Presynaptic and postsynaptic inhibition of spinal motoneurones

A thesis submitted for the degree of Doctor of Philosophy
of The Australian National University
December 1990

by

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STATEMENT

All the work described in this thesis is original. The experiments were performed by me between May 1987 and July 1990 under the supervision of Prof. Steve Redman. During this time a number of presentations were made at scientific meetings in Australia. The following abstracts were published in conjunction with these presentations.

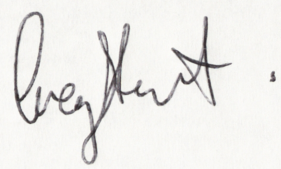
Stuart, G.J. & Redman, S.J. (1988). Voltage dependence of reciprocal inhibitory post-synaptic currents in cat spinal motoneurones. Proceedings of the Australian Physiological and Pharmacological Society. 19: 44*P*.

Stuart, G.J. & Redman, S.J. (1989). Synaptic currents generated by Ia reciprocal inhibitory interneurones in cat spinal motoneurones. Neuroscience Letters Supplement. 34: S157.

Stuart, G.J. & Redman, S.J. (1990). The role of $GABA_A$ and $GABA_B$ receptors in presynaptic inhibition of Ia EPSPs in cat spinal motoneurones. Proceedings of the Australian Neuroscience Society. 1: 132.

In addition, the following paper related to some of the work described in this thesis has been published:

Stuart, G.J. & Redman, S.J. (1990). Voltage dependence of Ia reciprocal inhibitory currents in cat spinal motoneurones. Journal of Physiology. 420: 111-125.



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ACKNOWLEDGEMENTS

First I must thank my supervisor Prof. Steve Redman for all his help, encouragement and guidance over the last three and a half years. I would particularly like to thank Steve for always finding the time to answer my numerous questions.

I would also like to thank Prof. Curtis for discussions on the iontophoretic work described in Chapter four and for his generous gifts of various compounds.

In addition, I would like to acknowledge the invaluable technical assistance given to me by Garry Rodda and Terrina Thompson during the preparation of experiments. Thanks also to Rosemary, Annie, Suse and Prof. Curtis for proof-reading various chapters of this thesis and to everybody in the department for making these last years enjoyable and interesting.

Also thanks to Suse for the blue rice and other distractions.

Last, but not least, I would like to thank my parents for bringing me up in an environment that enabled me to realise my own goals.

SUMMARY

This thesis describes an *in vivo* study of presynaptic and postsynaptic inhibition in the lumbar spinal cord of the cat.

Postsynaptic inhibition was examined using current and voltage clamp techniques to record inhibitory postsynaptic potentials (IPSPs) and currents (IPSCs) in motoneurones during reciprocal inhibition. IPSPs evoked in motoneurones during reciprocal inhibition were blocked by the iontophoretic application of strychnine hydrochloride providing strong evidence that this inhibition is mediated by the neurotransmitter glycine. A single electrode voltage clamp was used to record IPSCs in motoneurones generated by a population of Ia reciprocal inhibitory interneurones (population IPSCs), and by individual Ia reciprocal inhibitory interneurones (unitary IPSCs). Both population and unitary IPSCs were recorded over a range of membrane potentials and the voltage dependence of their amplitude and time course determined.

Population IPSCs had an average rise time of 0.51 ± 0.02 ms (±S.E.M., $n=22$) and decayed exponentially with an average time constant of 0.99 ± 0.04 ms at resting membrane potentials (37°C). Unitary IPSCs, recorded following spike triggered averaging from single Ia reciprocal interneurones, had amplitudes of 120 to 220 pA and an average rise time of 0.40 ± 0.06 ms ($n=5$). The decay of these unitary currents was exponential with an average time constant of 0.82 ± 0.07 ms at resting membrane potentials (37°C). If the rate of decay of these unitary IPSCs is determined by the open time of the channels activated following the release of glycine, then the average channel open time of glycine activated channels at this synapse will be ~0.8 ms at resting membrane potentials. The time course of IPSCs was unaffected by either pentobarbitone or α-chloralose at concentrations necessary for deep anaesthesia.

The peak synaptic current varied linearly with the membrane potential over the range -90 to -30 mV, and had an average reversal potential of -80.7 ± 1.5 mV (±S.E.M., $n=6$) when measured using KCH_3SO_4 filled electrodes. The reversal potential for the IPSC was used to calculate $[Cl^-]_i$ from the Nernst equation. This was estimated to be 6.5 mM assuming that the inhibitory synaptic current was carried purely by Cl^- ions. The possibilty that HCO_3^- ions contribute to the ionic current that flows during reciprocal inhibition is discussed.

The rate of decay of both population and unitary IPSCs was exponentially dependent on the membrane potential, the decay time constant increasing *e*-fold for a 91 mV depolarisation. This result was independent of $[Cl^-]_i$ or the magnitude of the inhibitory synaptic current and was interpreted as a voltage dependence of the glycine channel open time. The voltage dependence of the channel open time, together with the direct effect of the membrane potential on the peak inhibitory current, will act to enhance the strength of reciprocal inhibition as the motoneurone membrane potential approaches threshold.

The average peak conductance of unitary IPSCs was 9.1 ± 1.7 nS ($n=5$). This was approximately twenty times smaller than the average peak conductance of population IPSCs (167 ± 30 nS; $n=11$) suggesting than on average twenty Ia reciprocal interneurones synapse with each motoneurone. Assuming a mainstate glycine single channel conductance of 46 pS (Bormann, Hamill & Sakmann, 1987), it was calculated that following activation of a single Ia reciprocal interneurone approximately 200 postsynaptic Cl^- channels will be opened at the synaptic contacts formed by with a motoneurone. The number of Cl^- channels opened by the release of a single quantum of glycine at each release site could be ten times smaller than the above figure of 200, i.e. a single quantum of glycine probably opens as few as 20 postsynaptic Cl^- channels at this synapse.

A detailed study was made of the pharmacology and mechanisms of presynaptic inhibition of synaptic transmission at the Ia afferent/motoneurone synapse.

The role of $GABA_A$ and $GABA_B$ receptors in presynaptic inhibition was studied by the application of specific $GABA_A$ and $GABA_B$ receptor antagonists by iontophoresis during intracellular recording in motoneurones of presynaptic inhibition of group Ia afferent excitatory postsynaptic potentials (EPSPs).

The $GABA_A$ receptor antagonist bicuculline methochloride (BMC) was found to block presynaptic inhibition of both compound and unitary Ia EPSPs by up to 85%. BMC also substantially reduced, and occasionally abolished, the late part of the IPSP evoked in motoneurones during presynaptic inhibition. The early part of this IPSP was found to be sensitive to strychnine.

The $GABA_B$ receptor antagonist 2-OH-saclofen was used to investigate whether $GABA_B$ receptors were involved in presynaptic inhibition. To first establish that 2-OH-saclofen could antagonize $GABA_B$ receptors, its ability to antagonize the effect of the $GABA_B$ agonist baclofen was examined. 2-OH-saclofen was found to antagonize the reduction in amplitude of Ia EPSPs produced by baclofen and also to cause a slight reduction in presynaptic inhibition of Ia EPSPs. In contrast, baclofen caused a large decrease in the amplitude of Ia EPSPs, but was relatively ineffective in reducing presynaptic inhibition.

It was concluded that at the Ia afferent/motoneurone synapse presynaptic inhibition is mediated primarily through the activation of $GABA_A$ receptors. The activation of $GABA_B$ receptors appears to play only a minor role in presynaptic inhibition at this synapse. This contrasts with the relative ease with which baclofen, presumably via the activation of $GABA_B$ receptors, can reduce transmitter release from Ia afferent terminals and suggests that the receptors which are activated by baclofen are predominantly extrasynaptic.

The interaction between paired-pulse facilitation and presynaptic inhibition of Ia EPSPs was also investigated. The main finding from this study was that paired-pulse facilitation was enhanced during presynaptic inhibition. This finding is analogous to that seen at other synapses *in vitro* when the probability of transmitter release is lowered by reducing the extracellular calcium or raising the extracellular magnesium concentration.

The results from this study provide further evidence that presynaptic inhibition is associated with a reduction in the probability of transmitter release. By analogy with the effects of reduced calcium influx on paired-pulse facilitation at other synapses, the results support the idea that presynaptic inhibition is associated with a decrease in calcium influx into Ia afferent terminals.

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Chapter one:

GENERAL INTRODUCTION

Inhibition of synaptic transmission in the mammalian central nervous system (CNS) can be mediated in two different ways. One way decreases the depolarising effects of excitatory synaptic currents, and the other decreases transmitter release from excitatory nerve terminals. The first type of inhibition is called 'postsynaptic inhibition' and involves an increase in membrane conductance of a postsynaptic cell to an ion (or ions) with a reversal potential more negative than the membrane potential required to reach threshold. The second type of inhibition is called 'presynaptic inhibition' and is thought to occur following a reduction in the probability of transmitter release. This introduction gives a brief overview of some of the properties of these two types of inhibition.

Historical perspective

The first documented observation of inhibition in the nervous system dates back to 1845 when the Weber brothers demonstrated that stimulation of the vagus nerve could stop the heart. By the early 1900's Sherrington had greatly extended our knowledge of inhibition in the CNS, particularly in the spinal cord. One of the fundamental inhibitory reflex pathways described by Sherrington was what he termed 'reciprocal innervation'. This inhibition, which is now usually termed reciprocal inhibition, describes the inhibition of motoneurones innervating antagonistic muscles by the activation of stretch receptors in the agonist muscle. The properties of the synaptic currents generated in motoneurones during reciprocal inhibition will be described in detail in Chapter three of this thesis.

Although the importance of inhibition in the CNS was well established by the 1940's, the mechanisms involved in mediating inhibition were not. There were two schools of thought; one believed that inhibition was mediated electrically, the

other believed inhibition was mediated by chemical means. Despite a considerable amount of evidence for the chemical hypothesis (Loewi demonstrating the chemical nature of vagal inhibition in 1921), Brooks and Eccles (1947) came out in favour of an electrical hypothesis for inhibition called the Golgi-cell hypothesis. They proposed that a Golgi cell (or interneurone) was interposed between the excitatory fibres and the inhibited cell in such a way that following excitation of the Golgi cell, outward current from the synaptic knobs (terminals) of its axon would lead to a hyperpolarisation of the area of contact with the postsynaptic cell. There would be an accompanying depolarisation of equal magnitude distributed diffusely over the remainder of the postsynaptic cell and no net change in membrane potential. This somewhat far-fetched idea was abandoned after the first intracellular recordings showed that postsynaptic inhibition was associated with a direct hyperpolarisation of the postsynaptic cell (Brock, Coombs & Eccles, 1952*a*,*b*). This left the chemical hypothesis to explain inhibition. Brock, Coombs and Eccles (1952*a*,*b*) proposed that inhibition was mediated by a specific inhibitory transmitter which caused an active hyperpolarisation of the postsynaptic cell. This hyperpolarisation is known as the inhibitory postsynaptic potential, or IPSP.

Postsynaptic inhibition

The first direct evidence of a change in membrane properties during postsynaptic inhibition was obtained by Fatt and Katz (1953) at the crustacean neuromuscular junction. They showed that during postsynaptic inhibition, which often occurred in the absence of postsynaptic hyperpolarisation, there was an increase in the rate of decay of the end-plate potential. Fatt and Katz also found that depolarisation of the postsynaptic cell could unmasked an IPSP, whereas hyperpolarisation could reverse the IPSP into a depolarising potential. These findings were explained by an increase in membrane conductance of the postsynaptic cell to an ion, or ions, that were at equilibrium at a membrane potential close to the resting membrane potential of crustacean muscle. (It was

later shown by Dudel and Kuffler (1961*b*) that some of the inhibition observed by Fatt and Katz was presynaptic.)

A subsequent, more detailed study by Coombs, Eccles and Fatt (1955*a*) on postsynaptic inhibition in motoneurones concluded that "the inhibitory transmitter substance that is liberated from the inhibitory presynaptic terminals acts on inhibitory patches of the post-synaptic membrane and greatly increases their permeability to all ions below a critical size". They also suggested that this increase in permeability was to both K^+ and Cl^- ions.

Mechanisms of postsynaptic inhibition

These early studies lead to the idea that postsynaptic inhibition reduces the excitability of the postsynaptic cell via an increase in membrane conductance which is often accompanied by a hyperpolarisation. An increase in membrane conductance will shunt excitatory synaptic currents, decreasing the ability of excitatory inputs to bring the cell to threshold, whereas hyperpolarisation of the postsynaptic cell shifts the membrane potential away from that required for initiation of an action potential.

The ability of postsynaptic inhibition to shunt excitatory synaptic currents will depend not only on the size and duration of the inhibitory conductance, but also on its location and on the location of the excitatory input. Theoretical studies on the passive cable properties of nerve cells, originally developed by Rall in the early sixties, suggest that in general postsynaptic inhibition is most effective in reducing all excitatory inputs to the postsynaptic cell, independent of their location, if the inhibitory synapses are located on or near the soma (Rall, 1964; Jack, Noble & Tsien, 1975). This is exactly where most postsynaptic inhibition in the mammalian CNS is found to be located. For postsynaptic inhibition to be selective for a particular excitatory input it is best located directly adjacent to that excitatory input (Jack *et al.* 1975). This type of selective postsynaptic inhibition is most suited to excitatory inputs which are generated on the most distal parts of the dendritic tree.

Inhibitory transmitters and their actions

The classical inhibitory transmitters in the mammalian CNS are the amino acids γ-aminobutyric acid (GABA) and glycine. Their identification as putative inhibitory transmitters began with the work of Curtis and Watkins (1960) who showed that these amino acids reduced the excitability of neurones in the spinal cord. It was later found that the distribution of GABA and glycine in the spinal cord was consistent with a role of these amino acids as inhibitory transmitters (Aprison, Graham, Baxter & Werman, 1965; Aprison & Werman, 1965). Convincing evidence came from the observations that when applied iontophoretically GABA and glycine could mimic the postsynaptic response of the naturally released transmitter (Werman, Davidoff & Aprison, 1966; Krnjevic & Schwartz, 1967). For example, the glycine induced hyperpolarisation of motoneurones reverses at the same potential as IPSPs generated during reciprocal inhibition and shows the same dependence on the intracellular chloride concentration (Curtis, Hosli, Johnston & Johnston, 1968).

Glycinergic inhibition can be selectively blocked by strychnine (Bradley, Easton & Eccles, 1953; Curtis *et al.* 1968; Akaike & Kaneda, 1989), whereas classically GABA mediated inhibition is selectively blocked by picrotoxin or bicuculline (van der Kloot, Robbins & Cook, 1958; Curtis, Duggan, Felix & Johnston, 1971). More recent studies have shown that there are at least two types of pharmacologically distinct GABA receptors. The classical bicuculline-sensitive $GABA_A$ receptor and the bicuculline-insensitive $GABA_B$ receptor. $GABA_B$ receptors can be selectively activated by (−)-baclofen, a *β-p*-chlorophenyl derivative of GABA (Bowery, Dobel, Hill, Hudson, Shaw & Turnbull, 1979; Hill & Bowery, 1981; Bowery, Dobel, Hill, Hudson, Shaw, Turnbull & Warrington, 1981) and blocked by the antagonists phaclofen (Kerr, Ong Prager, Gynther & Curtis, 1987; Dutar & Nicoll, 1988*a*) and the more potent 2-OH-saclofen (Kerr, Ong, Johnston, Abbenante & Prager, 1988; Curtis, Gynther, Beattie, Kerr & Prager, 1988). A new $GABA_B$ antagonist which can cross the blood brain barrier has also recently been found (Olpe, Karlsson, Pozza, Brugger, Steinmann, Riezen, Fagg,

Hall, Froestl & Bittiger, 1990). In addition to $GABA_B$ receptors, some recent reports have suggested that there may be other types of bicuculline-insensitive GABA receptors (Muller & Misgeld, 1989; Solis & Nicoll, 1990).

Due to similarities in the primary structures of the $GABA_A$ and glycine receptors it has been suggested that these ligand-gated receptors form part of a superfamily of ion channel/receptor proteins (Schofield, Darlison, Fujita, Burt, Stephenson, Rodriguez, Rhee, Ramachandran, Reale, Glencorse, Seeburg & Barnard, 1987; Grenningloh, Rienitz, Schmitt, Methfessel, Zensen, Beyreuther, Gundelfinger & Betz, 1987; Barnard, Darlison & Seeburg, 1987). The $GABA_B$ receptor is thought to belong to a different superfamily of receptor proteins in which the receptor is coupled to a GTP-binding protein (Hill, Bowery & Hudson, 1984; Holz, Rane & Dunlap, 1986; Andrade, Malenka & Nicoll, 1986; Dolphin & Scott, 1987; Birnbaumer, Abramowitz & Brown, 1990).

Activation of both $GABA_A$ and glycine receptors causes a transient increase in membrane conductance to Cl^- ions (Bormann, Hamill & Sakmann, 1987). This increase in membrane conductance is selective for Cl^- ions and impermeable to K^+ ions (Bormann *et al.* 1987). The increase in Cl^- conductance produced by GABA and glycine are thought to be very similar, as they show almost identical single channel sub-conductance states and ion permeability (Bormann *et al.* 1987). However, the main conductance state of GABA activated Cl^- channels (30 pS) is thought it be different from that of glycine activated Cl^- channels (46 pS; Bormann *et al.* 1987).

The conformational change leading to the opening of these channels is thought to require the binding to two molecules of GABA or glycine to the $GABA_A$ and glycine receptors (Akaike, Inoue & Krishtal, 1986; Akaike & Kaneda, 1989). The K_D for binding of GABA to the $GABA_A$ receptor is approximately 10 μM (Akaike *et al.* 1986), whereas glycine is thought to bind to its receptor with a K_D of approximately 90 μM (Akaike & Kaneda, 1989). The $GABA_A$ and glycine receptors differ, not only in their selectivity for different agonist and antagonists, but also because the increase in Cl^- conductance activated

by $GABA_A$ receptors can be enhanced by clinically important drugs such as benzodiazepines and barbituates (Olsen, 1982). This potentiation is thought to occur due to an increase in the $GABA_A$ single channel open time and burst duration (Study & Barker, 1981; MacDonald, Rogers & Twyman, 1989).

Activation of $GABA_B$ receptors by either baclofen or GABA, in the presence of bicuculline, decrease a voltage dependent calcium conductance in the cell bodies of dorsal root ganglion cells (Dunlap & Fischbach, 1978, 1981; Dunlap, 1981*a,b*; Deisz & Lux, 1985; Dolphin & Scott, 1986, 1987; Robertson & Taylor, 1986). This effect has been shown to be mediated through a pertussis toxin sensitive GTP-binding protein (Holz *et al.* 1986; Dolphin & Scott, 1987) and may involve a protein kinase C-dependent phosphorylation of voltage dependent calcium channels (Rane & Dunlap, 1986; Rane, Walsh, McDonald & Dunlap, 1989). The reduction in calcium conductance in the presence of GABA or baclofen is thought to be due to a transmitter-induced change in the voltage-dependence with which these channels are opened (Bean, 1989; Grassi & Lux, 1989).

The activation of $GABA_B$ receptors on nerve terminals could cause a reduction in calcium influx during the presynaptic action potential. As the relationship between the intracellular calcium concentration in nerve terminals and transmitter release is thought to be highly nonlinear (Dodge & Rahamimoff, 1967; Augustine, Charlton & Smith, 1985), a small reduction in calcium influx would be expected to cause a substantial reduction in transmitter release. Although it has not been directly demonstrated, the ability of baclofen to cause a reduction in a voltage dependent calcium conductance on nerve terminals can probably explain the presynaptic actions of baclofen in the CNS (see Discussion in Chapter four). A recent report has shown that the reduction in calcium conductance produced by baclofen can be partially blocked by phaclofen, but curiously not by 2-OH-saclofen (Huston, Scott & Dolphin, 1990).

Activation of $GABA_B$ receptors can also cause an increase in a potassium conductance in many central neurones (Newberry & Nicoll, 1985; Inoue, Matsuo & Ogata, 1985; Gahwiler & Brown, 1985). This effect has also been shown to be

mediated through a pertussis toxin sensitive GTP-binding protein (Andrade *et al.* 1986; Dutar & Nicoll, 1988*b*). It has been suggested that the increase in potassium conductance produced by GABA and baclofen may be mediated via a direct coupling of the GTP-binding protein to the potassium channel (Andrade *et al.* 1986). The increase in potassium conductance produced by baclofen can be completely blocked by $GABA_B$ antagonists (Duter & Nicoll, 1988*a*,*b*; Solis & Nicoll, 1990). A potassium dependent IPSP has been reported in many regions of the brain which can also be blocked by $GABA_B$ antagonists (Dutar & Nicoll, 1988a; Soltesz, Haby, Leresche & Crunelli, 1988; Hasuo & Gallagher, 1988; Lambert, Harrison, Kerr, Ong, Prager & Teyler, 1989). This IPSP is thought to be mediated by the activation of $GABA_B$ receptors.

A recent report also suggests that GABA and baclofen can shift the voltage dependence of inactivation of a transient potassium current, recorded in the cell bodies of cultured hippocampal neurones, to more depolarised potentials (Saint, Thomas & Gage, 1990). If this occurs in nerve terminals it could shorten the duration of the presynaptic action potential, reducing calcium influx and transmitter release.

Beside GABA and glycine, there are also a number of other putative transmitters which have been shown to decrease neuronal excitability. These include acetylcholine, norepinephrine, dopamine, serotonin, adenosine and the neuropeptides enkephalin and somatostatin (reviewed by Nicoll, Malenka & Kauer, 1990). The inhibitory effect of these transmitters is mediated through an increase in a potassium conductance which is qualitatively similar to that produced by GABA acting at $GABA_B$ receptors. In many cases this increase in potassium conductance is thought to be mediated through a GTP-binding protein. Acetylcholine, norepinephrine, adenosine and the neuropeptides dynorphin and neuropeptide Y can also reduce voltage dependent calcium conductances in dorsal root ganglion cells and so could potentially reduce calcium influx into nerve terminals and reduce transmitter release. For many of these putative transmitters this effect has been shown to be mediated through a pertussis toxin sensitive GTP-

binding protein and may involve a protein kinase C-dependent phosphorylation of voltage dependent calcium channels.

Inhibitory postsynaptic currents

The time course of the conductance change underlying an IPSP can be measured by a technique introduced by Cole in 1949 called the voltage clamp. Accurate measurement of this conductance change can only be obtained if the membrane through which the ionic current flows is isopotential with the voltage clamp. Under these conditions the current that flows during an IPSP will be identical to the current required to clamp the membrane potential. This current can be measured and is called the inhibitory postsynaptic current, or IPSC. The time course of the IPSC will be the same as the time course of the increase in membrane conductance underlying the IPSP.

Araki and Terzuolo (1962) were the first to record IPSCs in mammalian central neurones. They showed that the IPSC recorded in motoneurones during reciprocal inhibition was brief, lasting only a few milliseconds, and reversed at approximately -80 mV. This reversal potential was the same as had been previously reported for the reversal potential of IPSPs recorded during reciprocal inhibition (Coombs *et al.* 1955*a*). The brief nature of the conductance change underlying these IPSPs was in agreement with earlier attempts to measure the time course of this conductance change by investigating the interaction between these IPSPs and either EPSPs or antidromically activated action potentials (Coombs *et al.* 1955*b*; Curtis & Eccles, 1959). A study on the change in impedance during reciprocal inhibition also concluded that the increase in membrane conductance underlying IPSPs generated during reciprocal inhibition lasted only a few milliseconds (Smith, Wuerker & Frank, 1967).

If the decay of the inhibitory synaptic current is determined by the average open time of the synaptically activated channels, then a description of the time course of these currents, and effects which alter this time course, will provide

valuable information about the properties of the membrane channels underlying the increase in conductance which occurs during postsynaptic inhibition.

In the mammalian CNS, $GABA_A$ mediated IPSCs have been studied *in vitro* in slices of hippocampus (Collingridge, Gage & Robertson, 1984; Sakmann, Edwards, Konnerth & Takahashi, 1989; Edwards, Konnerth & Sakmann, 1990; Ropert, Miles & Korn, 1990) and neocortex (Kriegstein & LoTurco, 1990). $GABA_B$ receptor mediated IPSCs have also recently been recorded *in vitro* from CA1 neurones in the hippocampus (Davies, Davies & Collingridge, 1990). It is doubtful if the time course of these $GABA_B$ mediated IPSCs reflects the true time course of the synaptic current, as the synapses generating these IPSCs are thought to be located on the distal dendrites and so would not be isopotential with a somatically located voltage clamp.

Glycinergic IPSCs have previously been investigated in goldfish Mauthner cells (Faber & Korn, 1987, 1988) and lamprey Muller cells (Gold & Martin, 1983*a*), however have not been characterised in the mammalian CNS. Araki and Terzuolo (1962) recorded IPSCs evoked during strychnine-sensitive reciprocal inhibition in the mammalian spinal cord, but did not analyse the time course of these IPSCs or dependence on the membrane potential. Only a brief report on some properties of glycinergic IPSCs recorded in spinal cord slices *in vitro* has been published (Konnerth, Takahashi, Edwards & Sakmann, 1988). A detailed analysis of the properties of glycinergic IPSCs evoked during reciprocal inhibition in the mammalian spinal cord *in vivo* is described in Chapter three.

Presynaptic inhibition

Inhibition of synaptic transmission can also be mediated via a reduction in the amount of excitatory transmitter released from nerve terminals. In the mammalian CNS this type of inhibition was first described by Frank and Fuortes in 1957. They reported that prior conditioning stimulation could reduce the amplitude of monosynaptic Ia EPSPs recorded in cat spinal motoneurones without causing a hyperpolarisation or a change in the motoneurone's excitability. It was

concluded that this inhibition occurred presynaptically. In a later report Frank (1959) suggested the alternative explanation that the reduction in Ia EPSPs could occur via postsynaptic inhibition "at a distance from the cell body where the effect of the inhibitory volley alone cannot be 'seen' by the microelectrode." Frank termed this type of inhibition remote inhibition.

Work by Eccles in the early 60's supported the original hypothesis of Frank and Fuortes that the reduction in Ia EPSPs occurred via presynaptic inhibition. This inhibition was correlated with the presence of the dorsal root potential, which was found to have a similar duration as the time course of the inhibition of Ia EPSPs (Eccles, Eccles & Magni, 1961). These findings lead Eccles, Eccles & Magni (1961) to postulate that "presynaptic depolarization results in EPSP depression because it depresses the size of the presynaptic impulse and hence decreases the liberation of excitatory transmitter". Possible mechanisms involved in mediating presynaptic inhibition will be discussed later.

A morphological basis for presynaptic inhibition came from observations by Gray (1962) of the presence of axo-axonic synapses (axon terminals forming synaptic contacts with other axon terminals) in an electron microscopic study of cat spinal cord. Axo-axonic synapses have since been shown to occur on Ia afferent terminals in the ventral horn (Conradi, 1969; Fyffe & Light, 1984), on Ia and Ib afferent terminals in Clark's column (Walmsley, Wieniawa-Narkiewicz & Nicol, 1987) and in the dorsal horn (Barber, Vaughn, Saito, McLaughlin & Roberts, 1978; Zhu, Sandri & Alkert, 1981). Axo-axonic synapses have not, however, been found on nerve terminals of neurones which have their cell bodies within the CNS.

Additional evidence for presynaptic inhibition was supplied by Eide, Jurna and Lundberg (1968) who showed that the same conditioning stimulation which depressed Ia EPSPs did not affect a monosynaptic EPSP evoked in motoneurones by stimulation of descending spinal tracts.

Frank's suggestion that the inhibition of Ia EPSPs could be produced by remote inhibition gained some support following the findings of Kellerth (1968) and Cook and Cangiano (1972) that the conditioning stimulation used to produced

presynaptic inhibition could also caused postsynaptic inhibition. This postsynaptic inhibition was often associated with a small IPSP in motoneurones and a small or undetectable change in membrane conductance. It was concluded that these properties were consistent with a remote dendritic origin of this postsynaptic inhibition (Cook & Cangiano, 1972). However, as was pointed out by Cook and Cangiano, this postsynaptic inhibition could not account for all of the reduction in Ia EPSPs produced by the conditioning stimulation.

Recent evidence for presynaptic inhibition comes from the work of Clements, Forsythe and Redman (1987) using quantal analysis of the fluctuations in amplitude of unitary Ia EPSPs before and after the conditioning stimulus used to evoke presynaptic inhibition (see also Kuno, 1964). To avoid problems associated with postsynaptic inhibition, quantal analysis was only performed on somatic or near somatic unitary EPSPs evoked at latencies of 70 to 100 ms after the conditioning stimulus. Due to the brief nature of the synaptic current underlying somatic Ia EPSPs (Finkel & Redman, 1983*a*), the peak voltage of a somatic Ia EPSP will be determined largely by the flow of current across the membrane capacitance and so will be insensitive to any increase in membrane conductance that may occur during the conditioning stimulation used to evoke presynaptic inhibition (see Gage & McBurney, 1973; Edwards, Hirst & Silinsky, 1976). It has been shown that the peak amplitude of a somatic Ia EPSP will be unaffected by an increase in the membrane conductance, unless the membrane conductance is increased to more than ten times its original valve (Edwards, Redman & Walmsley, 1976*c*). Quantal analysis of EPSPs before and after conditioning showed that the conditioning stimulation decreased the number of quanta contributing to the inhibited EPSP without changing the quantal size, thereby confirming the original proposal of Frank and Fuortes (1957) that the reduction in amplitude of Ia EPSPs occurs via presynaptic inhibition.

