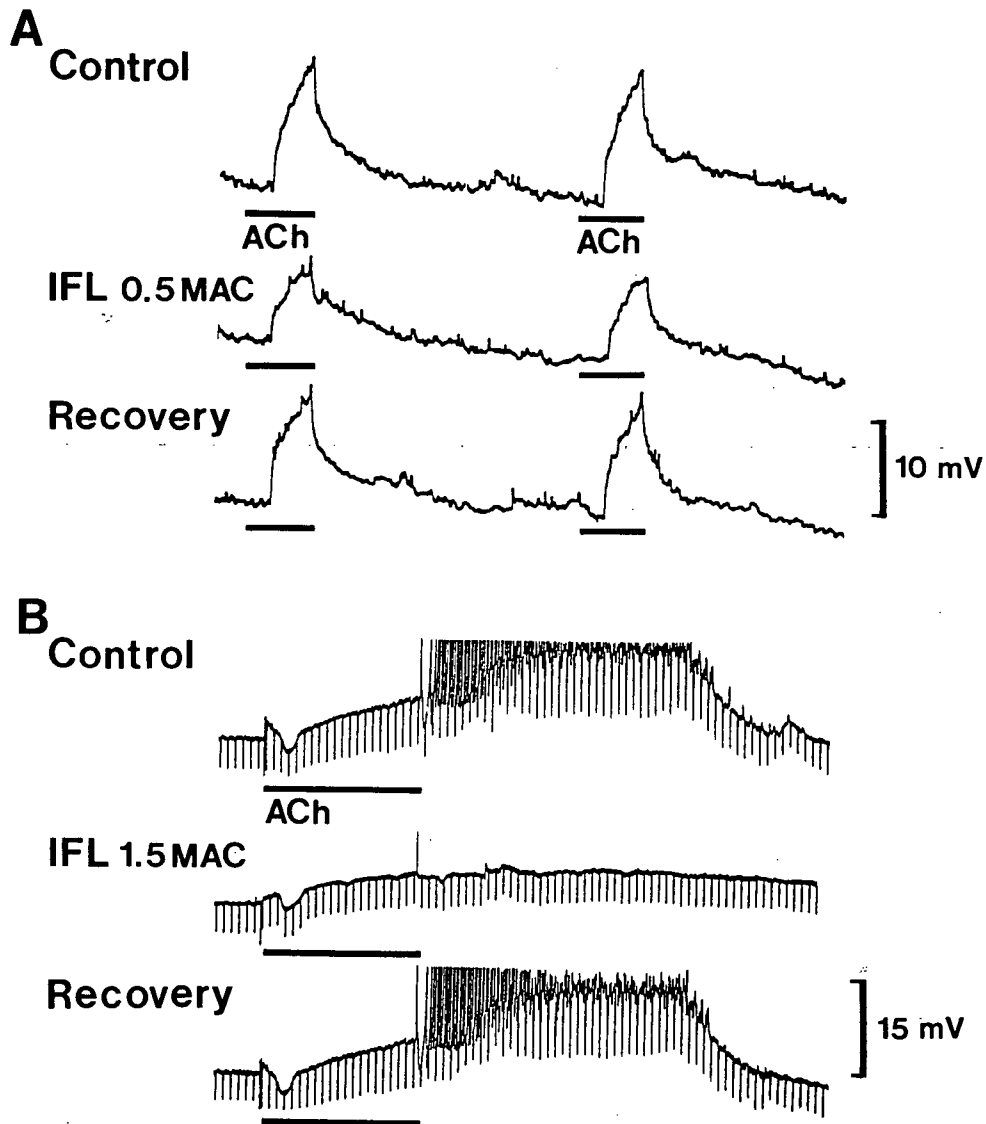


Fig. 24 Depression by isoflurane (IFL) of responses to acetylcholine (ACh; 150 nA for 18s in A, 100 nA for 20s in B) in 2 neurons (A, $V_m = -70$ mV; B, $V_m = -68$ mV). TTX ($1.5 \mu\text{M}$) was applied in A. Middle records (A,B) were obtained at 9 min of IFL application.

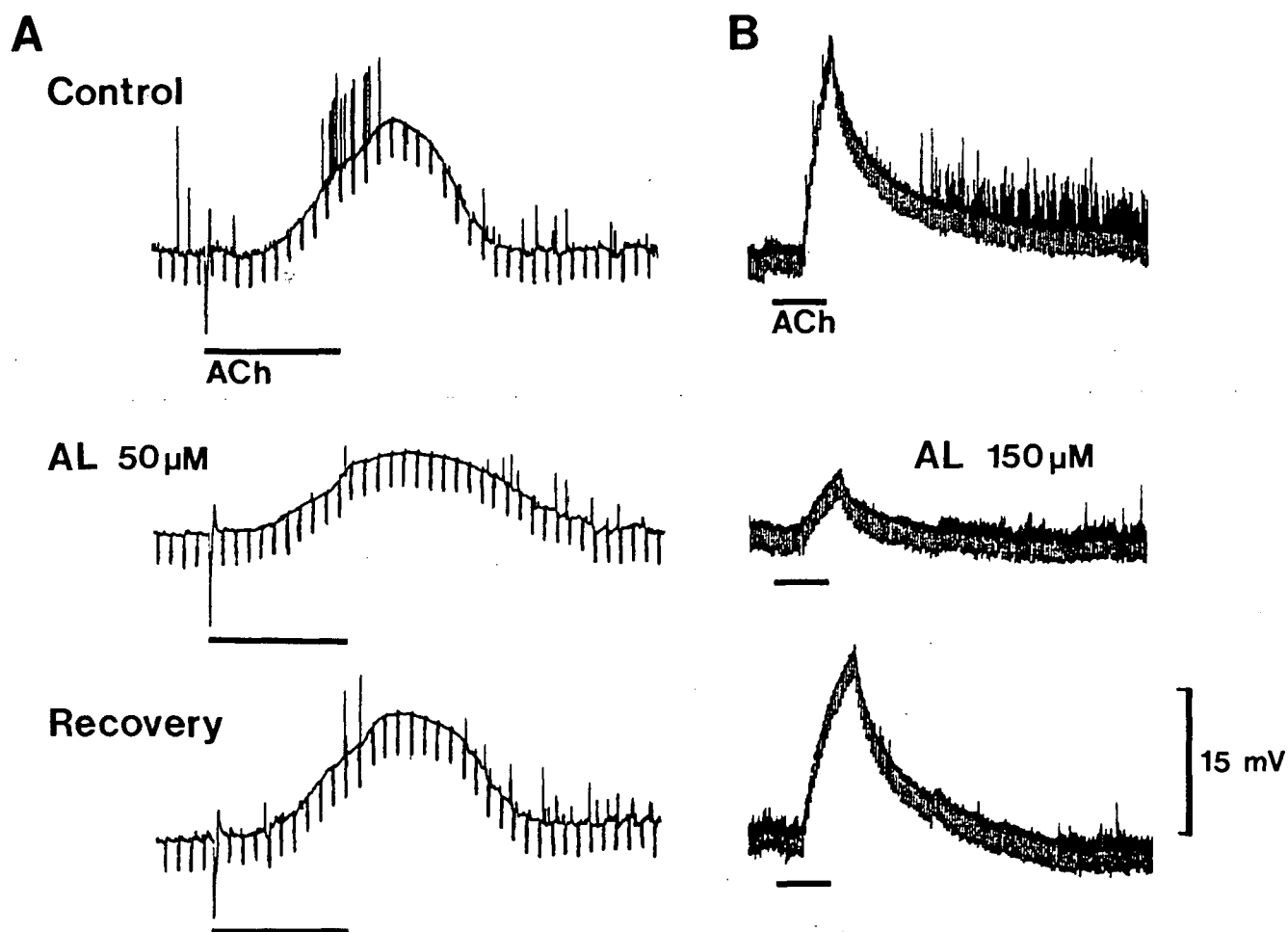


Fig. 25 Depression by Althesin (AL) of responses to ACh (125 nA for 9s in A, 95 nA for 25s in B) in 2 neurons (A, $V_m = -72$ mV; B, $V_m = -70$ mV). Negative voltage deflections (thick record in B) represent tests for changes in input resistance. TTX (1.5 μ M) was applied in B. Middle records were obtained at 8 min (A) and 6 min (B) of AL application.

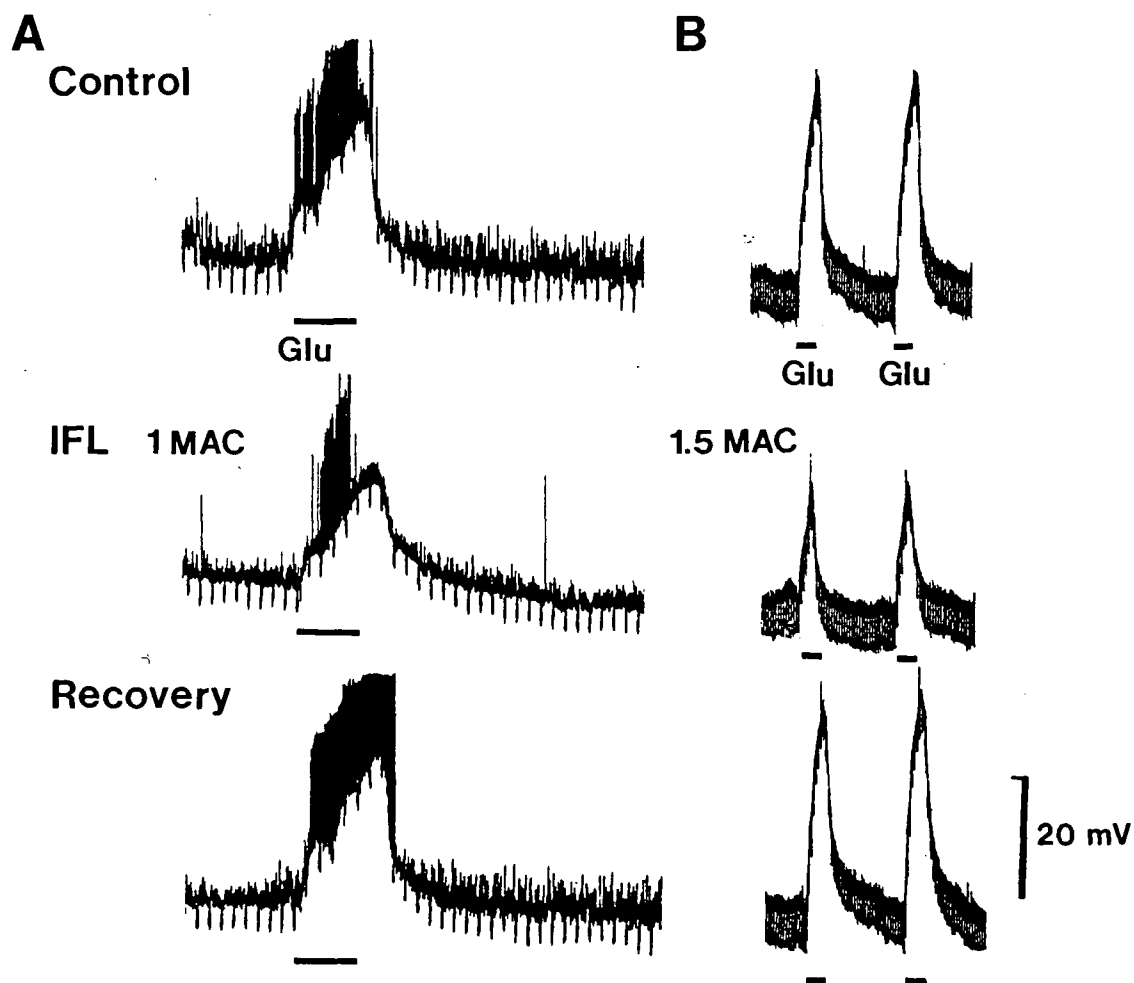


Fig. 26 Depression by isoflurane (IFL) of responses to glutamate (Glu; 100 nA for 5s in A, 95 nA for 8s in B) in 2 neurons ($V_m = -70$ mV in A and B). Negative voltage deflections (thick record in B) represent tests for changes in input resistance. TTX ($1 \mu\text{M}$) was applied in B. Middle records were obtained at 9 min (A) and 10 min (B) of IFL application.

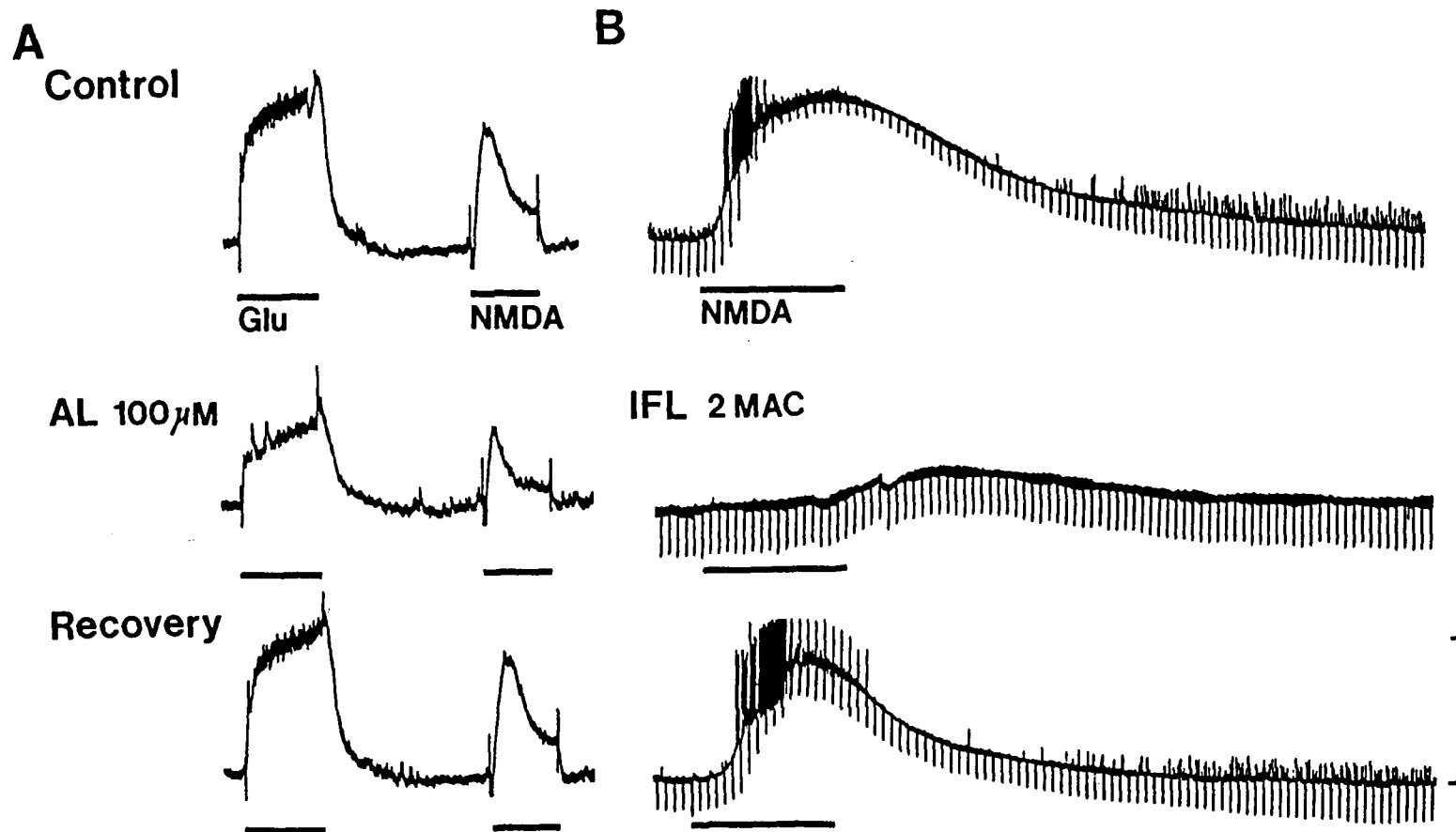


Fig. 27 Anaesthetic depression of responses to Glu (35 nA) and NMDA (55 nA for 5s in A, 110 nA for 15s in B) in 2 neurons ($V_m = -75$ mV in A, -69 mV in B). Resistance test pulses were applied only in B. TTX (1.5μ M) was applied in A. Middle traces were obtained at 10 min (A) and 9 min (B) of anaesthetic application. Voltage calibration: 20 mV in A, 30 mV in B.

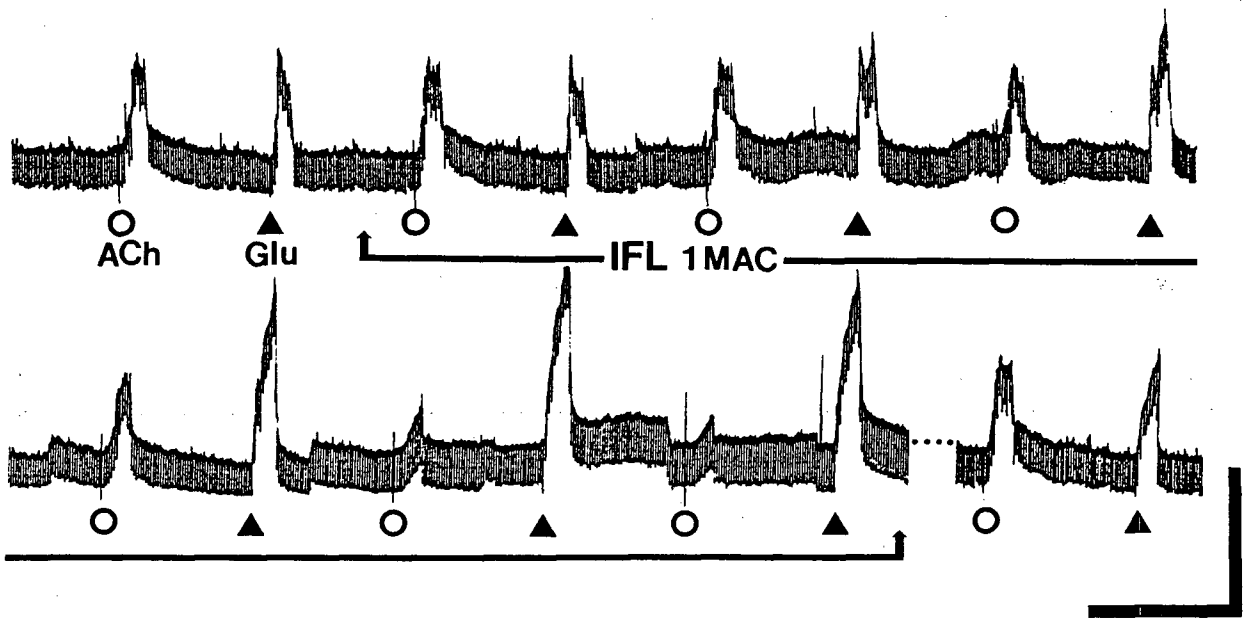


Fig. 28 Potentiation of Glu actions and attenuation of ACh responses by isoflurane (1MAC) in an anterior cingulate neuron. Recovery was evident after 16 min. V_m was kept constant at -72 mV by continuous DC-injection.

Because of the the poor sensitivities of the neurons (layers IV and V) to iontophoretically applied NMDA observed in the presence of external Mg^{+2} (2 mM), a full dose-response relationship could not be constructed in these experiments. Nonetheless, both anaesthetics attenuated the NMDA-evoked depolarizations in a dose-dependent manner (Fig. 27) and to approximately the same extent as the Glu-induced responses.

Application of halothane (1.5 MAC; 6 neurons) suppressed the Glu-, NMDA- and ACh-induced responses in a manner similar to that observed with isoflurane administration.

5.1.2.2 Responses to GABA. Isoflurane administration at 1.5 MAC (5 neurons; Fig. 29A) had no effect on the hyperpolarizing responses evoked by GABA applications. However, 2 MAC isoflurane suppressed the responses to GABA by ~15%. Administration of isoflurane or Althesin slightly reduced the depolarizing responses to GABA in the low dose range (0.5-1.5 MAC isoflurane; 25-100 μ M Althesin, Figs. 29B and 30). The maximum depression in these responses was not more that 40% even when doses as high as 2.5 MAC isoflurane and 200 μ M Althesin were applied (Fig. 31).

5.1.3 Selectivity in the depression of responses to transmitter substances

5.1.3.1 Anaesthetic interactions with transmitters. In most neurons anaesthetic application produced reductions in the responses to both ACh and Glu (Table 1). However, acetylcholine actions were more susceptible to depression by either isoflurane or Althesin. This selectivity was most apparent in neurons where initially, these transmitter responses were approximately equal in amplitude (Figs. 29B and 32). The selectivity was much more pronounced with isoflurane than after Althesin application (Fig. 32) and was not observed on perfusion of the vehicle, cremophor EL. In 7

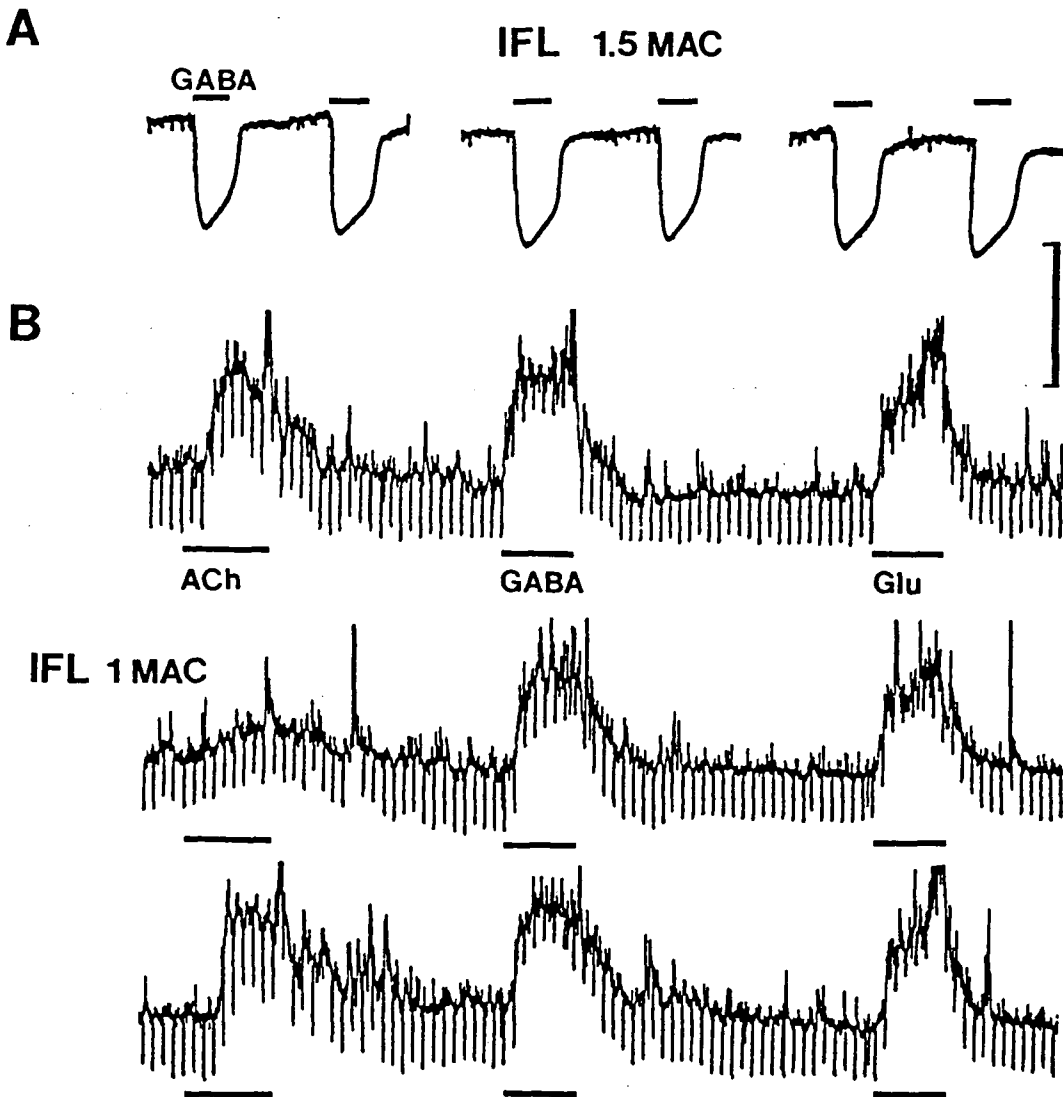


Fig. 29 Effects of isoflurane (IFL) application on equi-amplitude responses to GABA (100 nA for 5s in A, 75 nA for 6s in B), Glu, and ACh in 2 neurons ($V_m = -75$ mV in A, -72 mV in B). TTX ($1 \mu\text{M}$) was applied in A. Resistance test pulses were applied only in B. Glu (85 nA) and ACh (90 nA) were applied in B. Middle traces in A and B were obtained at 9 min of IFL application. Voltage calibration: 10 mV in A, 20 mV in B.