Pharmacology of presynaptic inhibition

Before reviewing the literature on the pharmacology of presynaptic inhibition, it should be stressed that while it is now generally accepted that in the mammalian spinal cord presynaptic inhibition of transmitter release from primary afferent fibres is mediated by the release of GABA at axo-axonic synapses, much of the evidence for this is indirect.

Evidence for the role of GABA as the transmitter mediating presynaptic inhibition comes from a number of different sources. In the first study on the pharmacology of presynaptic inhibition in the mammalian CNS, Eccles, Schmidt and Willis (1963*a*) showed that picrotoxin could reduce both the 'prolonged inhibition' of ventral root reflexes and the dorsal root potential evoked by the conditioning stimulation. Picrotoxin has since been shown to antagonize the $GABA_A$ activated increase in Cl^- conductance by a direct block of the Cl^- channel (Gallagher, Higashi & Nishi, 1978; Akaike, Yakushiji, Tokutomi & Carpenter, 1987). The more specific $GABA_A$ receptor antagonist bicuculline has also been shown to reduce both prolonged inhibition and dorsal root potentials (Curtis *et al.* 1971).

GABA was found to depolarize primary afferent fibres, mimicking the depolarising dorsal root potential (Eccles *et al.* 1963*a*). In agreement with this finding GABA has also been found to depolarize the cell bodies of dorsal root ganglion cells (Feltz & Rasminsky, 1974; Nishi, Minota & Karczmar, 1974; Gallagher *et al.* 1978). This depolarisation, which has a reversal potential of approximately -30 mV, is associated with an increase in Cl^- conductance and can be blocked by $GABA_A$ antagonists. Of perhaps more physiological significance is the finding that GABA can also cause a bicuculline-sensitive depolarisation of the terminals of primary afferent fibres (Gmelin & Cerletti, 1976; Sastry, 1979*a*; Curtis & Lodge, 1982). This depolarisation is presumably caused by the outward movement of Cl^- ions following activation of $GABA_A$ receptors on primary afferent terminals. These findings suggests that a high intracellular Cl^-

concentration is maintained in the cell bodies and nerve terminals of dorsal root ganglion cells by an *inwardly* directed Cl^- pump.

Eccles, Schmidt and Willis (1963*a*) also found that prolonged inhibition and the dorsal root potential were not reduced by strychnine, and that the barbiturate sodium pentobarbitone could enhance prolonged inhibition and increase the size of dorsal root potentials. The significance of this later finding, while not appreciated at the time, can be explained by the ability of barbituates to potentiate the effect of GABA acting at $GABA_A$ receptors. More recent studies have also shown that prolonged inhibition and dorsal root potentials can be enhanced by benzodiazepines (Haefely & Polc, 1986).

Additional pharmacological evidence for the proposal that GABA mediates presynaptic inhibition comes from the finding that depletion of GABA from the CNS by inhibitors of the production of glutamate decarboxylase (GAD), the enzyme responsible for the synthesis of GABA, reduce both prolonged inhibition and dorsal root potentials (Bell & Anderson, 1972). Immunocytochemical evidence for a role of GABA in presynaptic inhibition has also been demonstrated, with both GAD (a proposed marker for GABA) and GABA being localised in nerve terminals presynaptic to terminals of primary afferent fibres (Barber *et al.* 1978; Magoul, Onteniente, Geffard & Calas, 1987; Maxwell, Christie, Short & Brown, 1990).

These studies provide good pharmacological and morphological evidence that GABA is the transmitter released from axo-axonic synapses during presynaptic inhibition. There is also good evidence that the release of GABA during presynaptic inhibition causes a bicuculline-sensitive depolarisation of the terminals of primary afferent fibres.

While there have been many studies on the pharmacology of prolonged inhibition of ventral root reflexes, to date there have been no pharmacological studies on presynaptic inhibition. While much of the evidence suggests that prolonged inhibition of ventral root reflexes is mediated, at least in part, through the activation of $GABA_A$ receptors, this inhibition is complicated by the

postsynaptic inhibition that occurs in conjunction with presynaptic inhibition (Kellerth, 1968; Cook & Cangiano, 1972). There is clearly a need for a reassessment of the pharmacology of presynaptic inhibition in the mammalian CNS using more direct techniques to monitor presynaptic inhibition. This has become even more important following the discovery over the last ten years that GABA can activate at least two different types of GABA receptors, $GABA_A$ and $GABA_B$. While it has been traditionally thought that presynaptic inhibition is mediated through the classical $GABA_A$ receptor, the possibility that GABA reduces the release of transmitter from primary afferent terminals by acting at bicuculline-insensitive $GABA_B$ receptors must be considered. Chapter four of this thesis involves a pharmacological investigation into the role of $GABA_A$ and $GABA_B$ receptors in presynaptic inhibition of intracellularly recorded Ia EPSPs.

Mechanisms of presynaptic inhibition

The mechanisms involved in mediating presynaptic inhibition in the mammalian CNS are poorly understood. Fundamental to our understanding of presynaptic inhibition is the knowledge of what type(s) of receptors mediate this inhibition. As discussed above, there is now considerable evidence which suggests that presynaptic inhibition of synaptic transmission is mediated by the release of GABA at axo-axonic synapses. However, we know very little about how GABA causes presynaptic inhibition.

Presynaptic inhibition of transmitter release from the terminals of Ia afferents has been shown to be associated with a reduction in the number of quantal events contributing to the inhibited EPSP without a change in the quantal size (Kuno, 1964; Clements *et al.* 1987). It is most likely that this occurs due to reduced calcium influx into the nerve terminals of Ia afferent fibres during presynaptic inhibition. Some evidence for this is give in Chapter five. At many synapses the relationship between calcium influx into nerve terminals and transmitter release is highly nonlinear (Dodge & Rahamimoff, 1967; Augustine *et al.* 1985). If the same relationship exists at primary afferent terminals, then a small

reduction in calcium influx could lead to a substantial reduction in transmitter release. The question becomes how does synaptically released GABA cause a reduction in calcium influx into primary afferent nerve terminals during presynaptic inhibition?

Calcium influx into nerve terminals is thought to enter through voltage activated calcium channels (VACCs). These channels, which are believed to be present on nerve terminals close to the site of transmitter release, are thought to be activated by the voltage change associated with action potential invasion of the nerve terminal. There are three ways that presynaptic inhibition could reduce calcium influx through VACCs. Firstly, presynaptic inhibition could reduce the amplitude and/or duration of the presynaptic action potential, causing a subsequent reduction in activation of VACCs. Secondly, prior depolarisation of the presynaptic terminal could cause inactivation of VACCs, reducing the number of VACCs available to be activated during the presynaptic action potential and thirdly, presynaptic inhibition could directly affect activation of VACCs.

A reduction in the amplitude and/or duration of the presynaptic action potential could occur following an increase in membrane conductance of primary afferent terminals (Segev, 1990). As the relationship between the size of the presynaptic action potential and transmitter release is highly nonlinear (Hagiwara & Tasaki, 1958; Takeuchi & Takeuchi, 1962; Katz & Miledi, 1967), a small decrease in the amplitude of the presynaptic action potential would be expected to cause a marked reduction in transmitter release. A steep relationship is also thought to exist between the duration of the presynaptic action potential and transmitter release (Katz & Miledi, 1967; Dudel, 1984). As an increase in the membrane conductance has been observed during primary afferent depolarisation (Curtis & Lodge, 1982; Padjen & Hashiguchi, 1983; Curtis, Gynther & Malik, 1986), it is likely that presynaptic inhibition is mediated, at least in part, by this mechanism.

It is also possible that the depolarisation of primary afferent terminals that occurs in conjunction with presynaptic inhibition (Wall, 1958; Eccles, Magni &

Willis, 1962; Gmelin & Creletti, 1976; Curtis & Lodge, 1982) could lead to the inactivation voltage dependent sodium channels located on presynaptic terminals or at the last node(s) of Ranvier. Voltage activated sodium channels on the cell bodies of dorsal root ganglion cells are 50% inactivated at a membrane potential of ~-40 mV (Carbone & Lux, 1986). As the conditioning stimulus used to evoke presynaptic inhibition is thought to depolarize the terminals of primary afferent fibres by at least 5 to 10 mV from a resting membrane potential of ~-70 mV (Eccles *et al.* 1962; Padjen & Hashiguchi, 1983; Bagust, Forsythe & Kerkut, 1985), this depolarisation could cause some inactivation of these channels. This would be expected to decrease the amplitude of the presynaptic action potential and reduce transmitter release. At many synapses depolarisation of the nerve terminal has been shown to decrease the size of the presynaptic action potential and reduce transmitter release (Hagiwara & Tasaki, 1958; Takeuchi & Takeuchi, 1962; Eccles, Kostyuk & Schmidt, 1962*b*; Hubbard & Willis, 1968; Llinas, 1968).

The depolarisation of primary afferent terminals could also cause inactivation of VACCs. This would depend on both the size of the depolarisation that occurs in primary afferent terminals during presynaptic inhibition and on the voltage dependence of inactivation of the VACCs which contribute to the calcium influx that triggers release.

Three different types of VACCs have been identified in the cell bodies of dorsal root ganglion cells (Fox, Nowycky & Tsien, 1987*a*,*b*; Kostyuk, Shuba & Savchenko, 1988). A low-threshold, transient VACC (T-type), which is activated at membrane potentials more positive that ~-70 mV, and two high-threshold VACCs. A non-inactivating high-threshold VACC (L-type), activated at membrane potentials more positive than~-10 mV, and a slowly inactivating VACC (N-type), activated at membrane potentials more positive than ~-30 mV. Recent studies suggest that vertebrate nerve terminals contain only high-threshold VACCs (Lindgren & Moore, 1989; Lemos & Nowycky, 1989; Yawo, 1990). Consistent with this finding, there is some evidence that in the mammalian CNS transmitter release is mediated primarily through the activation of high-threshold N-type

VACCs (Kamiya, Sawada & Yamamoto, 1988; Rascol, Duter, Potier & Lamour, 1990). N-type VACCs are thought to be 50% inactivated at membrane potentials of between -60 and -70 mV (Fox *et al.* 1987*a,b*). A depolarisation of 5 to 10 mV during presynaptic inhibition could cause an increase the fraction of N-type VACCs which are inactivated, and therefore could reduce calcium influx and transmitter release.

Finally, the transmitter released during presynaptic inhibition could reduce the activation of VACCs via an action on the channels themselves. This effect could occur through the activation of $GABA_B$ receptors on primary afferent terminals. As mentioned earlier, activation of these receptors has been shown to reduce a voltage dependent calcium conductance in the cell bodies of dorsal root ganglion cells.

The contribution of $GABA_A$ and $GABA_B$ receptors to these different possible mechanisms underlying presynaptic inhibition is discussed in detail in Chapter four.

Chapter two:

GENERAL METHODS

This chapter deals with the general methods which were used in the experiments described in Chapters three, four and five. Each of these experimental chapters also has its own methods section which deals with specific details related to particular experimental work described in that chapter.

The preparation

All experiments were preformed on male or female adult cats weighing between 1.5 and 3.5 kg. Cats were initially anaesthetised either by an intra-peritoneal injection of sodium pentobarbitone (40 mg kg^{-1}) or by breathing a mixture of halothane and nitrous oxide with oxygen. The trachea, left common carotid artery and left cephalic vein were then cannulated, and anaesthesia continued by either intravenous injection of sodium pentobarbitone in animals initially anaesthetised with pentobarbitone (approximately 6mg/hour, or as required), or by α-chloralose (initial dose 60 mg kg^{-1}) in animals initially anaesthetised with halothane and nitrous oxide. Ten to twenty minutes were given for this dose of α-chloralose to take full effect and then the halothane/nitrous oxide anaesthesia was slowly discontinued and anaesthesia maintained by intravenous injection of α-chloralose (5 mg kg^{-1} as required). In addition, some experiments in Chapter three were performed on animals initially anaesthetised with halothane and nitrous oxide followed by an intercollicular decerebration.

Blood pressure, heart rate, and end-tidal CO_2 levels were monitored continuously throughout the experiment. Body temperature was measured by a thermocouple place behind the scapula and maintained at 37±1 °C by a feedback controlled electric blanket. A bilateral pneumothorax was induced and the animals were artificially respired. End tidal CO_2 levels were maintained at 4%. The

neuromuscular blocking agent pancuronium bromide (Pavulon, Organon, Holland) was given to maintain stability of recordings; supplementary doses were given only after recovery from the previous dose, and only after the level of anaesthesia had been checked by corneal and toe pinch reflexes. The level of anaesthesia was also monitored by the level of muscular tone in the jaw and the degree of pupillary constriction.

The left hindlimb was dissected to expose various muscle nerves for stimulation. As the particular muscle nerves exposed depended on the experimental requirements a description of the different hindlimb nerve dissections will be given in the methods section of each experimental chapter.

Following the hindlimb dissection the cat was mounted in a rigid steel frame and a laminectomy was performed to expose the spinal segments from L4 to S1. The dura was cut longitudinally and a dural sling constructed by gentle retraction of the cut edges of the dura using 5-0 silk sutures. A silver/silver chloride electrode was secured to the exposed muscle next to the spinal cord to act as the indifferent electrode and this was connected to the equipment ground. The spinal cord and surrounding tissue were then covered with warm liquid paraffin and heated by radiant heat. A separate leg pool was formed and the hindlimb muscle nerves were covered with warm liquid paraffin. In some experiments different ventral roots on the left side of the spinal cord were cut and mounted on stimulating electrodes.

Stimulation procedures

All electrical stimuli to leg muscle nerves and ventral roots were applied via bipolar stimulating electrodes using isolated stimulators (Digitimer, Model DS2). Stimulus pulses of 0.2 ms duration were used. The isolated stimulators were triggered with either a time sequence generator (designed by Prof. Redman) or Digitimers (Model D4030).

Recording procedures

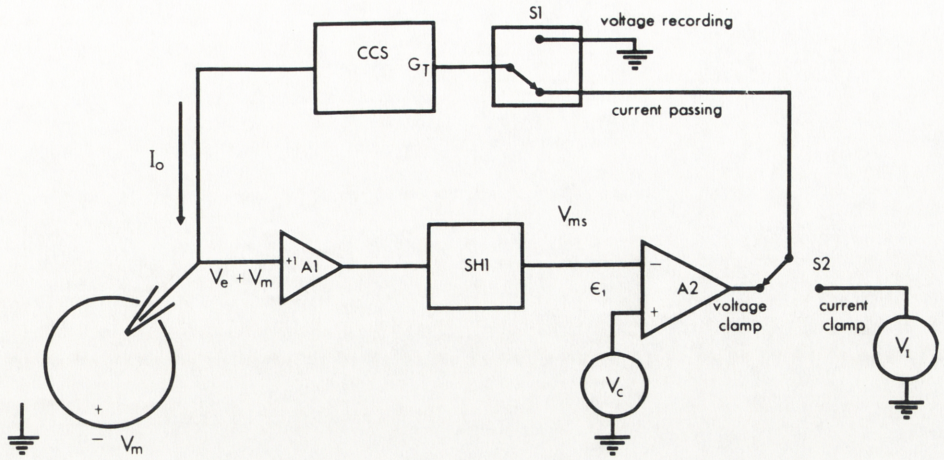
All intracellular and extracellular recording was performed using Axoclamp 2A amplifiers and headstages (Axon instruments). Intracellular, intra-axonal and extracellular recordings were made using conventional microelectrodes with the aid of a stepping motor-drive. These were pulled from 1.5 mm (o.d) thin walled glass capillary tubing (Clark Electromedical Instruments, GC150TF-15) using a vertical puller (Narishige Scientific). For intracellular recording from motoneurones and extracellular recording from interneurones the tips of these electrodes were broken back to approximately 1 μm by advancing them into a glass bead under high magnification. These electrodes typically had resistances of approximately 10 MΩ when filled with 2M KCH_3SO_4. Intra-axonal recordings were performed with electrodes with unbroken tips, usually with resistances of 20 to 30 MΩ when filled with 3M KCl.

A ball electrode was routinely used to record the arrival of the afferent volley from the cord dorsum. This ball electrode was also used to gently retract the L7 dorsal root medially, allowing access to the motoneurone pools in the ventral horn of the L7/S1 spinal segments.

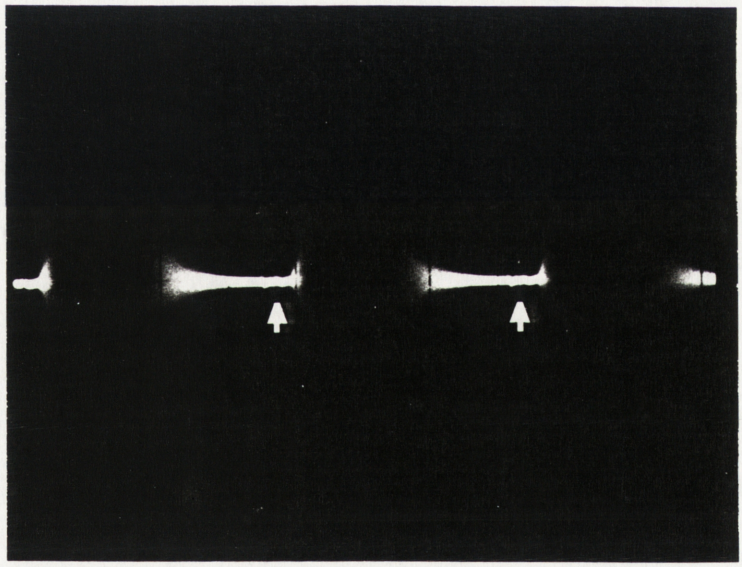
Voltage and current clamping procedures and electrodes

Motoneurones were voltage or current clamped using a discontinuous single electrode voltage or current clamp. A block diagram of the single electrode voltage clamp (SEVC) used is shown in Fig. 2.1*A*. This figure is taken from Finkel and Redman (1985). A brief description of the operation of the SEVC is outlined below. The membrane voltage (V_m), combined with the voltage drop across the electrode resistance and capacitance (V_e) was fed into a unity-gain high-speed amplifier (A1) and then into a sample-and-hold device (SH1). The sampled voltage (V_{ms}) was then compared to the command voltage (V_c) in a differential amplifier (A2). The output of this amplifier, which is proportional to the difference between the sampled voltage and the command voltage, was then used to drive a controlled current source (CCS) if the switch S1 was in the current passing

A



B



10 µs

Fig. 2.1. Single electrode voltage clamp (SEVC). *A*, block diagram of the SEVC circuit from Finkel and Redman (1985). *B*, multiple sweeps of the input to the sample and hold device during SEVC (switching at 20 kHz). The voltage can be seen to settle back to the baseline before it is sampled by the clamp circuitry (at the white arrows). Vertical calibration voltage (arbitrary units).

position. The output of the CSS was regulated by the gain G_T. A current I_o will then be applied to the cell via the electrode. The sampled voltage (V_{ms}) and the current I_o were recorded and give the membrane voltage and current. The input to the sample and hold device was also monitored to determine if it had settled back to the baseline before being sampled, as shown in Fig. 2.1*B* (white arrows).

The protocol used during voltage clamping experiments is outlined below. After obtaining a stable intracellular recording the amplifier was switched to discontinuous current clamp mode (DCC). A few nanoampers of constant hyperpolarising current were then injected into the cell and, while monitoring the input to the sample and hold device (Fig. 2.1*B*), the capacitance neutralization and switching rate were adjusted to their optimum settings (Finkel & Redman, 1985). The potentiometer which controls the holding potential during voltage clamp was adjusted to the resting membrane potential. The gain control on the CCS (G_T) and the anti-alias filter were set to zero. The amplifier was then switched to SEVC. The clamp gain (G_T) was then increased as much as possible (usually to between 8 and 25 nA/mV) while monitoring the input to the sample and hold device. Adjustment of the capacitance neutralization was made if necessary. The membrane voltage and current were then averaged. The membrane potential was voltage clamped at different membrane potentials by adjustment of the holding potential to the desired level.

The voltage clamp work described in Chapter three required specially designed electrodes which could switch at fast rates (up to 30 kHz) while clamping the membrane of neurones several millimetres below the surface of the spinal cord. These electrodes needed to have low resistance and capacitance. The electrode resistance was reduced by using an electrode of large tip diameter. The transmural electrode capacitance was reduced by shielding the electrode almost to its tip and connecting this shield to the unity gain output of the voltage clamping amplifier. This unity gain output is related to the output of the CSS such that whenever the current I_o is passed into the voltage clamping electrode a current equal in magnitude will be passed to the driven shield. This driven shield must be

electrically isolated from the bathing solution or tissue due to the large voltage changes which will occur during current passing (up to ±15 V).

The design and manufacture of these electrodes is described in detail by Finkel and Redman (1983*b*) and only a brief summary will be given here. Manufacture started with a conventional electrode which was coated with a very thin gold film using a vacuum sputterer. The electrode was filled with electrolyte and the gold etched back from the tip approximately 150 μm by advancing the electrode under microscopic control into a droplet of a non-fuming gold etch (KI solution) (Fig. 2.2*A*). A second electrode was pulled using thin walled soft glass tubing (Kimax 51) with a taper that exceeded the taper of the gold coated electrode by approximately 30% over the first 10 mm back from the tip. The tip of this electrode was broken back to give an outside diameter of ~10 μm, and a 10 mm length of the tapered region back from the tip snapped off. This small glass jacket was fixed to a metal rod attached to a micromanipulator using modelling clay. The gold coated electrode (attached to another micromanipulator) was then advanced through the glass jacket, using a binocular dissecting microscope for guidance, until it fitted tightly. The gold shield usually ended about 20 μm from the tip of the glass jacket. If it ended any less than this the jacket was discarded and another one used. Once a tight fit was established the gold coated electrode was withdrawn a few hundred microns (Fig. 2.2*B*) and a droplet of fast setting epoxy glue (5 minute Araldite, Ciba-Geigy) introduced to the tip of the glass jacket from were it moved rapidly back down the inside of the glass jacket via capillary action. Once the glue had moved back approximately 100 μm from the tip of the glass jacket the gold coated electrode was advanced through the glue until a tight seal was again formed between the gold coated electrode and the glass jacket (Fig. 2.2*C*). Excess glue was removed by placing a droplet of glue over the electrode tip and slowly withdrawing the droplet. Once dry, the broad end of the glass jacket was secured to the inner electrode and a fine wire was then wound around the gold coated recording electrode and electrical continuity with the gold coat formed by a drop of silver paint. This wire was connected to the unitary gain output of the

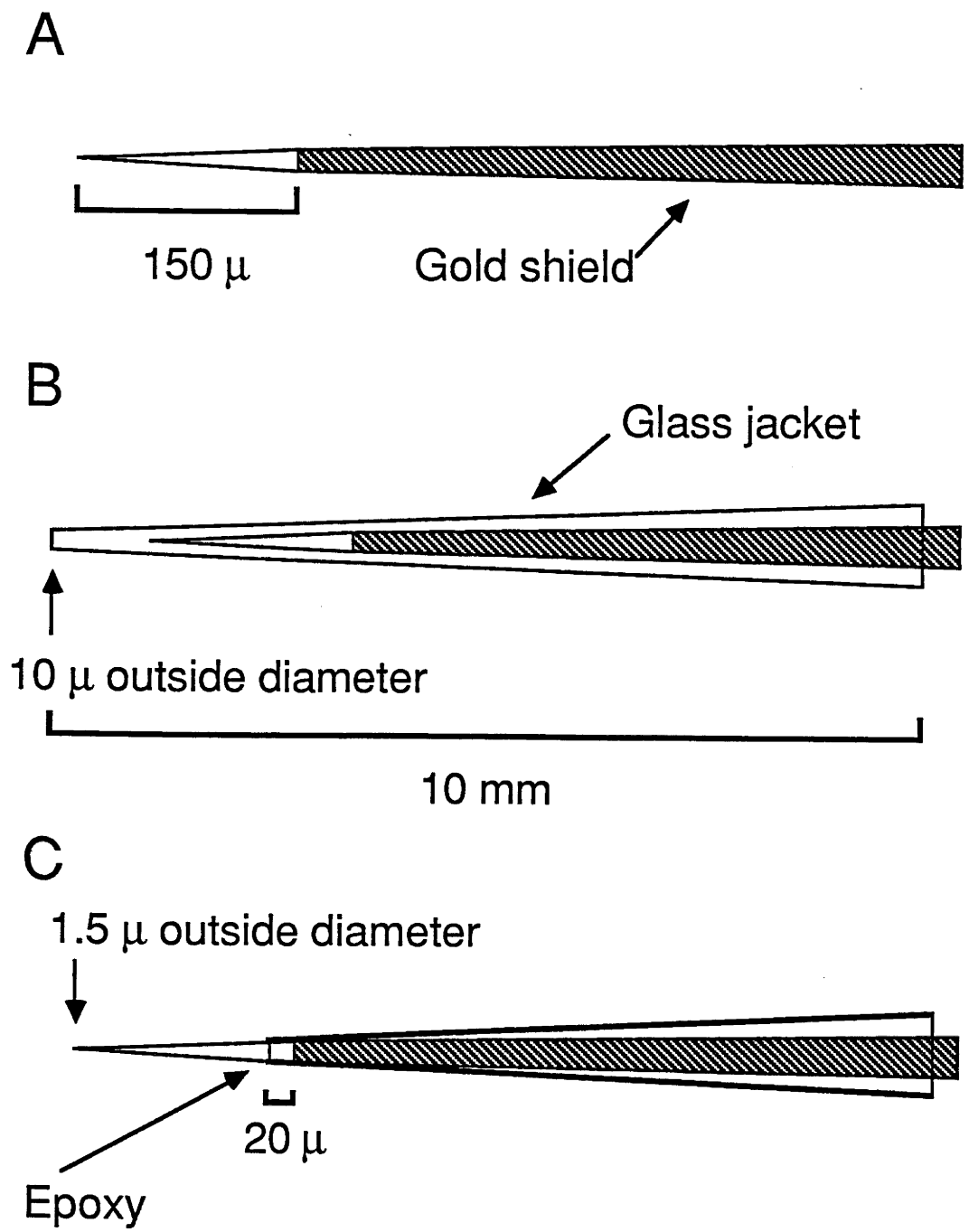


Fig. 2.2. A schematic diagram of the construction of a single barrel voltage clamping electrode. *A*, shows a conventional microelectrode which has been gold coated using a vacuum splutterer and then had the gold film etched back 150 μm from the tip. *B*, shows placement of the glass jacket over the gold coated electrode. *C*, shows the tip region of the completed SEVC electrode.

microelectrode amplifier. The tip of the microelectrode was then either bevelled or broken back to give a tip diameter of approximately 1.5 μm. The resistance of these electrodes was around 5 MΩ when filled with either 2M KCH_3SO_4 or 3M KCl. Finally, the microelectrode tip was immersed several millimetres below the surface of a saline bath and the insulation resistance between the shield and the bath was measured. Microelectrodes with insulation resistances of less than 1000 MΩ were discarded.

Iontophoretic electrodes and current passing procedures

The iontophoretic work described in Chapters three and four required intracellular recording from motoneurones during extracellular iontophoresis of different compounds. The electrodes used contained two extracellular iontophoretic barrels and were largely based on the "parallel" micropipettes used and designed by Prof. D.R. Curtis in the late sixties (Curtis, 1968). A brief description of the construction of these electrodes is given below.

First, a double barrelled iontophoretic electrode was pulled from double barrel 1.5 mm (o.d.) thick walled capillary tubing (Clark Electromedical Instruments, 2GC150F-15). The tip of this electrode was broken back to approximately 6 μm, giving an outside diameter of 2 to 3 microns per barrel and the final 5 mm of this electrode bent to an angle of approximately 15° to the axis of the shaft using a micro-forge. The micro-forge consisted of a thin piece of platinum wire suspended tightly between two metal rods and connected to a variable transformer (Zenth). A conventional electrode was then pulled as described previously and the two microelectrodes were axially aligned using separate micro-manipulators with the aid of a small surface mirror, angled at 45°, and a binocular microscope. The double barrelled extracellular iontophoretic electrode was offset approximately 100 μm back from the tip of the intracellular recording electrode (Fig 2.3). Once the two electrodes were aligned correctly a droplet of fast setting epoxy glue was placed near the bend of the iontophoretic electrode and moved several hundred microns towards the tips of the electrodes by capillary action.