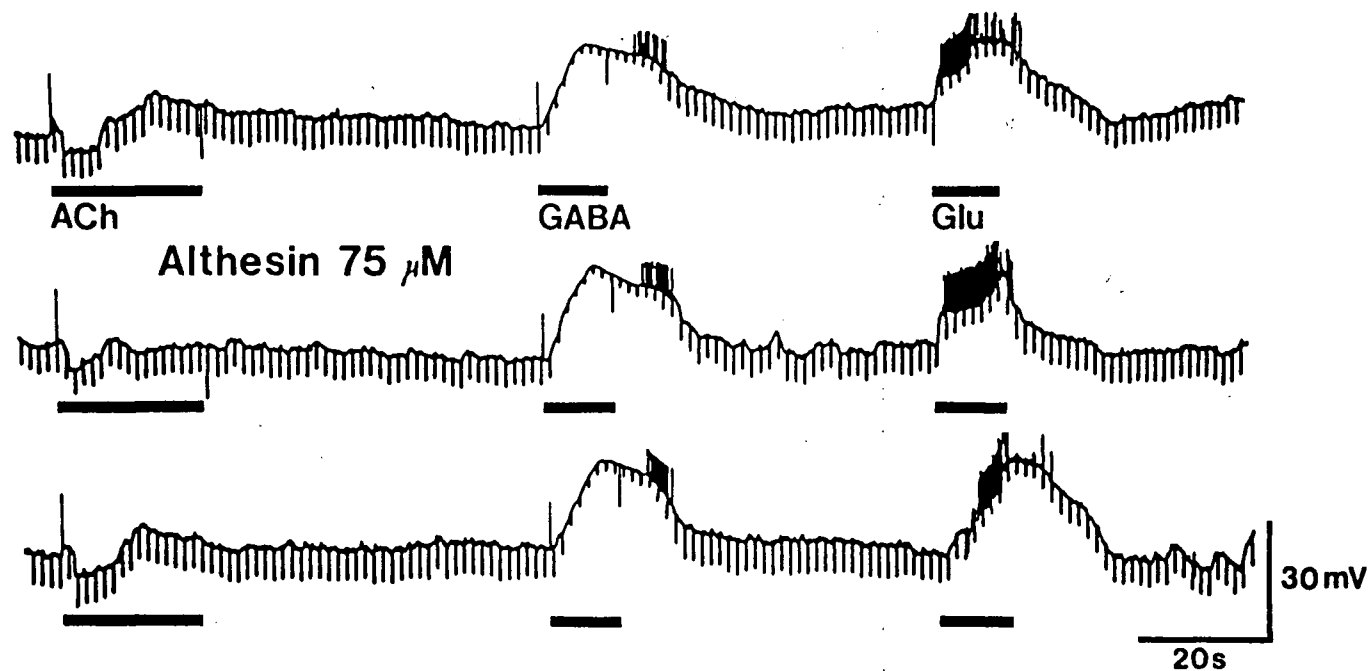


Fig. 30 Effects of Althesin application on the ACh, GABA and Glu-evoked responses (ACh 70 nA for 23s; Glu 90 nA for 10s; GABA 110 nA for 10s). Note that, initially, a prominent hyperpolarizing response to ACh was present. Anaesthetic application depressed the depolarization induced by ACh, slightly attenuated the Glu-response and had no effect on the response to GABA.

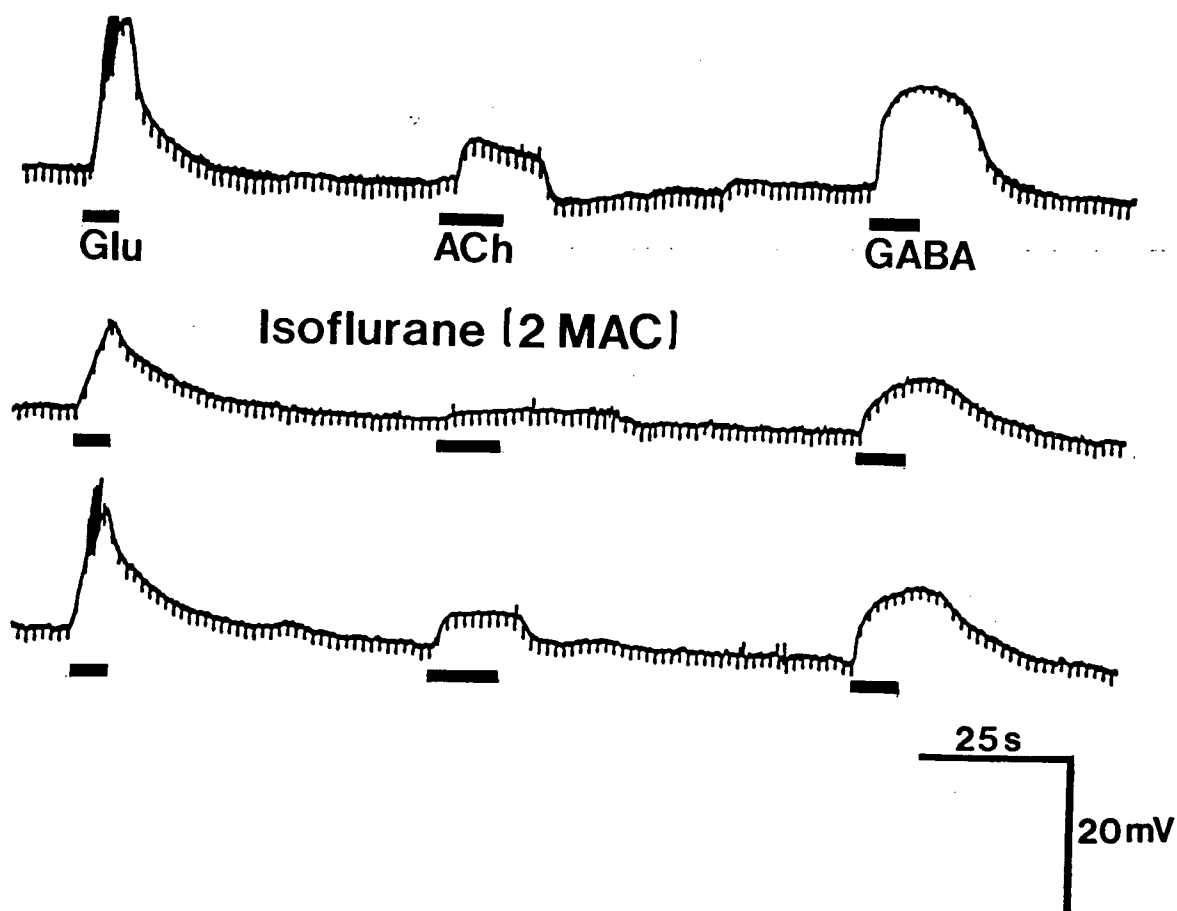


Fig. 31 Depression of ACh, Glu and GABA responses by high dose of isoflurane in neuron ($V_m = -68$ mV ; ACh 85 nA for 10s; Glu 100 nA for 5s; GABA 75 nA for 8s).

Table 1. Anaesthetic depression of neuronal responses to transmitter substances

<u>Anaesthetic applied</u>	<u>Depolarization evoked by</u>			<u>GABA evoked</u>	
	ACh (n = 36)	Glu (n = 65)	NMDA (n = 15)	Hyperpolarization (n = 9)	Depolarization (n = 29)
Isoflurane	18/18	30/40	8/10	4/9	10/17
Althesin	15/16	25/32	7/9		11/15
Halothane	3/4	5/7			2/4

Depression was defined as >15% attenuation of control responses by the anaesthetic. The total number of the neurons investigated was 65, i.e., all neurons were tested to Glu and to at least one other transmitter substance. The anaesthetic doses were 0.5-2.5 MAC for isoflurane, 25-200 μ M for Althesin and 0.5-2 MAC for halothane.

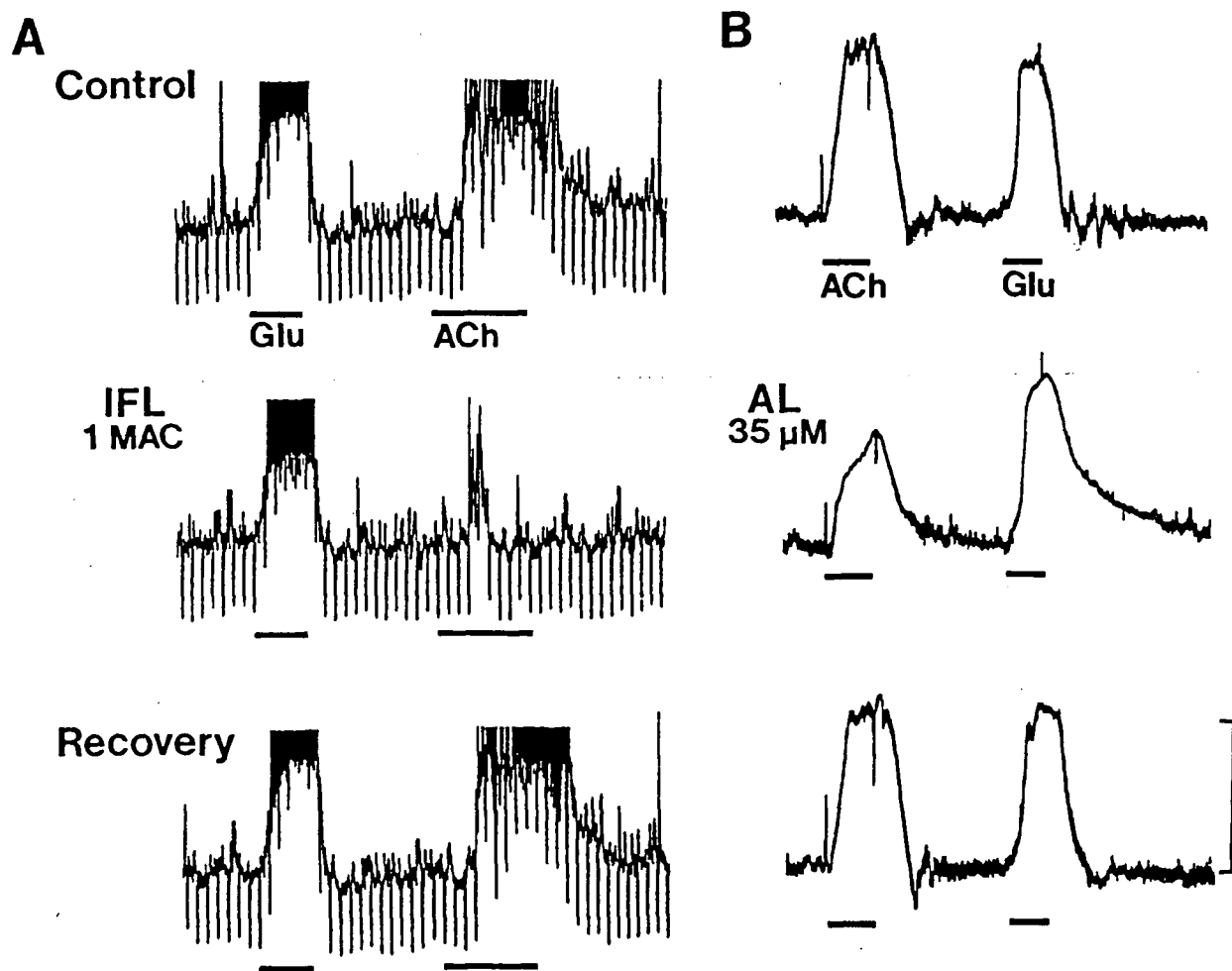


Fig. 32 Selective anaesthetic depression of ACh-evoked responses in 2 neurons ($V_m = -70$ mV in A and B). Equi-amplitude responses were evoked by $\sim \text{ED}_{50}$ s of ACh (85 nA for 10s in A, 110 nA for 7s in B) and Glu (70 nA in A, 100 nA in B). Resistance test pulses were applied only in A. Voltage calibration: 20 mV in A, 10 mV in B.

neurons, low dose administration of isoflurane or Althesin produced no depression of the Glu-depolarization and sometimes prolonged the duration of the Glu response (cf. Fig. 32B).

When GABA, Ach and/or Glu were applied alternatively to the same neuron, the additional application of isoflurane or Althesin did not significantly alter the depolarizing effects of GABA, slightly reduced those of Glu and markedly attenuated the responses to Ach (Figs. 29B-31).

5.1.3.2 Time course of anaesthetic actions. The depressions produced by isoflurane or Althesin had rapid onsets. The Ach-evoked response usually was suppressed after ~3 min, and was maximally reduced after ~8-12 min of anaesthetic application (Fig. 33). In 5 neurons, the reductions in the responses to Ach were evident at 30-90s, i.e., before detectable attenuation of the Glu responses. Administration of isoflurane, Althesin or suppressed the responses to NMDA (15 of the 19 neurons) with time courses that were similar to the anaesthetic reductions in Glu responses. Full recovery from anaesthetic effects on the Glu depolarization usually was observed several minutes earlier than a complete recovery of the Ach responses to control amplitude (Fig. 33).

5.1.3.3 Dose-response relationships. The dose-response relationships for the actions of isoflurane and Althesin on the just-maximal responses evoked by the transmitter substances are shown in Figs. 34 and 35. Each point on the curves represents an averaged depression produced by 4 applications of either anaesthetic. The EC_{50} for the isoflurane depression of the Ach responses was 0.9 MAC compared with 1.9 MAC for the suppression of Glu responses. Such differences in selectivity also were apparent in a comparison of Glu with the NMDA responses, although the data were more limited. The selectivity was less pronounced in the case

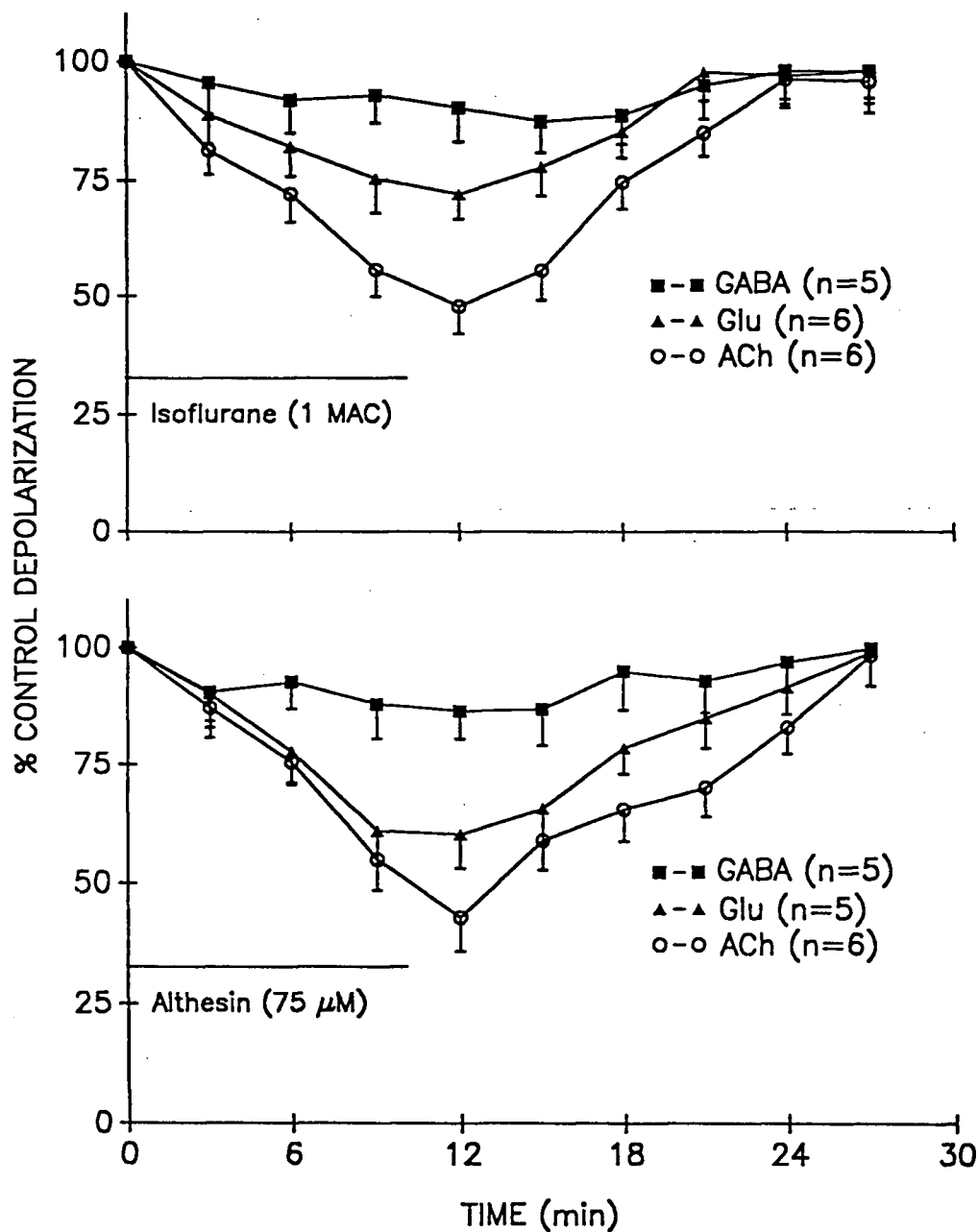


Fig. 33 Time courses for anaesthetic depression of just-maximal responses to GABA, Glu and ACh in the presence of TTX (1 μ M). Two or 3 transmitter substances were applied alternatively to each neuron. Error bars indicate \pm S.E. mean.

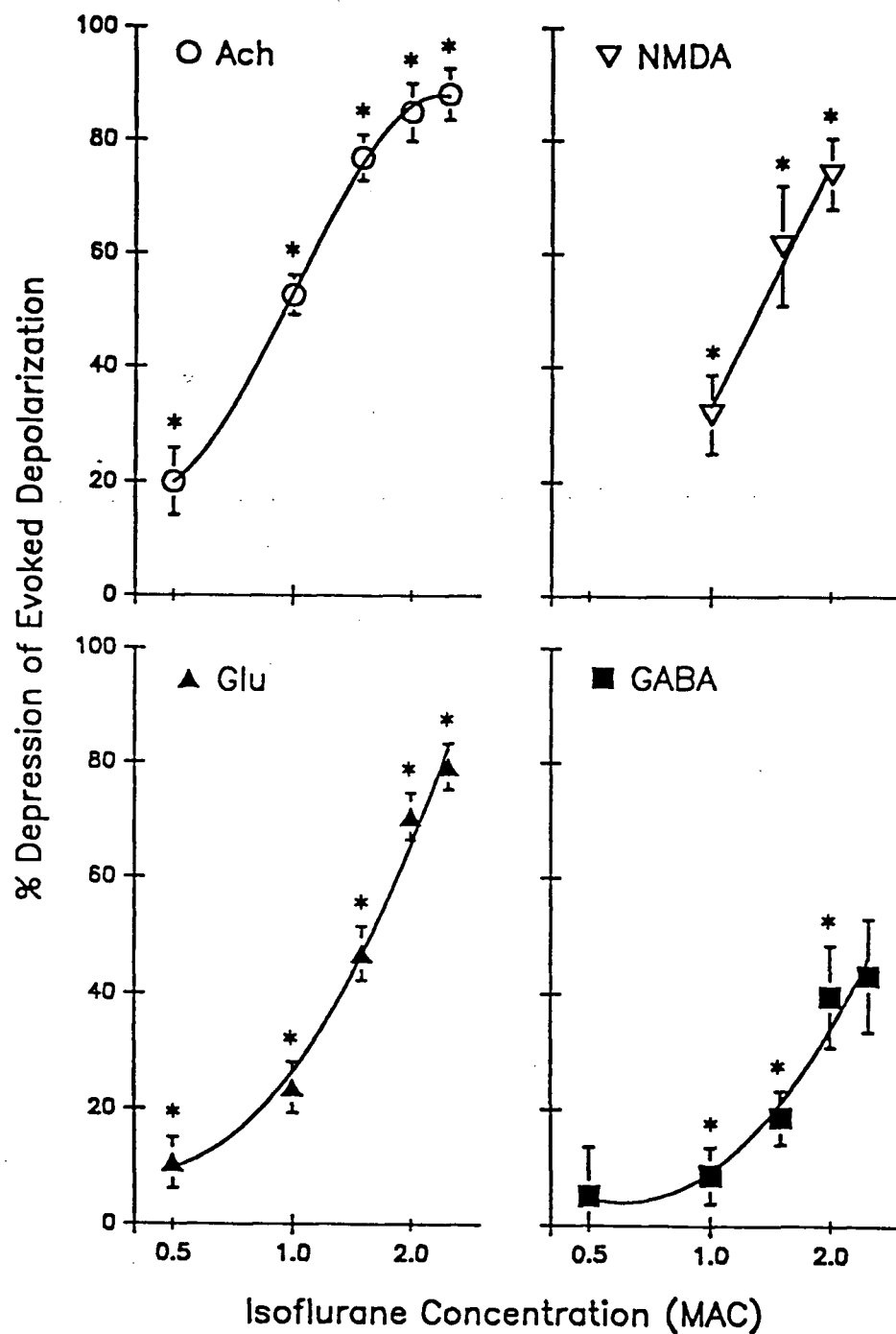


Fig. 34 Pooled data show dose-response relationships for isoflurane-induced depressions of depolarizations evoked by transmitter substances. Each point on the curve is the mean response to at least 4 applications ($n = 22$). Symbols (*) indicate significant difference from control at $p < 0.05$. Overall significance was determined by ANOVA. Error bars indicate \pm S.E. mean.

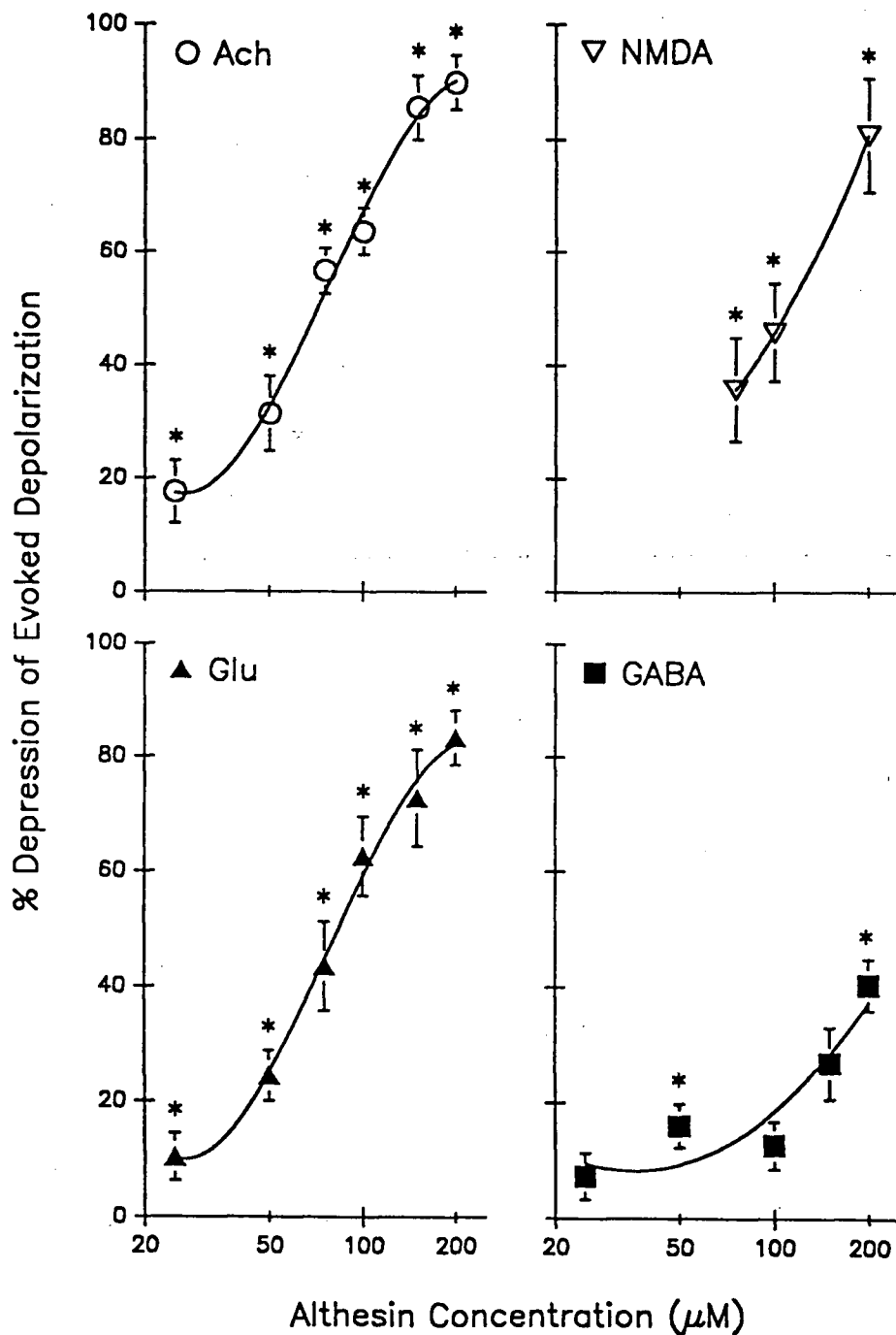


Fig. 35 Dose-response relationships for Althesin-induced depression of depolarizations evoked by transmitter substances. Each point on the curve is the mean response to at least 4 applications ($n = 20$). Symbols (*) indicate significant difference from control at $p < 0.05$. Overall significance was determined by ANOVA. Error bars indicate \pm S.E. mean.

of Althesin where the EC_{50} s were 75 μ M for the depression of the ACh responses and 90 μ M for the depression of Glu responses (Fig. 35). The maximal reductions in the depolarizing responses to GABA application were only about 40% at 2.5 MAC isoflurane or 200 μ M Althesin.

The effects of anaesthetics were further quantified in cases where the $\sim ED_{50}$ of a transmitter substance had been applied to the same neuron (Fig. 36A). the acetylcholine/glutamate response ratio (ACh/Glu) was depressed more by isoflurane than by Althesin application, although the ACh-evoked depolarizations were more susceptible to the depressant actions of both anaesthetics. This susceptibility also was revealed by investigations of anaesthetic effects on the just-maximal responses to ACh, Glu and GABA which were applied for comparison in the same neurons (Fig. 36B).

5.1.3.4 Anaesthetic effects on the potentiation of Glu-responses by ACh. This possibility was examined in 7 neurons by applying Glu ($\sim ED_{50}$) for 6s during longer applications of ACh ($\sim ED_{50}$) lasting 120-180 s, and then concomitantly applying the anaesthetic (isoflurane in 4, and Althesin in 3 neurons). As can be seen from Figs. 37 and 38, the application of ACh enhanced the effects of Glu i.e., the depolarizations were additive but there was a qualitative change in the discharge which appeared to be disproportionate to the magnitude of the combined depolarization. When 1.5 MAC isoflurane or 75 μ M Althesin was additionally administered, this enhancement was no longer evident. Note that such anaesthetic applications also reduced the Glu responses which recovered at about the same time as the ACh effects from the anaesthetic depression.

5.2 Discussion

5.2.1 Responses to transmitter and related agents. Although there were no fundamental differences between the responsiveness of neocortical neurons

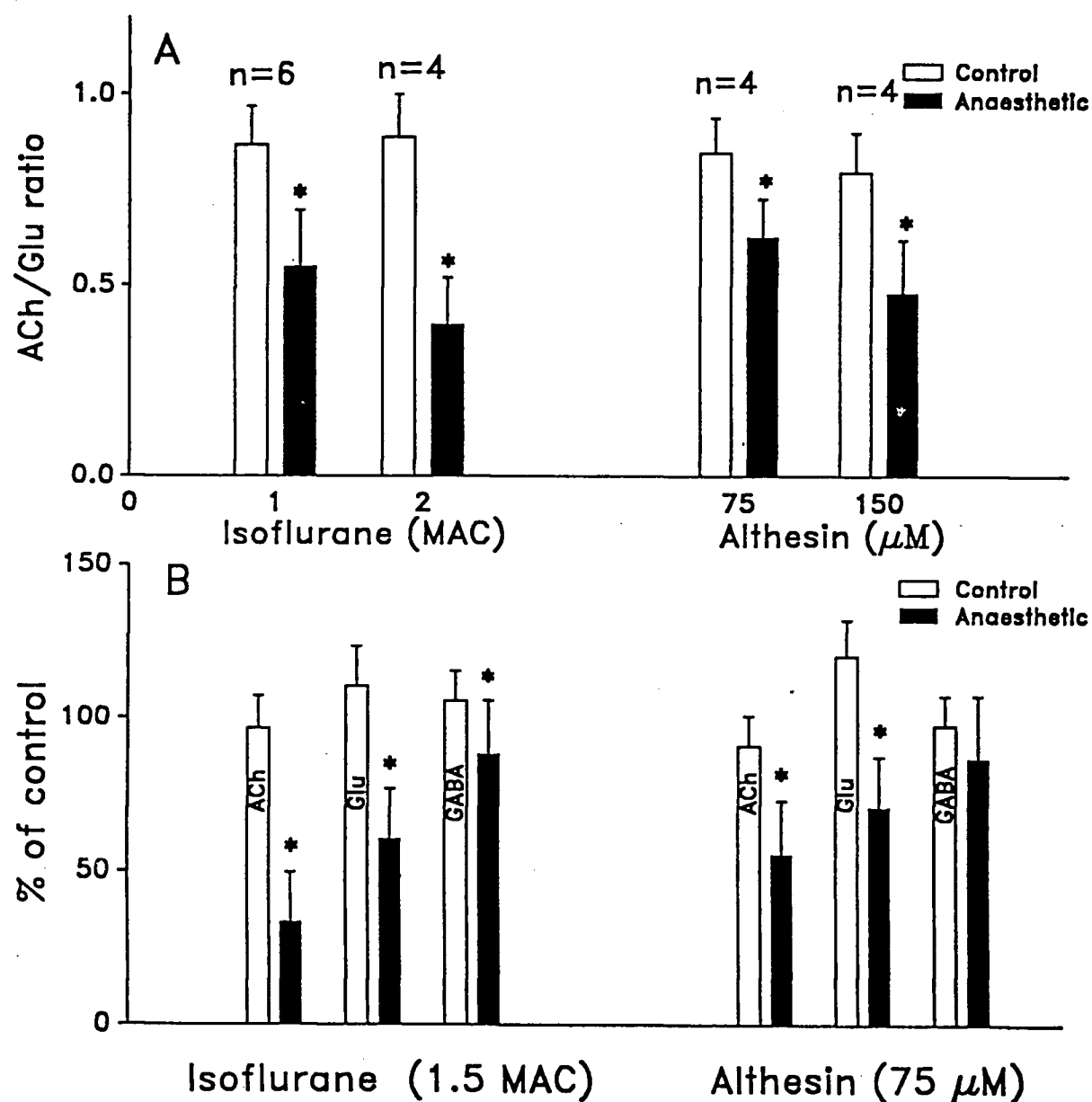


Fig. 36 Selectivity in isoflurane and Althesin-evoked depression of neuronal responsiveness to ACh, Glu and GABA. (A) ACh/Glu ratio of depolarizing responses was depressed by anaesthetic applications indicating greater susceptibility of the ACh responses. Error bars show \pm S.E. mean. Symbols (*) indicate significant difference from control at $p < 0.05$. (B) pooled data from 12 neurons in which at least 2 transmitter substances evoked just-maximal response. Error bars show \pm S.E. mean. Post-hoc comparison test was used. Symbols (*) indicate significant difference from control at $p < 0.05$ as determined by Student's t-test. Overall significance was determined by ANOVA.

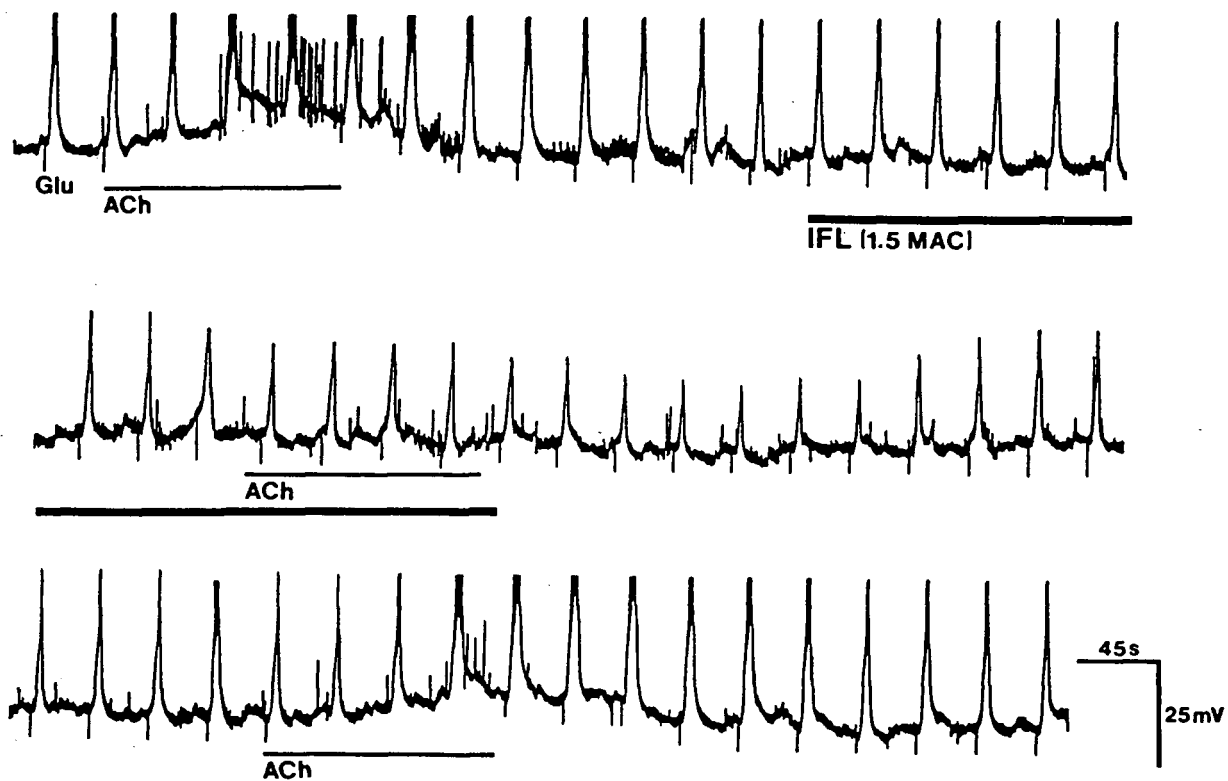


Fig. 37 Continuous record of isoflurane (IFL)-induced depression of the ACh-potentiation of Glu-actions. Depolarizations evoked by Glu (cf. negative artefacts at onset of 70 nA current) and ACh (50 nA) are ~ 50% of their maximal responses in this neuron. IFL was applied for 5 min (thick bar).

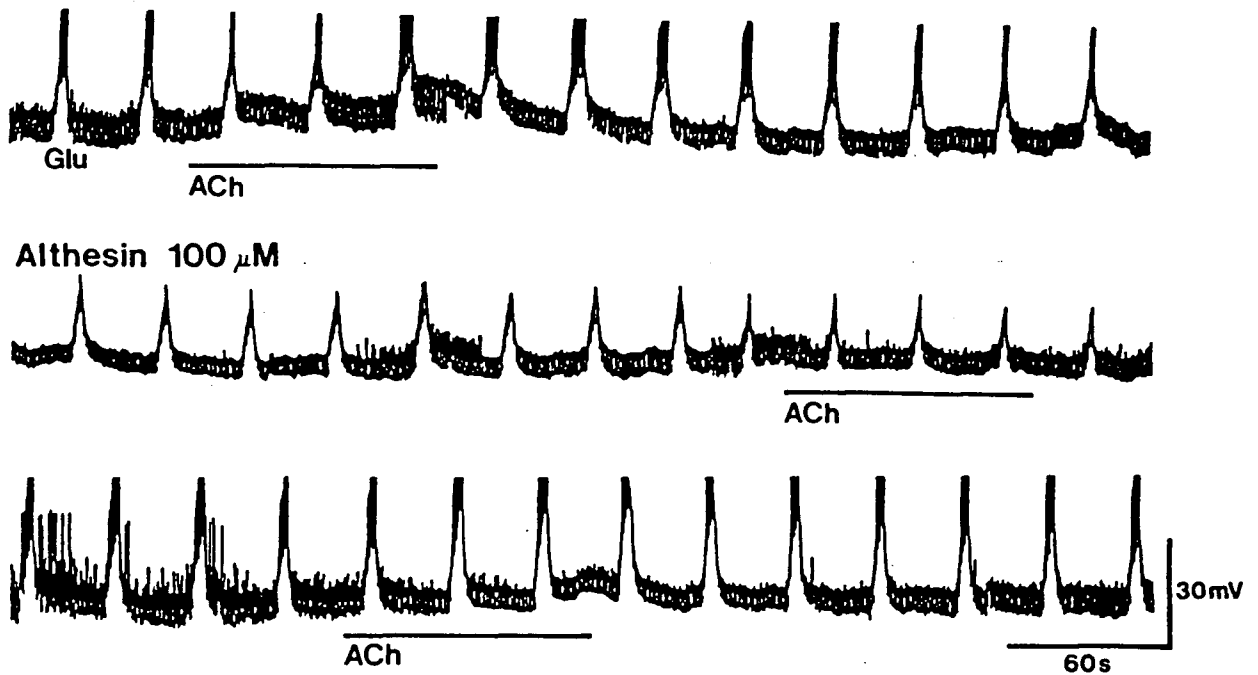


Fig. 38 Althesin-induced depression of the ACh-potentiation of Glu-actions. Depolarizations evoked by Glu (60 nA) and ACh (40 nA) were depressed by Althesin application at 8 min; recovery was evident after 15 min (lower trace).

in these investigations and the sensitivities of other neuronal cell types in other areas of the CNS (Krnjević 1974b) to the transmitter substances, some qualitative differences have been identified by these investigations.

5.2.1.1 Acetylcholine. The depolarization and increased input resistance induced by ACh were described previously for neocortical neurons in decerebrate (Krnjević et al. 1971b) and awake cats (Woody et al. 1978). In neocortical slice preparations, McCormick and Prince (1986) also observed that ACh evoked a rather small long-lasting depolarization with spike burst firing that was preceded by a brief hyperpolarization and an increased conductance. The initial hyperpolarization was attributed to activation of an interneuron by the ACh "puffed" from an independent micropipette because it could be blocked selectively by concomitant TTX application. In the present investigations, both types of responses were observed after ACh application from two types of electrode placements. The possibility of activating an interneuron is likely to be less with the compound electrode assemblies having intertip distances of $\sim 50 \mu\text{m}$ because the transmitter is ejected mostly near the soma of the neuron (Puil 1974). With such assemblies only slow depolarizations were observed, whereas ACh application from an independent iontophoretic pipette, evoked a short-lasting hyperpolarization which could be blocked with TTX and was always followed by a depolarizing response. The "afterburst" of synaptic activity recorded only occasionally in the presence of TTX (cf. Fig. 17C) could be due to presynaptic actions on nicotinic receptors of nerve terminals (Ropert and Krnjević 1982; Rovira et al. 1983) or spontaneous release of excitatory transmitters.

Not all the neurons (presumably pyramidal cells), were responsive to ACh applications ($\sim 70\%$ were responsive) despite their high sensitivities to Glu. Part of this ACh-insensitivity could be explained by the damage of the

neurons produced by electrode penetration by the recording electrode, particularly in view of the action of ACh to increase neuronal input resistance by decreasing membrane conductance for K^+ . Functional differences between neurons recorded in vitro and those recorded in the intact or decerebrate preparations where the pathways are undisturbed may provide other reasons for the insensitivity observed in 30% of the neurons. The relatively long-lasting effects of ACh applied on the neurons (cf. Results) may be attributable to a long-term modulation of muscarinic secondary messenger systems (cf. Woody and Gruen 1988; Muller et al. 1988; Dutar and Nicoll 1988).

5.2.1.2 Glutamate and N-methyl-D-aspartate. An outstanding feature in the Glu-induced responses was their consistency both qualitatively and quantitatively despite the length of the experimental period (e.g., 3-4 hours). Unlike Glu, NMDA-evoked depolarizations exhibited both acute and long-term forms of desensitization, probably due to the presence of external Mg^{+2} (Mayer and Westbrook 1987; MacDonald et al. 1987). The occasional difficulty in inducing equivalent responses to Glu and ACh (especially in the case of applications from the compound electrode assemblies) could be explained by the known topographical distribution of the glutamatergic and cholinergic (in this case, muscarinic) receptors. For example, the locations of high sensitivity or the "hot spots" for Glu may be on dendrites whereas those for ACh are located mainly on the soma.

5.2.1.3 γ -aminobutyrate. GABA applications to neocortical neurons produced hyperpolarizing and depolarizing responses. The hyperpolarizing responses could be evoked only by GABA application from the compound electrodes and were readily blocked by bicuculline (50 μ M); these could

result from interactions with somatic GABA_A receptors (cf. Bormann 1988). The depolarizing responses observed exclusively with the separate application and recording electrodes are probably attributable to stimulation of another subpopulation of receptors for GABA that are located on the dendrites of the pyramidal neurons (cf. Alger and Nicoll 1982). Depolarizing responses to GABA have been reported in the spinal cord (Barker and Ransom 1978b; Curtis and Gynther 1987) and after dendritic application in the hippocampus (Andersen et al. 1980; Thalmann et al. 1981; Alger and Nicoll 1982; Avoli and Perreault 1987; Blaxter and Carlen 1988). In cortical neurons this type of response can be blocked by bicuculline and is mediated mostly by an increase in Cl⁻ conductance (Krnjević and Schwartz 1967; Alger and Nicoll 1979, 1982; Andersen et al. 1980; Thalmann et al. 1981; Misgeld et al. 1986b). In the present investigations, the GABA depolarizations were only blocked by large doses (>100 μM) of bicuculline and then only after prolonged applications (>15 min; 2ml/min flow rate). Moreover, a reversal potential of these responses could not be attained when the resting potential was shifted by DC-current injection in the range of -100 to -30 mV in the presence of TTX (cf. Fig. 21C). Despite the uncertainties as to the ionic mechanisms and the subtype of GABA-receptor mediating this response, the response evoked by GABA was capable of "shunting" spikes elicited by application of Glu to the same neuron. In several neurons spike activities were observed on the rising and falling phases of the depolarizing response to GABA; similar actions of GABA may underlie a paradoxical excitatory phenomena described earlier by Puil et al. (1974) in the isolated neocortical slab investigations or reported following stimulation of cortical neurons by release of H⁺ concomitantly with the GABA ejections (cf. Krnjević and Schwartz 1967).

5.2.2 Anaesthetic-induced alterations in the chemosensitivity of extracellularly applied transmitter agents. Although Krnjević and Phillis (1963a) found many years ago, that cerebral cortical neurons showed a lower sensitivity to ACh in barbiturate-anaesthetized animals in comparison to the cerveau isolé preparations, only a few investigations reportedly have explored the anaesthetic-induced alterations in the responsiveness of neocortical neurons to various transmitters and related substances. Subsequent reports by Krnjević and his group reported that a wide variety of intravenous and volatile anaesthetics selectively depressed the excitatory actions of ACh while the Glu- and the GABA-responses were preserved in decerebrate animals (Krnjević and Phillis 1963b, c; Krnjević et al. 1966; Catchlove et al. 1972; Krnjević and Puil 1975; Puil and Krnjević 1978). However, this preferential attenuation of ACh actions on neocortical cells has not been confirmed by other investigators (Crawford and Curtis 1966; Crawford 1970; cf. Bazil and Minnemann 1989). Moreover, halothane or thiopental application have been shown to depress, uniformly, the excitatory responses elicited by applications of ACh or monoamines (5-hydroxytryptamine, noradrenaline and isoprenaline) in neocortical neurons of decerebrate cats (Johnson et al. 1969). A major disadvantage in the use of extracellular recording techniques for unit discharge activity in the abovementioned reports is the inherent problem with the interactions of effects from nearby neurons excited by the transmitter applications. This may provide a reason for the dissimilarities with the results of the present studies. Another reason may be related to the varying degrees that the intact ascending inhibitory and excitatory pathways radiate to the cerebral cortex as well as to the variability in the effects of different anaesthetics on the cerebral blood flow of the intact animals.

Unfortunately, only incomplete information is available about the effects of anaesthetics on the chemical responsiveness of other neurons at different levels in the central neuraxis of mammals. In the cerebellar cortex of in vivo cat preparations, barbiturates selectively depress ACh-evoked excitation without attenuating Glu actions on spontaneously discharging neuronal units recorded extracellularly (McCance and Phillis 1964). Despite the suppression of the effects of ACh, noradrenaline and serotonin on unit responses of the olfactory bulb in in vivo experiments, inhalational agents depress Glu-firing and potentiate ACh-excitation recorded extracellularly in the olfactory cortex in vitro (Richards and Smaje 1976; Smaje 1976). Responses to Glu, Quis, ka and NMDA in hippocampal CA1 neurons in vitro are suppressed to varying degrees by barbiturates (Sawada and Yamamoto 1985). However, Glu- and GABA-elicited depolarizations are not affected by halothane or isoflurane application in low doses. Higher doses of these agents depressed only the Glu-responses and did not have any effect on the responses to GABA (Brooks et al. 1986). Barbiturates and various ethers attenuate ACh- and not Glu-induced discharges in the thalamus (Phillis and Tebecis 1967) and basal ganglia (Bloom et al. 1965; Spencer and Havlicek 1974). Brainstem neurons and spinal motoneurons exhibit a selective decrease in their chemosensitivities to ACh applied intermittently with monoamines and Glu (Bradley and Dray 1973). Interpeduncular neuronal responses to ACh are less affected than substance P-evoked excitations during anaesthetic application of intravenous or volatile agents (Sastry 1978). The ACh-facilitation of unit activity of medullary neurons (Salmoiraghi and Steiner 1963) and Renshaw cells (Biscoe and Krnjević 1963) is not attenuated by anaesthetics administered in in vivo cat preparations. Although the

available evidence may be interpreted only with some uncertainties, the possibility that anaesthetics have selective effects on the sensitivities to suspected transmitters on subcortical neurons that have some relevance to the production of the anaesthetic state should be subjected to extensive re-investigation.

In the present experiments, the use of the in vitro slice preparation and TTX-blockade of Na-spike genesis for the investigations of anaesthetic modifications in the chemical responsiveness of the neocortical neurons reduced possible inhibitory or excitatory influences external to the recorded neuron. Differences in neuronal metabolism in vitro and in vivo also should be considered in comparing these results with the information obtained from intact animals. An interesting observation in the present studies is the greater attenuation by isoflurane or Althesin application, of the ACh-induced responses compared to the Glu- or the NMDA-evoked depolarizations. Under conditions of synaptic blockade by TTX and in the absence of significant changes induced by the anaesthetic in the passive membrane properties, the suppression of the chemosensitivity of the neurons to the excitatory transmitter agents could be due to anaesthetic effects on the ionic channel-receptor complex or on the modulation of the secondary messenger system responsible for the agonist/receptor binding impulse transduction.