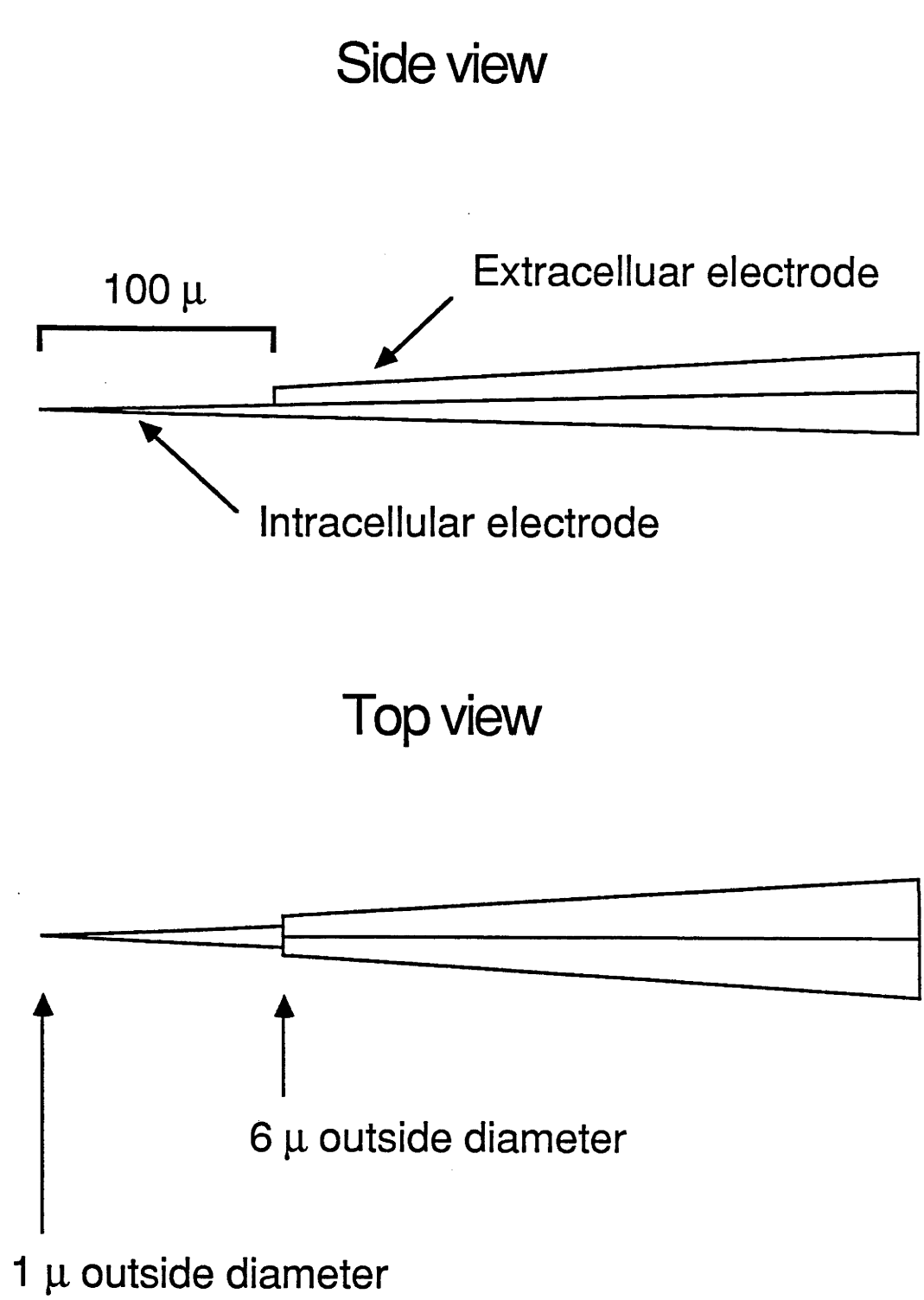


Fig. 2.3. A schematic diagram of the side and top views of the tip region of an iontophoretic electrode used for intracellular recording from motoneurones during extracellular iontophoresis of different compounds.

Small lengths of electrode glass were glued across both microelectrode barrels to provide mechanical support. Once the glue was dry, alignment of the two electrodes in both the horizontal and vertical directions was checked under high magnification. If aligned correctly the intracellular recording electrode was filled with 2M KCH_3SO_4 and the tip broken back to approximately 1 μm.

Iontophoretic barrels were filled with either (−)-baclofen (5 mM in 150 mM NaCl, pH 3.0), bicuculline methochloride (BMC, 10 mM in 150 mM NaCl, pH 3.0), 2-OH-saclofen (20 mM in 80 mM NaCl, pH 3.0) or strychnine hydrochloride (5 mM in 150 mM NaCl, pH 3.0). The pH of these solutions was adjusted with weak hydrochloric acid. The baclofen, BMC and strychnine were gifts from Prof. D.R. Curtis and the 2-OH-Saclofen was purchased from Tocris Neuramin (England). All compounds were ejected as cations. Iontophoretic electrodes typically had resistances of 50 MΩ and retaining currents of -20 nA were routinely used. The magnitude of ejection currents was measured (and is quoted) in nanoamperes (nA). The coupling resistance between the iontophoretic barrels and the intracellular recording barrel was less than 10 kΩ. Hence, the passage of a cationic current of 100 nA resulted in a coupling artifact on the intracellular electrode of less than 1 mV in the depolarising direction.

The intracellular barrel was connected to one input of a two channel microelectrode amplifier (Axoclamp 2A). One of the extracellular iontophoretic barrels was connected to the other input of the same amplifier. This extracellular iontophoretic electrode was used for both iontophoretic application of compounds (the Axoclamp 2A amplifier can pass up to ±100 nA of constant current) and extracellular recording. The other iontophoretic barrel was connected to a more powerful current passing unit. This current passing unit was made by the author based on a design by Prof. D.R. Curtis. It could be used to pass currents of up to ±600 nA, with a working input voltage range of ±90 V (a circuit diagram and more detailed description of this current passing unit is given in Fig. 2.4).

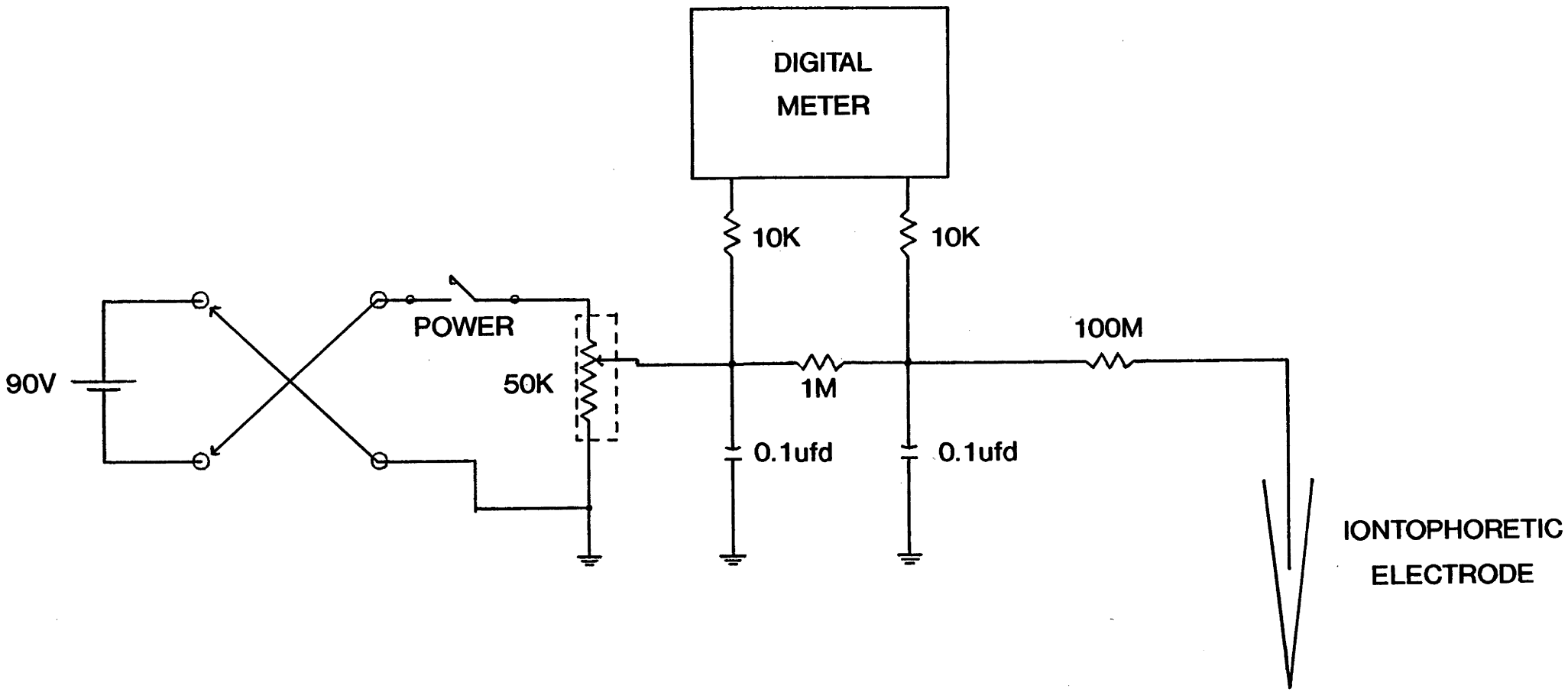


Fig. 2.4. Circuit diagram of the current passing unit used during iontophoresis. A variable 50 MΩ resistor was used as a voltage divider to change the voltage across a 100 MΩ resistor in series with the electrode resistance (typically 50 MΩ). The maximum voltage across this resistance was ±90V (supplied by ten 9V batteries in series) giving iontophoretic currents of up to ±600 nA. This current was measured using a digital volt meter (±2V scale, 3 decimal places) as the voltage drop across a 1 MΩ resistor in series with the 100 MΩ and electrodes resistance.

Data acquisition and analysis

High gain voltage and current signals were filtered by a single pole filter at 3 kHz, passed through a sample-and-hold device and displayed differentially on a digital oscilloscope (Tektronix 5223). A low gain DC recording of the membrane potential was displayed on another channel of the same oscilloscope and also monitored on a chart recorder. The high gain voltage and current signals were then passed via the analog output of the oscilloscope to a 12 bit A/D board in a microcomputer (Cromemco, System One). The collection programs for the microcomputer were written by Dr. F. Edwards. Records were digitised at either 10 or 20 kHz and displayed on a monitor (Type 611, Tektronix). After each experiment the data were transferred to a Mainframe computer (Pyramid 90X, and later Sun 4, 280) for off-line analysis. The software programs for data analysis and graphics plotting were written in Fortran mainly by Dr. J. Clements with some modifications made by the author.

Chapter three:

VOLTAGE DEPENDENCE OF INHIBITORY GLYCINERGIC SYNAPTIC CURRENTS IN SPINAL MOTONEURONES

Introduction

Glycine is considered to be an important inhibitory neurotransmitter in the mammalian CNS (Aprison & Werman, 1965; Werman *et al.* 1966; Curtis *et al.* 1968; van den Pol & Gorcs, 1988). However, while glycine activated channels have been studied in cultured and acutely isolated neurones (Barker & McBurney, 1979; Barker, McBurney & MacDonald, 1982; Hamill, Bormann & Sakmann, 1983; Bormann *et al.* 1987; Krishtal, Osipchuk & Vrublevsky, 1988; Akaike & Kaneda, 1989), *Xenopus* oocytes (Gundersen, Miledi & Parker, 1984, 1986), lamprey Müller cells (Gold & Martin, 1982, 1983*a,b*), the goldfish Mauthner cell (Faber & Korn, 1982, 1987, 1988) and recently in mammalian spinal cord slices, *in vitro* (Konnerth *et al.* 1988), there is still very little known about the channels opened by the synaptic release of glycine in the mammal, *in vivo*.

The synaptic current generated at synapses mediating reciprocal inhibition of spinal motoneurones provides an opportunity to study glycine channels in the intact mammalian CNS. In the cat this inhibition is mediated by a disynaptic pathway (Eccles, Fatt & Landgren, 1956; Eccles & Lundberg, 1958) involving Ia reciprocal interneurones (Hultborn, Jankowska & Lindstrom, 1971*a,b*; Jankowska & Roberts, 1972*a,b*). Primarily on the basis of antagonism by strychnine, the inhibitory neurotransmitter released by these interneurones is considered to be glycine (Bradley *et al.* 1953; Curtis *et al.* 1968). In addition, electrophysiological and histological studies suggest that Ia reciprocal interneurones synapse predominantly on the soma of motoneurones (Fig. 3.1; Smith *et al.* 1967; Burke, Fedina & Lundberg, 1968, 1971; Jankowska & Roberts, 1972*b*; Fyffe, 1981, 1987),

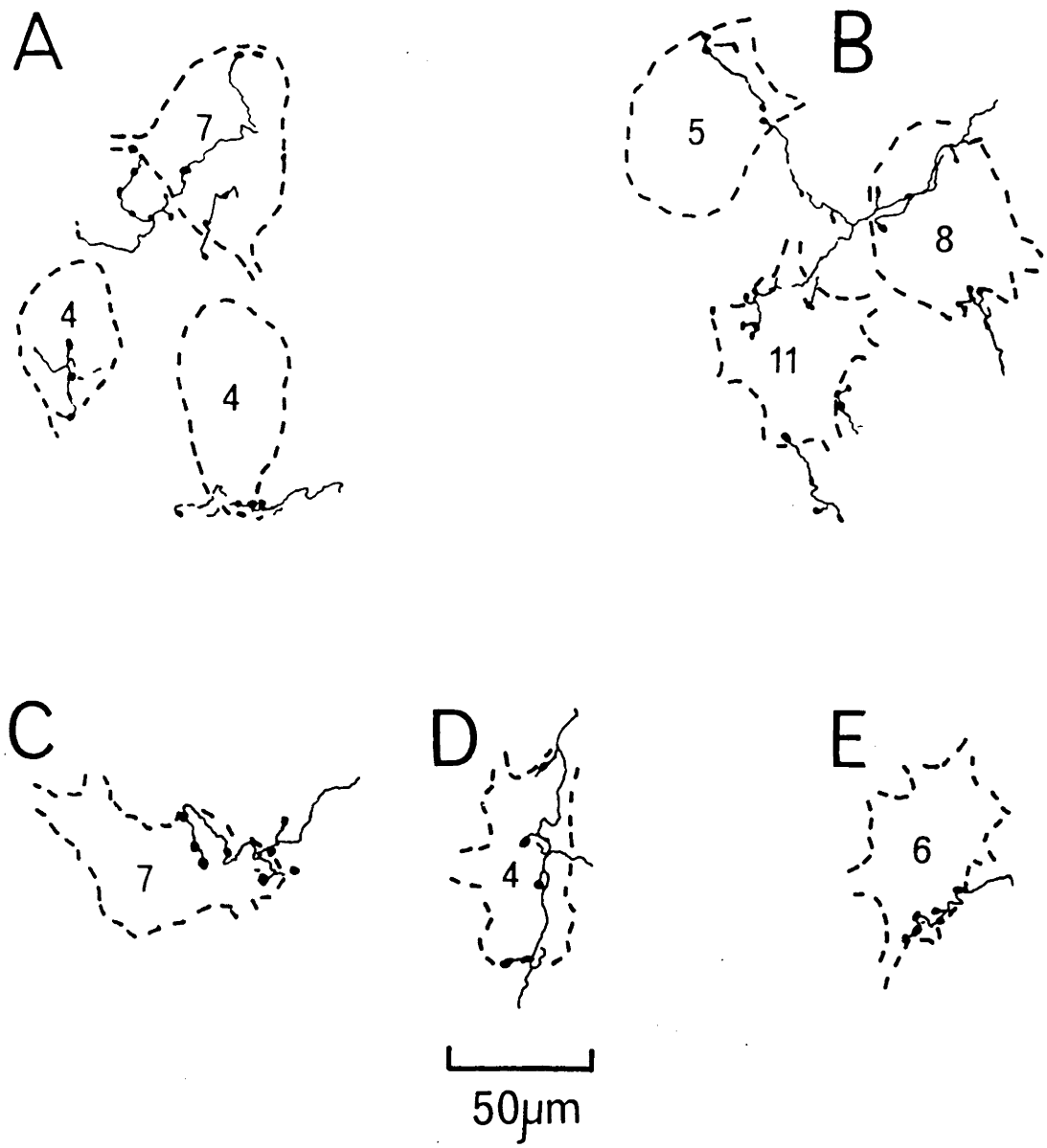


Fig. 3.1. Locations of synaptic contacts made by axon collaterals of a HRP stained Ia reciprocal interneurone onto counterstained motoneurones in the lumbar spinal cord of the cat. The dashed lines represent motoneurone cell bodies and the numbers represent the presumed number of synaptic contacts made by the Ia reciprocal interneurone with each motoneurone. It is clear that juxtasomatic connections predominate in this system. This figure is taken from Fyffe, 1981.

thereby allowing the undistorted measurement of synaptic currents by a somatically located voltage clamp. In the present study inhibitory postsynaptic currents generated by single Ia reciprocal interneurones, and by a population of these interneurones, have been recorded in motoneurones over a range of membrane potentials and the voltage dependence of their amplitude and time course determined.

Methods

The experiments were performed on adult cats anaesthetised with either pentobarbitone or α-chloralose, or on unanaesthetised decerebrated preparations. Initial surgery was performed as described in Chapter two. The hindlimb was dissected as follows. The nerves to posterior biceps and semitendinosus (PBSt) in the left hind limb were separated from surrounding tissue, cut distally and mounted on a stimulating electrode. All other branches of the sciatic nerve were cut and crushed. The ipsilateral quadriceps (Q) muscle nerve was also freed from the surrounding tissue, cut distally, and mounted on a buried electrode. In experiments where individual Q Ia reciprocal interneurones were identified, the L6 ventral root was cut and mounted on a stimulating electrode.

Recording

Intracellular recordings were made from antidromically identified PBSt motoneurones (resting membrane potentials more negative than -55 mV; spike height greater than 60 mV) using single barrel voltage clamping electrodes previously described in Chapter two. These electrodes were filled with either 2M KCH_3SO_4 or 3M KCl.

Experiments involving iontophoresis used iontophoretic electrodes and current passing procedures also described in Chapter two. One of the two extracellular iontophoretic barrels was filled with a 5 mM solution of strychnine hydrochloride and connected to the current passing unit. The other extracellular

barrel and the intracellular microelectrode were both filled with 2M KCH_3SO_4 and were connected to different headstages of the same microelectrode amplifier.

A schematic diagram of the experimental set-up is shown in Fig. 3.2. Inhibitory postsynaptic potentials (IPSPs) were evoked in PBSt motoneurones by stimulation of the Q muscle nerve at 1.5 x group I threshold (T, as determined from the cord dorsum potential), giving a compound or population IPSP. Unitary IPSPs were recorded by spike triggered averaging from the extracellular spikes of an identified Q Ia reciprocal interneurone (see below, Jankowska & Roberts, 1972*a,b*). The motoneurone membrane potential was then voltage clamped and the current record averaged to reveal the underlying population or unitary inhibitory postsynaptic current (IPSC).

Unitary IPSCs were obtained in the following way. Extracellular recordings were obtained from interneurones dorsomedial to antidromically identified Q motoneurones using a second microelectrode filled with a 1M solution of the excitatory amino acid DL-homocysteic acid (DLH) in 1M NaCl, and were identified as Q Ia reciprocal interneurones by the following criteria: 1) monosynaptic, low threshold activation by the Q muscle nerve, 2) inhibition by Renshaw cells activated following stimulation of the L6 ventral root and 3) ability to follow Q nerve stimulation at frequencies greater than 100 Hz (Fig. 3.3; Hultborn *et al.* 1971*b*). A single barrel voltage clamping electrode was then placed in the previously identified PBSt motoneurone pool and brief current pulses (up to 10 μA, 100 μs duration), generated by an isolated constant current source, were used in an attempt to antidromically activate the identified interneurone. Once antidromic activation was achieved the position of the microelectrode was adjusted until a location was found where the identified interneurone could be antidromically activated with only 1 to 3 μA of current. The SEVC electrode was then switched from stimulation mode to record mode by a remote electronic relay and PBSt motoneurones in this region were impaled and the extracellular spikes recorded from the interneurone (occurring spontaneously or by iontophoresis of DLH) were used as a trigger to determine if a unitary IPSP could be recorded in

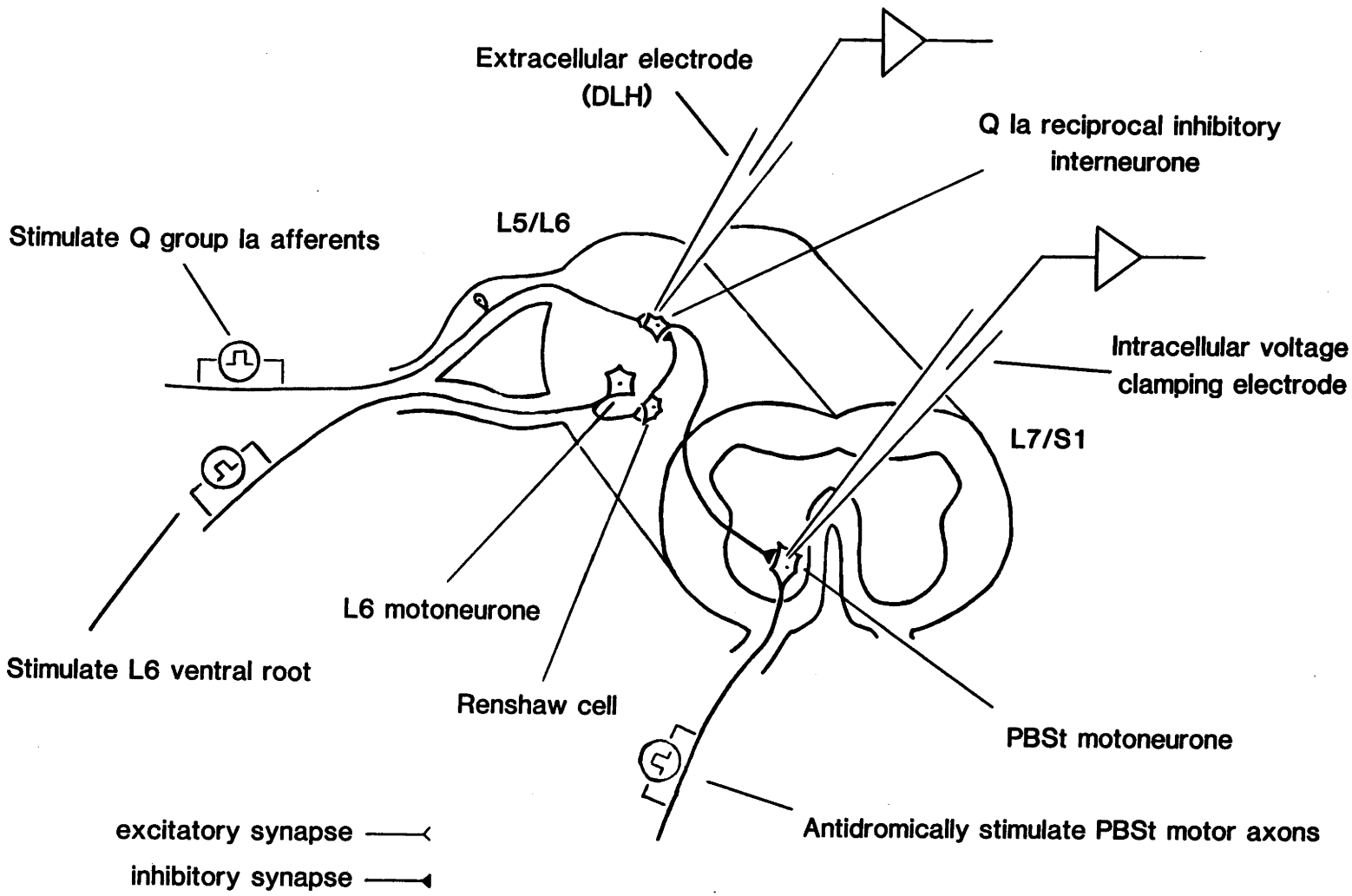


Fig. 3.2. A schematic diagram of the lumbar spinal cord showing the experimental arrangement as described in the text. See Jankowska & Roberts (1972*a,b*) for more detail.

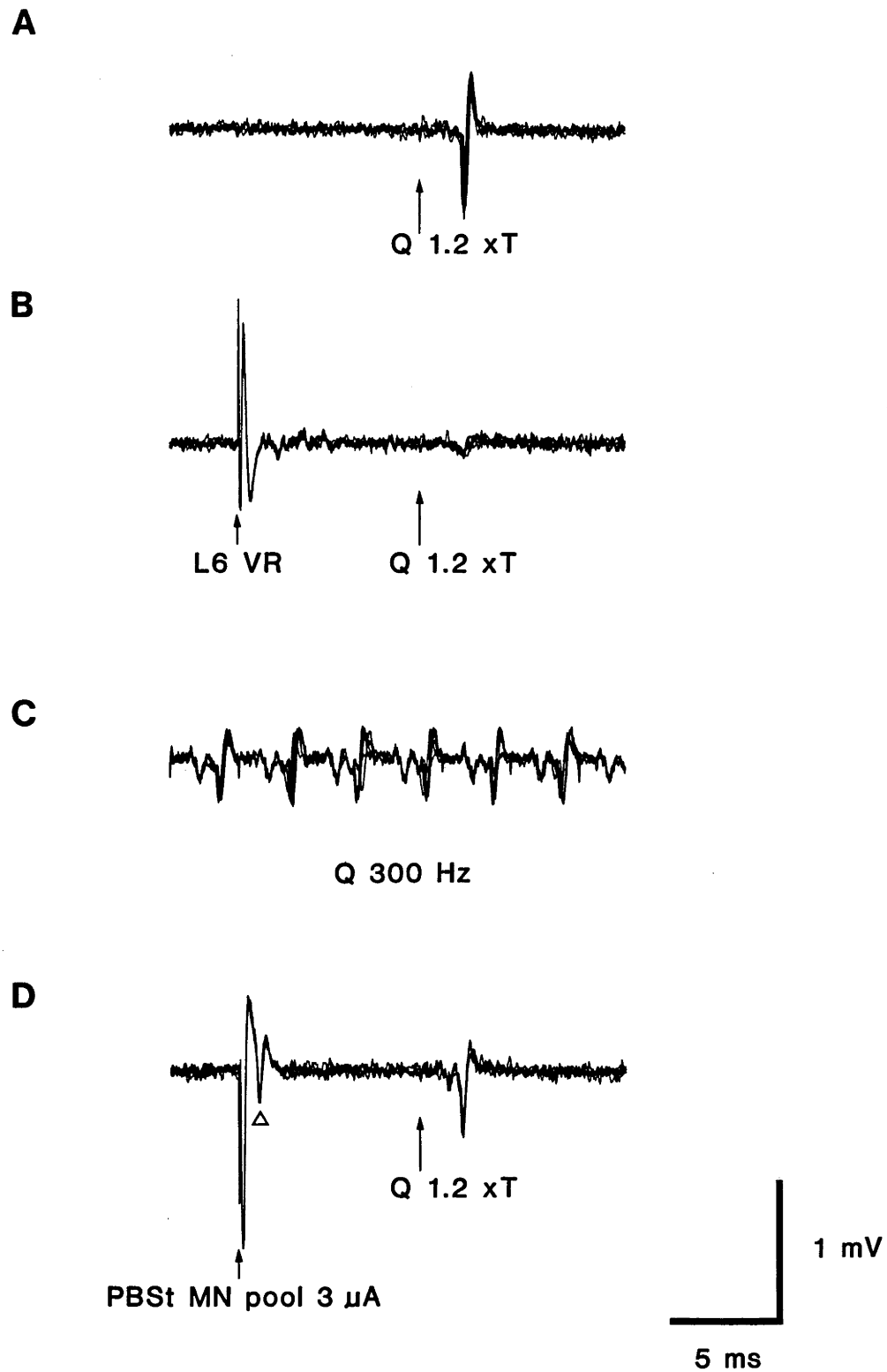


Fig. 3.3. Identification of a Ia reciprocal interneurone. *A*, extracellular recording from an interneurone receiving short latency, low threshold excitation following stimulation of the quadriceps (Q) nerve. *B*, Q evoked activity is inhibited by prior activation of the L6 ventral root (VR). *C*, the interneurone could follow Q nerve stimulation at 300 Hz. *D*, this interneurone could also be antidromically activated (△) from the PBSt motoneurone pool by brief (100 µs), 3 µA, negative going current pulses. Each record is made up of 5 to 10 superimposed single sweeps.

the impaled PBST motoneurone. If a unitary IPSP was present the membrane potential was voltage clamped and the current record averaged to give the unitary IPSC.

Data analysis

Five hundred to 1000 trials were averaged and the data stored on a microcomputer as described in Chapter two. The averaged IPSCs were analysed to obtain the magnitude of the peak current, the 10 to 90% rise time and the time constant of decay (τ_D). This time constant was measured by displaying the IPSC semi-logarithmically and fitting a linear regression line to the decay phase of the IPSC using a least squares procedure for the best fit.

Results

Pharmacology of IPSPs generated during reciprocal inhibition

An example of the effect of iontophoresis of strychnine hydrochloride on the population IPSP evoked in a motoneurone during reciprocal inhibition is shown in Fig. 3.4. This application of strychnine almost completely abolish this IPSP, providing strong evidence that the receptors mediating this inhibition are glycinergic.

Time course of population IPSCs

An example of the current underlying a population IPSP is illustrated in Fig. 3.5. The upper record shows the population IPSC, the middle record the clamped voltage and the lower record the unclamped population IPSP. The synaptic current at -50 mV (using a KCH_3SO_4 filled electrode) was outward, rising steeply and decayed with a single exponential as shown by the continuous line. The 10 to 90% rise time was 0.47 ms and the IPSC decayed with a time constant of 1.0 ms.