Recent patch- and voltage-clamp studies on different systems (Lechleiter and Gruener 1984; Lechleiter et al. 1986; Arimura and Ikemoto 1986; Flanigan and Brett 1987; Ikemoto et al. 1988) suggest that the cholinergic depression produced by anaesthetics may result from a decrease in the maximum number of channels activatable by ACh. This may be due to a stabilization of the closed state of the channel or to an allosteric

modification of the channel proteins leading to a shortening of the rate constants governing transitions between the open state and one or more normally occult closed states, with a subsequent excessive flickering of the ionophore. Binding studies have shown that a wide range of general anaesthetics bind with the ACh-receptor allosterically and inhibit the specific binding or stabilize the high-affinity state of the receptor; the outcome of these actions probably corresponds to the non-desensitized state in the in vivo situation (Young and Sigman 1981). In addition, halothane has been found to convert G protein-coupled and -uncoupled muscarinic receptor to states of lower agonist affinity and to lower the affinity of receptor-G protein complexes for guanine nucleotides (Aronstam et al. 1986; Dennison et al. 1987; Anthony et al. 1989).

On the other hand, the depression of the Glu-induced depolarizations by isoflurane and Althesin could be attributed in part to an increased affinity of Glu to its receptors and partly to a decrease in the maximal number of activatable receptor-channel complexes which would result in the formation of either a blocked-closed or desensitized state (cf. Ikemoto et al. 1988). However, in the absence of supportive biochemical and patch-clamp data such suggestions remain highly speculative. More recently, Puil and Baimbridge (1989) observed that isoflurane attenuated the Glu-evoked increase in $[Ca^{2+}]_i$ in cultured hippocampal neurons. They concluded that the actions of isoflurane and halothane most likely involve a blockade of Ca-channels activated by interactions of Glu with either Quis or NMDA types of receptors and a direct blockade of voltage-gated Ca-channels.

A suppression of the GABA-evoked depolarizations has been reported for pentobarbital in doses higher than 300 μ M, anaesthetic doses of alphaxalone, and high doses of enflurane (Connors 1980; Simmonds 1981; Cullen and

Martin 1982; Parker et al. 1986; Brooks et al. 1986). The reasons for the effects observed previously and in the present studies are not clear. Possibilities include the plugging of the chloride channels by high concentrations of the anaesthetic, similar to that observed at ACh-activated endplate channels (Adams 1976) or the occupation of the active sites on the GABA receptor by the anaesthetic molecules.

Qualitatively, the present investigations are concordant with the results of extracellular studies performed many years ago on mammalian neocortical neurons in vivo (Krnjević and Phillis 1963a,b,c; Catchlove et al., 1972). The observed dose-response relationships allude to the possibility that the anaesthetic depressions of excitatory and inhibitory transmitter actions in the intact animal are a continuum of interference beginning with ACh-suppression and ending with complete attenuation of the Glu (and NMDA-) responses. This is in contrast to the generalized concept of depression of the chemosensitivities of CNS neurons during anaesthesia, and suggests much more selective anaesthetic actions.

The results of these experiments are not in agreement with the observations in olfactory cortical neurons where inhalational anaesthetics did not depress unit discharge evoked by ACh application (Smaje 1976). In addition to the inherent differences in the membrane excitabilities of neocortical and olfactory neurons, the low doses of anaesthetics applied to the olfactory cortical slices may explain the discrepant results. In the olfactory investigations, halothane, diethyl ether and trichloroethylene had been administered in doses of <0.5 MAC values for rodents. Increasing the concentrations of these agents in the atmosphere of the slice suppressed the firing rate of the neurons (cf. Fig. 4 in Smaje 1976).

In conclusion, the chemosensitivities of neocortical neurons in the in vitro preparation are reduced by applications of isoflurane or Althesin in a selective and gradual manner; these effects begin with ACh and end with complete attenuation of the ACh- and Glu-responses approximately at the anaesthetic ED_{50} and ED_{95} s respectively. This selective attenuation of the ACh-induced responses at low doses, with preservation of the Glu-depolarizations, may reflect different affinities for the anaesthetic molecules to their sites (membrane receptors) of action. The mechanism of such an interference may be related to the channel-receptor complex protein and/or to the secondary messenger systems responsible for the agonist-receptor binding impulse transduction.

6 EFFECTS OF HYPOMAGNESIA ON TRANSMITTER AND ANAESTHETIC ACTIONS

6.1 Results

The effects of a reduction in the $[Mg^{2+}]_o$ were investigated in 28 sensorimotor neurons on their responses to extracellular applications of ACh, Glu and GABA before, during, and after an application of isoflurane or Althesin. These neurons had a mean V_m and R_i resistance of -76.3 (± 5.97 ; $\pm S.D.$) mV and 44.96 (± 16.46) $M\Omega$, respectively. Intracellular current injections evoked spikes with amplitudes of more than 80 mV and durations of less than 2 ms. The current-voltage curves in 85% of the neurons revealed inward rectification. Applications of ACh, Glu and GABA (50-300 nA for 4-20 s) produced mean depolarizations of 13.12 (± 3.6), 22.5 (± 8.7) and 20.5 (± 6.8) mV respectively. The input resistances of these neurons were either increased ($\sim 15\%$) or unaffected near the peak

depolarizing response induced by ACh. Input resistances were always decreased (35-90%) by Glu or GABA applications despite the presence ($n = 18$) or absence ($n = 10$) of TTX ($1-1.5 \mu\text{M}$).

6.1.1 Effects of bath application of Mg-free ACSF. The calculated $[\text{Mg}^{2+}]$ in the nominally Mg-free media was $\sim 1-3 \mu\text{M}$ because of trace impurities in the salts used (Mayer and Westbrook 1985; Sutor et al. 1987). Perfusion of the slices with this "Mg-free" solution, in the presence or absence of TTX, induced slight hyperpolarization (3-5 mV) in 8 neurons. However, the R_i of the neurons were either unchanged or decreased by $\sim 10\%$ (5/8 and 3/8 neurons respectively). In the absence of the Na^+ -channel blocker the spontaneous and the background synaptic activities were greatly enhanced. After $\sim 1-3$ hrs incubation in nominally Mg-free medium and TTX perfusion abrupt periods of spontaneous slow depolarizing waves were recorded in 4 neurons (cf. Fig. 39A).

Applications of ACh (5/6 neurons) and GABA (10/10 neurons) induced depolarizations that were reduced in Mg-free solutions with or without TTX present (Fig. 39B). On the other hand, Glu responses were inconsistently affected during perfusion with solutions containing zero mM Mg. The depolarizations evoked by Glu were either depressed (Figs. 39B and 40) unchanged or, on other occasions, potentiated. Responses to NMDA that were comparable to the Glu responses could not be obtained during perfusion with control solution (10/10 neurons). During continuous perfusion of Mg-free medium, a brief application of NMDA evoked a depolarization of ~ 20 mV in 6/10 neurons (Fig. 40). These results are summarized in Table 2.

6.1.2 Effects of gradual removal of $[\text{Mg}^{2+}]_o$. The effects of different levels of hypomagnesemia, were studied in slices that were gradually depleted from their initial extracellular Mg^{2+} content by sequential

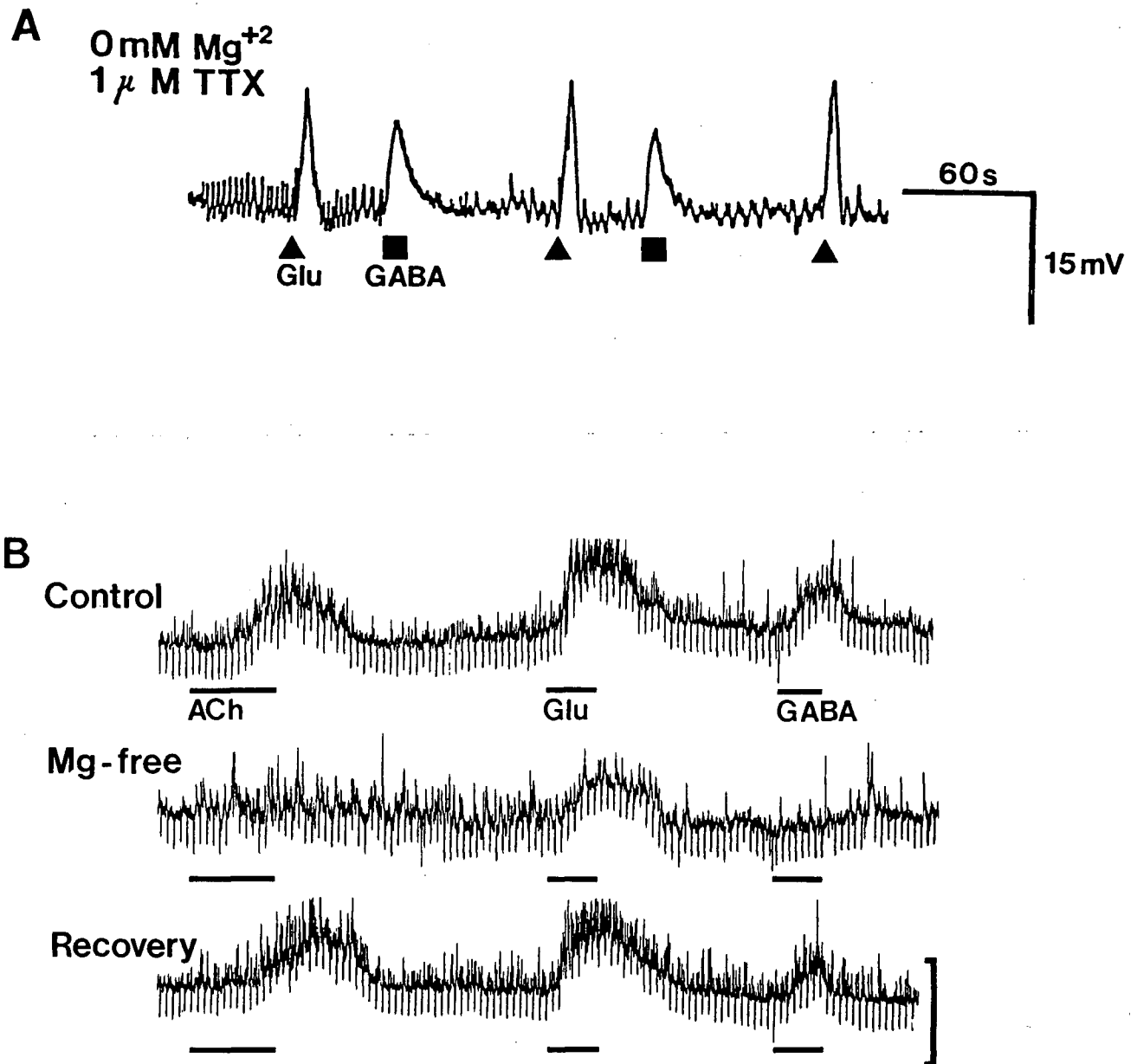


Fig. 39 Blockade of ACh and GABA actions in Mg-free perfusate. (A) spontaneous depolarizing waves evoked in a sensorimotor neuron ($V_m = -70$ mV) from a neocortical slice incubated in Mg^{2+} -free media. (B) ACh and GABA responses were attenuated in neuron ($V_m = -75$ mV) by removal of Mg^{2+} from the media. Vertical bar indicates 15 mV in B.

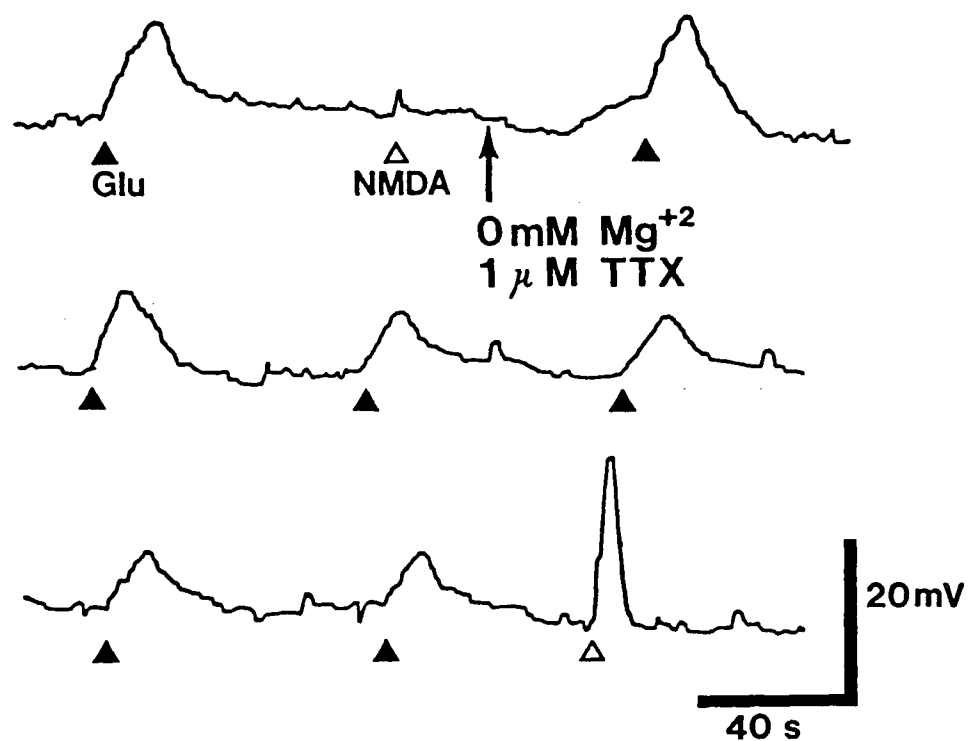


Fig. 40 Potentiation of NMDA responses in the Mg-free bathing solution containing TTX. NMDA-responses were greatly potentiated, while the Glu-evoked depolarizations were depressed.

Table 2 Effects of Mg-free media on sensorimotor neurons*

ACh responses	Depression of**		Potentiation of*** NMDA responses	Glutamate Responses		
	GABA responses			Depres- sion	No change	Poten- tiation
7/7	13/13		10/10	3/9	4/9	2/9

*Hyperpolarization was observed in 8/16 of the neurons. A decrease in input resistance ($\pm 10\%$) was detected in 5/16 neurons.

**Depression was defined as $>20\%$ attenuation of control amplitudes of ACh- or GABA-induced depolarization.

***Potentiation was defined as $>20\%$ increase of control amplitudes of glutamate or NMDA.

perfusion of ACSF solutions containing low $[Mg^{2+}]$. The effects of removal of the Mg-cation from the extracellular environment on the Glu induced depolarizations are shown in Fig. 41. A gradual depression of the Glu responses were not observed in other neurons (7/10) that showed no change or a slight potentiation of the Glu actions.

However, the effects of GABA were consistently reduced by the gradual depletion of Mg^{2+} in a dose-dependent manner in 11/11 neurons. Recovery was slow but usually was observed within the experimental period. A single application of low Mg^{2+} solution (0.5 mM) for 15-20 min produced an attenuation of the GABA responses that was completely reversible (Fig. 42A). On the other hand, increasing the $[Mg^{2+}]$ to 3.5 mM in the perfusing media had no effect on the GABA-evoked depolarizations (Fig. 42B).

6.1.3 Effects of increasing $[Mg^{2+}]$. Neurons ($n = 8$) in slices that were incubated in Mg-free ACSF solution for 3-7 hours showed extensive spontaneous and synaptic activities. During perfusion with increasing $[Mg^{2+}]_o$, the neurons exhibited a corresponding progressive suppression of the exaggerated spontaneous firing and synaptic background which had been present just after impalement. A slight depolarization (± 4 mV) which was not associated with a change in the input resistance was observed in 6/8 neurons. Under these conditions the neurons did not respond to ACh applications. However, sequential perfusion with 0.5, 1 and 2 mM Mg^{2+} concentrations produced a steady increase in the ACh-evoked depolarizations (5/8 neurons; Fig. 43). Applications of Glu evoked depolarizing responses in all neurons (8/8). However, the Glu responses were unaffected (4/8) or enhanced (4/8) by the systematic increase in $[Mg^{2+}]_o$ (cf. Fig. 43). The GABA-induced depolarizations were gradually potentiated in a dose-dependent manner by the increments in $[Mg^{2+}]_o$ (Fig. 44).

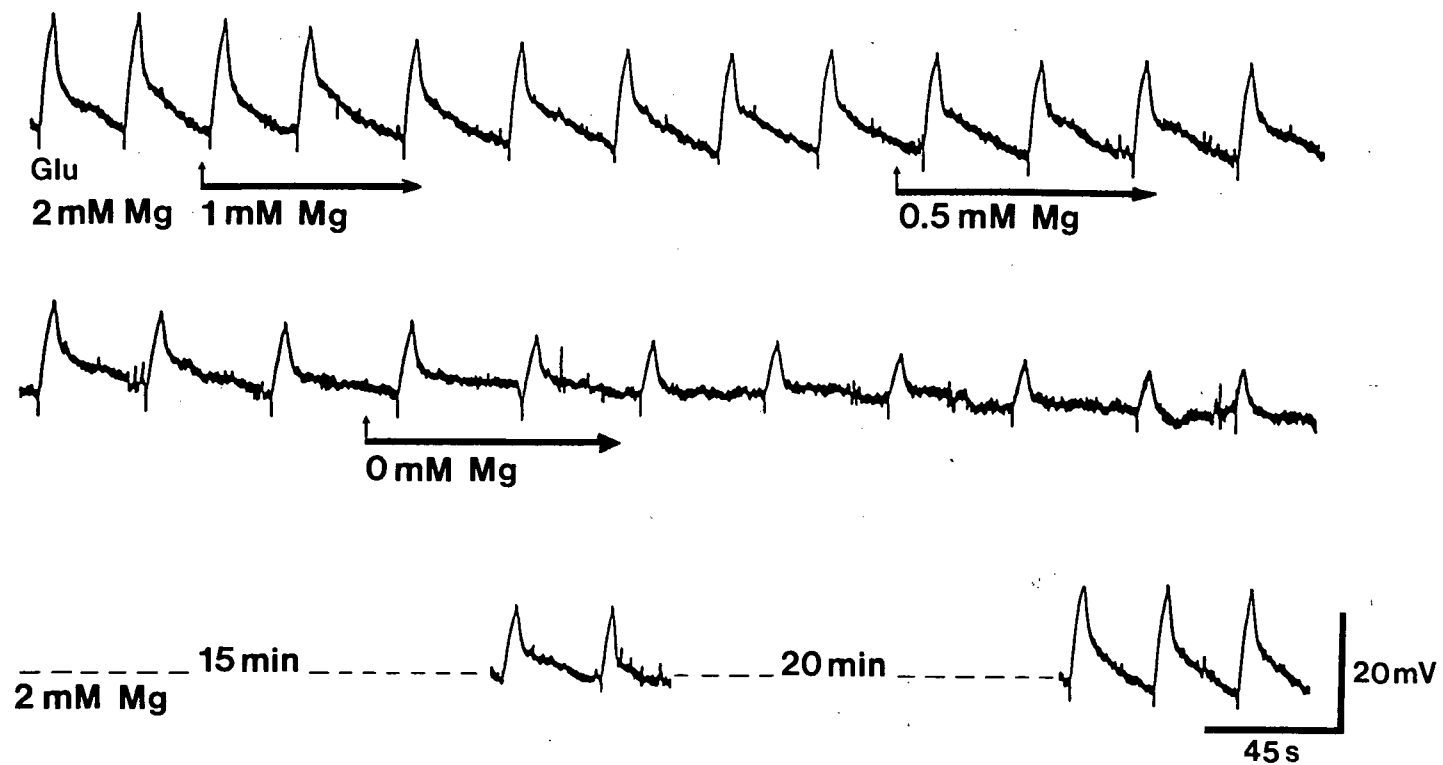


Fig 41 Depression of Glu-responses by gradual removal of $[Mg^{2+}]_o$ in a neuron ($V_m = -65$ mV). Complete recovery was obtained after 20 min in control solutions (2 mM Mg^{2+}).

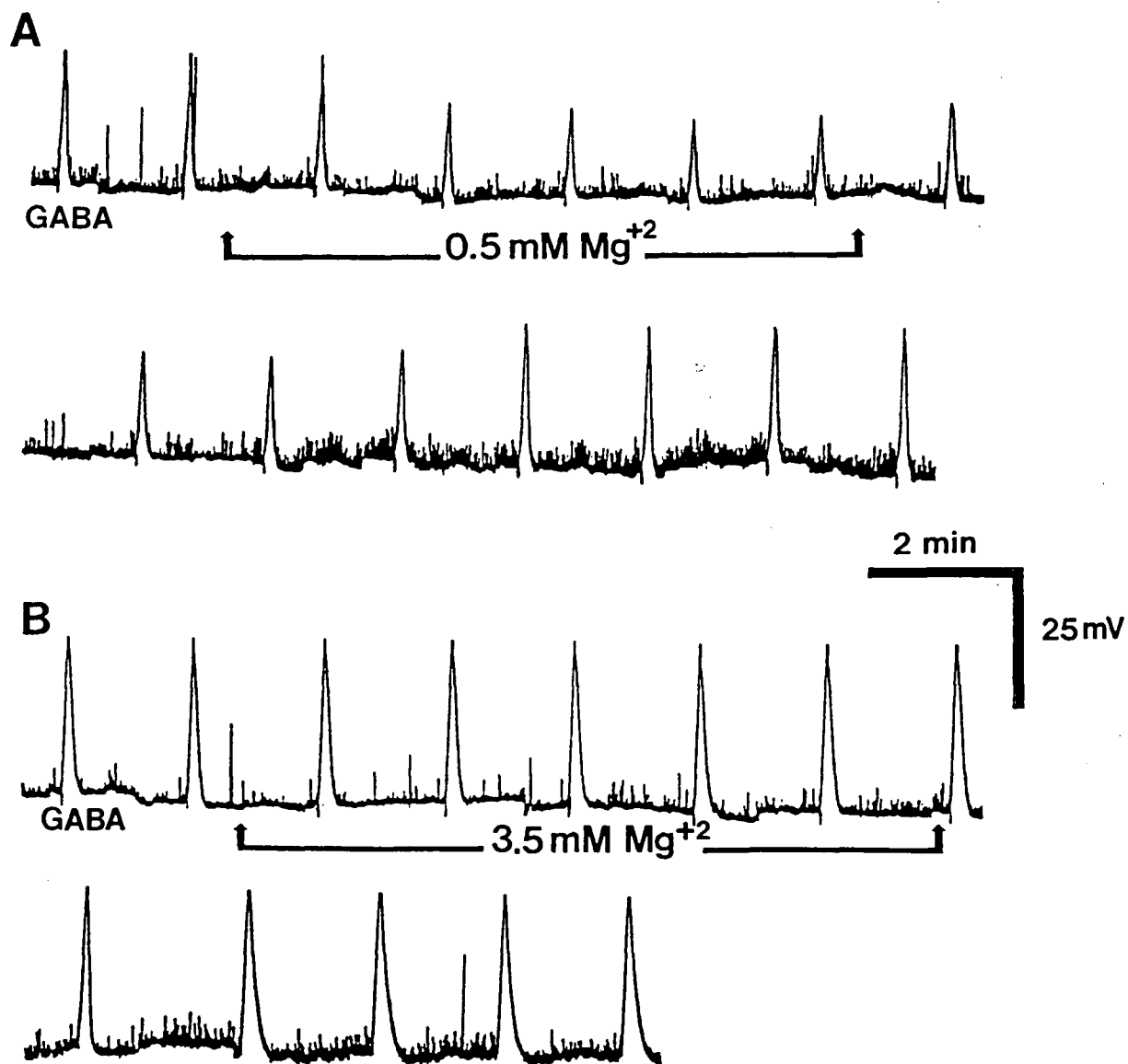


Fig. 42 Depression of GABA-evoked depolarization by low $[Mg^{2+}]_o$. (A) perfusion with 0.5 mM Mg^{2+} containing media depressed the responses to GABA in an anterior cingulate neuron ($V_m = -68$ mV). (B) increasing levels of $[Mg^{2+}]_o$ had no effect on GABA-induced depolarizations.