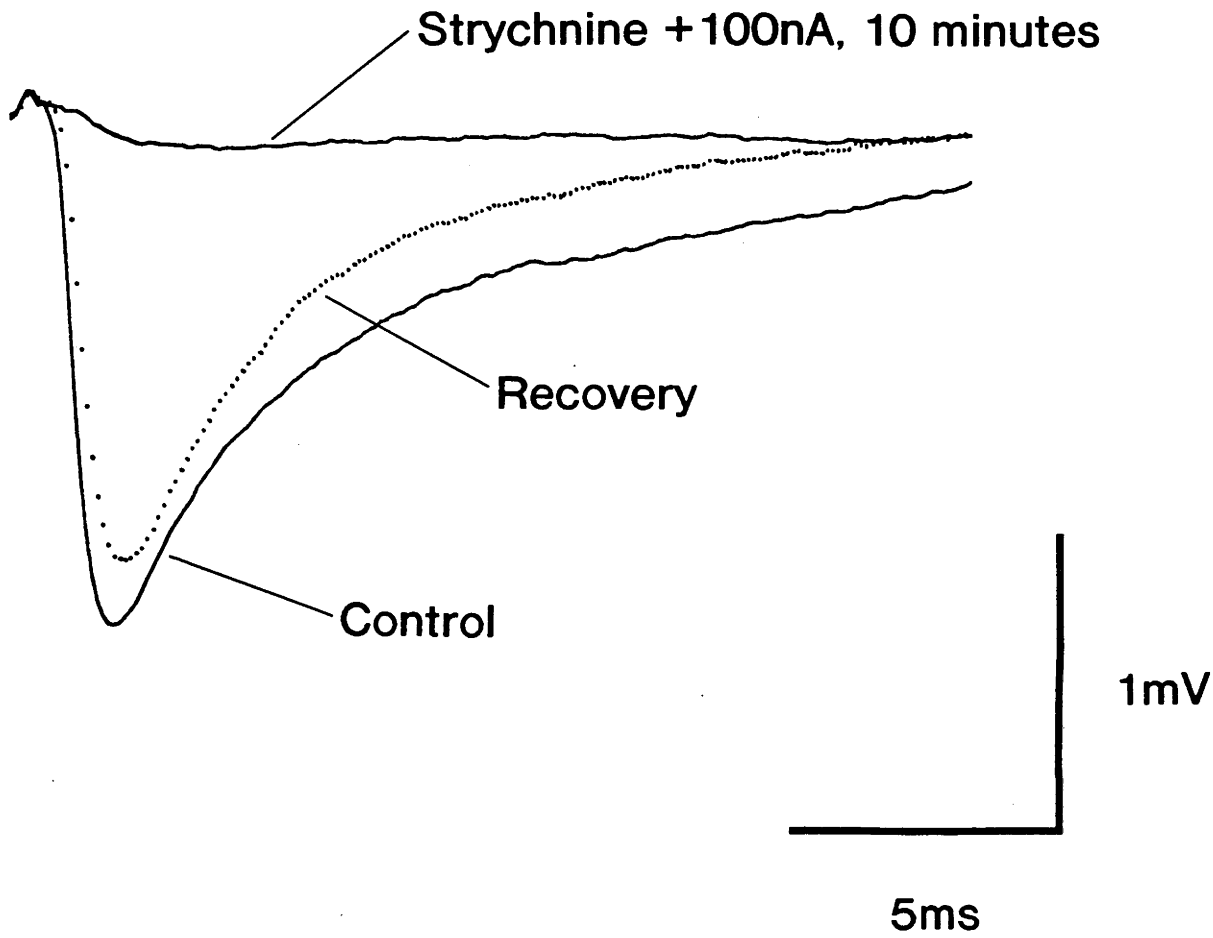


Fig. 3.4. Antagonism of population IPSPs evoked during reciprocal inhibition by iontophoretic application of strychnine. The control record shows a population IPSP recorded intracellularly in a PBSt motoneurone (KCH_3SO_4 filled electrode) following activation of the whole Q muscle nerve at 1.5 x threshold. This IPSP was almost completely abolished by the local application of strychnine (+100 nA, 10 minutes) from an extracellular iontophoretic electrode. Recovery was slow and still incomplete after 75 minutes.

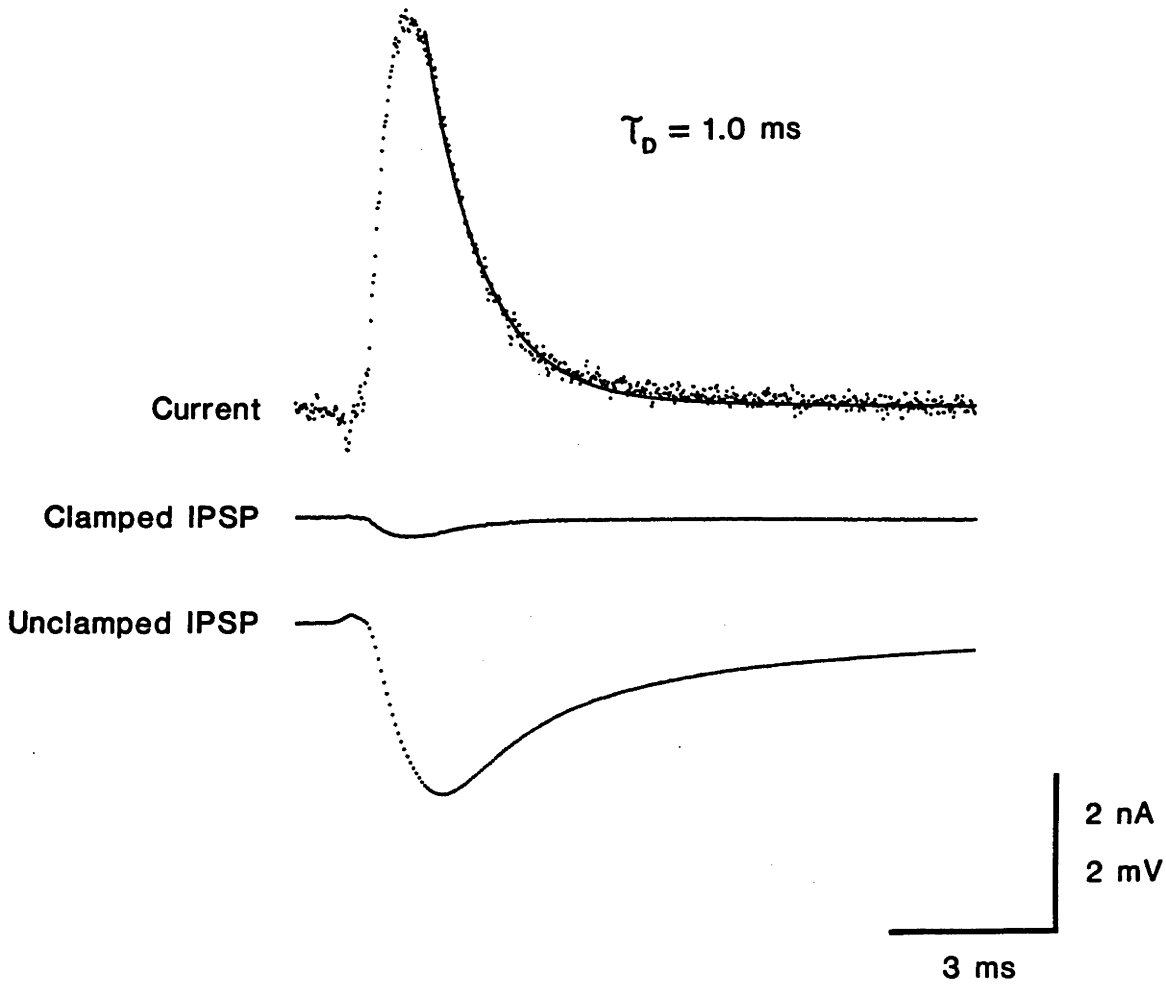


Fig. 3.5. Recording of the population inhibitory current. The upper record is the synaptic current, the middle record the clamped IPSP and the lower record the unclamped population IPSP. Both the clamped and unclamped records were obtained at a membrane potential of -50 mV, using a KCH_3SO_4 filled electrode. 1000 trials were averaged to obtain the clamp records. The decay phase of the synaptic current has been fitted by a single exponential with a time constant (τ_D) of 1.0 ms.

Population IPSCs were recorded in 22 different voltage clamped PBSt motoneurones using both KCH_3SO_4 and KCl filled electrodes. The average 10 to 90% rise times and τ_D for all 22 population IPSCs, obtained over a range of resting membrane potentials from -56 to -68 mV, was 0.51 ± 0.02 ms and 0.99 ± 0.04 ms (±S.E.M., $n=22$) respectively. There was no difference in the time courses of IPSCs recorded with KCl or KCH_3SO_4 electrodes.

As reciprocal inhibition is mediated by a disynaptic pathway, the synaptic current evoked by stimulation of the whole Q muscle nerve may contain some spatial and temporal distortion which would tend to overestimate both the rise time and τ_D. The amount of spatial distortion arising from the presence of synapses which are not isopotential with the voltage clamp should be small as Ia reciprocal interneurones synapse predominantly on the soma of motoneurones (Introduction, and below). However, differences in the activation times and conduction velocities of single Ia afferent fibres coupled with variability in synaptic delays and in the excitability of individual Ia reciprocal interneurones will lead to some temporal dispersion of the population current. These temporal problems can be avoided by obtaining unitary synaptic currents by spike triggered averaging from the extracellular spikes of identified Q Ia reciprocal interneurones. Results from these experiments show that, despite the above reservations, the population current gives a good approximation of the time course of the true synaptic current.

Unitary IPSPs

Unitary IPSPs were recorded in twelve different PBSt motoneurones using KCH_3SO_4 filled electrodes. They ranged in size from 25 to 215 μV with a mean of 68.5 ± 15.7 μV (±S.E.M). An example of unitary IPSPs recorded in three different PBSt motoneurones following spike triggered averaging from the extracellular spikes of the *same* Ia reciprocal interneurone is shown in Fig. 3.6.

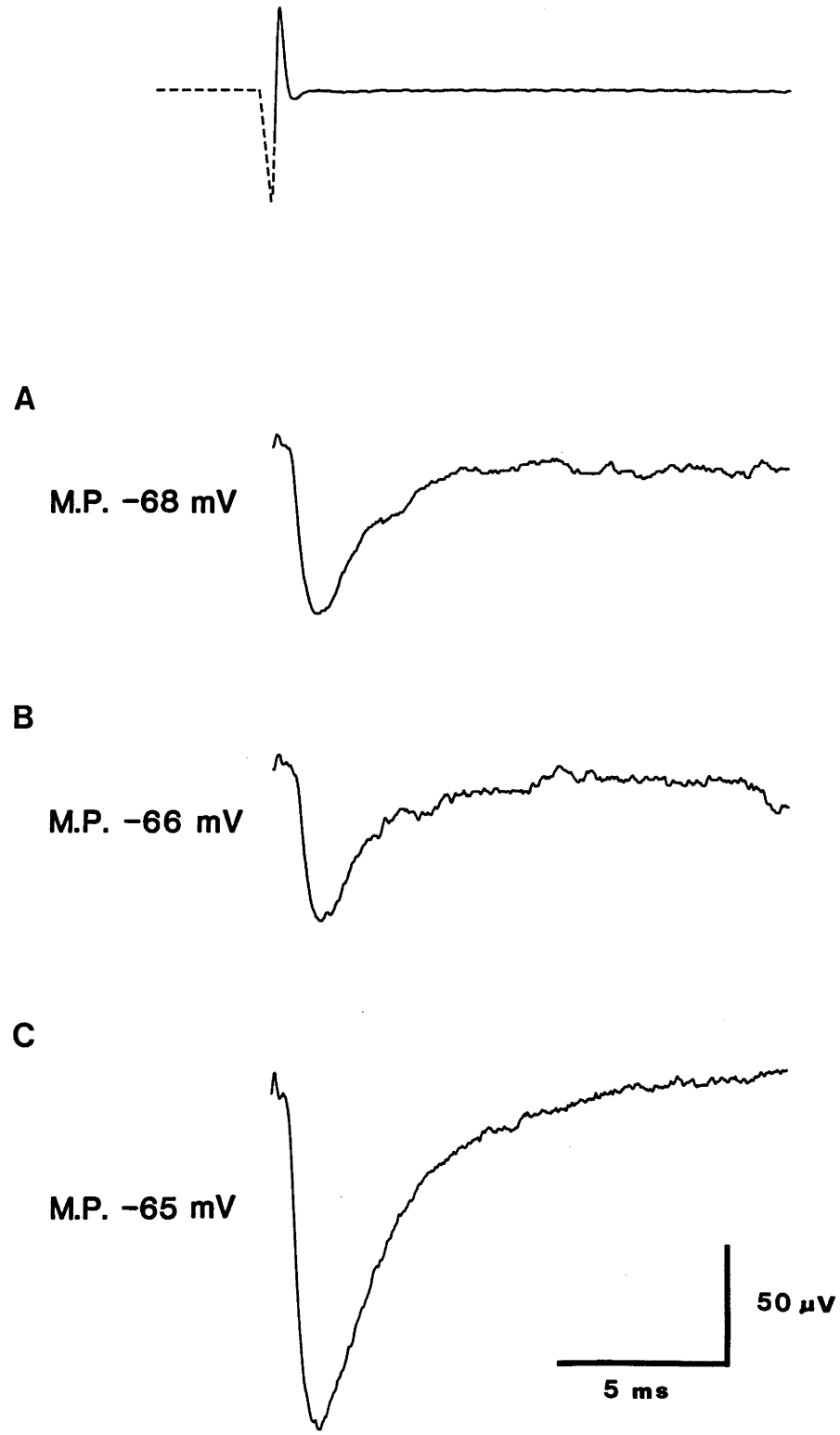


Fig. 3.6. Unitary IPSPs. *A* - *C* show examples of unitary IPSPs recorded in different PBSt motoneurones at the membrane potentials indicated following spike triggered averaging from the extracellular spikes of the same Ia reciprocal interneurone (shown in the top record). n.b. the variability in size, but similarity in time course of the different unitary IPSPs. All IPSPs were recorded using KCH_3SO_4 filled electrodes.

Time course of unitary IPSCs

The membrane potential of five motoneurones in which unitary IPSPs were recorded was successfully voltage clamped to reveal the underlying unitary IPSC. An example of one of these successful experiments is shown in Fig. 3.7. In this case the bottom record shows the population IPSP evoked in a PBSt motoneurone following stimulation of the whole Q muscle nerve. After identification of an interneurone excited at short latency by low threshold stimulation of the Q muscle nerve and inhibited by L6 ventral root stimulation, spike triggered averaging from spontaneous extracellular spikes recorded from this interneurone revealed a unitary IPSP in the same motoneurone. This unitary IPSP (Fig. 3.7*C*) had a similar time course to the population IPSP, but its amplitude was approximately twenty times smaller. Fig. 3.7*B* shows the clamped voltage record and the corresponding underlying unitary current is shown in Fig. 3.7*A*. The unitary synaptic current recorded at -67 mV rose from 10 to 90% of its peak of 150 pA in only 0.29 ms and the decay was fitted with a single exponential having a time constant of 0.67 ms as shown by the continuous line.

At resting membrane potentials (-55 to -67 mV) unitary IPSCs had peak amplitudes of between 120 pA and 220 pA, mean 160 ± 17 pA (±S.E.M., n=5), and 10 to 90% rise times of between 0.25 to 0.57 ms, mean 0.40 ± 0.06 ms. These measured values overestimate the true rise time and underestimate the peak current because of inadequate clamping of the fast rising phase of the unitary IPSP. The τ_D of unitary IPSCs ranged from 0.67 to 1.01 ms, with a mean of 0.82 ± 0.07 ms. When the data for both population and unitary IPSCs were compared the rise times of the unitary currents appeared to be only slightly faster and the τ_D slightly shorter than those measured for the population IPSCs (Fig. 3.8).

Location of synapses from Ia reciprocal interneurones

The similarity in the rise times and τ_D of population and unitary IPSCs (Fig. 3.8) suggests that the ionic current generated during reciprocal inhibition is localised to a very spatially restricted area of the motoneurone surface membrane,

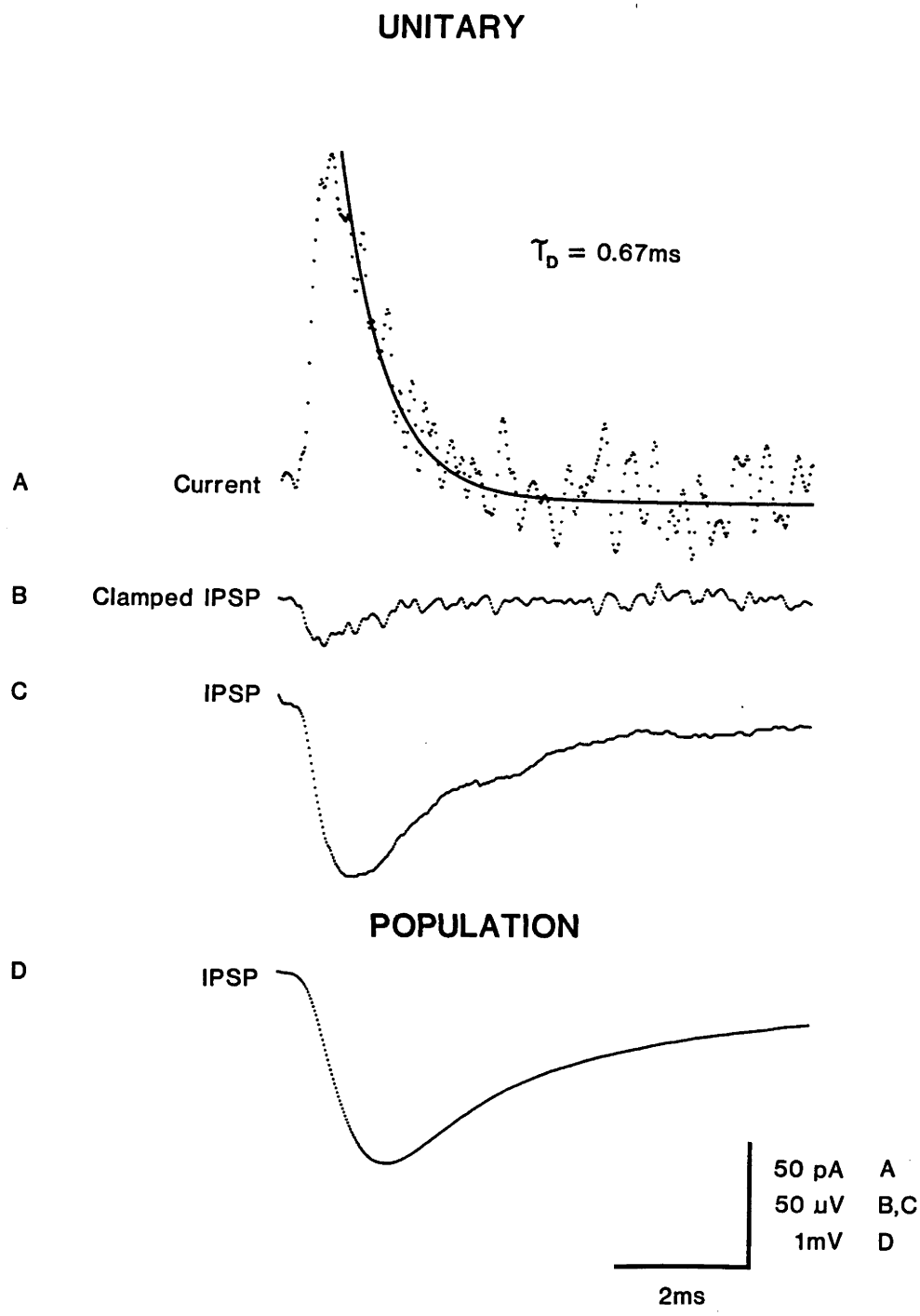


Fig. 3.7. Recording of the unitary inhibitory current. *A*, the unitary synaptic current. *B*, the clamped unitary IPSP. *C*, the unclamped unitary IPSP. *D*, the averaged population IPSP. All records were obtained at -67 mV in the same motoneurone, using a KCH_3SO_4 filled electrode. 1000 responses were used to obtain the clamped records. The decay phase of the synaptic current has been fitted by a single exponential with a time constant (τ_D) of 0.67 ms.

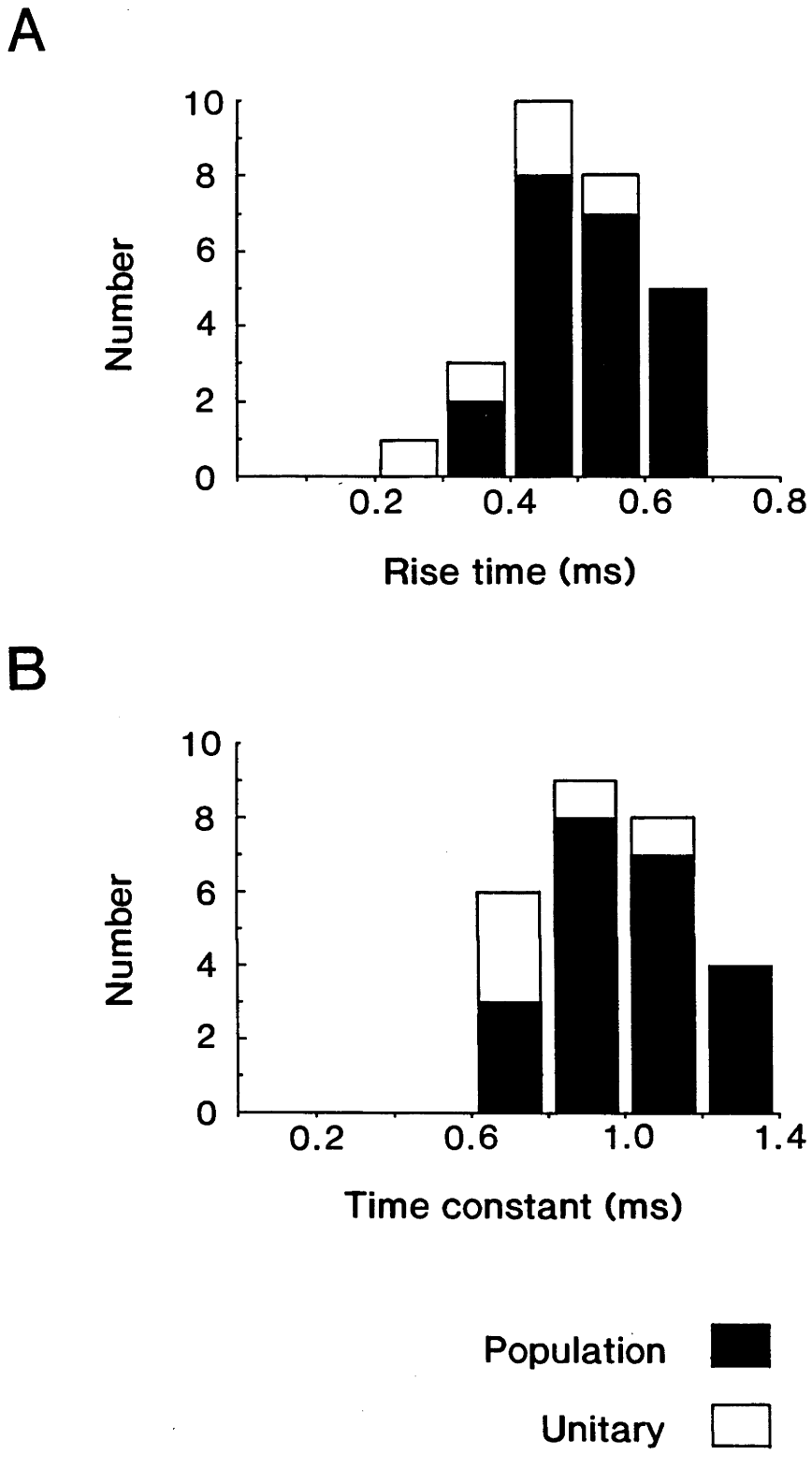


Fig. 3.8. Pooled time course data for 22 population and 5 unitary IPSCs. *A*, histogram of the 10-90% rise times of population (filled) and unitary (open) IPSCs recorded at their resting membrane potentials (-55 to -68 mV). *B*, histogram of the decay time constants of population (filled) and unitary (open) IPSCs recorded at their resting membrane potentials (-55 to -68 mV).

implying that individual Ia reciprocal interneurones must synapse at very similar electrotonic distances from the motoneurone soma.

Evidence for a predominantly somatic location of these synapses comes from an analysis of the rise times and durations at half peak amplitude (half-widths) of population and unitary IPSPs (Jankowska & Roberts, 1972*b*). The unnormalised rise time and half-width data for a sample of 11 population IPSPs (filled circles) and all 12 unitary IPSPs (open circles) has been plotted in Fig. 3.9 and is compared to the data previously obtained for unitary EPSPs recorded in motoneurones following the activation of single Ia afferents (Jack, Miller, Porter & Redman, 1971). This comparison is made on the shape indices of unnormalised data as a reliable measure of the membrane time constant was not obtained during the IPSP experiments. It can be seen that most of the IPSPs have shape indices comparable to the fastest unitary EPSPs which are thought to be generated by synapses on the soma of motoneurones (Rall, Burke, Smith, Nelson & Frank, 1967; Jack *et al.* 1971). That the rise times and half-widths of the unitary IPSPs are slightly longer than that of the fastest EPSPs can be accounted for in part by the finding that the duration of synaptic current underlying unitary IPSPs is approximately twice as long as the synaptic current underlying unitary Ia EPSPs (Rall, 1967; Jack & Redman, 1971; Finkel & Redman, 1983*a*). Given this difference the data shown in Fig. 3.9 suggests that the synapses which generate unitary IPSPs have shape indices consistent with a location on, or very close to, the soma of motoneurones.

Effects of anaesthetics

While most experiments were performed on cats anaesthetised with α-chloralose, some measurements were also obtained in animals anaesthetised with pentobarbitone and in unanaesthetised, decerebrated preparations. When the rise times and τ_D for population IPSCs recorded from voltage clamped PBSt motoneurones in these different preparations were compared there was no apparent difference between decerebrated, α-chloralose or pentobarbitone

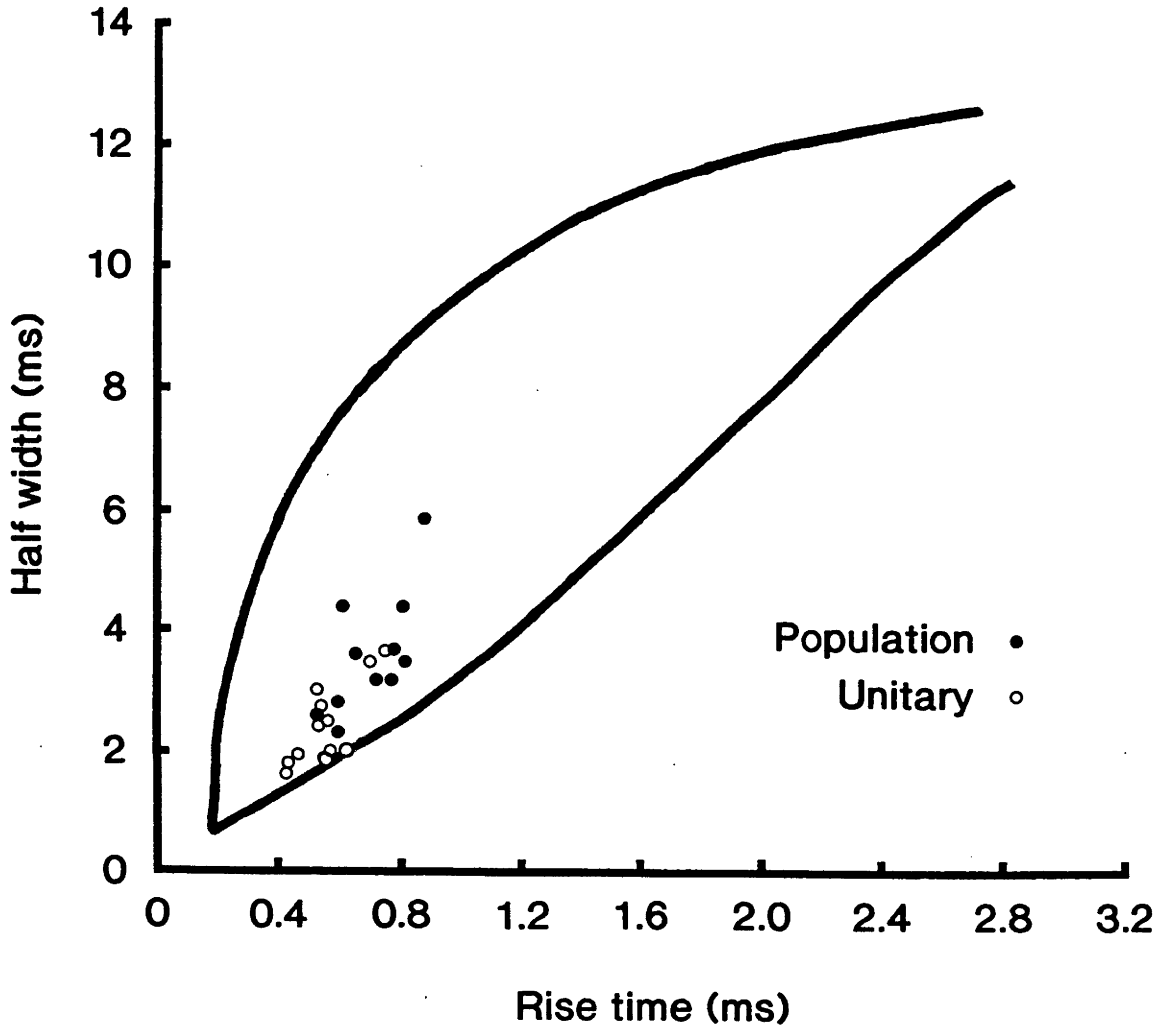


Fig. 3.9. Plot of the 10-90% rise time against half-width for 11 population IPSPs (filled circles) and 12 unitary IPSPs (open circles). These points are compared with the range of 10-90% rise times and half-widths of unitary Ia EPSPs recorded in lumbar motoneurones by Jack, Miller, Porter & Redman (1971), falling within the bounds of the continuous line. All IPSPs were recorded using KCH_3SO_4 filled electrodes.

anaesthetised preparations (Fig. 3.10). This suggests that the time course of the synaptic current was unaffected by the presence of either α-chloralose or pentobarbitone at concentrations necessary for deep anaesthesia.