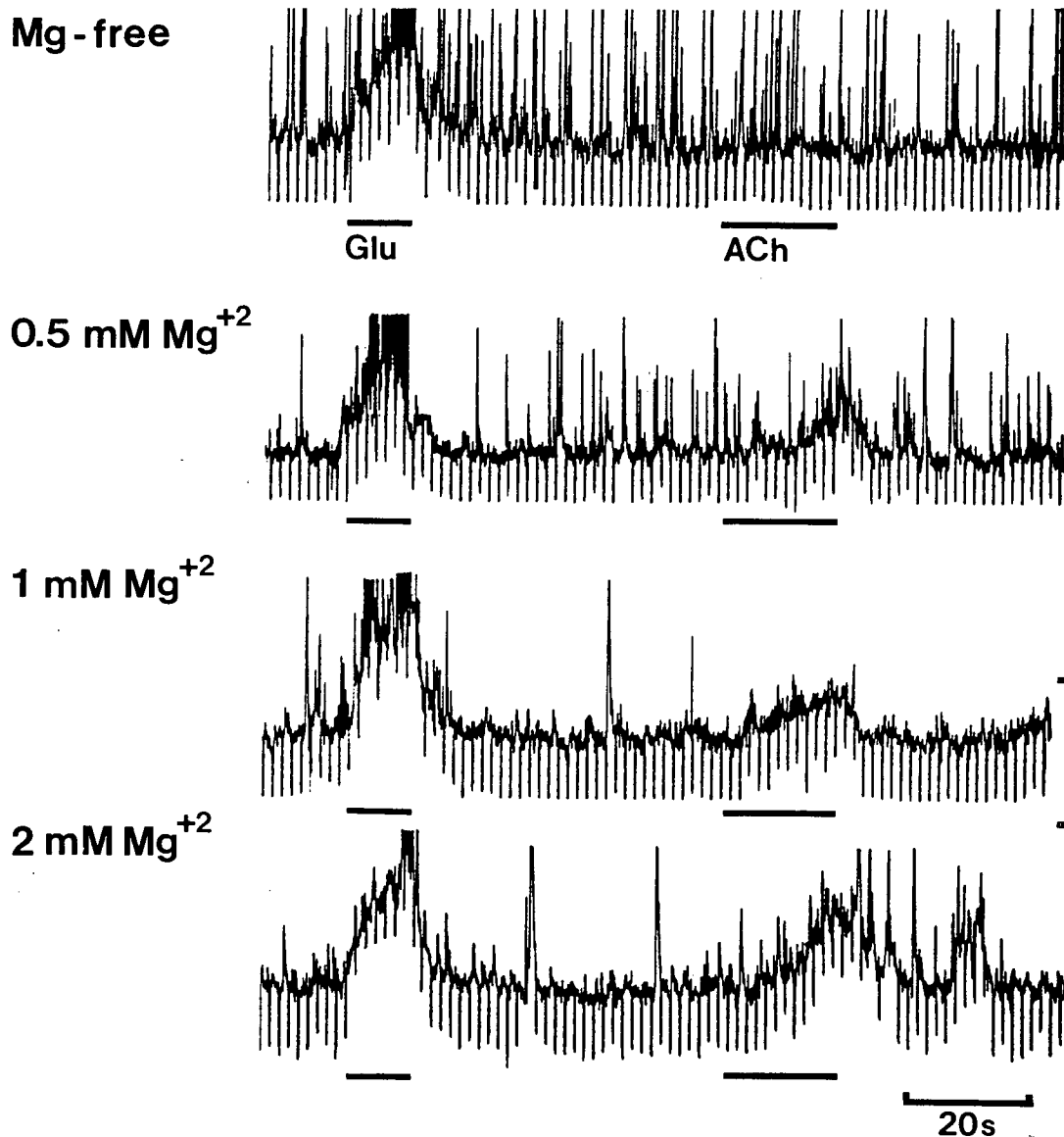


Fig. 43 Gradual potentiation of ACh-actions in a neuron ($V_m = -70$ mV) due to a sequential increase of $[Mg^{2+}]_o$. Recordings were obtained in a slice that had been incubated in free Mg-media for ~3 hours. Vertical bar indicates 15 mV.

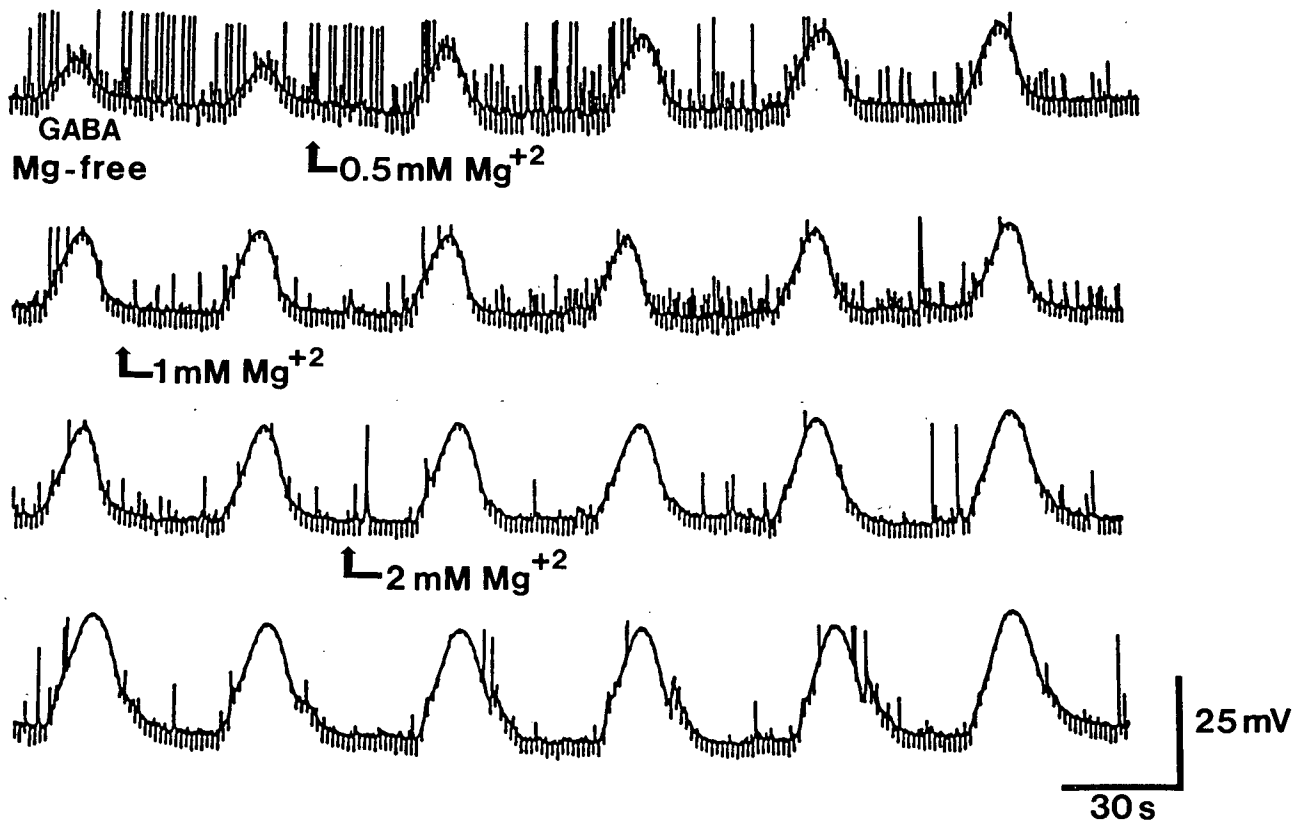


Fig. 44 GABA actions were gradually potentiated by sequential increase in $[Mg^{2+}]_o$ concentration in the perfusing media. Recordings were obtained from a neuron in a slice that had been incubated in Mg-free ACSF for ~4 hours.

The magnitudes of the depolarizations evoked by Ach, Glu and GABA at each $[Mg^{2+}]_o$ were averaged in 18 neurons (Fig. 45). The changes in the responses to ACh and GABA were larger and approached their respective maxima sooner with increasing levels of $[Mg^{2+}]_o$. However, the rate of rise and magnitude of the Glu responses were largely independent of the $[Mg^{2+}]_o$.

6.1.4 Effects of external Ca^{2+} and Mg^{2+} exclusion on the Glu responses. Because of the variability in the effects of Mg^{2+} removal and buildup on the Glu responses, a separate set of experiments was designed to explore the alterations in Glu-responses by manipulating the divalent cation environment without changing the total cationic concentration. These were carried out in the presence of TTX. Exclusion of both Ca^{2+} and Mg^{2+} from the perfusing media and their substitutions with 4 mM Co^{2+} attenuated the Glu-induced depolarizations by 38.8 (± 7.5)% in 3 out of 4 neurons (Fig. 46A). Substitution of Ca^{2+} with Co^{2+} suppressed the Glu responses by 15 (± 5.7)% in 4 out of 4 neurons (Fig. 46B). Recovery was complete after 15-20 min.

6.1.5 Anaesthetic actions during Mg-free perfusion. Anaesthetics were applied after perfusing the slice with Mg-free solution for 25-35 min. Isoflurane (1.5 MAC; 4 neurons) applications suppressed the Glu- and GABA-evoked depolarizations by 35.5 (± 10)% and 55.4 (± 7.2)% respectively (Fig. 47A). Althesin (75 μ M; 5 neurons) attenuated Glu- and GABA-induced responses by 32.8 (± 6.4)% and 51.2 (± 8.5)% respectively (Fig. 47B). The GABAergic actions were more vulnerable to suppression by the anaesthetics than the responses evoked by Glu. This was in contrast to the observations obtained in 2 mM Mg^{2+} (cf. Sections 5.1 and Figs. 34 and 35). However, in neurons where ACh elicited depolarizations of 5-8 mV amplitude in zero mM Mg^{2+} , isoflurane completely blocked the effects of ACh (cf. Fig. 47A).

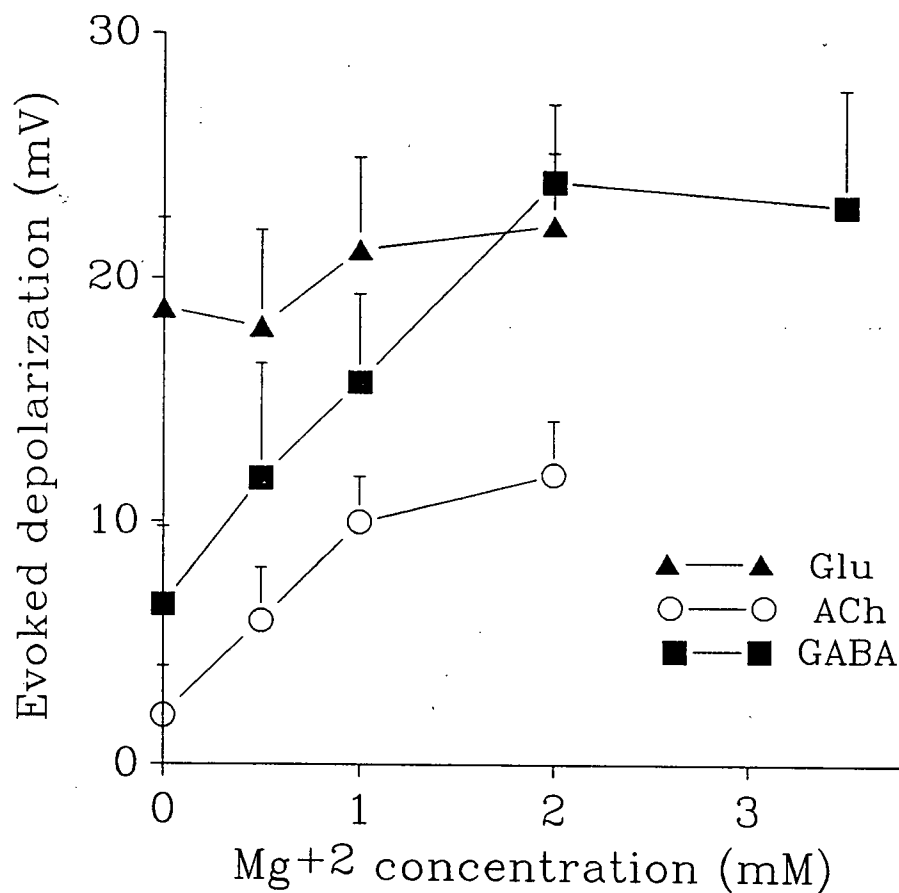


Fig. 45 Dose-response curves showing the effects of $[Mg^{2+}]_0$ on the depolarizations evoked by ACh, Glu and GABA. Raw data were obtained from 18 neurons subjected to different levels of $[Mg^{2+}]_0$.

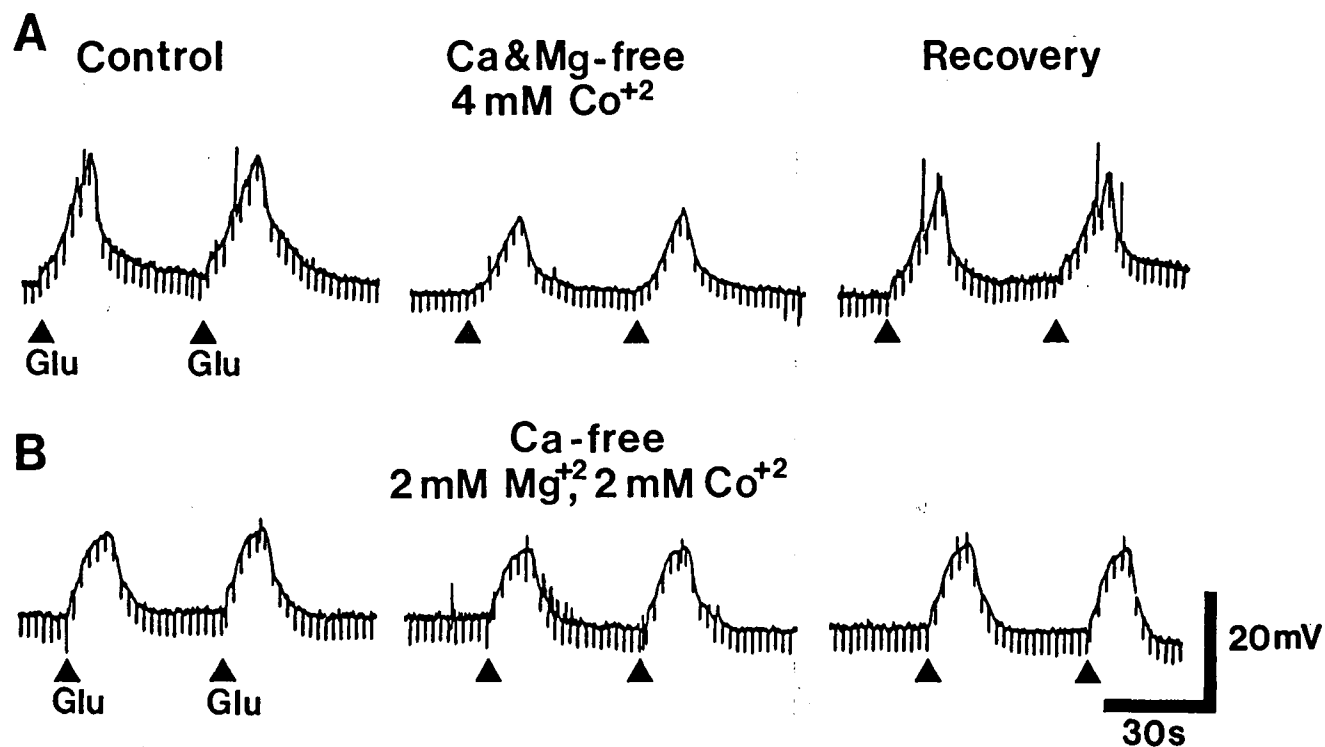


Fig. 46 Effects of changing Ca- and Mg-concentrations on glutamate actions in two neurons ($V_m = -68$ mV in A and -79 mV in B). Responses Glu were depressed in Ca- and Mg-free solutions (A) more than in the absence of Ca^{2+} alone (B).

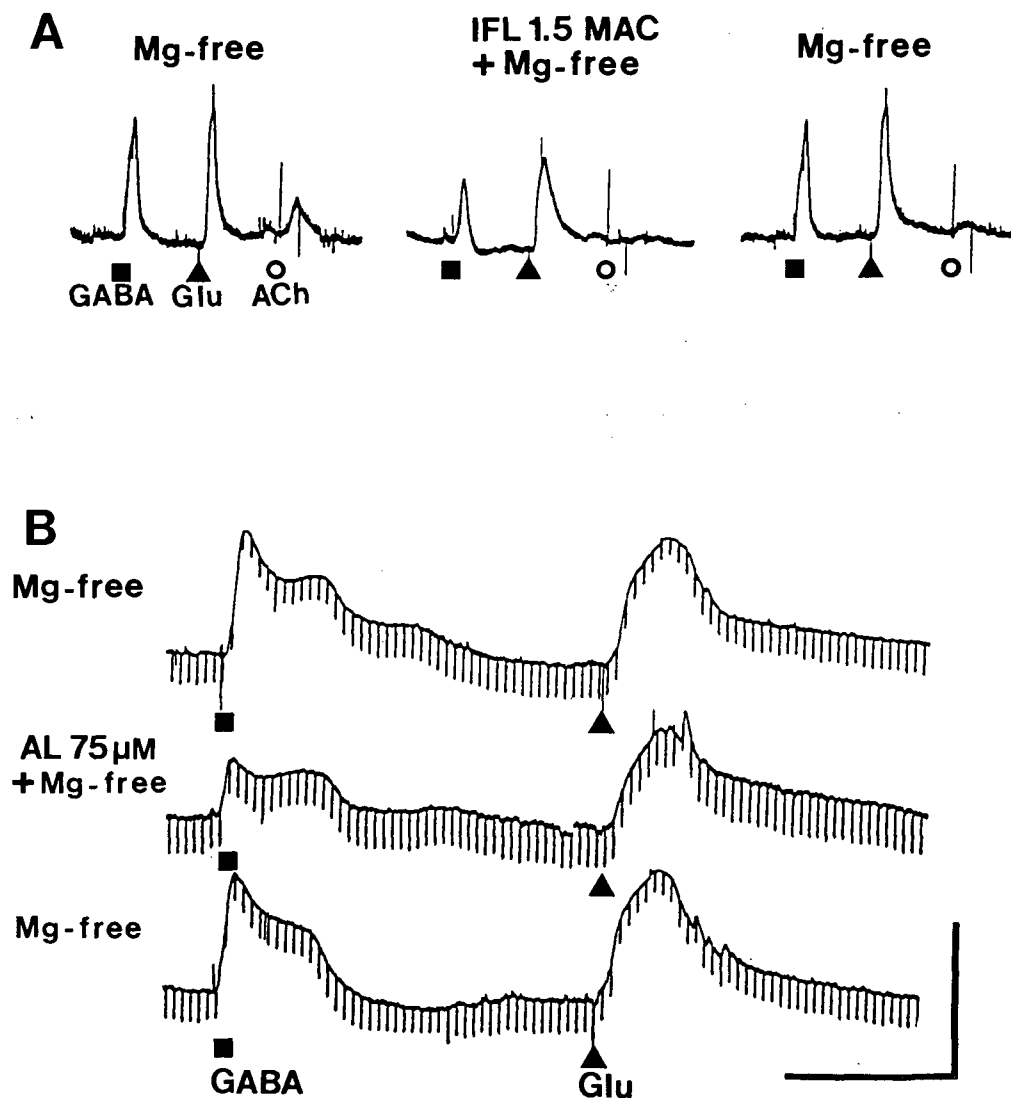


Fig. 47 Anaesthetic actions on ACh-, Glu- and GABA-induced responses during application of Mg-free ACSF in two neurons ($V_m = -65$ mV in A and -70 in B). Isoflurane (IFL) and Althesin (AL) depressed GABA- more than Glu-responses. The residual ACh-depolarization in (A) was completely blocked by Althesin. Calibration bars indicate 25 s in A and 60 s in B, and 20 mV in A and B.

6.2 Discussion

Although manifestations of hyperexcitability of the CNS that are apparent clinically during hypomagnesaemia modifies the anaesthetic requirements perioperatively (Martindale and Heaton 1964; Agus et al 1982; Aldrete 1987; Gambling et al. 1988), the underlying pathophysiological mechanisms have not yet received much attention. In these experiments, hypomagnesia was produced by substituting Ca^{2+} for Mg^{2+} in the perfusing media such that the total divalent cation concentration was kept constant (4 mM). Consequently, any interference with the surface charge screening of the membrane that can facilitate the activation of inward currents was avoided (Frankenhauser and Hodgkin 1957; Llinàs and Walton 1980; Mayer et al. 1984). As a result, alterations in the passive and active membrane properties induced by the simulated hypomagnesaemia are almost entirely due to changes in the $[\text{Mg}^{2+}]_o$ (Frankenhauser and Hodgkin 1957; McLaughlin et al. 1971; Lüttgau and Glitsch 1976).

Unlike hippocampal neurons (Mody et al. 1987), exposure of the neocortical slices to Mg-free media hyperpolarized the neurons in these investigations and decreased their input resistances. In contrast, depolarizations of comparable magnitude were observed on several occasions during the Mg^{2+} -buildup experiments. The latter effects were probably due to the interference with a tonic blocking action of Mg^{2+} on Ca-activated K-channels (Llinàs and Walton 1980; cf. Iseri and French 1984 and Begenisich 1988). According to this scheme, the Ca-channels would be relieved from the sustained blockade by Mg-cations resulting in an enhancement of the resting Ca-activated K-conductance and thereby a hyperpolarization.

6.2.1 Suppression of the ACh-induced responses. The dose-dependent suppression of the ACh-induced responses by removal of Mg^{2+} from the media

has not been reported previously for neocortical neurons. However, iontophoretic applications of Mg^{2+} have been found to depress the ACh- and carbachol-evoked responses recorded extracellularly in the spinal cord and dorsal root ganglia (Davies and Watkins 1977; Evans and Watkins 1978). Recent reports describe a blockade of ACh action by a reduction in $[Mg^{2+}]_o$ in cardiac muscle (Kurachi et al. 1986; 1988) and a failure of Mg^{2+} to antagonize nicotinic actions on cerebellar neurons (Garza et al. 1987). In neocortex, the depression of ACh responses in the present investigations could be attributed to one or a combination of the following mechanisms:

(1) a decrease in $[Mg^{2+}]_o$ would remove the tonic inhibition of an inwardly directed Ca conductance, thereby increasing Ca-dependent K-efflux (Wanke et al. 1987); this would decrease the ACh-induced depression of the input conductance and the time- and voltage-dependent M-current,

(2) decreasing $[Mg^{2+}]_o$ may cause a decrement in intraneuronal $[Mg^{2+}]$ (Baker and Crawford 1972; Heinonen and Akerman 1986) thereby enhancing the inwardly rectifying K-conductance which would interfere with the action of ACh (cf. Krnjević et al 1967 and Stanfield 1988),

(3) low $[Mg^{2+}]_o$ favours the conversion of the muscarinic receptor with high affinity binding sites to low affinity conformational states; this is suggested by the observed shift to the left of the occupancy-concentration curves for carbachol binding in the cerebral cortex whenever Mg^{2+} was added to the incubating medium (Hulme et al. 1980; Gurwitz and Sokolovsky 1980; Birdsall et al. 1984; Aronstam et al 1985).

6.2.2 Changes in $[Mg^{2+}]_o$ do not significantly affect Glu-induced responses. Previous intracellular investigations in rat neocortical slices showed no substantial changes in the responses evoked by Glu during

application of Mg-free solutions (Thomson et al. 1985; Sutor et al. 1987). In the frog and rat spinal cords, alterations of $[Mg^{2+}]_o$ in both directions had no appreciable effects on the intra- or extracellularly recorded Glu responses (Evans et al. 1977; Ault et al. 1980; Lacey and Nistri 1988). The pooled data in the present studies indicated a trend towards slight depression of the depolarizations evoked by Glu; this was not, however, statistically significant. The absence of an effect could be due to an inability of Mg^{2+} to significantly influence the binding of Glu to its receptors (Baudry and Lynch 1979) and/or may reflect Glu interactions predominantly with Quis and ka (not NMDA sites) sensitive sites at layers IV-V of the cortex as suggested by several autoradiographic studies (Greenamyre et al. 1985; Monaghan and Cotman 1985). The observed trend in depression of the Glu-responses, if confirmed by systematic studies, would suggest that Mg^{2+} could be a charge carrier contributing to the Glu-evoked depolarization. The decrease in extracellular Mg^{2+} activity recorded with ion-selective electrodes during local application of Glu to hippocampal pyramidal neurons supports this possibility (Pumain et al. 1987; 1988). In addition, the larger depression of the Glu responses observed in the absence of Mg^{2+} and Ca^{2+} than in the presence of Mg^{2+} in the perfusing media (cf. Results) also may be interpreted that Mg^{2+} cations, when present, permeated through Glu-operated ionophores.