Effects of membrane potential on IPSPs

Changing the membrane potential was found to effect both the peak amplitude and the time course of the IPSP. In agreement with Coombs, Eccles and Fatt (1955*a*) the peak of the IPSP was found to decrease as the membrane potential was hyperpolarised and reversed at a membrane potential close to -80 mV (Fig. 3.11).

Interestingly, Fig. 3.11*A* shows that the rate of decay of the IPSP was changed by membrane depolarisation. At membrane potentials more depolarised than approximately -50 mV IPSPs decayed faster than would be expected by the membrane time constant, the decay phase of these IPSPs often overshooting the baseline. This suggests that inward current is flowing during the decay of the IPSP at depolarised membrane potentials. The increase in the rate of decay of IPSPs at depolarised potentials probably occurs following the activation of a voltage dependent conductance. There are two possibilities. The first is that a voltage dependent conductance leading to the flow of inward current is turned on by repolarisation of the membrane potential during the decay of the IPSP. The second possibility is that a non-inactivating outward current is turned off by the rapid hyperpolarisation at the start of the IPSP. The most obvious candidate for the latter possibility is the delayed rectifier current, which in motoneurones is present at membrane potentials more positive than -50 mV (Takahashi, 1990).

Effects of membrane potential on IPSCs

The membrane potential was found to affect both the peak amplitude and the time course of IPSCs. An example of the effect of different membrane potentials on the peak current is shown in Fig. 3.12. In agreement with Araki & Terzuolo (1962) the peak current decreased as the membrane potential became

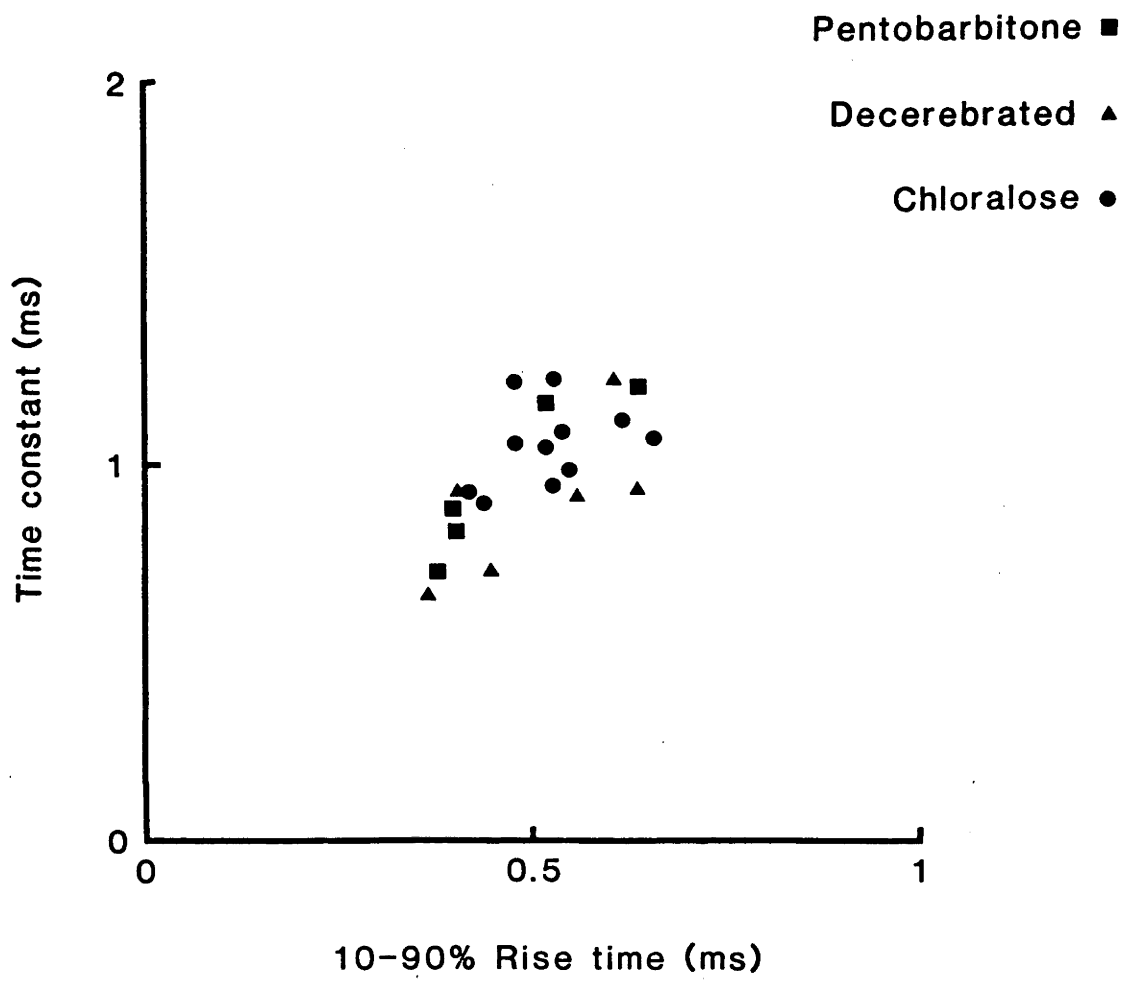


Fig. 3.10. The rise time and decay time constant of 22 population IPSCs recorded in different motoneurones at their resting membrane potentials (-56 to -68 mV) in either pentobarbitone (squares) or α-chloralose (circles) anaesthetised preparations, or in unanaesthetised, decerebrated preparations (triangles).

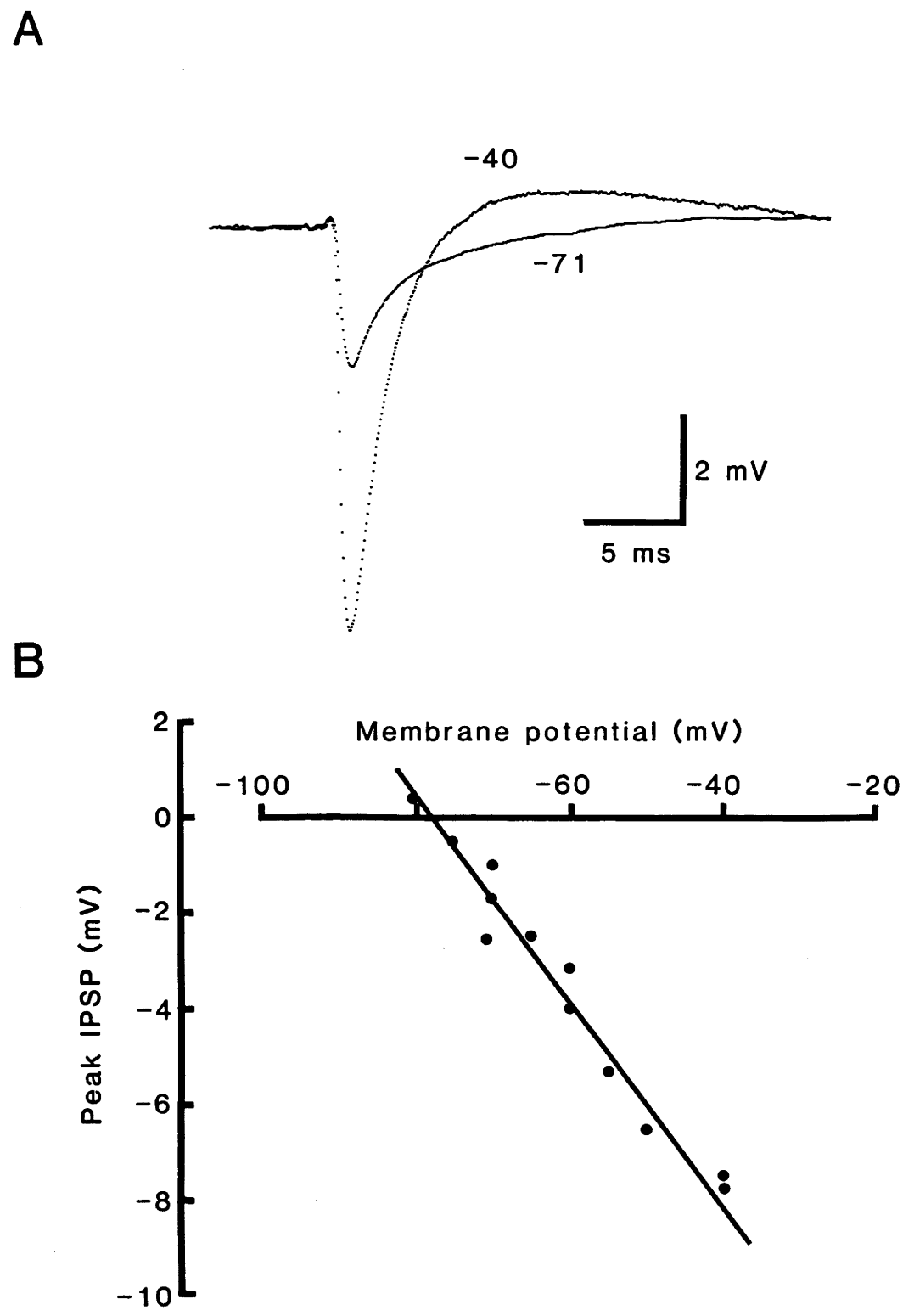


Fig. 3.11. The effect of membrane potential on the IPSP. *A*, population IPSPs were recorded using a KCH_3SO_4 filled electrode at the membrane potentials indicated on each record in discontinuous current clamp. n.b. the decay of the IPSP recorded at -40 mV overshoots the baseline. *B*, the voltage at the peak of the IPSP shown in *A* is plotted against the holding potential. The data have been fitted with straight line with a correlation coefficient of -0.98.

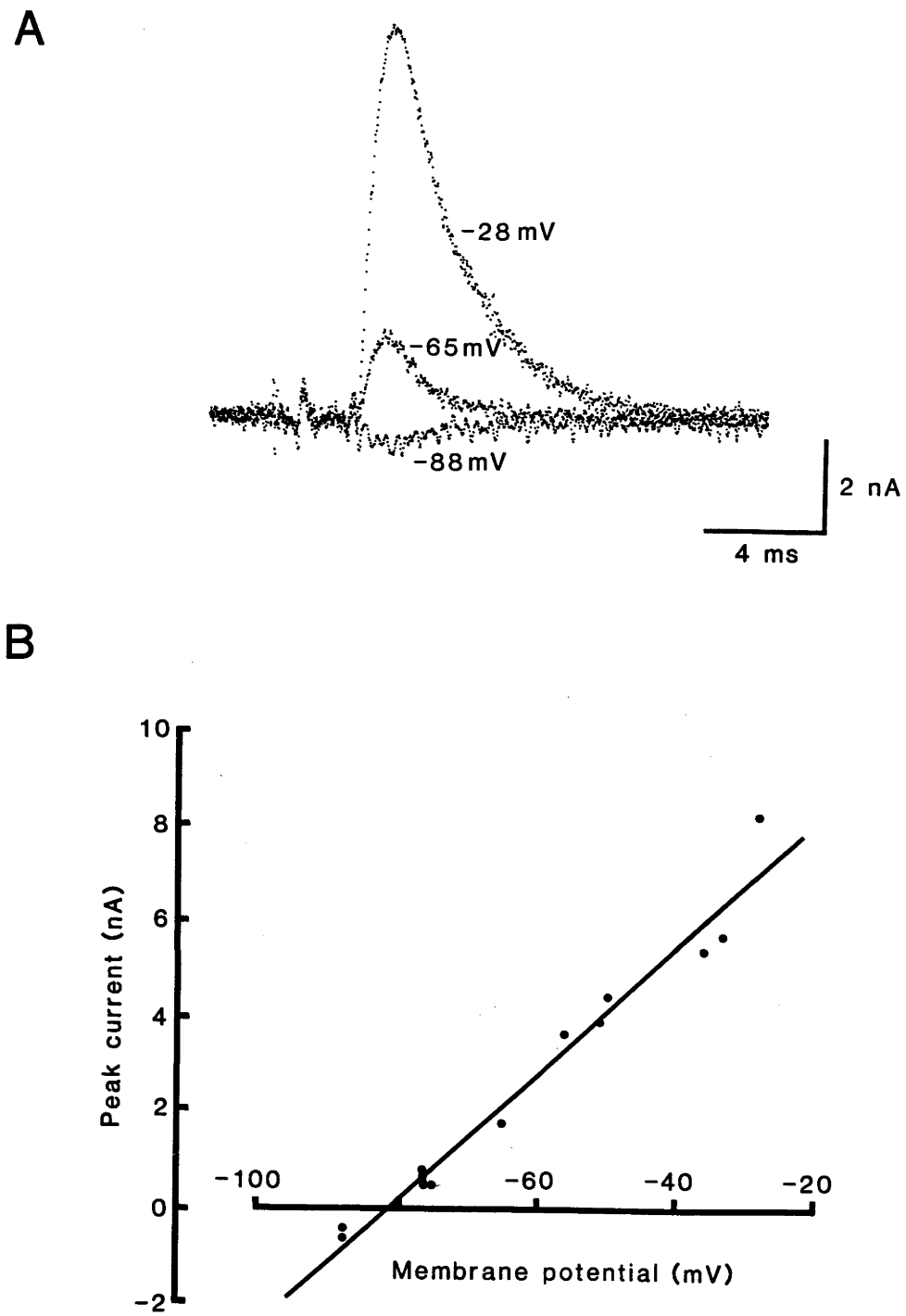


Fig. 3.12. The effect of membrane potential on peak current. *A*, population IPSCs were recorded at the membrane potentials indicated on each record using a KCH_3SO_4 filled electrode. 500 responses were used to obtain each record. Peak current measurements have been made from these and other averaged records from the same motoneurone and plotted in *B* as a function of membrane potential. The magnitude of the peak synaptic current at -76 mV was monitored frequently during recording. The straight line is the linear regression fit to these points (correlation coefficient of 0.98) and indicates a reversal potential of ~-82 mV.

more negative (using a KCH_3SO_4 filled electrode) and at -88 mV had clearly reversed to become an inward current.

The peak current/voltage relationship for the population IPSC shown Fig. 3.12*A* is shown in Fig. 3.12*B*. The data were well fitted by a straight line with a correlation coefficient of 0.98. The regression fit through these points is given by the equation:

$$I_p = 0.13\ V_m + 10.9$$

where I_p is the peak current (nA) and V_m the membrane potential (mV). The calculated reversal potential is ~ -82 mV. Data from six different motoneurones where complete reversal of the synaptic current was achieved gave reversal potentials of between -75 and -84 mV, with an average reversal potential of -80.7 ± 1.5 mV (±S.E.M., $n=6$). The slope of the regression line in Fig. 3.12*B* gives a measure of the peak conductance change generated by the population IPSC which, in this example, was 133 nS. The slopes of the peak current/voltage relationships for population IPSCs from 11 cells gave an average peak conductance of 167 ± 30 nS (± S.E.M.)

To establish if the quality of the voltage clamp was affected by changing the membrane potential, the residual IPSP voltage during voltage clamp (the error voltage) was routinely compared to the peak IPSC at each clamped membrane potential (Fig. 3.13). In the example shown in Fig. 3.13, and in all cells in which it was examined, the relationship between the error voltage and the peak current was linear. A linear relationship implies that there has been no change in the ability of the voltage clamp to clamp the membrane at different membrane potentials.

The decay of the synaptic current was slowed by depolarisation. This is shown clearly in Fig. 3.14*A*, where τ_D of a population IPSC increased from 0.97 ms to 1.79 ms following a depolarisation from -76 mV to + 4 mV. In this case the PBSt motoneurone had been impaled with a 3M KCl filled electrode. Passive diffusion of chloride from the electrode into the motoneurone shifted the reversal

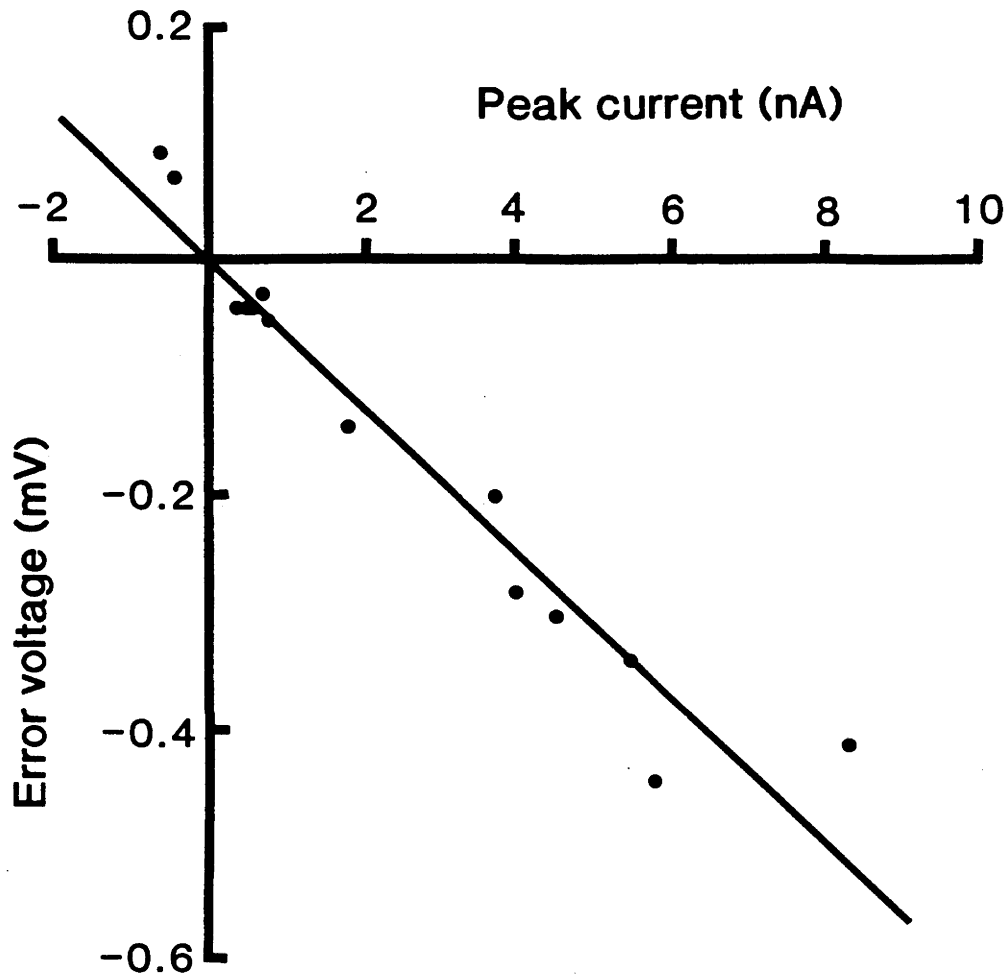


Fig. 3.13. The error voltage left during voltage clamp was measured and has been plotted against the peak current for the same cell as shown in Fig. 3.12. The relationship between the error voltage and the peak current is linear with a correlation coefficient of 0.97.

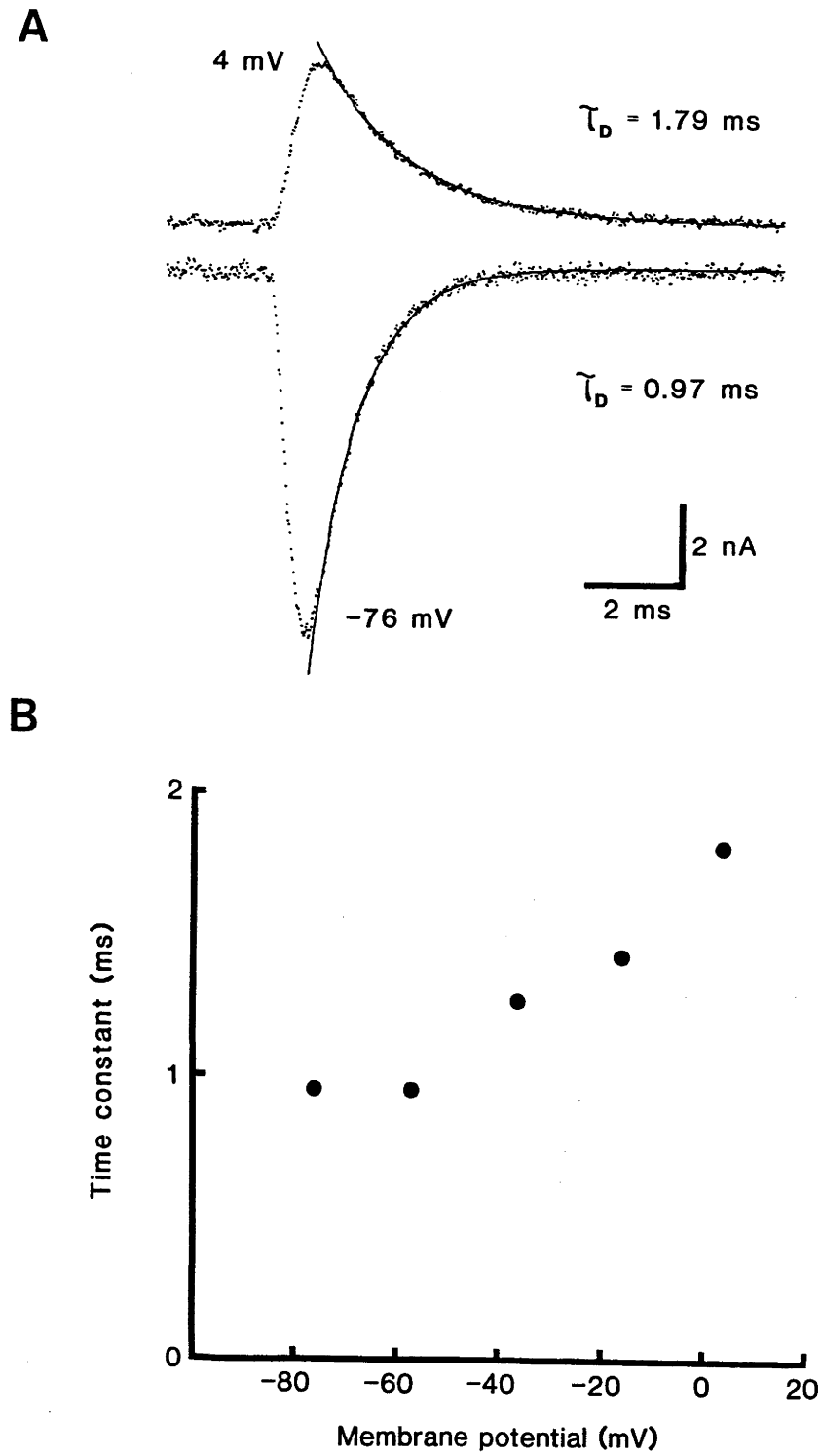


Fig. 3.14. Effect of membrane potential on the decay of the population IPSC. *A*, the upper record is the population current recorded at a membrane potential of +4 mV and the lower record is the current recorded at a membrane potential of -76 mV, using a KCl filled electrode. The decay phase of the synaptic currents have been fitted with a single exponential of time constant, τ_D. *B*, the time constant of decay of population IPSCs recorded in the same cell as shown in *A* have been plotted against the holding potential.

potential for the IPSC to about -25 mV. The relationship between τ_D and the membrane potential for the population IPSC recorded in this motoneurone is shown in Fig. 3.14*B*.

The decay of unitary IPSCs was also slowed by membrane depolarisation. This is shown in Fig. 15*A*, where τ_D of a unitary IPSC increased from 0.70 ms to 1.09 ms following a depolarisation from -68 mV to -40 mV (using a KCH_3SO_4 filled electrode). The relationship between τ_D and the membrane potential for this unitary IPSC is shown in Fig. 3.15*B*.

When the data from population IPSCs and unitary IPSCs at different membrane potentials were pooled the rate of decay of IPSCs was found to be exponentially dependent on the membrane potential. This is shown in Fig. 3.16. Here, the natural logarithm of the normalised decay time constant (τ_D') for population IPSCs (14 neurones; filled circles) and unitary IPSCs (five neurones; open circles) has been plotted against the corresponding clamped membrane potential (V_m). Decay time constants were normalised to the τ_D measured at the resting membrane potential for each neurone (between -55 and -68 mV). This reduce scatter introduced by variation between different neurones and preparations. The data were well fitted by a straight line with a correlation coefficient of 0.89. The regression fit through these points is given by the equation:

$$\ln \tau_D' = 0.74 + V_m / 91$$

This result indicates that the time constant of decay of the synaptic current is exponentially dependent on the postsynaptic membrane potential, increasing *e*-fold for a 91 mV depolarisation.

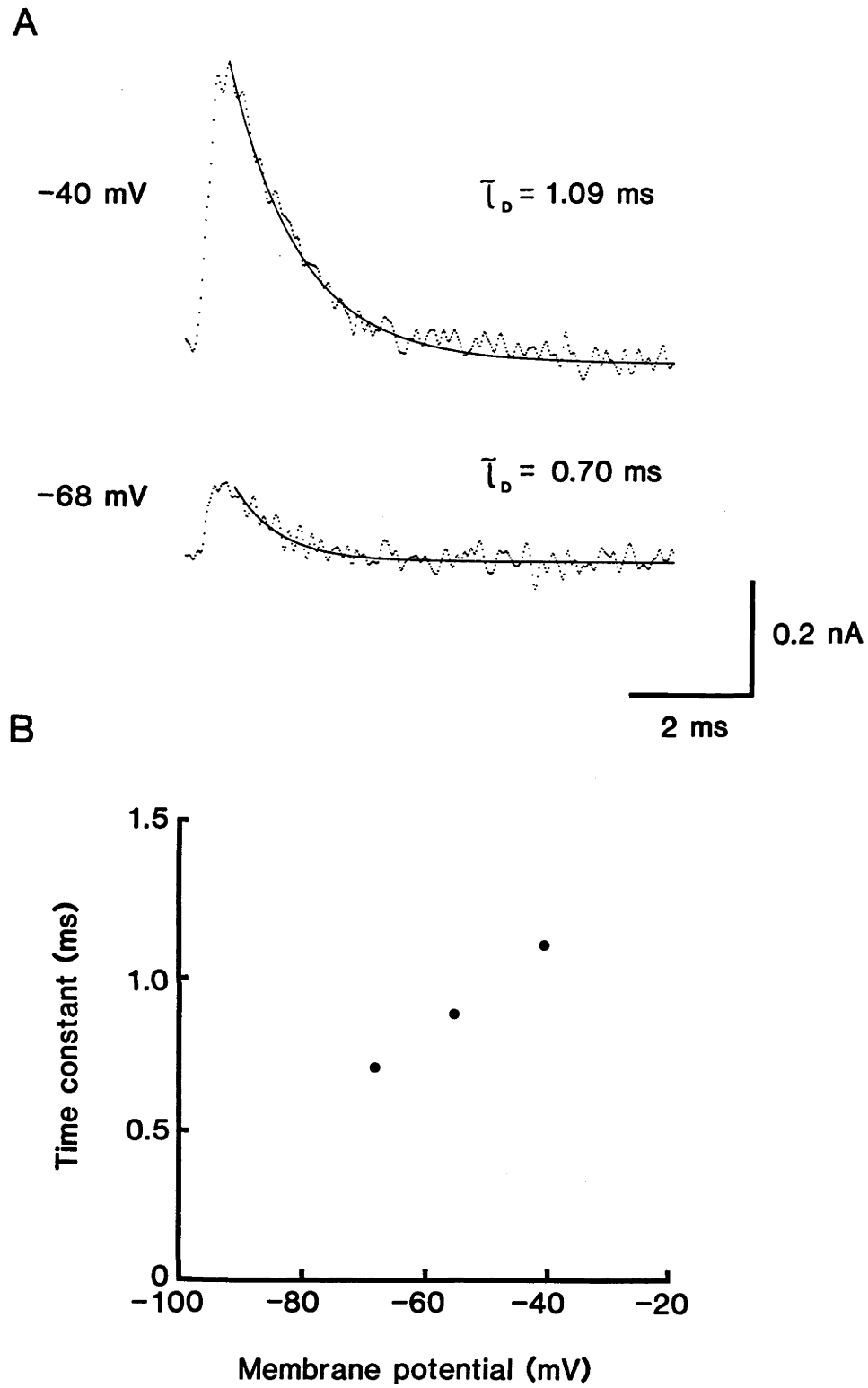


Fig. 3.15. Effect of membrane potential on the decay of the unitary IPSC. *A*, the upper record is the unitary current recorded at a membrane potential of -40 mV and the lower record is the current recorded at a membrane potential of -68 mV, using a KCH_3SO_4 filled electrode. The decay phase of the synaptic currents have been fitted with a single exponential of time constant, τ_D. *B*, the time constant of decay of unitary IPSCs recorded in the same cell as shown in *A* have been plotted against the corresponding holding potential.