6.2.3 GABA-depolarizations are consistently attenuated by the removal of $[Mg^{2+}]_o$. $[Mg^{2+}]$ has been suggested to be an essential factor for the prevention of "rundown" in $GABA_A$ -receptor function (Stelzer et al. 1988; Gyenes et al. 1988). The effects of $[Mg^{2+}]$ on postsynaptically GABA-induced Cl^- -currents have not been previously reported for mammalian central neurons. However, a wide variety of divalent cations (Zn^{2+} ,

Co^{2+} , Ni^{2+} and Cd^{2+}) antagonize responses to GABA in different vertebrate species (Kaneko and Tachibana 1986). In the absence of conclusive biochemical evidence that $[\text{Mg}^{2+}]_o$ affects the binding characteristics of GABA_A receptors, it may be assumed that the dose-dependent inhibition of the depolarizations produced by GABA applications in the present investigations is due to a decrease in the intracellular Mg^{2+} concentration ($[\text{Mg}^{2+}]_i$) subsequent to the removal of $[\text{Mg}^{2+}]_o$ (Baker and Crawford 1972; Heinonen and Akerman 1986; cf. Fry 1986) which leads to a "rundown" of GABA_A receptor function.

6.2.4 Enhancement of neuronal excitability by the removal of $[\text{Mg}^{2+}]_o$.

The reduction of $[\text{Mg}^{2+}]_o$ is known to enhance neuronal excitation in many cortical systems including hippocampus and entorhinal cortex (Walter et al. 1986; Stanton et al. 1987; Mody et al. 1987; Hamon et al. 1987; Tancredi et al. 1990) as well as neocortex (Thomson 1986; Avoli et al. 1987; Aram et al. 1989). Several mechanisms have been proposed for these actions, including:

- (1) removal of antagonistic actions of Mg^{2+} on pre- and postsynaptic Ca^{2+} entry (Katz and Miledi 1969; Llinás and Walton 1980; Lambert and Heineman 1986; Czeh and Somjen 1989),
- (2) reduced surface charge screening and an associated facilitation of inward currents and action potentials (Frankenhaeuser and Hodgkin 1957; Llinás and Walton 1980; Mayer et al. 1984) and
- (3) removal of the voltage-dependent Mg-blockade of NMDA receptor associated ionophores (Davies and Watkins 1977; Thomson 1986; Walter et al. 1986; Mody et al. 1987; Stanton et al. 1987; MacDonald et al. 1987; Mayer et al. 1988;).

In the present investigations, the potentiation of the NMDA responses in low $[Mg^{2+}]_0$ is consistent with the above considerations. An additional mechanism that may contribute to the neuronal hyperexcitability under conditions of hypomagnesemia is the dose-dependent depression of the GABA-ergic transmission; thus, the pyramidal neurons would be relieved from the negative feedback loop inhibition by interneurons.

6.2.5 Anaesthetic-induced depression of Glu and GABA responses in Mg^{2+} -free media. These investigations demonstrate, for the first time, the depressant actions of isoflurane and Althesin on the Glu- and GABA-evoked depolarizations in simulated hypomagnesaemia. An interesting finding was the reversed pattern in suppression of the transmitter-induced responses. Both anaesthetic agents preferentially depressed GABA- compared with Glu-actions. Whereas in normal $[Mg^{2+}]_0$, the amount of attenuation of the GABA-responses was insignificant at similar anaesthetic concentrations. Though the mechanism of such "selectivity" is not clear, these observations partly explain the increased anaesthetic requirements in patients suffering from conditions associated with hypomagnesaemia, e.g., in chronic alcoholism.

7 ANAESTHETIC-INDUCED ATTENUATION OF POSTPIKE AFTERHYPERPOLARIZATIONS

MEDIATED BY Ca-ACTIVATED K-CONDUCTANCE

7.1 Results

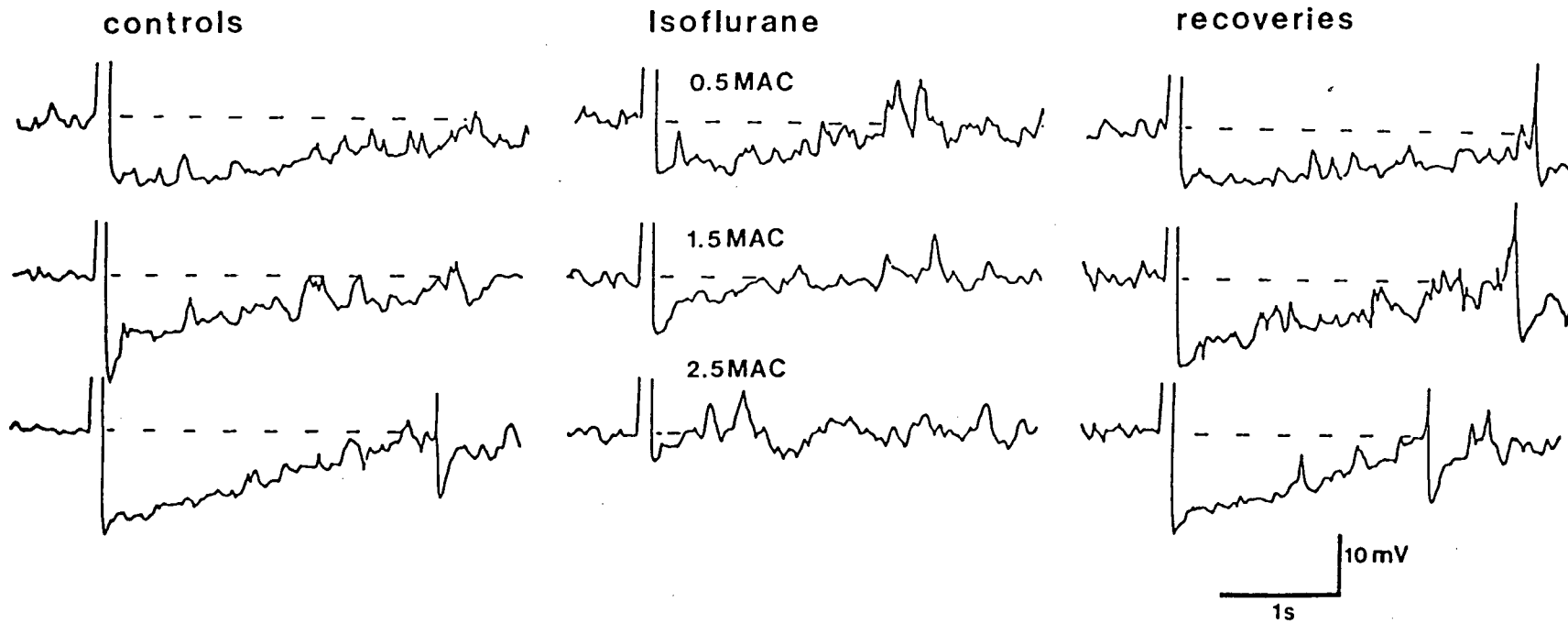
The 52 neurons selected for anaesthetic applications in this series of experiments had resting potentials more negative than -65 mV, spike amplitudes usually of 70-75 mV, and ability to fire 5 such spikes repeti-

tively during current-pulse injections. The depths of successful impalements corresponded to layers IV and V of typical cortex. The input resistances (mean \pm SD; $65.7 \pm 20.5 \text{ M}\Omega$) and resting potentials ($-73.2 \pm 7.9 \text{ mV}$) remained stable for periods of 50 to 220 min.

7.1.1 Depression of AHPs. The effects of anaesthetic application on the AHPs accompanying 5 (in a few cases, 6) spikes evoked by current-pulse injections (cf. Methods) were studied in the 52 neurons. The control AHPs ranged from 3.5-11 mV in amplitude and 700-2800 ms in duration.

Applications of isoflurane or Althesin produced dose-dependent, reversible depressions in the amplitudes and durations of the AHPs in all cases (Figs. 48-50). These effects were observed during DC-compensation for any anaesthetic evoked changes (usually 2-5 mV, if present) in resting potential (cf. Methods). Isoflurane administered at 1 MAC reduced the mean amplitude by $\sim 23 \pm 4.81\%$ and the mean duration by $\sim 26 \pm 4\%$ (Fig. 50). At an isoflurane concentration of 2.5 MAC, the mean amplitude and duration of the AHPs were reduced by $\sim 68\%$ and $\sim 75\%$, respectively (Fig. 50). Complete recovery was observed 9-15 min after discontinuing isoflurane application. The EC_{50} for attenuation of the AHP amplitude by Althesin application was $150 \mu\text{M}$. The EC_{50} for the shortening of AHP durations was $70 \mu\text{M}$ which corresponds approximately to the concentrations determined in rat brain after a single intravenous dose of Althesin that induces an anaesthetic state in vivo (Smith et al., 1974). Recovery was observed at 10 to 12 min after terminating applications of 15-300 μM doses. Although Althesin applications at the 1500 μM dose reduced the mean amplitude and duration by $85.5 \pm 13.6\%$ and $95.5 \pm 18.5\%$, respectively (Fig. 50), no recovery was observed even at 30 min after returning to control perfusion at a rate of 3 ml/min (Fig. 49, last panel). The percent changes in the amplitude and duration of AHPs were significant statistically at all doses of both agents (Fig. 50).

Fig. 48 Isoflurane induced an attenuation of the afterhyperpolarizations (AHPs). Isoflurane applications to three neurons in 0.5, 1.5, 2.5 MAC doses produced dose-dependent and reversible reductions in the duration and amplitude of the AHPs.



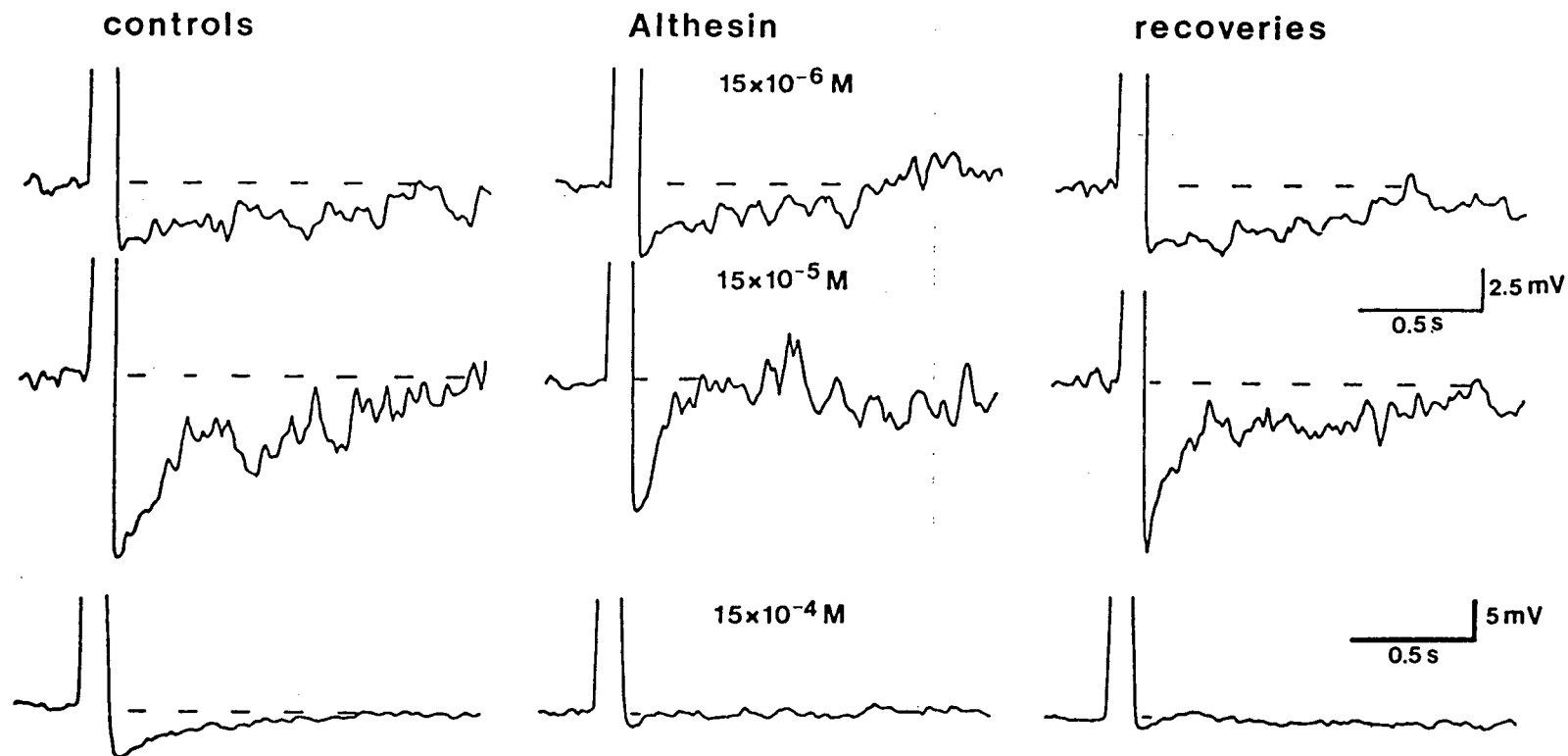


Fig. 49 Depression of the afterhyperpolarizations (AHPs) by applications of Althesin. The effects were evident at different doses (within two log units of alphaxalone content). The depression by the highest dose of Althesin was irreversible even after 30 min in control solution.

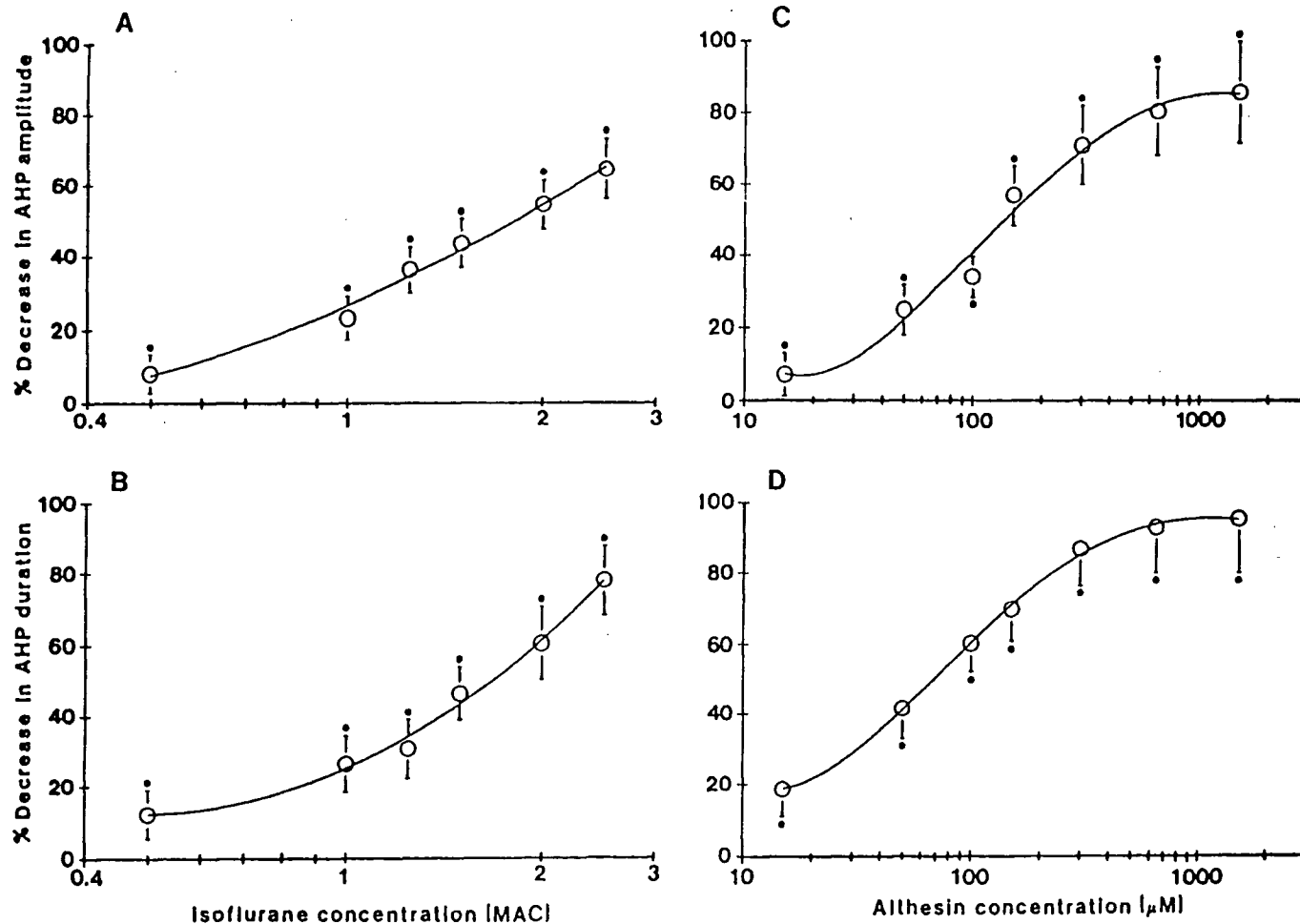


Fig. 50 Dose-response relationships for the depression of the afterhyperpolarizations produced by applications of isoflurane (6-9 min; A,C) and Althesin (4-6 min; B,D). The data points were fitted by 3rd order regression analysis. Symbols (●) indicate statistically significant differences from the mean control values at $p < 0.05$.

7.1.2 Bicuculline-blockade of inhibitory postsynaptic potentials

Bicuculline applications were used in 8 non-spontaneously active neurons to reduce "contamination" of the AHPs by synaptic and DC potentials generated through Cl-conductances mediated by receptors for endogenous GABA (cf. McCormick and Prince 1986). For example, a summation of AHPs with IPSPs could have occurred if the current-pulse injections had activated, through recurrent collaterals, a synaptically connected interneuron that released GABA onto the recorded neuron. A 50 μ M concentration of bicuculline was chosen because of observations that IPSPs evoked by epicortical electrical stimulation were completely blocked in such slice preparations (cf. Section 2). The doses of isoflurane (1.5 MAC) and Althesin (100 μ M) were chosen for fast recovery times as well as for their clinical relevance (Smith et al. 1974; Cullen 1986).

Bicuculline application alone did not greatly affect V_m and facilitated repetitive spike bursts evoked on current-pulse injections. A small increase (~10%) in R_i was observed during bicuculline application. When isoflurane or Althesin was applied concomitantly with bicuculline, the AHPs were reduced much more than with the sole administration of the anaesthetic (Fig. 51). The anaesthetic actions on the AHPs had approximately the same time courses in the absence and presence of bicuculline (Fig. 52). The changes in V_m and R_i induced by Althesin were less in the presence of bicuculline than in its absence (Fig. 52B).

7.2 Discussion

The most striking effects of both agents on all neurons in this series of investigations were the dose-dependent attenuations in the amplitudes, as well as the shorter durations of the AHPs associated with the repetitive spike discharges. These postsynaptic effects could not be attributed to

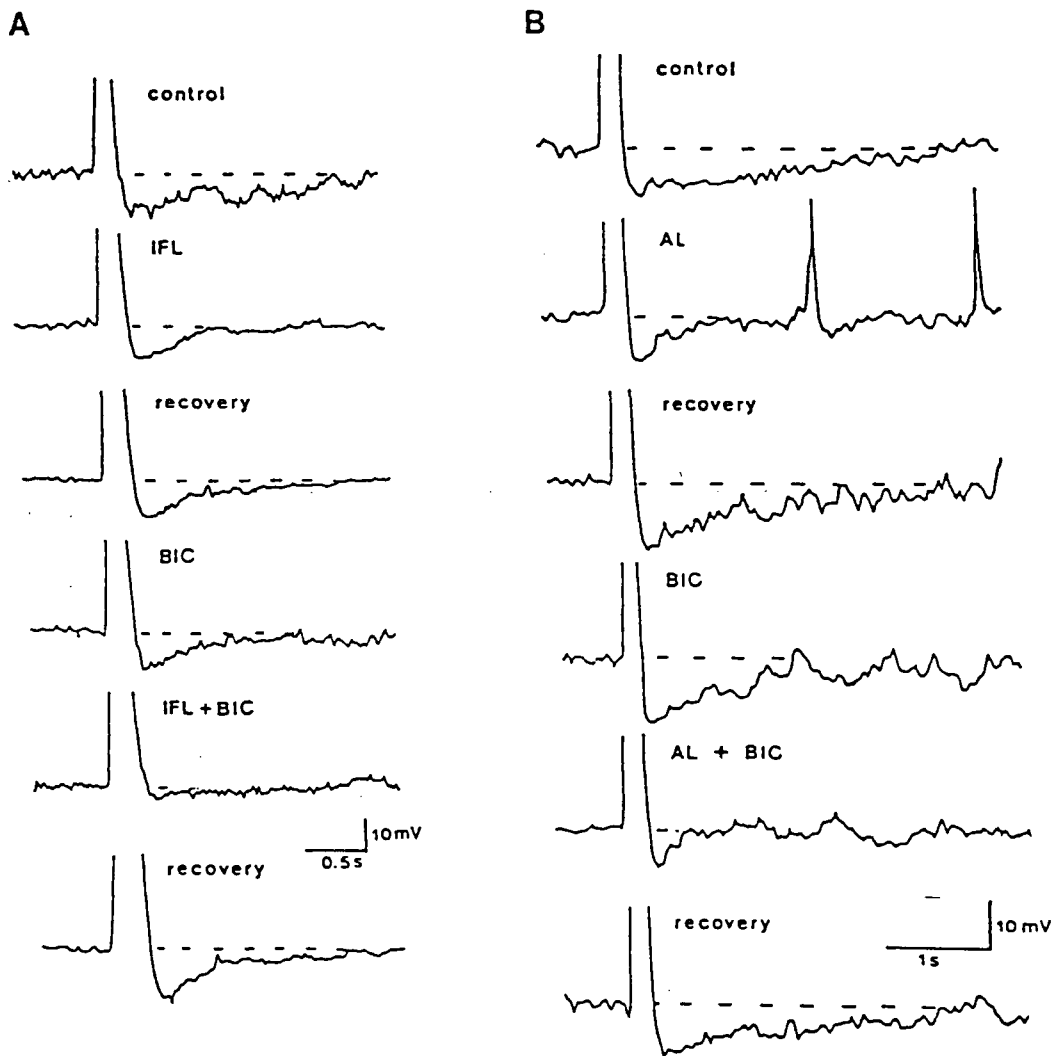


Fig. 51 Isoflurane (IFL; 1.5 MAC for 6 min) and Althesin (AL; 100 μ M for 4 min) induced depressions of the afterhyperpolarizations (AHPs) in two neurons (A,B) where bicuculline methiodide (BIC; 50 μ M) had been additionally applied. The AHPs were attenuated more in the presence of bicuculline than in its absence. Note that a depression of the AHPs occurred with the application of bicuculline alone.