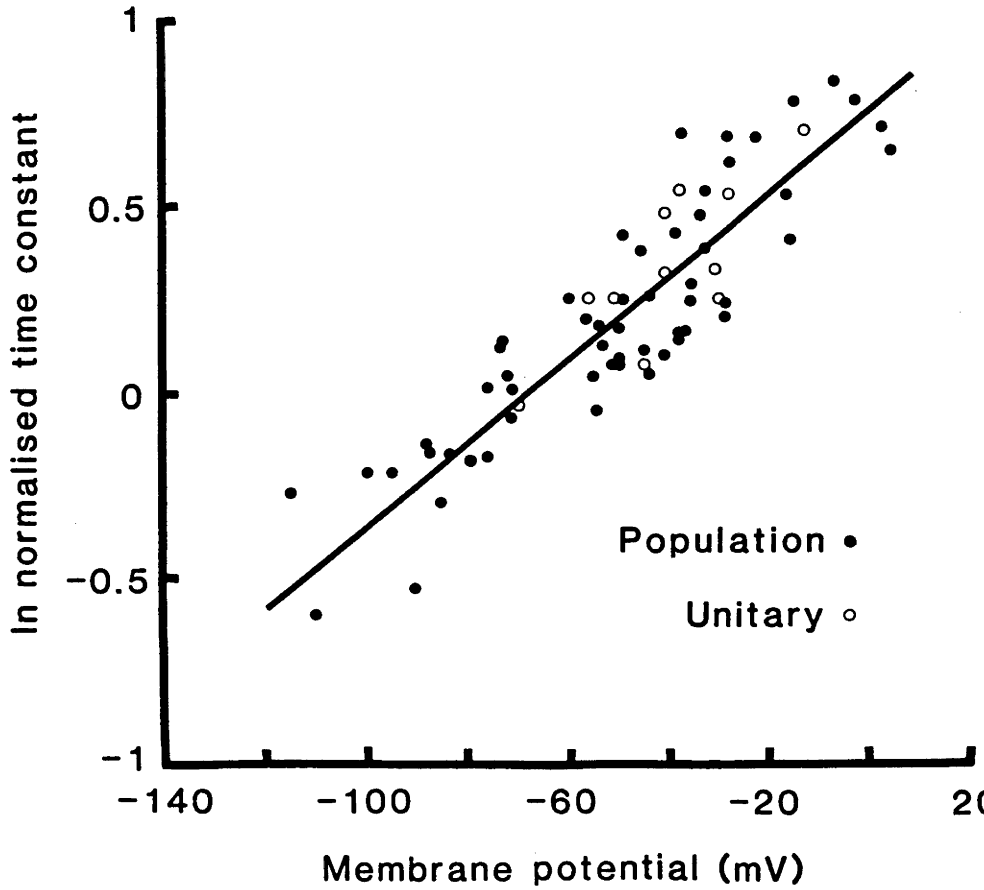


Fig. 3.16 The natural logarithm of the normalised decay time constant of population (filled circles) and unitary (open circles) IPSCs has been plotted against the corresponding clamped membrane potential. The straight line through these points has a correlation coefficient 0.89.

Five of the 14 population IPSCs in Fig. 3.16 were recorded with 3M KCl filled electrodes, shifting the reversal potential for these IPSCs to more positive membrane potentials. This increased the size of IPSCs recorded at hyperpolarised membrane potentials and also allowed investigation of the effect of changing the intracellular chloride concentration ($[Cl^-]_i$) on the time course of the IPSC. Despite large changes in $[Cl^-]_i$ (reversal potentials for IPSCs recorded with KCl electrodes ranged from -70 to -20 mV) these IPSCs had similar rise times and τ_D to those recorded with KCH_3SO_4 electrodes.

If the ionic current flowing during the IPSC is carried purely by Cl^- ions, then the reversal potential for the IPSC (E_{rev}) will be identical to the Cl^- equilibrium potential and can be used to calculate $[Cl^-]_i$ from the Nernst equation assuming $[Cl^-]_o$ is 134 mM (Vogh & Maren, 1975).

The τ_D of population IPSCs measured at the resting membrane potential for six neurones where reversal of IPSCs was observed using 2M KCH_3SO_4 electrodes and for five neurones where reversal was observed with 3M KCl electrodes has been plotted against the calculated $[Cl^-]_i$ in Fig. 3.17. This figure shows that despite large changes in $[Cl^-]_i$ (between 5.4 and 63.4 mM) all IPSCs had similar decay time constants. The mean τ_D for population IPSCs recorded with KCH_3SO_4 electrodes was 0.99 ± 0.07 ms (± S.E.M., n=6), whereas the mean τ_D for population IPSCs recorded with KCl electrodes was 0.96 ± 0.08 ms (± S.E.M., n=5). These means were not significantly different (Students *t*-test, $P > 0.25$). Recordings obtained with KCl electrodes also showed the same voltage dependent properties as those obtained with KCH_3SO_4 electrodes indicating that the voltage sensitivity in the decay of these IPSCs was not dependent on the intracellular chloride concentration or on the magnitude of the synaptic current.

The effect of membrane potential on the rise times of the synaptic currents was also investigated. The normalised IPSC rise time (RT') for population IPSCs (14 neurones; filled circles) and unitary IPSCs (five neurones; open circles) has been plotted against the corresponding clamped membrane potential (V_m) in Fig. 3.18. Rise times were normalised to the rise time measured at the resting

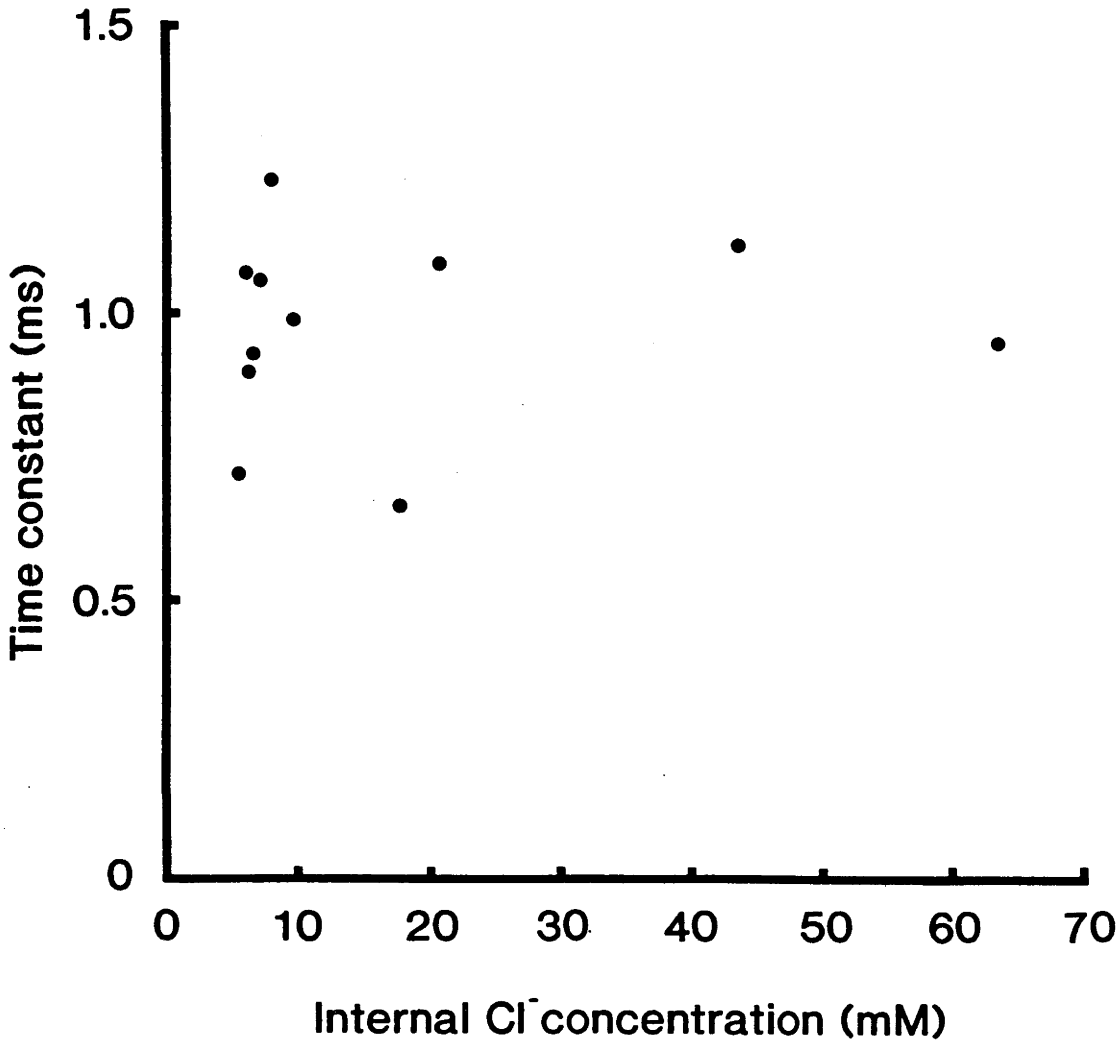


Fig. 3.17. The time constant of decay of population IPSCs recorded at resting membrane potentials (-55 to -68 mV) for six neurones where complete reversal of the IPSC was observed using KCH_3SO_4 filled electrodes and for five neurones recorded with KCl filled electrodes have been plotted against the internal Cl^- concentration calculated from the Nernst equation (see text) .

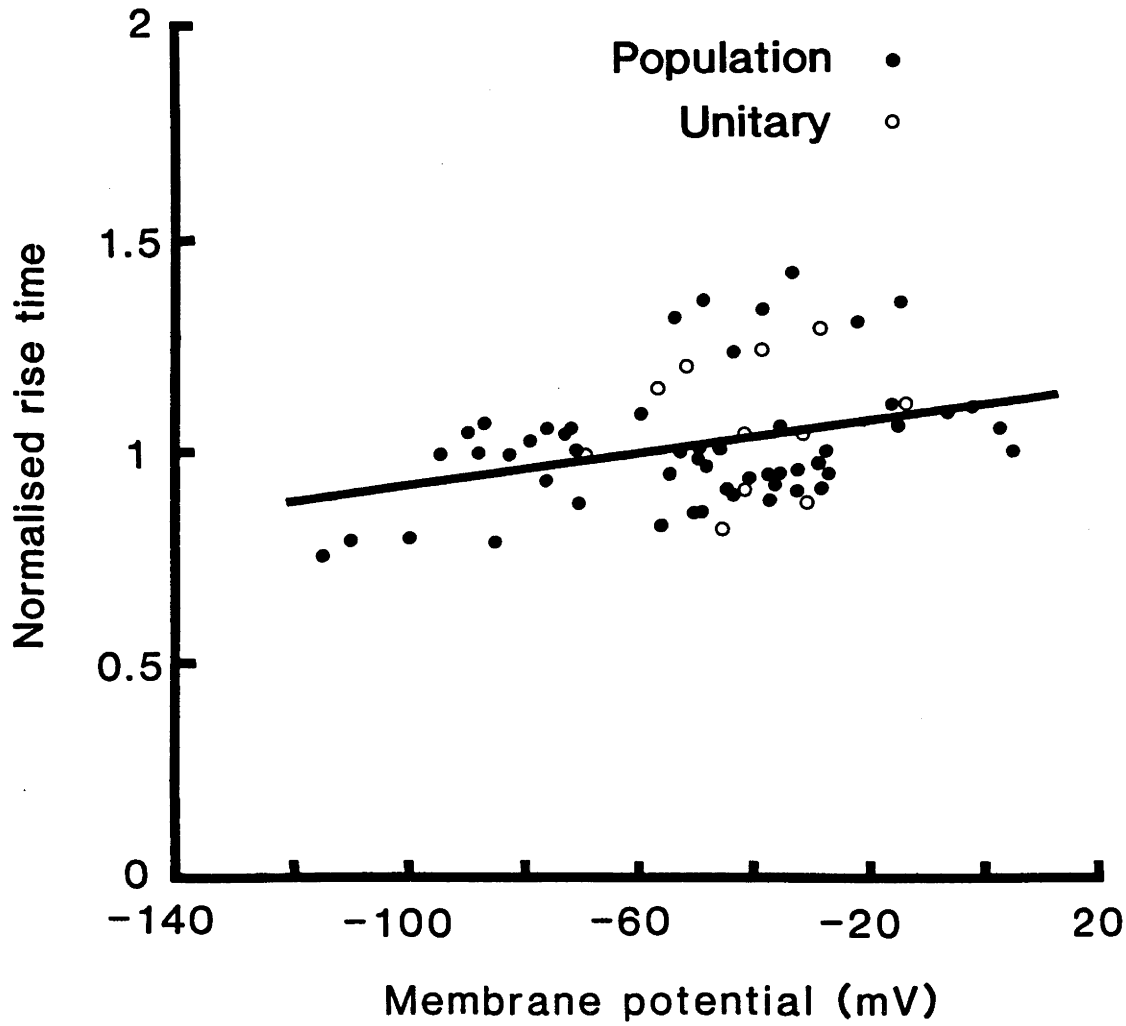


Fig. 3.18. The normalised 10-90% rise times of population (filled circles) and unitary (open circles) IPSCs have been plotted against the corresponding clamped membrane potential. The straight line through these points has a correlation coefficient 0.34.

membrane potential for each neurone (between -55 and -68 mV). The data were fitted by a straight line with a correlation coefficient of 0.34 and a slope of ~0.02. The regression fit through these points is given by the equation:

$$RT' = 1.9 \times 10^{-3} V_m + 1.12$$

This relationship suggests a slight trend for longer rise times at more depolarised membrane potentials.

Discussion

Time course of the inhibitory synaptic current

Both population and unitary currents were recorded in voltage clamped motoneurones during reciprocal inhibition. Unitary currents peaked in only 0.4 ms and decayed exponentially with an average time constant of ~0.8 ms at resting membrane potentials. This decay was fitted with a single exponential as have been the decays of glycinergic IPSCs recorded in the goldfish (Faber & Korn, 1987, 1988) and the lamprey (Gold & Martin, 1983*a*). The decay of glycinergic IPSCs recorded in rat spinal cord slices was fitted with a double exponential (Konnerth *et al.* 1988). The glycinergic IPSCs recorded in the present study were considerably briefer than glycinergic synaptic currents recorded previously (Faber & Korn, 1987, 1988; Gold & Martin, 1983*a*; Konnerth *et al.* 1988). This difference can, however, be attributed largely to the lower temperatures used in other preparations. A comparison of the decay time constants of glycinergic IPSCs recorded in different preparations adjusted to 37°C is shown in Table 3.1.

Some distortion of the population IPSC may have occurred. Temporal dispersion of the timing of transmitter release at the many release sites activated by stimulation of the whole Q nerve will distort or prolong the rising phase and, to a lesser extent, the decay phase of the population IPSC. In addition, inadequate voltage control of the fast rising phase of both population and unitary IPSPs will

TABLE 3.1. Comparison of the τ_D of glycinergic IPSCs recorded at the resting membrane potential in different preparations adjusted to 37°C.

Preparation	Average τ_D (ms)	Temperature (°C)	τ_D adjusted to 37°C (ms)*
Gold fish Mauthner cell[1]	5.7	17-20	0.7-1.0
Lamprey Müller cell[2]	32	5	2.2
Rat spinal cord slices[3]	τ_1= 4.6	22-24	1.0-1.2
	τ_2=25.8	22-24	5.5-6.8
Cat spinal motoneurones[4]	0.8	37	0.8

[1]Faber & Korn, 1980; [2]Gold & Martin, 1983*a*; [3]Konnerth *et al.* 1988; [4]this report.

* An estimate of the τ_D of IPSCs at 37°C was calculated using a Q_{10} of 2.8. This is an average Q_{10} of the τ_D of IPSCs and EPSCs recorded at many different synapses (Magleby & Stevens, 1972*b*; Anderson & Stevens, 1973; Onodera & Takeuchi, 1979; Collingridge *et al.* 1984).

distort and prolong the rise time of the recorded synaptic current. For these reasons, the measured rise times and time constants of decay should be considered as maximum values.

Large changes in the membrane potential, especially in the depolarising direction, may affect the spatial extent of the voltage clamp. This could occur if activation of voltage dependent conductances cause a significant decrease in the membrane resistance. A decrease in the specific membrane resistance (R_m) will decrease the steady state space constant (λ) according to the following relationship:

$$\lambda \propto \sqrt{(R_m / R_i)}$$

where R_i is the specific resistance of the cytoplasm. A decrease in λ means that the cell will be less electrically compact and will reduce the spatial extent of the voltage clamp. This would only become important if some inhibitory synapses located on proximal dendrites and isopotential at the resting membrane potential become non-isopotential during large changes in the membrane potential. That both unitary and population IPSCs consistently showed the same voltage dependent properties suggests this did not occur. The finding that population IPSCs rose and decayed slightly more slowly than the unitary IPSCs can be accounted for by temporal dispersion of the population current.

The time constant of decay of many synaptic currents has been shown to reflect the average open time of the transmitter activated channels (Anderson & Stevens, 1973; Crawford & McBurney, 1976; Faber & Korn, 1980, 1982; Gold & Martin, 1983*a,b*; Segal & Barker, 1984*a,b*; Konnerth *et al.* 1988). This is generally thought to only occur if the concentration of transmitter within the synaptic cleft decreases rapidly compared with the average channel open time (Magleby & Stevens, 1972*b*; Katz & Miledi, 1973). However, recent experiments using the rapid application of glutamate agonists has lead some authors to suggest that the rate of decay of fast excitatory synaptic currents can be determine by the rate of

desensitization of the synaptically activated channels (Trussell & Fischbach, 1989; Dudel, Franke & Hatt, 1990). This idea has been recently challenged by the finding that concanavalin A, a tetrametric lectin which binds to specific carbohydrate residues, reduces desensitization of channels activated by externally applied glutamate, but has no effect on the decay of fast excitatory synaptic currents (Mayer & Vyklicky, 1989). Comparable data on the desensitization of glycine activated currents shows that desensitization takes hundreds of milliseconds (Krishtal *et al.* 1988; Akaike & Kaneda, 1989). As this is many orders of magnitude longer than the decay time constant of the IPSCs recorded in the present study it suggests that desensitization does not play an important role in determining of the decay of glycinergic IPSCs.

If we assume that the decay of the IPSCs generated during reciprocal inhibition reflects the average channel open time, then the average channel open time at 37°C for glycine activated channels at the synapse between Ia reciprocal interneurones and motoneurones will be approximately 0.8 ms at the resting membrane potential. Available data on the estimated open times of glycine activated channels in different preparations adjusted to 37°C is shown in Table 3.2. This table shows that the estimated glycine single channel open time from the present study (0.8 ms) is comparable to that estimated from fluctuation analysis or single channel recording in other systems. This finding is consistent with the idea that the decay of the glycinergic IPSCs recorded in motoneurones during reciprocal inhibition is determined by the single channel open time of the glycine activated channels. Presumably this occurs as following transmitter release the concentration of glycine in the synaptic cleft falls rapidly below the K_D for binding to its receptor (90 μM; Akaike & Kaneda, 1989).

TABLE 3.2. Comparison of the estimated glycine single channel open time in different preparations adjusted to 37°C.

Preparation	Open time (ms)*	Temperature (°C)	Open time adjusted to 37°C (ms)†
Cultured spinal cord neurones[1]	~5	26	1.6
Gold fish Mauthner cell[2]	7.2	17-20	0.9-1.2
Cultured spinal cord neurones[3]	~8	24	2.1
Lamprey Müller cell[4]	34	5	2.4
Cultured spinal cord neurones[5]	<10‡	22-24	<2.1-2.9
Rat spinal cord slices[6]	τ_1= 2.5	22-24	0.5-0.7
	τ_2= 27	22-24	5.8-7.1

[1]Barker & McBurney, 1979; [2]Faber & Korn 1980; [3]Barker, McBurney & MacDonald, 1982; [4]Gold & Martin 1983*b*; [5]Hamill *et al.* 1983; [6]Konnerth *et al.* 1988.

* Open time estimated from fluctuation analysis studies 1 to 4, from single channel recording studies 5 and 6.

† Where the Q_{10} of the single channel open time was not measured an estimate of the open time at 37°C was calculated using a Q_{10} of 2.8. This value is the Q_{10} of the open time of ACh gated channels at the neuromuscular junction (Anderson & Stevens, 1973).

‡ extracellular glycine concentration 5 μM

Number of channels opened by transmitter release from a single Ia reciprocal interneurone

Unitary IPSCs recorded at resting membrane potentials ranged in size from 120 to 220 pA. The synaptic conductance associated with these unitary IPSC (g_{IPSC}) can be calculated from the equation:

$$g_{IPSC} = I_{IPSC} / (V_H - E_{IPSC})$$

where I_{IPSC} is the peak unitary current, V_H is the holding potential and E_{IPSC} is the reversal potential for the IPSC. Unitary peak conductances ranged from 6 to 15 nS, with an average unitary peak conductance of 9.1 ± 1.7 nS (±S.E.M., n=5). Comparison of the average unitary peak conductance to the average peak conductance associated with the population IPSC (167 nS) suggests that, on average, each PBSt motoneurone receives input from approximately 20 Q Ia reciprocal interneurones. This is fewer than previously reported (Jankowska & Roberts, 1972*b*; maximum of 70 Ia reciprocal interneurones in contact with each motoneurone).

If we assume a mainstate glycine single channel conductance of 46 pS (mouse cultured spinal neurones; Bormann *et al.* 1987), then following activation of a single Ia reciprocal interneurone on average approximately 200 postsynaptic chloride channels will be opened by the release of glycine at the synapses formed with a motoneurone.

Due to high noise levels miniature IPSCs could not be identified in the present study (unitary IPSCs were usually obtained from averages of 500 to 1000 single sweeps, see Fig. 3.7). This makes it difficult to estimate the conductance change generated at an individual release site by the release of a single quantum of the glycine. However, it is known that Ia reciprocal interneurones make multiple synaptic contacts with each motoneurone (see Fig. 3.1; range: 4 to 11 contacts per motoneurone, also Gad, Jankowska, McCrea & Rastad, 1983) and that each synaptic contact contains from one to three possible release sites (Rastad, 1981).

In addition, the average probability of transmitter release at each release site has been estimated to be ~0.5 (Kuno & Weakly, 1972). From this it can be estimated that the number of channels opened by the release of a single quantum of glycine could be ten times smaller than the number opened at the termination of a single Ia reciprocal interneurone with a motoneurone. For example, if there are on average 20 release sites associated with each Ia reciprocal interneurone/motoneurone connection, each with an average probability of transmitter release of 0.5, then the average conductance change generated at an individual release site following the release of a single quantum of glycine will be (9 nS / 20 release sites) / 0.5. This value of 0.9 nS corresponds to the opening of only ~20 postsynaptic channels per quantum of glycine, assuming a single channel conductance of 46 pS. This simplistic calculation is used to demonstrate that the quantal response at this glycinergic inhibitory synapse probably involves the activation of only a small number of channels.

This estimate is considerably lower than the 1,500 to 2,000 chloride channels thought to be opened by a single quantum of glycine in the goldfish (Korn, Mallet, Triller & Faber, 1982; Korn, Burnod & Faber, 1987) and the lamprey (Gold & Martin, 1983*a*). However, it is of similar magnitude to the 15 to 20 chloride channels thought to be opened by a single quantum of glycine in mammalian spinal cord slices (Konnerth *et al.* 1988). The release of a single quantum of GABA is also thought to open only a small number (12 to 30) of chloride channels (Ropert *et al.* 1990; Edwards *et al.* 1990; Kriegstein & Lo Turco, 1990).

Gundersen, Miledi & Parker (1984) using human glycine receptors expressed in *Xenopus* oocytes have suggested that the binding of three molecules of glycine to its receptor may be required to open a single glycine activated membrane channel. However, a more recent study using acutely isolated hypothalamic neurones has suggested that only two molecules of glycine are required to bind to its receptor to allow activation of the associated membrane channel (Akaike & Kaneda, 1989). Given that only 200 postsynaptic channels are

opened by transmitter release at the termination of a single Ia reciprocal interneurone with a motoneurone and that as few as 20 channels are probably opened at each individual release site, then only 40 to 60 molecules of glycine would be required to open all postsynaptic channels at each release site. This is considerably smaller than the number of transmitter molecules thought to be contained within a single presynaptic vesicle (~5000; Kuffler & Yoshikami, 1975; Riveros, Fiedler, Lagos, Munoz & Orrego, 1986) and suggests that at this mammalian central synapse the contents of a single vesicle should be more than sufficient to open all available postsynaptic channels.

Effects of anaesthetics

Although the effects of different anaesthetics were not studied in detail, it was observed that there was no apparent difference in the time course of IPSCs recorded in pentobarbitone or α-chloralose anaesthetised preparations compared to those recorded in unanaesthetised, decerebrated preparations (Fig. 3.10). This suggests that neither pentobarbitone nor α-chloralose acts directly to modify the kinetics of glycine activated channels.

In contrast, $GABA_A$ activated IPSPs and IPSCs are prolonged by pentobarbitone (Nicoll, Eccles, Oshima & Rubia, 1975; Segal & Barker, 1984*b*; Collingridge *et al.* 1984). This has been attributed to an increase in the $GABA_A$ single channel open time (Study & Barker, 1981; Segal & Barker, 1984*b*). A more recent study has shown that barbiturates alter the rate constants which regulate entry into different open states in such a way that entry into the longest open state is favoured over shorter open states (MacDonald *et al.* 1989).

The difference in the effects of pentobarbitone on glycine and $GABA_A$ mediated events can probably be most simply explained by differences between the $GABA_A$ and glycine receptors, as it has been argued that both GABA and glycine open a common ion channel (Barker & McBurney, 1979; Hamill *et al.* 1983; McBurney, Smith & Zorec, 1985; Bormann *et al.* 1987). Presumably the $GABA_A$ receptor includes a barbiturate binding site whereas the glycine receptor does not.

Dependence of the peak current on membrane potential

The amplitude of the peak current varied linearly with the membrane potential over the range of potentials studied in these experiments (~-20 to -90 mV). While the effect of membrane potential on the peak current was not investigated at more hyperpolarised potentials, the amplitude of synaptically mediated glycinergic currents and whole cell currents activated by glycine have been seen to plateau at membrane potentials more negative than -90 to -100 mV (Gundersen *et al.* 1984, 1986; Faber & Korn, 1987, 1988; Bormman *et al.* 1987, Akaike & Kaneda, 1989). This outward rectification is thought to be caused by a voltage dependence of the glycine channel open time (Gundersen *et al.* 1986; Bormann *et al.* 1987).

Estimation of $[Cl^-]_i$

IPSCs reversed to become inward currents at an average membrane potential of -80.7 mV (using KCH_3SO_4 filled electrodes). This result agrees with earlier work of Araki and Terzuolo (1962). While it was originally suggested that the ionic current flowing at this synapse is carried by both Cl^- and K^+ ions (Coombs *et al.* 1955*a*), more recent work on glycinergic currents in cultured spinal neurones (Bormann *et al.* 1987) and isolated hypothalamic neurones (Akaike & Kaneda, 1989), suggests that glycine activated channels are impermeable to K^+ ions. If the ionic current flowing during the IPSC is carried purely by Cl^- ions then the reversal potential for the IPSC will be identical to the Cl^- equilibrium potential (E_{Cl}) and this can be used to calculate the intracellular chloride concentration, $[Cl^-]_i$, from the Nernst equation.

Assuming $[Cl^-]_o$ is 134 mM (Vogh & Maren, 1975), then at 37°C $[Cl^-]_i$ will be 6.5 mM. This is lower than would be expected if Cl^- was passive distributed across the membrane (Coombs *et al.* 1955*a*) and gives further evidence for the presence of an outwardly directed Cl^- pump in motoneurones (Lux, 1971). This value of 6.5 mM is close to the 6.6 mM estimated for $[Cl^-]_i$ in rat spinal motoneurones (Forsythe & Redman, 1988).

The above calculation will be in error if the reversal potential for the IPSC is not identical to the Cl^- equilibrium potential. This will occur if the ionic current flowing during the IPSC is partly carried by ions other than Cl^-. The only other small anion that is available in sufficient concentrations to contribute to this ionic current is the bicarbonate ion (HCO_3^-).