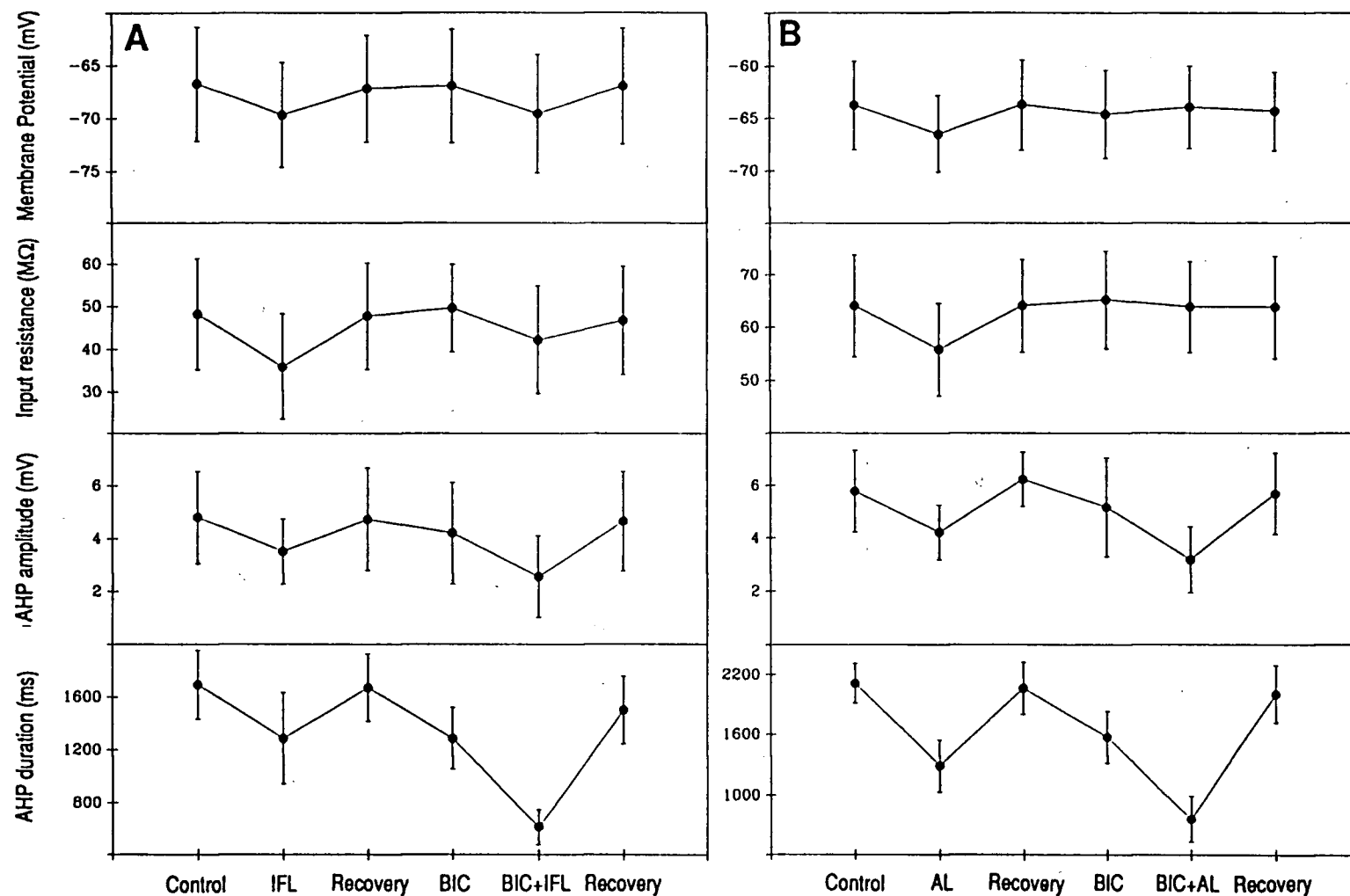


Fig. 52 The mean changes in resting membrane potential, input resistance, and afterhyperpolarization (AHP) amplitude or duration in eight neurons were induced by isoflurane (A; IFL, 1.5 MAC for 6-9 min, $n = 4$) and Althesin (B; AL, 100 μ M for 4-6 min, $n = 4$). Note the pronounced effects of IFL on the AHP in the presence of bicuculline (BIC) in A. (B) bicuculline partially blocked the changes in resting potential and input resistance, and potentiated the depression of the AHP induced by Althesin.

anaesthetic induced changes in resting membrane properties and also have been observed during similar applications of isoflurane to hippocampal CA₁ cells (Fujiwara et al 1988; Miu and Puil 1989) and human sympathetic ganglion neurons (Puil et al. 1988). A depression of directly or synaptically-evoked AHPs has been demonstrated during applications of other volatile anaesthetics, and barbiturates (Bosnjak et al. 1982; Werz and Macdonald 1985; Fujiwara et al. 1988; Miu and Puil 1989; Southan and Wann 1989). In some of the present studies, bicuculline was administered concomitantly with the anaesthetics to block the GABA-mediated Cl-conductances (cf. McCormick and Prince 1986). Hence, the likely summations of IPSPs with the AHPs as well as with endogenous GABA-actions that may have been potentiated by the anaesthetic, especially by Althesin (Barker et al. 1987) could be prevented in neocortical neurons. Bicuculline application alone decreased the amplitudes and durations of the AHPs suggesting a blockade of the endogenous GABAergic activity. In such cases, Althesin application produced a greater blockade of AHPs, presumably because an occlusion of the AHP-conductances due to anaesthetic potentiation of GABA-ergic activity had been avoided. Both anaesthetics had much more pronounced effects on the AHPs under conditions of blockade by bicuculline, suggesting some occlusion of the Ca-activated K-conductance (cf. Schwindt et al. 1988a, 1988b) by a GABA-mediated Cl-conductance in neurons where the GABA_A-antagonist had not been applied. It is unlikely that a non-Ca-dependent K-conductance interfered with, or contributed to, the genesis of the AHPs (cf. Schwindt et al. 1988a, 1988b). Therefore, the effects observed in these investigations could be attributed mainly to anaesthetic actions directly on the ionic mechanisms generating the AHPs.

7.2.1 Mechanism(s) of anaesthetic interference with AHP generation.

Normally, the AHP is a consequence of a K-conductance that is initiated by depolarization and Ca-entry. As a result, $[Ca^{2+}]_i$ rises, activates certain K-channels (cf. Krnjević et al. 1978b) and initiates an outward K-current. The decay of this current is partly contingent on the buildup of $[Ca^{2+}]_i$ that inactivates the Ca-channels (Eckert and Chad 1984) and on internal Ca-sequestration. Halothane, Althesin and barbiturates deplete intracellular energy stores thereby compromising Ca-buffering mechanisms (Smith et al. 1974; Morris 1986; Daniell and Harris 1988). Therefore, the interference with the Ca-activated K-conductance mechanism of the AHP in neocortical neurons that was produced by isoflurane or Althesin also could be a direct result of decreased inward Ca-currents (Krnjević and Puil 1988), or may be related to less specific anaesthetic alterations in the steady-state background of $[Ca^{2+}]_i$ (cf. Krnjević 1974a; Morris 1986).

7.2.2 Significance of AHP inhibition in neocortical neurons.

The importance of the slow AHPs in subcortical neurons (Baldissera and Gustafsson 1974; Krnjević et al. 1978b) and the medium-duration AHPs in neocortical neurons (Schwindt et al. 1988a,b) is well appreciated for regulating neuronal hyperexcitability. Carlen et al. (1985) observed that sedatives increased the AHPs and reduced repetitive firing in various subcortical neurons. The blockade of the slow AHP generation in such neurons by apamin (Zang and Krnjević 1987) or in aged hippocampal neurons by ethanol (Niesen et al. 1988), increases repetitive spike discharge. In neocortex, a depression of repetitive firing ability accompanied the anaesthetic blockade of the medium-duration AHP. The apparent paradox may be related to the observations that well-developed, fast AHPs in hippocampal neurons are not reduced by applications of apamin or ethanol (Zhang and

Krnjević 1987; Niesen et al. 1988). Such AHPs are not mediated by Ca^{2+} and are not prominent in neocortical neurons (Schwindt et al. 1988a,b). The anaesthetic depression of repetitive firing in neocortex could be a consequence of a removal of the major mechanism by which Na-channels are primed for activation. For example, depression of the medium-duration AHPs in neocortex by anaesthesia would prevent the late repolarizing shift of the membrane potential which normally decreases Na-inactivation and resets the excitation cycle for Na-activation, resulting in an attenuation of repetitive spike discharge.

8 EFFECTS OF ISOFLURANE ON THE GLUTAMATE- AND POTASSIUM-INDUCED

INCREASE IN INTRANEURONAL CALCIUM CONCENTRATION

The objectives of this series of investigations were:

- (1) to elucidate the mechanisms of the depressant actions of anaesthetics on the ACh- and Glu-evoked depolarization (cf. Section 5),
- (2) to monitor possible changes in the resting level of $[\text{Ca}^{2+}]_i$ induced by isoflurane, and
- (3) to study the effects of isoflurane on the pre- and postsynaptic activation of voltage dependent Ca-channels.

Cultured fetal hippocampal neurons were chosen for these investigations partly because they represent a well characterized model for the study of the Ca^{2+} homeostasis in the central nervous system (cf. Kudo and Ogura 1986).

8.1 Results

Resting $[\text{Ca}^{2+}]_i$ levels were $63.8 (\pm 38.9) \text{ nM}$ ($n = 30$). $[\text{Ca}^{2+}]_i$ were measured for ~2 min prior to agonist or drug application; neurons with

resting $[Ca^{2+}]_i$ values above 150 nM or those with resting $[Ca^{2+}]_i$ that fluctuated more than ± 20 nM were not included for analysis. [These conditions occurred rarely and seemed to be related to poor cell viability.] Similar volumes of control solution were injected on different occasions to test for injection (possibly mechanical) artifacts.

8.1.1 Response to ACh and carbachol. Small increases (~ 25 nM) in $[Ca^{2+}]_i$ were observed (3/12 neurons) when ACh or carbachol in the micromolar range (10-200 μ M) was applied in small volumes (25 μ l) by injection as a bolus into the recording chamber. The evoked responses had a latency of ~ 20 s and a duration of 100-150s. This is consistent with the observations of Benavides and associates (1988; cf. Kudo et al. 1988) in brain synaptoneurosome. The effects of isoflurane could not be well studied because the increments in $[Ca^{2+}]_i$ evoked by the cholinergic agonists were too small in this model.

8.1.2 Effects of isoflurane on resting $[Ca^{2+}]_i$ and Glu actions. Continuous perfusion of media saturated with 1.5 and 2.5 MAC isoflurane produced only small and inconsistent changes in the resting $[Ca^{2+}]_i$ over a period of 20 min. Application of 50 μ M Glu (25 μ l volumes) to 18 neurons evoked a mean increase of 220 ± 78 nM in $[Ca^{2+}]_i$. The responses were instantaneous and had an "on/off" type of pattern (Fig. 53). When TTX was added to the perfusing solution, similar responses were obtained with 50 μ M Glu application.

Perfusion of isoflurane 0.5 MAC had no effects on the $[Ca^{2+}]_i$ increases evoked by Glu (4/8 neurons) or potentiated the Glu responses (Fig. 53) in TTX free media. Attenuation of the Glu responses in media favouring NMDA or Quis was always observed on application of 1.75 and 2.5 MAC isoflurane (Figs. 54 and 55, Table 3). Isoflurane 2.5 MAC

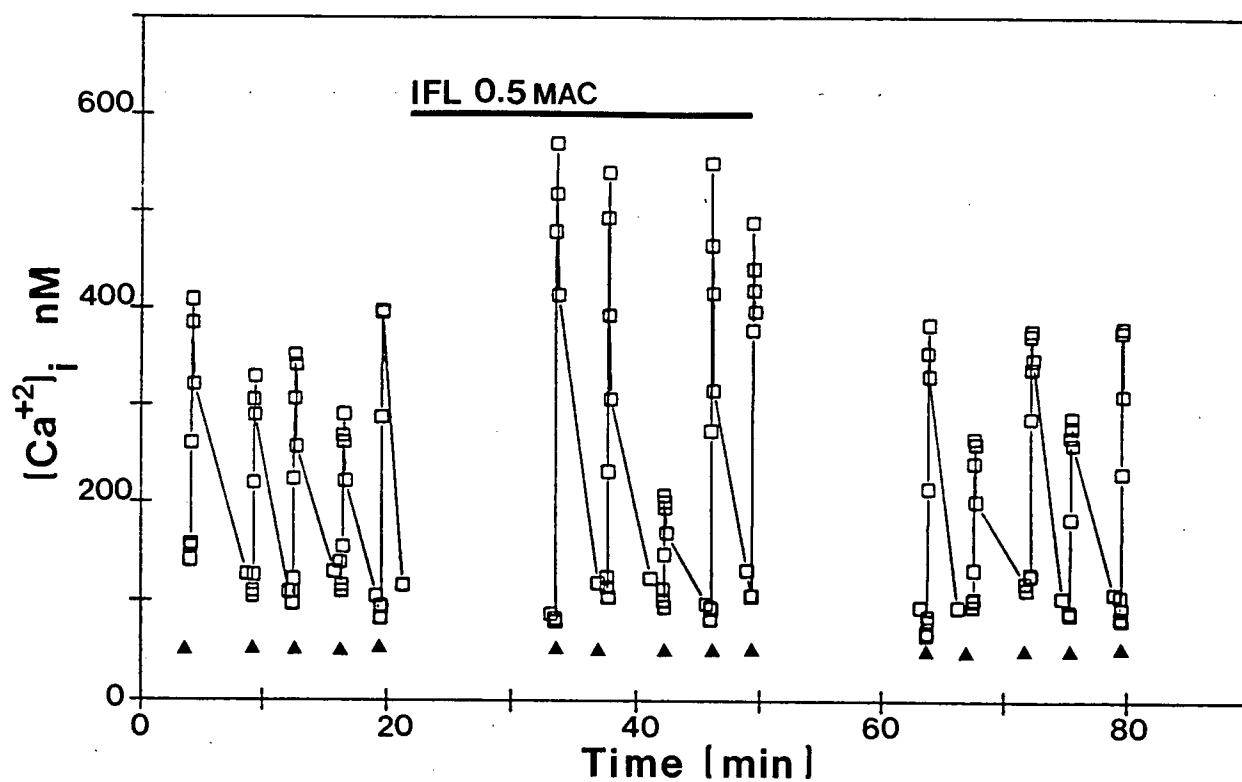


Fig. 53 Potentiation of responses to glutamate injections ($25 \mu\text{M}$; ▲) in 5 consecutively sampled neurons by 0.5 MAC isoflurane under QUIIS conditions.

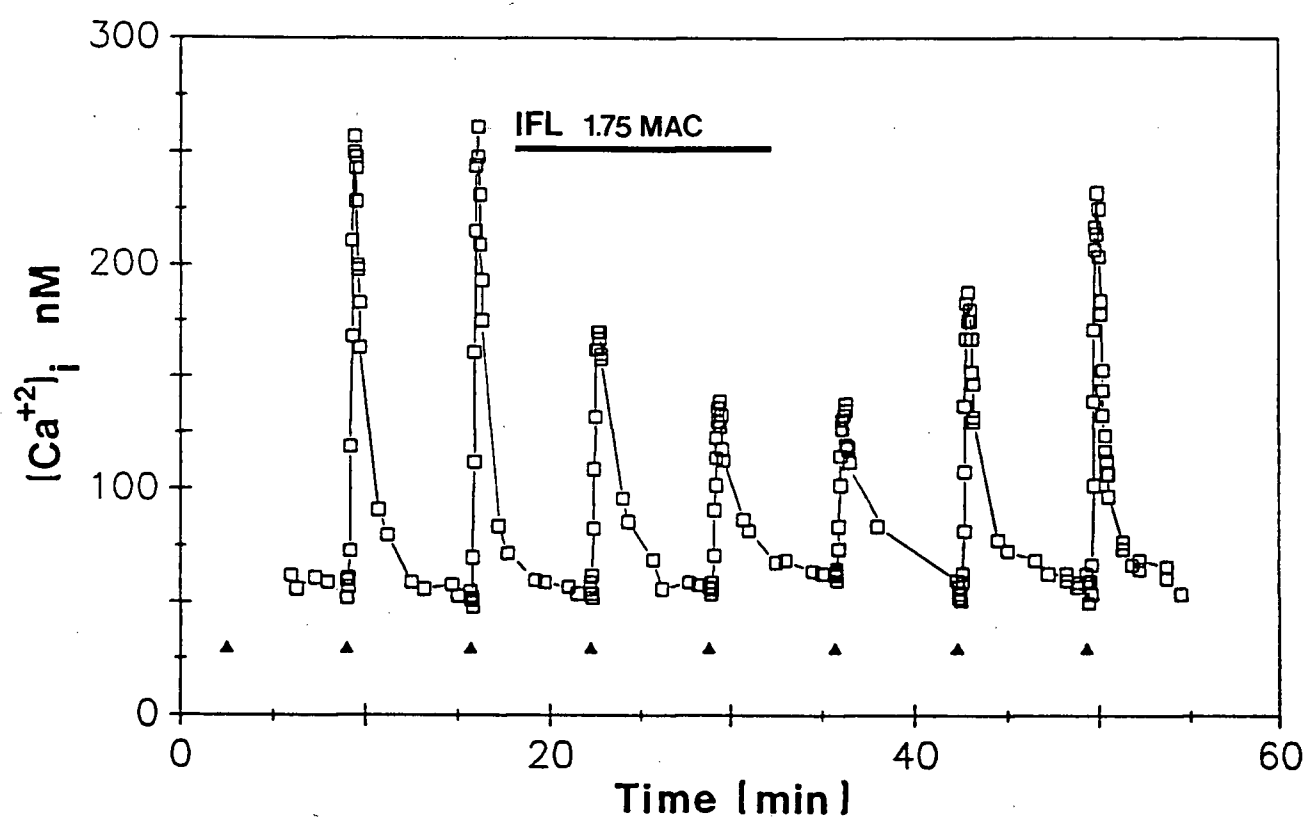


Fig. 54 Isoflurane (1.75 MAC) inhibition of increases in $[Ca^{2+}]_i$ produced by glutamate application ($50 \mu M$; ▲) under NMDA conditions.

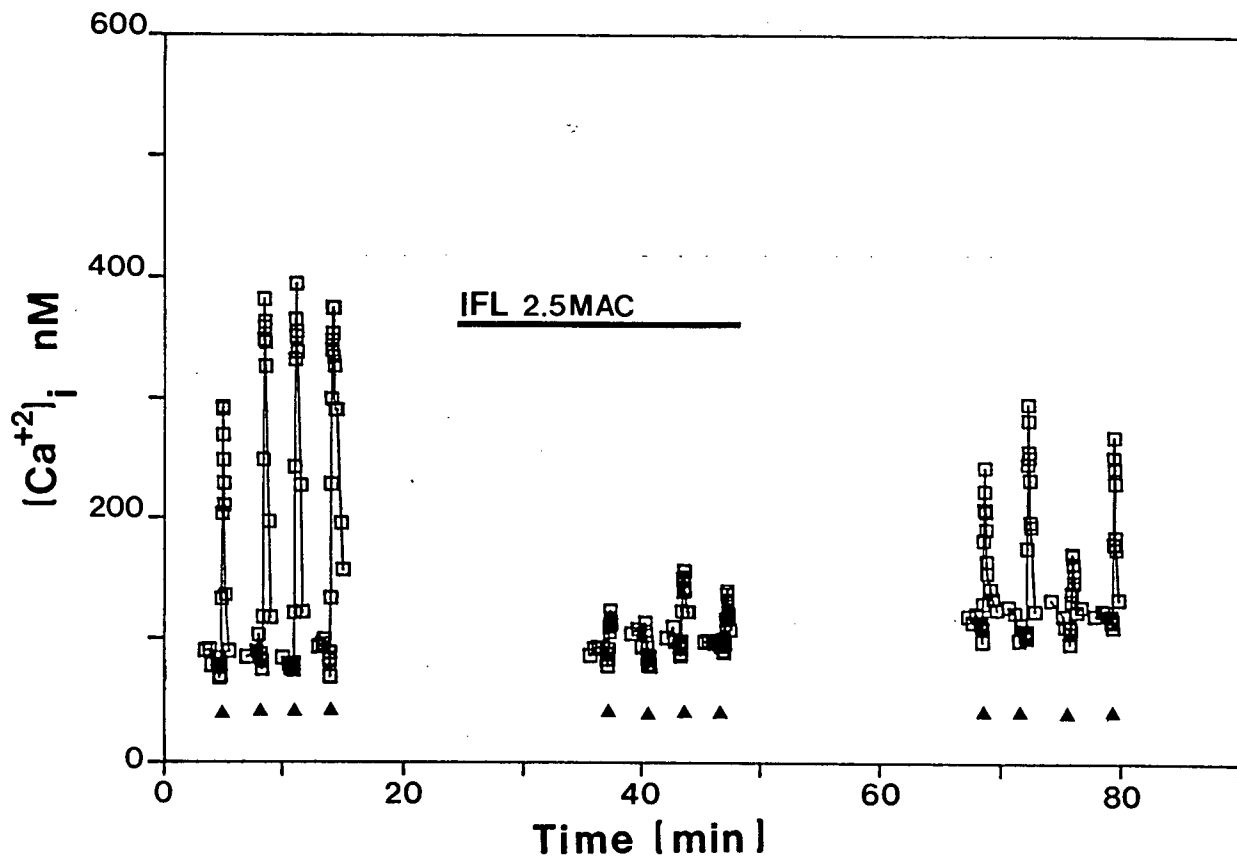


Fig. 55 Isoflurane (2.5 MAC) blockade of increase in $[Ca^{2+}]_i$ by glutamate application ($50 \mu M$; \blacktriangle) under Quis conditions.

Table 3 Effects of isoflurane on $[Ca^{2+}]_i$ increases evoked by glutamate and K^+ .

Increases in $[Ca^{2+}]_i$ evoked by		Depression* by isoflurane (MAC)			
		0.5	1.5	1.75	2.5
NMDA	(TTX present)			64 ± 14.4% (n = 4)	
	(No TTX)	No effect** (n = 3)	60 ± 10% (n = 8)		
QUIS	(TTX present)		52 ± 12.4% (n = 8)		85 ± 13.4% (n = 4)
	(No TTX)		41 ± 15.3% (n = 4)		
K^+	(TTX present)		45 ± 21% (n = 6)		
	(No TTX)				

*Depression was defined as >20% decrease in the responses induced by glutamate or K^+ injections than in control conditions.

**Potentiation of glutamate responses was observed in 5 neurons.

completely suppressed the Glu responses such that only partial recovery was observed within the experimental period (Fig. 55). These observations were consistent despite the presence or absence of TTX. Inhibition of the Glu responses required 6 min of anaesthetic application. Recovery was observed after 8-12 min.

8.1.3 Isoflurane actions on K-evoked increases in $[Ca^{2+}]_i$. K^+ (50 mM) slowly injected as a 25 μ l bolus into the recording chamber increased $[Ca^{2+}]_i$ by 300 ± 95 nM ($n = 15$) in the absence of TTX (Fig. 56). In the presence of TTX the increments of $[Ca^{2+}]_i$ were slightly greater in 7 neurons (Table 3).

Isoflurane (1.5 MAC) administration depressed the increase in $[Ca^{2+}]_i$ evoked by K^+ bolus injections by $45 \pm 22\%$ in the absence of TTX. Similar depressions were observed in the presence of the Na-channel blocker in 4 neurons.

8.2 Discussion

The failure of isoflurane application at high doses (2.5 MAC) to consistently increase basal $[Ca^{2+}]_i$ is not in agreement with other observations in rat thymocyte cell suspensions (Morris 1986) and brain synaptosomes (Daniell and Harris 1988). Different models used may account for the discrepant results. A drawback of synaptoneurosome, compared with cultured neurons, is their poorer survival stability and probable heterogeneity in size as well as receptors. In addition they possess significantly higher levels of resting $[Ca^{2+}]_i$ than those reported for intact cells (cf. Benavides et al. 1988).