While it was originally thought that the synaptic channels opened during reciprocal inhibition are impermeable to HCO_3^- (Coombs *et al.* 1955*a*), recent work by Bormann, Hamill and Sakmann (1987) on glycine activated currents in cultured mouse spinal neurones suggests that glycine activated channels are somewhat permeable to HCO_3^-. These authors give a permeability ratio of HCO_3^- to Cl^- ($P_{HCO3} : P_{Cl}$) of 0.11. If only Cl^- and HCO_3^- ions contribute to the ionic current flowing during the IPSC, then this permeability ratio can be used to determine $[Cl^-]_i$ using a modified form of the Goldman-Hogkin-Katz voltage equation:

$$E_{rev} = RT/F \ln [(P_{Cl} [Cl^-]_i + P_{HCO3} [HCO_3^-]_i)/(P_{Cl} [Cl^-]_o + P_{HCO3} [HCO_3^-]_o)]$$

where E_{rev} is the reversal potential for the IPSC and $[HCO_3^-]_o$, $[HCO_3^-]_i$, $[Cl^-]_o$ and $[Cl^-]_i$ are the extracellular and intracellular HCO_3^- and Cl^- concentrations. E_{rev} was found to be -80.7 mV and $[HCO_3^-]_o$ and $[Cl^-]_o$ are thought to be 22 and 134 mM respectively (Vogh & Maren, 1975). While the $[HCO_3^-]_i$ is unknown it can be calculated from the following equation, which comes from Gallard & Dupont (1990), given the intracellular and extracellular pH (pH_i and pH_o) and $[HCO_3^-]_o$:

$$[HCO_3^-]_i = [HCO_3^-]_o \times 10^{(pHi - pHo)}$$

If the pH_i is between 7.1 and 7.3 (Gallard & Dupont, 1990; Silver & Erecinska, 1990), pH_o is 7.4 and $[HCO_3^-]_o$ is 22 mM, then $[HCO_3^-]_i$ will range from 11 to 17 mM.

Substitution of these values into the modified form of the Goldman-Hogkin-Katz equation given above gives values of $[Cl^-]_i$ from 4.8 to 5.4 mM. Using this to recalculate E_{Cl} from the Nernst equation gives a Cl^- equilibrium potential of between -86 and -89 mV. Whether these alternative estimates of $[Cl^-]_i$ and E_{Cl} are correct depends largely on how permeable the chloride channels opened by the release of glycine from Ia reciprocal interneurones are to HCO_3^-. This is unknown; however, if these channels are permeable to HCO_3^-, then $[Cl^-]_i$ will be lower than previously estimated and E_{Cl} more negative than E_{rev}.

Some evidence that suggests this may be the case comes from work on GABA activated currents in crayfish muscle fibres where, in the presence of physiological concentrations of HCO_3^- ions, E_{Cl} is more negative than E_{GABA} by ~10 mV (Kaila & Voipio, 1987; Kaila, Pasternack, Saarikoski & Voipio, 1989). In addition, a recent report has shown that an intracellular alkalosis (and associated increase in $[HCO_3^-]_i$) causes a positive shift in the reversal potential of GABAergic IPSPs in mammalian cortical neurones (Kaila, Pasternack, Voipio & Deisz, 1990). This finding suggests that the inhibitory synaptic current underlying these IPSPs is carried in part by HCO_3^-.

Dependence of the decay time constant on membrane potential

Perhaps the most important finding from this work is that a clear voltage dependence in the rate of decay of glycinergic IPSCs was observed at a mammalian central synapse, *in vivo*. During this study Faber & Korn (1987) reported a similar voltage dependence in the decay of glycinergic IPSCs in the goldfish Mauthner cell; the decay time constant for these IPSCs increasing *e*-fold for a 45mV depolarisation. Curiously, however, the same authors report that the decay of spontaneous glycinergic IPSCs in the goldfish Mauthner cell is voltage insensitive (Korn *et al.* 1987). Gold and Martin (1983*a*) have also reported the absence of any voltage dependence in the rate of decay of spontaneous glycinergic IPSCs recorded in the lamprey Müller cells.

It is difficult to understand why there would appear to be a difference in the voltage dependence of the rate of decay of evoked and spontaneous IPSCs. One possibility is that spontaneous IPSCs are generated at distal sites, and so are not isopotential with the somatically located voltage clamp. If so spontaneous IPSCs would not 'see' the same voltage change as recorded at the soma and consequentially the rate of decay of these IPSCs may appear less sensitive voltage. It is also possible that a voltage dependence in the rate of decay of spontaneous glycinergic IPSCs was missed as only a narrow range of membrane potentials were examined in these studies. The rate of decay of both evoked and spontaneous synaptic currents generated by the release of GABA and acetylcholine have previously been shown to be voltage dependent (Gage, 1976; Onodera & Taheuchi, 1976, 1979; Dudel, 1977; Collingridge *et al.* 1984; Cull-Candy, 1986; Barker & Harrison, 1988; Kriegstein & Lo Turco, 1990).

If the decay of IPSCs generated during reciprocal inhibition reflects the open time of the synaptically activated channels, then a voltage dependence in the rate of decay of glycinergic IPSCs can be interpreted as a voltage dependence of the glycine channel open time. A voltage dependence of the glycine channel open time has been suggested by others (Gundersen *et al.* 1984, 1986; Bormann *et al.* 1987; Faber & Korn, 1987).

The decay of $GABA_A$ IPSCs and the open times of Cl^- channels gated by GABA are also prolonged by membrane depolarisation (Onodera & Taheuchi, 1976, 1979; Dudel, 1977; Collingridge *et al.* 1984; Segal & Barker, 1984*a*,*b*: Cull-Candy, 1986; Bormann *et al.* 1987; Barker & Harrison, 1988; Robertson, 1989; Kriegstein & Lo Turco, 1990), increasing *e*-fold for depolarisations of from 103 to 188 mV. This similarity in the voltage dependence of the glycine and GABA gated currents suggests that the channel associated with both the GABA and glycine receptors has similar properties.

In contrast, the rate of decay of excitatory synaptic currents is thought to be relatively insensitive to changes in the membrane potential (Onodera & Takeuchi, 1978; Finkel & Redman, 1983*a*; Nelson, Pun & Westbrook, 1986; Hestrin, Nicoll,

Perkel & Sah, 1990; however, see Dudel, 1974; Cull-Candy & Miledi, 1982; Konnerth, Kellar, Ballanyi & Yarri, 1990).

Voltage dependence in the rate of decay of transmitter activated currents was first observed at the neuromuscular junction (Takeuchi & Takeuchi, 1959; Gage & Armstrong, 1968; Kordas, 1969; Magelby & Stevens, 1972*a*) and can be explained by the following hypothesis originally developed by Magelby and Stevens (1972*b*). The gating of a channel into or out of the open state was proposed to require a conformational change in the receptor/channel molecule. This conformational change was proposed to be associated with a change in a dipole moment normal to the membrane surface. Changing the membrane potential would change the electric field surrounding this dipole moment and so could regulate the rate at which the channel enters or leaves the open state. If correct, this hypothesis predicts that the rate limiting step that determines the decay of transmitter activation currents is this conformational change in the receptor/channel molecule.

The voltage dependence of the channel open time, together with the direct effect of membrane potential on the peak inhibitory current, will act to enhance the strength of reciprocal inhibition as the motoneurone membrane potential approaches threshold. A change in membrane potential from -70 mV to -50 mV will cause a 3 fold increase in peak current and a 25% increase in the duration of the IPSC (calculated from the relationship shown in Fig. 3.16). This will result in a 275% increase in the total negative charge associated with the inhibitory current (see Fig. 3.19). While this enhancement is dominated by the increase in peak current, a significant contribution is made by the prolonged decay of the synaptic current at the more depolarised membrane potential.

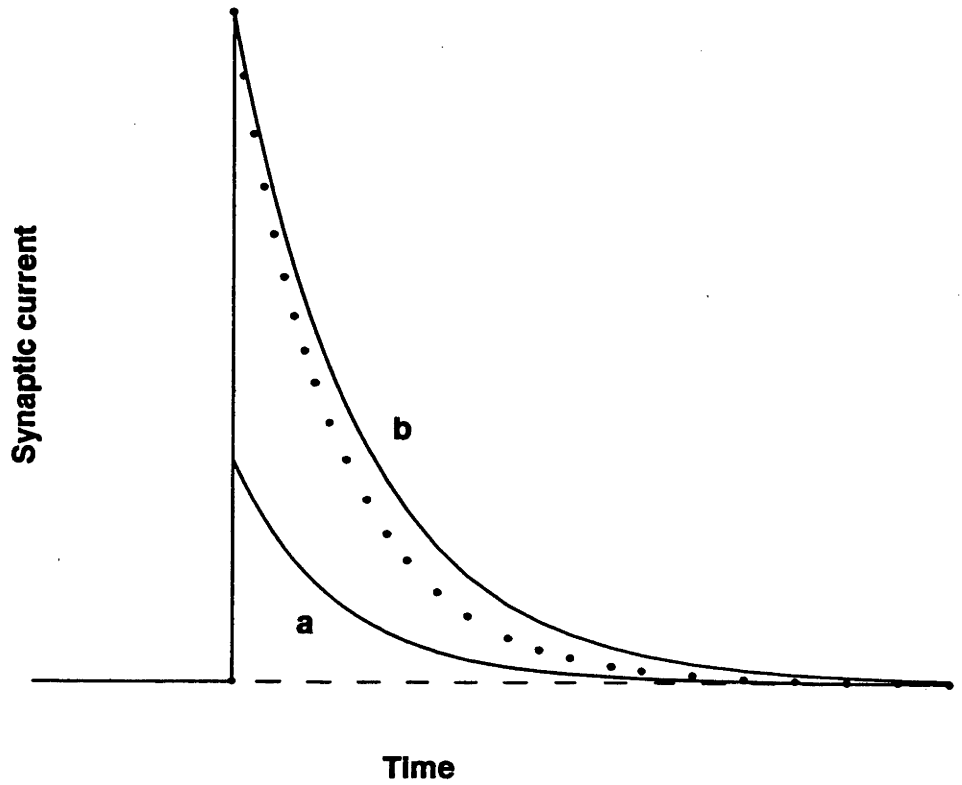


Fig. 3.19. Schematic diagram showing the effect of depolarisation on the IPSC peak and time course. *a*, shows an idealized IPSC of the form $y = e^{-t/\tau}$ at -70 mV, decaying with a time constant (τ) of 1 ms. *b*, the solid line shows the idealized IPSC at -50 mV, decaying now with a τ of 1.25 ms. The dotted line shows the IPSC at -50 mV decaying with a τ of 1 ms. i.e. decaying with the same τ as at -70 mV. The difference in the area below the solid and dotted lines in *b* gives the contribution to the synaptic current at -50 mV made by the increase in τ at the more depolarised membrane potential. The total negative charge transferred during the IPSC can be calculated by integrating the area under each curve. If the total negative charge transferred at -70 mV is 1 pC (assuming a peak current of 1 nA, τ = 1 ms), then the total negative charge transferred at -50 mV will be 3.75 pC, given a 3 fold increase in the peak current and a 25% increase in τ. The area under the dotted line in *b* gives the total negative charge transferred at -50 mV assuming τ is unchanged by the depolarisation and is 3 pC.

Chapter four:

THE ROLE OF $GABA_A$ AND $GABA_B$ RECEPTORS IN PRESYNAPTIC INHIBITION OF Ia EPSPS

Introduction

It is now generally accepted that in the mammalian spinal cord presynaptic inhibition of transmitter release from primary afferent fibres is mediated by the release of GABA at axo-axonic synapses (Eccles *et al.* 1963*a*; Curtis *et al.* 1971; Bell & Anderson, 1972; Gmelin & Cerletti, 1976; Barber *et al.* 1978; Curtis & Lodge, 1982; Curtis *et al.* 1986; Magoul *et al.* 1987; Maxwell *et al.* 1990). However, the mechanism by which GABA reduces transmitter release is unknown. As GABA has been shown to activate two types of receptors, $GABA_A$ and $GABA_B$ (Hill & Bowery, 1981), and both types of GABA receptors appear to be present on primary afferent terminals (Eccles *et al.* 1963*a*; Curtis *et al.* 1971; Pierau & Zimmermann, 1973; Sastry, 1979*a*; Davies, 1981; Curtis & Lodge, 1982; Curtis, Lodge, Bornstein & Peet, 1981; Price, Wilkin, Turnbull & Bowery, 1984; Price, Kelly & Bowery, 1987; Peng & Frank, 1989*a,b*; Edwards, Harrison & Jack, 1989; Curtis, 1990), it is possible that either, or both, of these GABA receptors may be involved in mediating presynaptic inhibition.

Activation of $GABA_A$ receptors is usually associated with an increase in chloride conductance (Bormann *et al.* 1987), whereas activation of $GABA_B$ receptors has been shown to increase a potassium conductance in many central neurones (Newberry & Nicoll, 1985; Inoue *et al.* 1985; Gahwiler & Brown, 1985) and also to decrease a voltage dependent calcium conductance in the cell bodies of dorsal root ganglion cells (Dunlap & Fischbach, 1978, 1981; Dunlap, 1981*a,b*; Deisz & Lux, 1985; Dolphin & Scott, 1986, 1987; Robertson & Taylor, 1986). A recent report also suggests that GABA and baclofen, a $GABA_B$ agonist (Bowery *et*

al. 1979; Bowery *et al.* 1981), can shift the voltage dependence of inactivation of a transient potassium current, recorded in the cell bodies of cultured hippocampal neurones, to more depolarised potentials (Saint *et al.* 1990). Any one of these actions, if present during invasion of an action potential into primary afferent terminals, could reduce calcium influx and decrease the probability of transmitter release.

Previous studies on the pharmacology of presynaptic inhibition in the mammalian spinal cord have used relatively indirect methods to assess "presynaptic" inhibition. Presynaptic inhibition has usually been measured by changes in the inhibition of ventral root reflexes or the dorsal root potential evoked by the conditioning stimulation (Eccles *et al.* 1963*a*; Curtis *et al.* 1971). The 'prolonged inhibition' of ventral root reflexes will undoubtedly be complicated by the postsynaptic hyperpolarisation which occurs in conjunction with presynaptic inhibition (see Results; Kellerth, 1968; Cook & Cangiano, 1972), whereas the dorsal root potential merely indicates depolarisation of primary afferent fibres and is not itself a measure of presynaptic inhibition. In addition, previous pharmacological studies have usually examined the effects of drugs administered either by systemic injection or topical application (however, see Gmelin & Cerletti, 1976; Curtis & Lodge, 1982; Curtis *et al.* 1986), which may modify synaptic transmission through the polysynaptic pathways which mediate presynaptic inhibition (Eccles, Kostyuk & Schmidt, 1962*a*; Jankowska, McCrea, Rudomin & Sykova, 1981; Rudomin, Solodkin & Jimenez, 1987).

The present study has attempted to overcome many of these problems by combining the local application of specific antagonists by iontophoresis with intracellular recording of presynaptic inhibition of monosynaptic excitatory postsynaptic potentials (EPSPs). The aim of the study was to evaluate the role of $GABA_A$ and $GABA_B$ receptors in presynaptic inhibition of Ia EPSPs in the mammalian spinal cord.

Methods

Initial surgery was performed on adult cats as described in Chapter two. All experiments were performed on cats anaesthetised with sodium pentobarbitone. As sodium pentobarbitone is known to enhance presynaptic inhibition (Eccles *et al.* 1963*a*), supplementary doses were only given after a recording session with a particular cell.

The hindlimb was dissected as follows. The nerves to posterior biceps and semitendinosus (PBSt) in the left hindlimb were separated from surrounding tissue, cut distally and mounted on a stimulating electrode. Usually the most proximal branch of posterior biceps, which usually runs together with a branch of anterior biceps, was not used. The nerve to medial gastrocnemius (MG) was also freed from surrounding tissue, mounted on a stimulating electrode and usually cut distally. In the experiments were single Ia afferent fibres were stimulated the MG muscle nerve was left intact and the MG muscle separated as much as possible from surrounding tissue. A thread was tied around the MG muscle tendon and the tendon cut distally.

Recording

Intracellular recordings were made from antidromically identified MG motoneurones (resting membrane potentials greater than -55 mV, spike height greater than 60 mV) using iontophoretic electrodes described in Chapter two. The intracellular recording electrode was filled with 2M KCH_3SO_4 and the extracellular iontophoretic barrels were filled with either (−)-baclofen (baclofen), bicuculline methochloride (BMC), 2-OH-saclofen, or strychnine hydrochloride (strychnine). See Chapter two for concentrations and current passing procedures.

Compound Ia EPSPs were recorded from MG motoneurones following stimulation of the MG muscle nerve at 1.5 x group I threshold (T, determined from the cord dorsum potential). Unitary EPSPs were recorded following the activation of single MG Ia afferent fibres and were obtained in the following way (see also

Honig, Collins & Mendell, 1983). Firstly, dorsal root filaments close to the L7/S1 entry zone were placed on a bipolar recording electrode and the number of Ia afferents in each filament assessed by the level of activity recorded during brief, passive stretch of the MG muscle. Once a filament was found which contained many Ia afferents, a small platform was positioned under the filament just distal to the bipolar recording electrode. This platform stabilized the filament during intra-axonal recording which was made using a second microelectrode filled with 3M KCl. The MG muscle nerve was stimulated and penetrated axons identified as MG Ia afferents by their conduction velocity, stimulus threshold and response to brief, passive stretch of the MG muscle. Once a stable recording from a MG Ia afferent was established action potentials were evoked in this axon by passing brief (1 to 2 ms in duration, up to 10 nA in magnitude) depolarising current pulses through the intra-axonal microelectrode. MG motoneurones were then impaled and intracellular records averaged to determine if a unitary Ia EPSP was present in response to axonal stimulation.

A schematic diagram of the experimental arrangement is shown in Fig. 4.1. Presynaptic inhibition of compound or unitary MG Ia EPSPs was produced by prior stimulation of the PBSt muscle nerve. This activates groups of inhibitory interneurones which synapse "presynaptically" onto MG Ia afferent terminals and "postsynaptically" onto MG motoneurones. Conditioning PBSt stimulation, composed of 3 stimuli at 300 Hz, 2 x T, always preceded EPSPs by at least 50 ms and was repeated at 1 second intervals. Alternate records of conditioned and unconditioned EPSPs were stored in separate buffers in a microcomputer and averaged. Usually 10 to 20 complete sequences of conditioned and unconditioned EPSPs were collected and averaged for compound EPSPs, whereas several hundred complete sequences were averaged for unitary EPSPs.

Data analysis

The peak amplitude, 10 to 90% rise time and duration at half peak amplitude (half-width) of unconditioned and conditioned compound and unitary

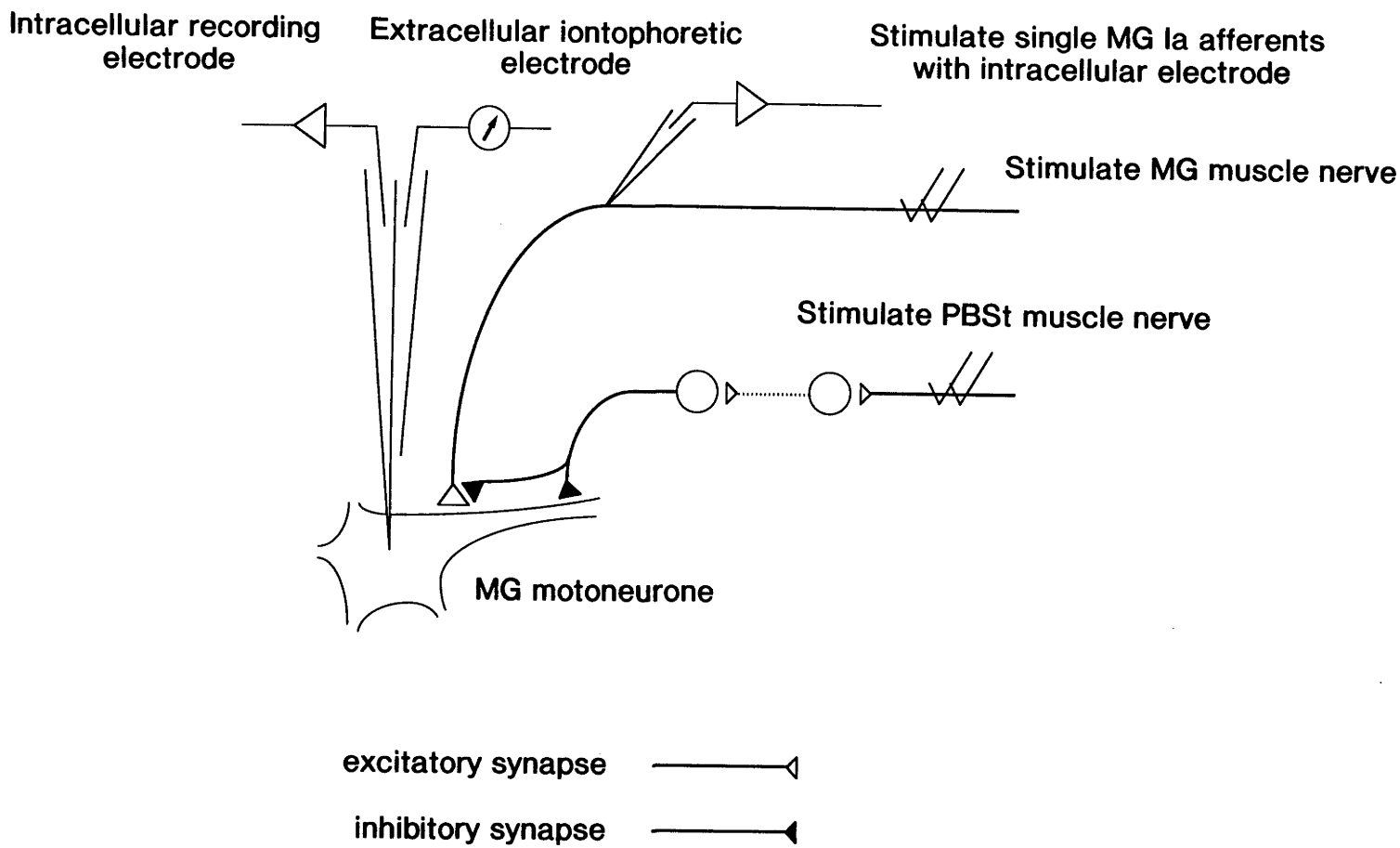


Fig. 4.1. A schematic diagram of the experimental arrangement used to study presynaptic inhibition as described in the text.

EPSPs was measured. The time constant of decay of EPSPs and membrane time constant were determined by displaying the decay phase of the EPSP semilogarithmically and fitting a linear regression line to the final decay phase, using a least-squares procedure for the best fit. Input resistance measurements were made using a discontinuous single electrode current clamp (see Chapter two) using switching rates of between 3 and 5 kHz. The input resistance was determined from the steady-state voltage response to 1 or 2 nA hyperpolarising current pulses of between 10 and 30 ms duration. This voltage response was also used to determine the membrane time constant using the same procedure as described for determining the membrane time constant from the decay of the EPSP.

As the conditioned EPSP was often superimposed on the repolarising phase of an inhibitory postsynaptic potential (IPSP), a linear, sloping baseline was used in an attempt to overcome the distortion of the conditioned EPSP caused by the repolarising IPSP.

The amount of presynaptic inhibition is expressed as the percentage decrease in the peak amplitude of the unconditioned EPSP produced by the conditioning PBSt stimulation. Unless otherwise stated, only the results obtained from cells where complete recovery from a particular drug application was established have been included.

Results

Presynaptic inhibition

The procedure for studing presynaptic inhibition of MG Ia EPSPs is shown in Fig. 4.2. Stimulation of the MG muscle nerve at 1.5 x T evoked a compound, monosynaptic EPSP in the MG motoneurone shown in Fig. 4.2*A*. Prior conditioning stimulation of the PBSt muscle nerve 50 ms before this EPSP (using three stimuli at 300 Hz, 2 x T) evoked an IPSP in this motoneurone and caused the EPSP to be reduced in amplitude (Fig. 4.2*B*). A comparison of the unconditioned and conditioned EPSPs indicated that the peak amplitude of the conditioned EPSP

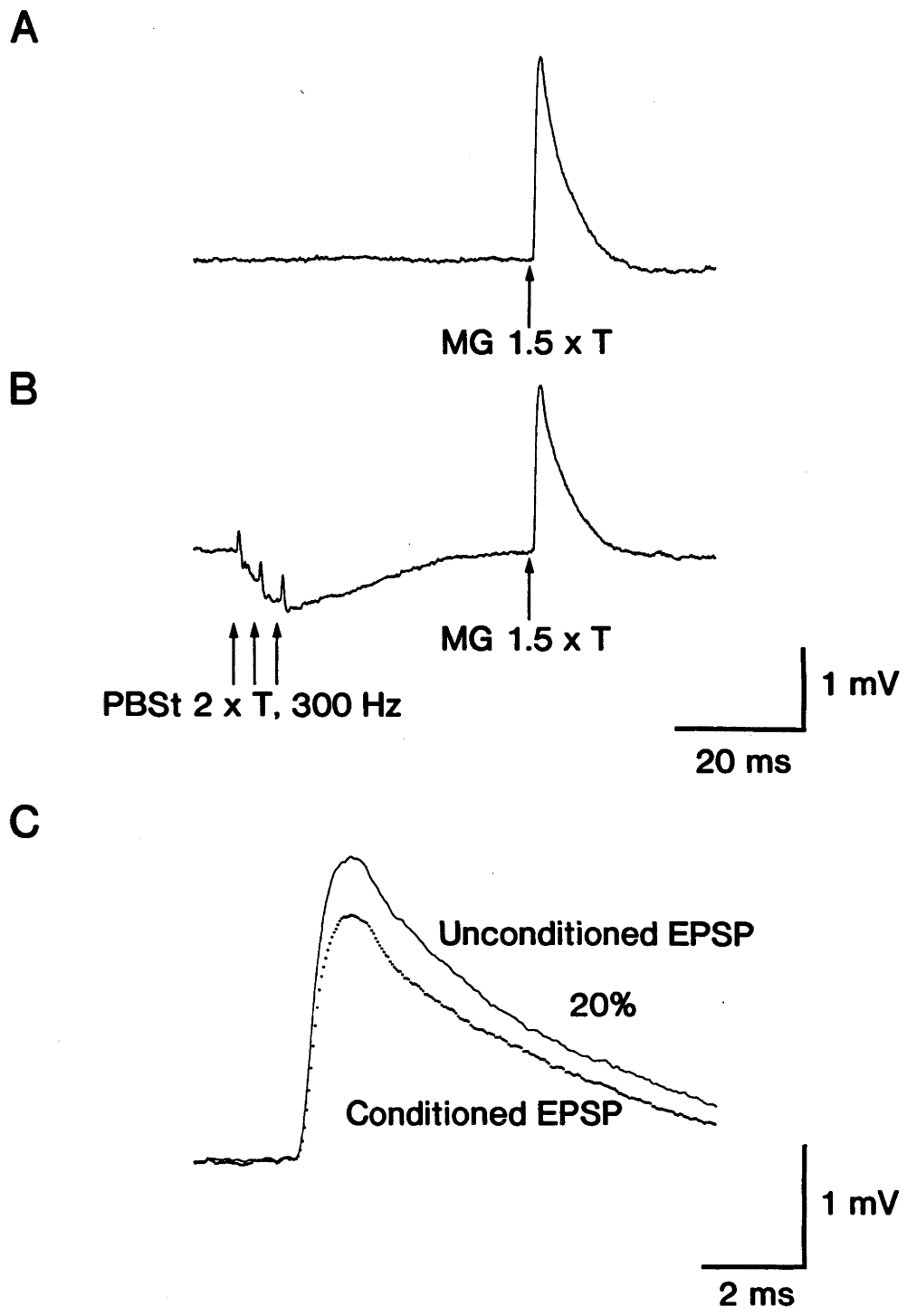


Fig. 4.2. Experimental protocol used to study presynaptic inhibition. *A*, unconditioned compound EPSP evoked in a MG motoneurone by stimulation of the MG muscle nerve. *B*, conditioning PBSt stimulation evokes an IPSP in this motoneurone and causes a reduction in the compound EPSP evoked 50 ms after the conditioning stimulus. *C*, the unconditioned and conditioned EPSPs are displayed and compared on an expanded time scale. The conditioned EPSP was reduced in amplitude by 20%.

was reduced by 20% (Fig. 4.2*C*). This reduction in the amplitude of the conditioned EPSP is considered to occur as a consequence of a presynaptic decrease in transmitter release, as originally proposed by Frank and Fuortes in 1957.

Supporting evidence for this idea comes from the finding that the input resistance measured 50 to 70 ms after the conditioning stimulation, often during the decay of the IPSP, was not significantly different from that measured without the conditioning stimulation. An example of this is shown in Fig. 4.3. The average input resistance measured with conditioning stimulation was 1.33 ± 0.19 MΩ (± S.E.M., $n=5$), compared to 1.34 ± 0.19 MΩ (± S.E.M., $n=5$) in unconditioned motoneurones. This finding is in agreement with previous findings by many authors (Eide *et al.* 1968; Kellerth, 1968; Cook & Cangiano, 1972; Sypert, Munson & Fleshman, 1980; however see also Carlen, Werman & Yaari, 1980). It does not, however, exclude the possibility that postsynaptic inhibition is involved in reducing the conditioned EPSP, as it may not be possible to detect a change in input resistance at the soma if the synapses which generate the IPSP are located on the distal parts of the motoneurone dendritic tree.