In the present investigations, Glu responses were evoked in the presence or absence of 800 μ M Mg in the bathing solution. This concentration is sufficient for blockade of the NMDA-type of receptors activated by Glu (Nowak

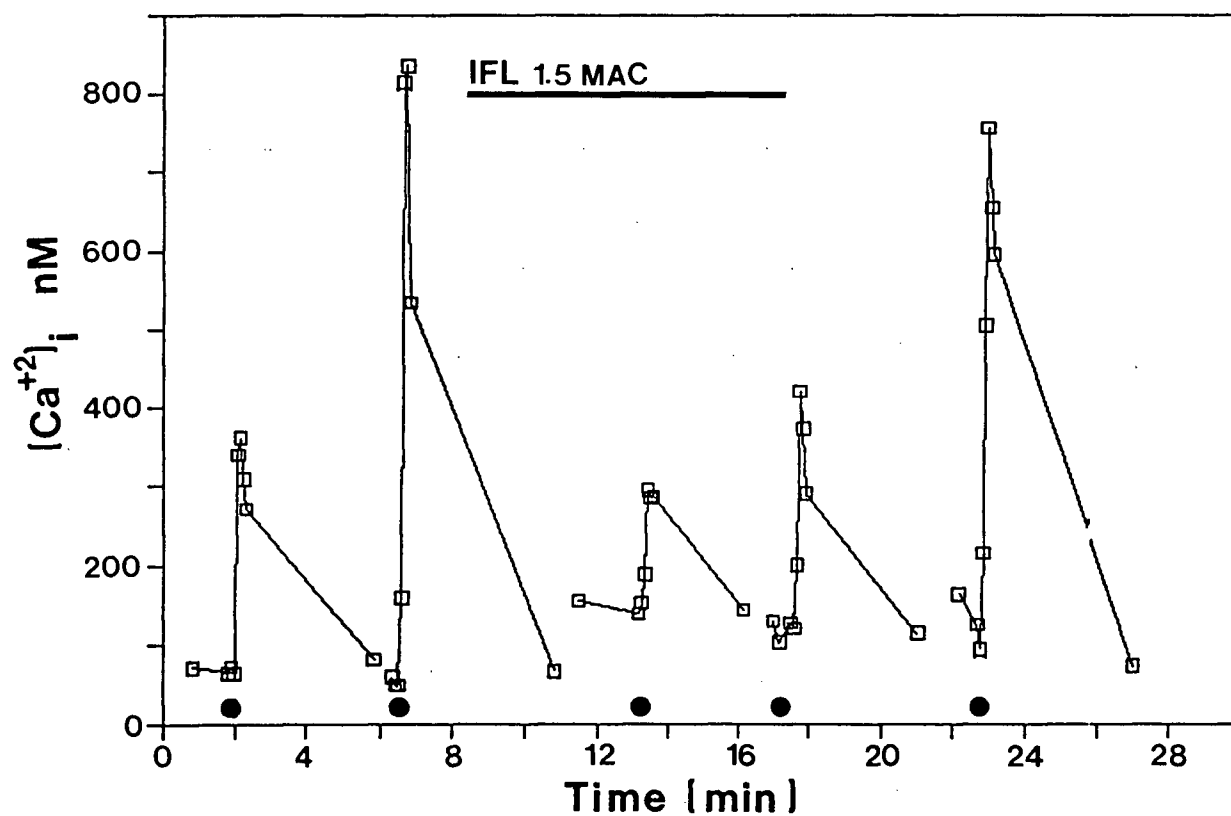


Fig. 56 Voltage-gated increase in $[Ca^{2+}]_i$ produced by K^+ (50 and 100 μM , \bullet) bolus injections that were depressed by isoflurane administration under Quis conditions. The anaesthetic depression was observed in the presence of TTX. Recovery was observed with the highest bolus of K^+ (100 μM).

et al. 1984). Consequently, the responses recorded were due mainly to activation of either the NMDA or the Quis-type of receptors (Murphy et al. 1987). The addition of TTX would have ensured an isolation of the neurons from impinging synaptic inputs. An attenuation of the Glu-induced rise in $[Ca^{2+}]_i$ has been reported previously for isoflurane as well as halothane (Puil and Baimbridge 1989). However, the mechanisms of interference of anaesthetics with the Glu actions are not well understood. Glu, by activating the Quis-subtype of receptors, induces two distinct responses in in vitro hippocampal neurons (Murphy and Miller 1989; cf. Furuya et al 1989.). A transient brief increase in $[Ca^{2+}]_i$ superimposed on top of a more maintained plateau response. The prolonged response was abolished by removal of Ca^{2+} or Na^+ from the external media or by blocking voltage-sensitive Ca-channels (Murphy and Miller 1989). In contrast, none of these manipulations abolished the transient $[Ca^{2+}]_i$ spike. Accordingly, the prolonged response has been suggested to result from activated voltage-dependent Ca-channels whereas the transient $[Ca^{2+}]_i$ spike resulted from Ca^{2+} mobilization from intracellular stores which in turn is due to agonist-induced inositol triphosphate (IP_3) synthesis (Berridge 1987; Nicoletti et al. 1988), mediated by phospholipase C effects at the receptor/G protein level (Katada et al. 1985; El-Fakahany et al. 1988). The actions of isoflurane on the Glu-evoked increases in $[Ca^{2+}]_i$ most likely involve blockade of voltage-dependent Ca-channels (cf. Lodge and Shaughnessy 1988; Krnjević and Puil 1988) and/or direct interference with the coupling of ionic channels triggered by the interactions of Glu with its receptors.

Increments of $[Ca^{2+}]_i$ evoked by K^+ applications were depressed by isoflurane despite the presence or absence of TTX. This provides presumptive

evidence that anaesthetics affect voltage-dependent Ca-channels at pre- and postsynaptic sites. Hence, the observed depression of EPSPs and IPSPs (cf. Section 4) could result from decreased transmitter release and attenuation of postsynaptic sensitivities to transmitters.

9 GENERAL DISCUSSION

These investigations provide, for the first time, comprehensive observations mainly on the effects of isoflurane and Althesin in neocortical neurons which are likely target sites in the production of the anaesthetic state. The neocortical in vitro slice preparation allowed well-controlled experiments to be carried out, despite certain generalized reservations about the in vitro slice preparation, e.g., qualitative and quantitative aspects of metabolism that may be different from that in the in vivo situation (cf. Kovachich and Mishra 1981), removal of the tonic influence of ascending arousal pathways and uncertainties about the achievement of optimal drug levels. For example, some of the pharmacokinetic parameters that complicate the interpretation of results obtained from studies in the intact or decerebrate animal (in vivo preparation) may be minimized in the slice preparation. Because neocortical slices are devoid of influences from extracortical afferent activity, the anaesthetic actions are attributable mostly to the neuron under investigation, particularly when TTX application had been employed to reduce activity emanating from the intracortical connections. In addition, the in vitro situation allowed manipulation of the ionic environment for the exploration of the mechanisms of drug-actions and for simulations of various pathophysiological conditions (e.g., hypomagnesaemia). Chemical determinations of drug concentrations in the bathing medium (cf. Methods) assured the presence of optimal levels in the slice, especially for dose-response studies.

9.1 Neocortical unit activity and general anaesthesia

Substantial evidence has been provided by many investigators that the spontaneous activity of neocortical neurons, depending on their exact location and animal species investigated, changes with time and according to the animal's degree of alertness (Evarts 1964; Noda and Adey 1970; Webb 1976 a,b; Foster et al. 1982). Moreover, Noda and Adey (1973) were not able to detect any differences between the effects of thiopental anaesthesia and normal sleep on the temporal pattern of discharge from single neurons in the cat associational cortex. Such modulation of neocortical spike activity during anaesthesia has been attributed to anaesthetic actions on the afferent pathways (Mountcastle et al. 1957; Merlis 1965; Berry and Hance 1965; Greenbaum and Merlis 1965). However, Robson (1967) reported that anaesthetics uniformly attenuated the activities of spontaneously firing neocortical units in decerebrate animals. The present investigations of slice preparations revealed that anaesthetic-induced depression of the spontaneous activity is due to direct actions on the cerebral cortical neurons. The reduction in spontaneous activity and the inabilities of the neurons to fire spikes repetitively would tend to disrupt the generation of meaningful spiking patterns and generally impede a transcortical spread of information. Accordingly, the prevailing level of cerebral cortical activation and generation of memory traces would be reduced to the extent that cortical perception of the internal and external environments would be obtunded, resulting in an anaesthetic (unconscious) state.

9.2 Anaesthetic actions: depression of excitation or potentiation of inhibition?

The observed selective interference with the actions of activating transmitters and cortical EPSPs observed in the present studies support the

concept that anaesthetic actions, at least in the neocortex, are due to depression of excitation and not a result of potentiation of inhibition. Enhancement or prolongation of inhibitory processes in the brain is a likely mechanism of action of certain soporific agents, e.g., benzodiazepines or barbiturates in sedative doses (Carlen et al. 1985). On the other hand, the strict intrinsic and extrinsic control of excitatory processes provides a direct and stringent regulation of neuronal excitability in the brain. Neocortical neurons receive sustained activation from intracortical synaptic inputs (cf. Section 3) as well as from ascending pathways, particularly those of the RAS (Matsumura et al. 1988). Hence, an attenuation of excitation particularly in the neocortex provides a plausible cellular basis for the anaesthetic state. This is not easy to reconcile with the non-specific lipid hypothesis that predicts a dissolution of anaesthetic molecules in the lipids of the plasma membrane which produces anaesthesia. The present results, however, are consistent with specific interactions of anaesthetics with hydrophobic proteins (Franks and Lieb, 1987).

The depression of the inhibitory responses to GABA demonstrated in neocortical neurons may underlie the excitatory phenomena that reportedly accompany induction of anaesthesia with Althesin or isoflurane; a logical preventive measure would be the premedication with a GABA-potentiating agent such as a benzodiazepine (Vickers et al. 1981; Richter 1981).

9.3 The anaesthetic state and suppression of consciousness mechanisms

The processes generating consciousness in the cerebral cortex are dependent on a high degree of spontaneous and repetitive activity (Libet 1965; Webb 1983) which is promoted by the actions of ACh (Woody et al. 1978) liberated onto the neurons as a consequence of the sustained activity of the ascending RAS (Szerb 1967; Krnjević 1969; Shute and Lewis 1963; Kimura et

al. 1981). ACh markedly enhances cortical responses to electrical stimulation and synaptically mediated depolarization (Krnjević et al. 1971b; Dodd et al. 1981). The facilitatory action of ACh at muscarinic receptors is slow to develop and confers a greater tendency of the neurons to discharge repetitively (Krnjević 1981, 1989; McLennan 1983). Consequently, the cholinergic RAS tonically raises the overall level of cortical activity and enhances the neuronal responsiveness to "on/off" transmitters like Glu for a fast, specific transfer of information (Puil and Benjamin 1988) between neurons in the conscious state. These actions are probably essential for the maintenance of alertness and contribute to the content of consciousness. A decrease in the background level of cortical activity can "tune" the conscious system to lower degrees of alertness and arousal. Hence, a complete suppression of the ascending cholinergic drive would obtund cortical perception of the sensory modalities arising from subcortical activities and result in an unconscious state (cf. Francesconi et al. 1988). Anaesthetics depress this tone either by blocking the intrinsic level of excitability of the RAS or by attenuating the responses of neocortical neurons to ACh synaptically released by the continuous excitant actions of the reticular formation.

The results of French et al. (1953; cf. also Morruzi and Magoun 1949) and others (Haugen and Melzack 1957; Davis et al. 1957) favoured the concept that anaesthetics interfere with the reticular formation in the processing of sensory inputs to the cortex. The findings were interpreted as being due to the greater number of synaptic connections that, according to Barany (1947), would render the RAS more vulnerable to anaesthetic actions than any other areas of the brain. This tacit assumption has remained the most widely accepted view of the essential central actions of anaesthetics, despite the

relative paucity of critical evidence. Several papers have reported that volatile and intravenous agents equally depress poly- as well as monosynaptic pathways in different areas of the neuraxis (Austin and Pask 1952; Esplin 1963; De Jong et al. 1968). Recent biochemical findings also indicate that the RAS is not a primary site of depressant action of anaesthetics, whereas a generalized or diffuse depression of the cerebral cortex is consistently observed (cf. Introduction).

The major hypothesis developed during the course of these investigations suggests that general anaesthetics depress chemical sensitivities of neocortical neurons to synaptically released activating transmitters thereby attenuating the sustained tone of the RAS and re-setting the cortical arousal system to a lower steady state level of activity which constitutes "the anaesthetic state". Partial verification of the hypothesis was achieved in the dose-response studies which showed that both isoflurane and Althesin selectively depressed the depolarizations evoked by extracellular applications of ACh in anaesthetic concentrations corresponding to those observed in the brain of anaesthetized animals. The attenuation of the fast responses induced by Glu produced lower slopes in the dose-response curves for both agents than the slopes of the dose-response curves characterizing the dose-dependent anaesthetic interference with the excitatory actions of ACh. Hence, both ACh- and Glu-evoked responses are depressed at a given dose with varying degrees of anaesthetic efficacy. By applying this interpretation to the in vivo situation, surgical levels of anaesthesia would be expressed when the tonic excitation produced by ACh in the cerebral cortex is depressed whereas the faster actions of Glu are only slightly affected. Deeper levels of the anaesthetic state would reflect interference with the actions of both activating transmitters.

The above observations are consistent with the recent conceptualization of the anaesthetic state as a cumulative continuum i.e., a series of transformations passing into each other, starting with analgesia and emerging into amnesia and loss of neurological responsiveness (White 1987). In order to explore the validity of the hypothesis, the conscious state was simulated by an in vitro neuronal model of neocortex and the effects of the anaesthetics was investigated therein. Both isoflurane and Althesin completely blocked the excitation sustained by a continuous ACh application with a low current intensity (cf. Fig. 37 and 38). During this time, the superimposed brief Glu-depolarizations evoked at consistent intervals were slightly depressed.

Because these structurally dissimilar anaesthetics produced nearly identical effects in this neuronal model, the following suggestions may be relevant to the mechanism of the anaesthetic state. That is, during anaesthesia, there are pharmacologically specific alterations of the sustained ascending cholinergic input in neurons within the cerebral cortex which resets their responsivities to the synaptically released transmitters. This modulation may be largely contingent on the abilities of anaesthetics to modulate the responsiveness of the postsynaptic receptors of neocortical neurons.

9.4 Ionic mechanisms of anaesthetic action

The effects of isoflurane and Althesin on neocortical neurons in these investigations can be summarized according to five basic observations:

- (1) depression of spontaneous activities of the neurons,
- (2) attenuation of evoked excitatory and inhibitory synaptic transients,
- (3) suppression of the "chemical responsiveness" to the extracellular application of ACh, Glu, NMDA and GABA near the somata,

(4) attenuation of the "pharmacologically isolated" Ca-activated K-conductance (i.e. AHP genesis) and

(5) blockade of Glu and K-evoked increases in the $[Ca^{2+}]_i$.

The most parsimonious explanation for these effects is that the anaesthetic agents interfered with Ca-influx through voltage-dependent and receptor-operated Ca-channels at pre- and postsynaptic sites. Consequently, transmitter release and slow inward postsynaptic Ca-currents responsible for the repetitive spiking activities of the neurons are decreased (cf. Stafstrom et al. 1985) whereas the Ca-dependent responses to ACh, Glu, NMDA and GABA would be attenuated (cf. Inoui et al 1986; Misgeld et al. 1986; Scholfield et al. 1987; Gahwiler and Brown 1987; Murphy and Miller 1988; Benavides et al. 1988; Bormann 1988, Akaike 1990). The dose-dependent suppression of the isolated Ca-activated K-conductance (cf. Section 5) together with the attenuation of Ca-spikes (Heyer and Macdonald 1982) and Ca-currents (Nishi and Oyama 1983; Krnjević and Puil 1988; Gross and Macdonald 1988; Gunderson et al 1988) suggest that voltage-dependent Ca-conductances are significantly reduced by the anaesthetic agents. Furthermore, it may be suggested that an anaesthetic-induced increase in $[Ca^{2+}]_i$ (Krnjević 1974b; 1986), may lead to inactivation of Ca-channels (cf. Eckert and Chad 1984). This would be expected on the basis that anaesthetics simulate the actions of metabolic inhibitors (Quastel 1952, 1962; Godfraind et al. 1971; Krnjević et al 1978a; Vanderkooi et al. 1981) and mimic the effects of reversible hypoxia (cf. Fujiwara et al. 1987; Lablond and Krnjević 1989; Krnjević and Walz 1990). Such an assumption is difficult to reconcile with the present investigations (cf. Section 6) as well as with recent reports that a significant increase in $[Ca^{2+}]_i$ was not observed during applications of anaesthetics (Daniell and Harris 1988; Puil and Baimbridge 1989).

Another possibility is that anaesthetic molecules bind to a cytosolic protein involved in secondary messenger transduction processes (cf. Firestone 1988). One such candidate is protein kinase C (PKC). PKC is a Ca-activated phospholipid-dependent enzyme that phosphorylates a variety of cellular proteins involved in transmembrane signaling. The activity of this enzyme has been firmly linked to signal transduction by the demonstrations that diacylglycerol, one of the initial products of muscarinic and glutamatergic receptor-activated inositol phospholipid hydrolysis (Berridge 1987; Peralta et al. 1988; Sladeczek et al. 1988), greatly increases the affinity of PKC for Ca, thereby causing its activation. In the brain this enzyme which is mostly associated with synapses (Kikkawa et al. 1982) affects receptor sensitivity (DeRiemer et al. 1985) and ion channel activity (Castagna et al. 1982; Conn et al. 1989). Moreover, Firestone and Firestone (1988) demonstrated that anaesthetics can inhibit PKC-activity in isolated rat brain homogenates. A simplified representation of the sequence of events may be schematized as below:

Anaesthetics bind with and inhibit the activity of protein kinase C. Voltage-dependent Ca-channels are no longer efficiently phosphorylated and hence inactivated. Glu (Sugiyama et al. 1987; Sladeczek et al. 1988) and NMDA (Berridge 1987) stimulate the phosphoinositide (PI) hydrolysis cycle thereby increasing intracellular Ca-concentration which activates protein kinase C to phosphorylate the receptor-operated channels. However, the protein kinase C already would be inhibited by the anaesthetic binding. Therefore the inositol phosphate diacylglycerol second messenger pathway that triggers a positive feedback mechanism for a further increase in Ca-influx would be blocked. Muscarinic receptors for ACh in the brain are known to activate PI-hydrolysis (Malenka et al. 1986; Peralta et al. 1988;

Dutar and Nicoll 1988) and their effects are thought to be mediated through a protein kinase in neocortical neurons (Woody and Gruen 1988; cf. El-Fakahany et al. 1989). Despite a lack of understanding of the link between the ionic basis of the muscarinic ACh induced responses and the secondary messenger systems, the presumed inhibition of the PKC by anaesthetic agents can explain the depression of ACh-evoked depolarizations during anaesthesia. In addition, their effects on GABA receptors are probably mediated through a protein kinase enzyme. Indeed the phosphorylation site on the cytoplasmic region of the GABA_A receptor has been identified (Scholfield et al. 1987; Sigel and Baur 1988; Gyenes et al. 1988). An anaesthetic induced inhibition of PKC may be partly responsible for the depression of the GABA-induced depolarizations observed in these investigations.

9.5 Molecular mechanisms of anaesthesia -- the lipid/protein controversy

Because of the difficulty in reconciling dissimilarities in chemical structures of anaesthetic agents with conventional 'lock and key' concepts of drug-receptor interactions, anaesthesiologists have not favoured specific or protein models of anaesthesia. Instead, non-specific or lipid-based models, have enjoyed most of the experimental attention (cf. Janoff 1982; Firestone and Kitz 1988; Firestone 1988). To some extent, the present investigations challenge the assumptions suggesting that the important actions of anaesthetics are induced by non-specific biophysical alterations of membrane properties. Moreover, the similar results obtained using chemically distinct agents (isoflurane and Althesin) indicate that anaesthetics could act specifically at similar hydrophobic sites rather than binding to different protein pockets as suggested earlier (cf. Richards 1975; 1983).

10 SUMMARY AND CONCLUSIONS

1. The effects of general anaesthetics were investigated on pyramidal neurons of layers IV and V of guinea pig neocortex in in vitro slice preparations. The anaesthetic actions were examined, using intracellular recording techniques, on passive and active membrane properties, the synaptic transients evoked by orthodromic stimulation, neuronal responsiveness to iontophoretically applied transmitter substances and postspike afterhyperpolarizing potentials.

2. Two structurally dissimilar anaesthetics, isoflurane - a halogenated ether and Althesin - a steroidal preparation, did not profoundly affect the passive membrane properties of the neurons, except at high doses. The highest concentrations used (isoflurane >2 MAC and Althesin >300 μM) hyperpolarized the neurons by 3-8 mV and increased their input conductance by 10-30%. However, clinically relevant induction doses of both agents produced a sharp, reversible decrease in the spontaneous activities of the neurons. The repetitive firing evoked by orthodromic stimulation or by intracellular current-pulse injections also were strongly inhibited by anaesthetic application.

3. A dose-dependent, reversible depression of neocortical EPSPs was observed on application of isoflurane and Althesin with EC_{50} s of 1 MAC and ~ 50 μM respectively. In order to eliminate a possible shunting effect on EPSPs by the conductances involved in the concomitant IPSPs, a GABA_A -antagonist (bicuculline) was applied together with the anaesthetics. After this IPSP blockade, the anaesthetics still depressed the EPSPs and were effective in reducing the epileptiform activities evoked by bicuculline

during subpial stimulation. The IPSPs also were evoked during blockade of K-conductances by internal Cs-application in order that the effects of anaesthetics could be studied more accurately on the Cl-mediated GABAergic IPSPs. Application of isoflurane or Althesin depressed these IPSPs in a dose-dependent manner.

4. Dendritic applications of acetylcholine, glutamate, NMDA and GABA depolarized the pyramidal neurons. Somatic application of GABA induced only hyperpolarizing responses that were either desensitizing or nondesensitizing and were long-lasting if the duration of GABA application was >10 s. The responses in layers IV and V neurons exhibited tachyphylaxis on frequent application of NMDA or GABA .

5. Isoflurane, Althesin and in some cases, halothane, markedly reduced the depolarizing responses and associated membrane conductance changes evoked by dendritic application of acetylcholine, glutamate, and NMDA. The hyperpolarization evoked by GABA was not affected whereas the GABA evoked depolarization was depressed slightly. The order of depression was acetylcholine > glutamate and NMDA >>> GABA. This selectivity also was assessed from the EC_{50} for the isoflurane-induced depression of the just-maximal responses to ACh which was 0.9 MAC compared with the EC_{50} of 1.9 MAC for the suppression of the glutamate-responses. The selectivity was less pronounced in the case of Althesin actions where the EC_{50} s were 75 μ M and 90 μ M for the depression of both transmitter responses respectively.

6. In order to investigate pathophysiological mechanisms of anaesthetic actions during hypomagnesaemia, a clinical condition that is known to be associated with increased anaesthetic requirements, the actions of anaesthetics were determined on the neuronal responses to putative transmitters

during simulated hypomagnesia in vitro. The depolarizations evoked by acetylcholine or GABA were blocked by lowering external $[Mg^{2+}]$ in a decremental manner. Hypomagnesia reversed the effects of isoflurane and Althesin on the GABA and glutamate responses, i.e., GABA actions were more depressed than those of glutamate.

7. A striking observation in this study was the dose-dependent suppression by anaesthetics of the postspike afterhyperpolarization (AHP). The same effect was observed under conditions of IPSP-blockade by bicuculline, which alleviated the AHPs (produced through activation of a Ca- activated K-conductance) from the hyperpolarizing, Cl-dependent influence due to GABAergic activity.

8. Using Fura-2 microspectrofluorimetry, the actions of isoflurane were studied on glutamate- and K-evoked increases in $[Ca^{2+}]_i$. The glutamate evoked increases in $[Ca^{2+}]_i$ under conditions where actions at NMDA- or quisqualate- receptors would be favoured, were attenuated by isoflurane. The volatile anaesthetic also depressed the voltage-dependent increases in $[Ca^{2+}]_i$ induced by extracellular K applications under conditions where Na-spike genesis was blocked by TTX.

9. These investigations provide evidence that the depression of neuronal excitability in neocortex induced by isoflurane and Althesin is due to attenuation of excitation rather than potentiation of inhibition. It is suggested that these agents decrease the level of cortical activity by interfering specifically with the actions of transmitters involved in mechanisms of behavioural arousal. The depression of the "isolated" post-synaptic Ca-conductance which is crucial to the generation of the AHPs together with attenuations of the EPSPs, IPSPs and the glutamate or

K-induced increases in $[Ca^{2+}]_i$, suggest that the anaesthetic actions on the processes generating consciousness in the neocortex may result from interference with Ca-influx through voltage-dependent and receptor-operated Ca-channels.

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