Additional evidence for a presynaptic decrease in transmitter release comes from the finding, also previously observed by many authors, that the time course of compound EPSPs is not significantly changed by the conditioning stimulation (Frank & Fuortes, 1957; Eccles et al. 1961*a*; Eide *et al.* 1968; Cook & Cangiano, 1972, McCrea, Shefchyk & Carlen, 1990, however also Carlen *et al.* 1980; Sypert *et al.* 1980; Lev-Tov, Fleshman & Burke, 1983). In a sample of eight compound EPSPs reduced in amplitude by an average of 18.3% during presynaptic inhibition, the average rise time was 0.62 ± 0.04 ms, compared with 0.64 ± 0.04 ms (± S.E.M., $n=8$) for unconditioned EPSPs. Half-widths for conditioned and unconditioned EPSPs were 4.0 ± 0.4 ms and 4.0 ± 0.5 ms (± S.E.M., $n=8$) respectively, and decay time constants were 5.0 ± 0.7 ms and 5.1 ± 0.8 ms (± S.E.M., $n=8$) respectively.

The most convincing evidence for a presynaptic locus for the inhibition of MG Ia EPSPs following the PBSt conditioning stimulation comes from the work of

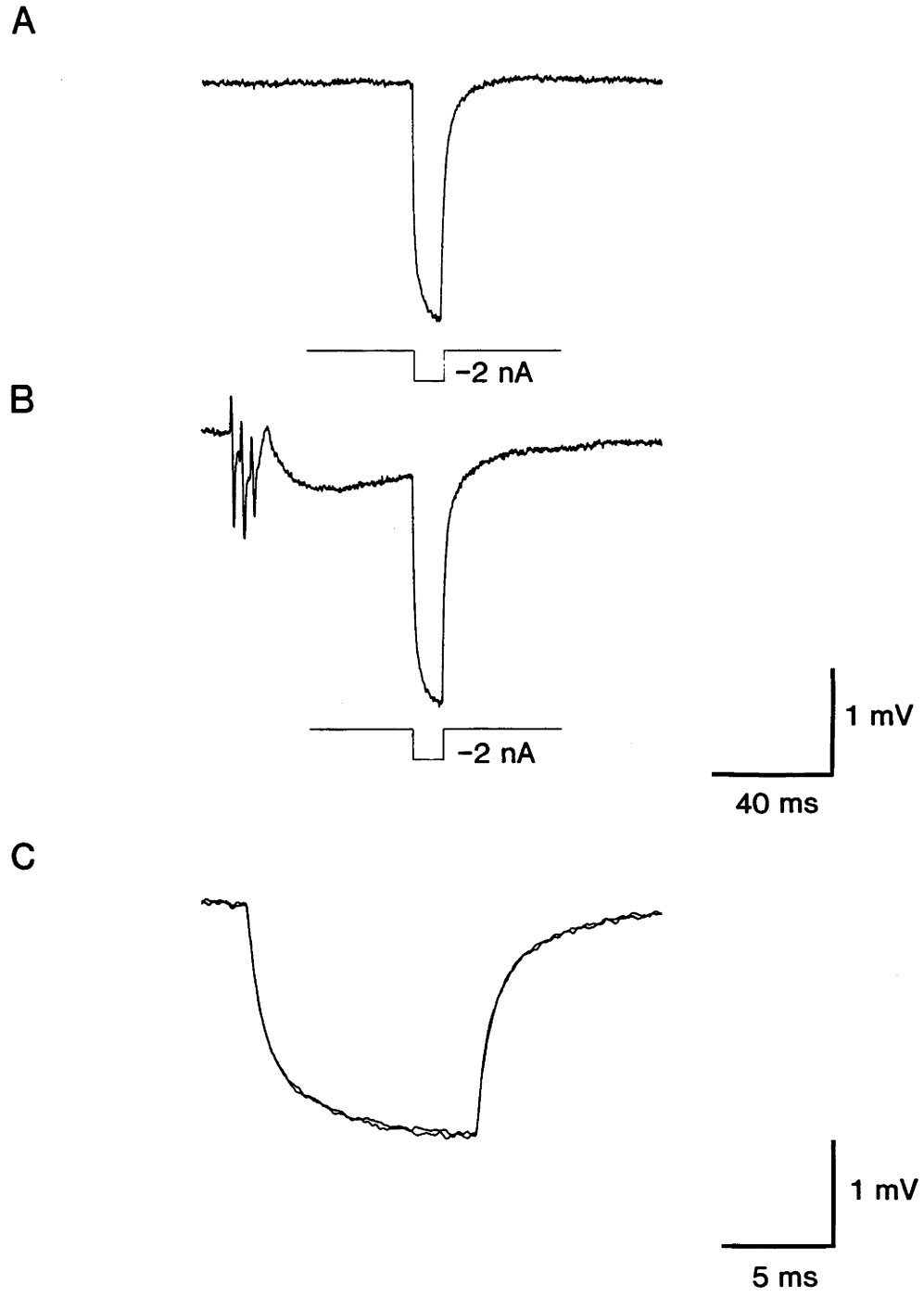


Fig. 4.3. Effect of the conditioning stimulation on the input resistance of a MG motoneurone. *A*, response of a MG motoneurone to a 10 ms, -2 nA current pulse. *B*, response to the same current pulse 70 ms after conditioning PBSt stimulation. *C*, the unconditioned and conditioned response to the current pulse in *A* and *B* are displayed on an expanded time scale and show that there was no effect of the conditioning stimulation on the input resistance of this motoneurone. Records were made using discontinuous current clamp switching at 4 KHz.

Clements, Forsythe and Redman (1987, see also Kuno, 1964), where quantal analysis of the fluctuations in amplitude of Ia EPSPs before and after conditioning showed that the conditioning stimulation decreased the number of quanta contributing to the conditioned EPSP without changing the quantal size.

The results from the quantal analysis experiments, together with the results from the present study (see also Chapter five), provide strong evidence that the reduction in the amplitude of MG Ia EPSPs evoked more than 50 ms after the conditioning PBSt muscle nerve stimulation occurs via a presynaptic decrease in the release of excitatory transmitter from the terminals of MG Ia afferent fibres.

Postsynaptic inhibition

As mentioned above, conditioning stimulation of the PBSt muscle nerve evoked a small IPSP in MG motoneurones (see also Kellerth, 1968; Cook & Cangiano, 1972). In the present study the conditioning stimulation almost always evoked an IPSP in every MG motoneurone examined. This IPSP varied in size from only a few hundred microvolts to up to several millivolts, had a latency to peak of between 20 to 30 ms measured from the start of the conditioning stimulus and usually had a duration of several hundred milliseconds.

Examples of different types of IPSPs recorded during the same experiment in different MG motoneurones are shown in Fig. 4.4. All responses were evoked by the same PBSt conditioning stimulation (3 pulses, 300 Hz, 2 x T), but had quite different time courses. The response in Fig 4.4*A* appeared to be a pure IPSP, whereas the response shown in Fig 4.4*B* was a mixture of an early EPSP and an IPSP. The IPSP shown in Fig 4.4*C* also suggests the presence of a early EPSP. These records were typical of those seen in this study and suggest that the postsynaptic response associated with the conditioning PBSt stimulation does not consist purely of an IPSP, but includes an early EPSP often masked by the more dominant IPSP. This finding is in agreement with the earlier work of Cook and Cangiano (1972, see also Wilson & Kato, 1965). The amplitude and the nature of the early part of the response to PBSt conditioning stimulation will depend on the

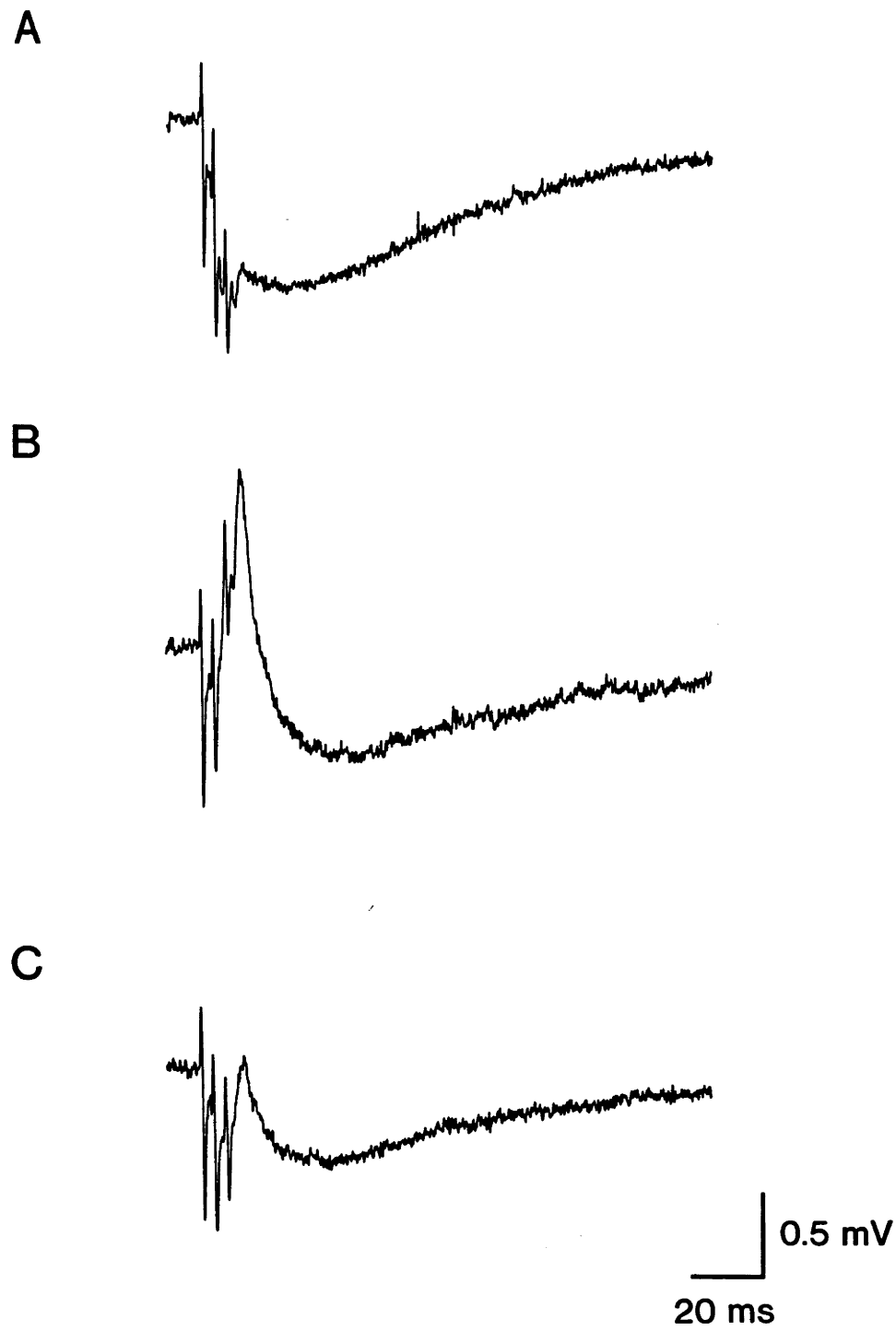


Fig. 4.4*A-C*. Examples of IPSPs evoked in different MG motoneurones by the same conditioning PBSt stimulation. Note the difference in the time course of responses and the appearance of an early EPSP in the response shown in *B*. The membrane potential of motoneurones at the time the responses shown in *A*, *B* and *C* were recorded was -61, -70 and -59 mV respectively.

membrane potential and on the relative strength of the synaptic projections mediating the early EPSP and the IPSP. As the membrane potential of a motoneurone approaches the reversal potential for the IPSP the early EPSP, if present, would be expected to become more dominant.

Pharmacology of postsynaptic inhibition

The early part of the IPSP appeared to be selectively reduced by the iontophoretic application of the glycine antagonist strychnine hydrochloride. An example of this is shown in Fig. 4.5. This application of strychnine had no effect on the late part of the IPSP and appeared to convert the early hyperpolarising response into a depolarising response, unmasking an early EPSP (c.f. Fig. 4.4*B*). The late part of the IPSP, as will be shown later, was found to be reduced by the $GABA_A$ receptor antagonist bicuculline methochloride (see Fig. 4.6, 4.7, 4.13).

As the ventral roots were left intact during most experiments the early IPSP will, in part, be mediated via Renshaw cells as MG motoneurones receive a small amount of presumably strychnine sensitive recurrent inhibition following antidromic activation of motor fibres in the posterior biceps muscle nerve (Eccles, Eccles, Iggo & Ito, 1961). The finding that there are strychnine and bicuculline sensitive components to the hyperpolarisation evoked in MG motoneurones by PBSt stimulation is consistent with the early work of Kellerth (1968).

Effect of the $GABA_A$ antagonist bicuculline methochloride on presynaptic inhibition of compound EPSPs

To investigate whether $GABA_A$ receptors were involved in mediating presynaptic inhibition the $GABA_A$ receptor antagonist bicuculline methochloride (BMC) was used. When applied iontophoretically during presynaptic inhibition BMC reduced both presynaptic inhibition of compound Ia EPSPs and the IPSP evoked in MG motoneurones by the conditioning PBSt stimulation. Examples of this are shown in Fig. 4.6 and Fig. 4.7. In Fig. 4.6*A* the application of +80 nA of BMC for 10 minutes reduced the amount of presynaptic inhibition from 20 to 10%,

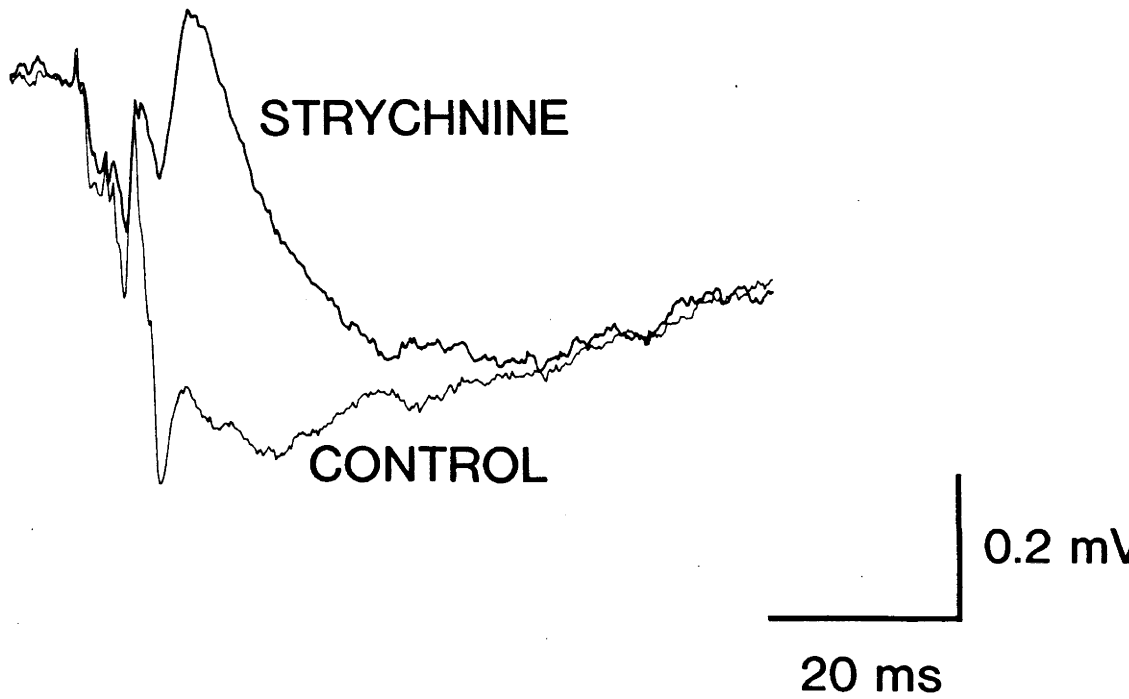


Fig. 4.5. The effect of the iontophoresis of strychnine hydrochloride (+50 nA for 5 minutes) on the IPSP evoked in a MG motoneurone by the conditioning PBSt stimulation. This application of strychnine selectively reduces the early part of the IPSP with no effect on the late part of this IPSP.

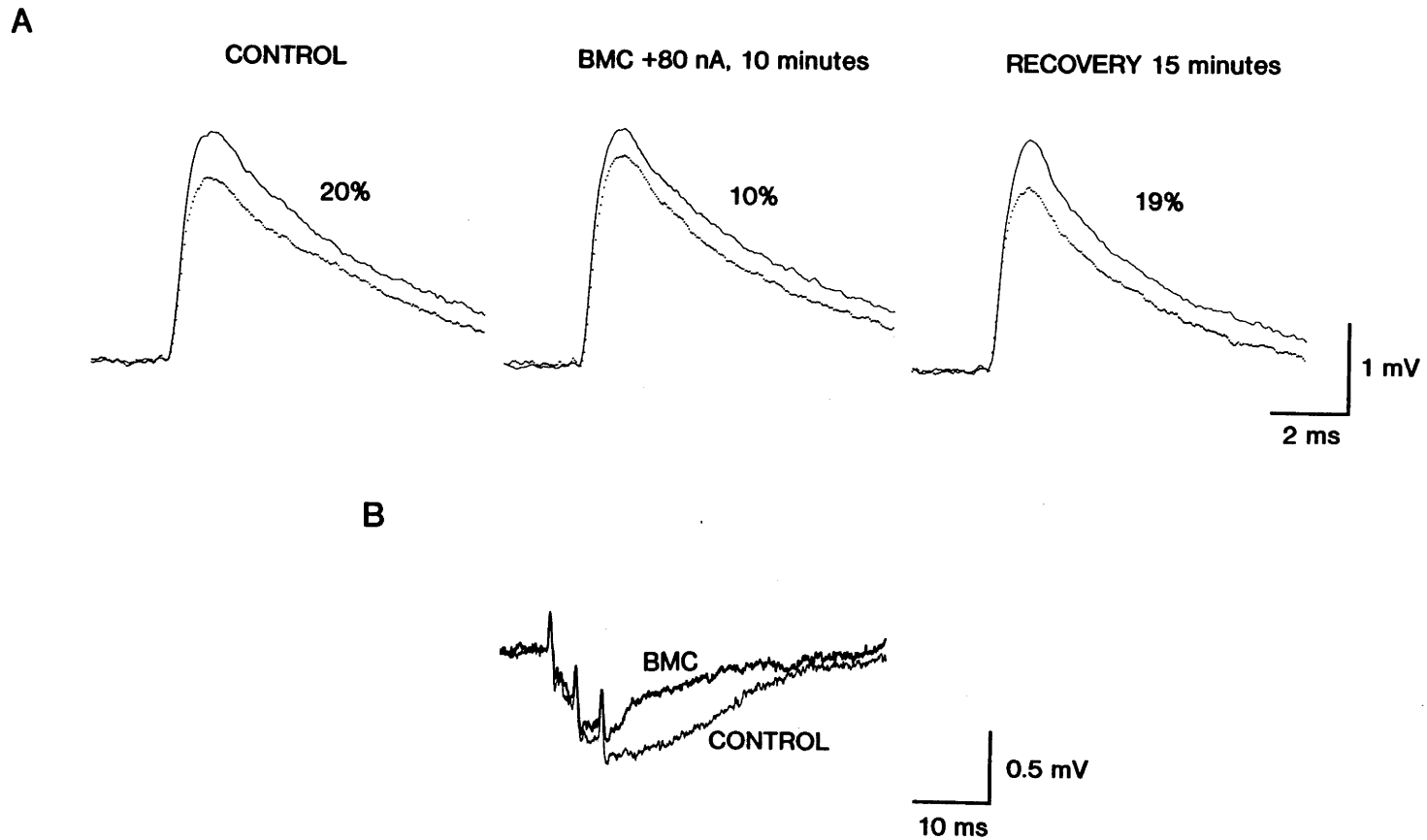


Fig. 4.6. Effect of bicuculline methochloride (BMC) on presynaptic inhibition of a compound EPSP. *A*, the conditioning PBSt stimulation reduced the amplitude of the control EPSP by 20%. The application of BMC (+80 nA for 10 minutes) reduced the amount of presynaptic inhibition of this EPSP to 10% and there was recovery 15 minutes later. *B*, the late part of the IPSP evoked in this motoneurone by the conditioning PBSt stimulation was reduced by the application of BMC.

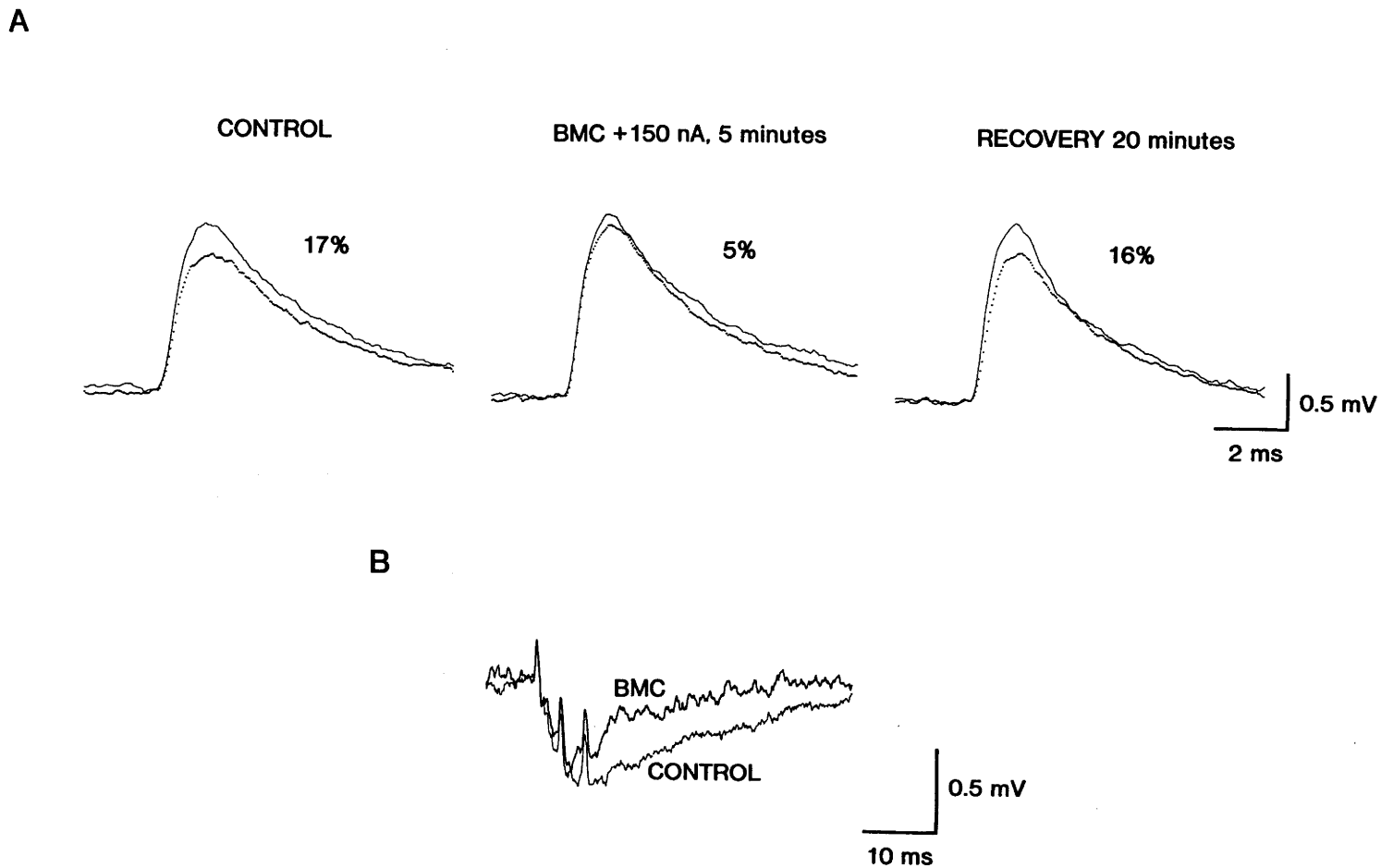


Fig. 4.7. Effect of bicuculline methochloride (BMC) on presynaptic inhibition of a compound EPSP. *A*, the conditioning PBSt stimulation reduced the amplitude of the control EPSP by 17%. The application of BMC (+150 nA for 5 minutes) reduced the amount of presynaptic inhibition of this EPSP to 5% and there was recovery 20 minutes later. *B*, the late part of the IPSP evoked in this motoneurone by the conditioning PBSt stimulation was reduced by the application of BMC.

a ~50% reduction in presynaptic inhibition, with complete recovery within 15 minutes. Iontophoresis of +150 nA of BMC for 5 minutes in a different motoneurone (Fig. 4.7*A*) substantially reduced the amount of presynaptic inhibition of this EPSP from 17 to 5%, a ~70% reduction in presynaptic inhibition, with full recovery in 20 minutes. Fig. 4.6*B* and Fig. 4.7*B* also clearly show than the same application of BMC markedly reduced the late part of the IPSP.

BMC also caused a slight increase in the peak amplitude, half-width and time constant of decay of the unconditioned EPSP. These increases were not statistically different from the control values, however suggest that the application of bicuculline may remove a small amount of tonic presynaptic and postsynaptic $GABA_A$ mediated inhibition.

Iontophoresis of BMC reduced presynaptic inhibition of compound EPSPs by between 42 and 76%, using iontophoretic currents of from 40 to 340 nA. The percentage decrease in presynaptic inhibition of compound EPSPs was found to be roughly related to the iontophoretic current used to eject BMC. This relationship is shown in Fig. 4.8. The line gives the linear regression fit to the data and has a correlation coefficient of 0.70. However, even the highest iontophoretic currents used could not completely abolish presynaptic inhibition.

There was also a roughly linear relationship between the percentage decrease in the amount of presynaptic inhibition of compound EPSPs during the application of BMC and the percentage decrease in the late part of the IPSP (measured between 20 and 30 ms after the start of the conditioning stimulus). This is shown in Fig. 4.9, the line gives the linear regression fit to the data and has a correlation coefficient of 0.78. Presynaptic inhibition could not, however, be completely abolished by the iontophoretic application of BMC, even when BMC completely abolished the late part of the IPSP.

There are two possible explanations for the inability of the iontophoretic application of BMC to completely abolish presynaptic inhibition of compound EPSPs. The most likely explanation is that with such a localised application of BMC, presumably close to the soma of the motoneurone, it was not possible to

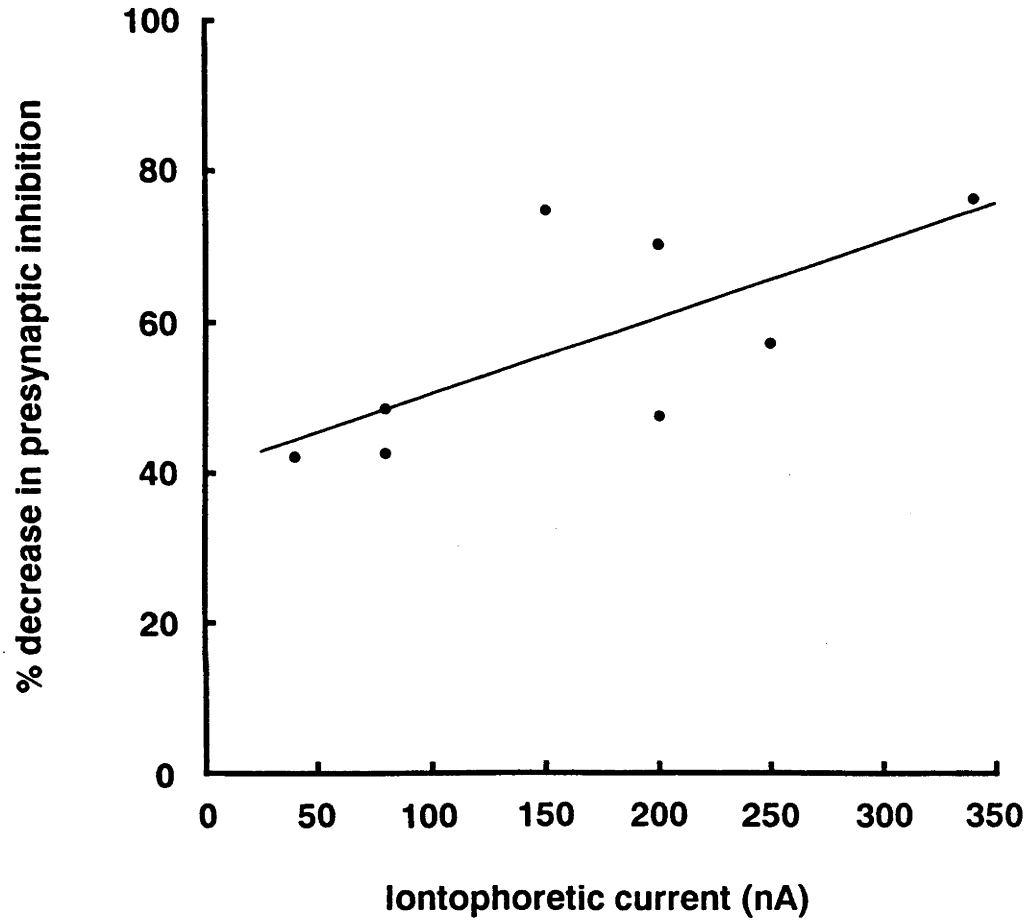


Fig. 4.8. Relationship between the iontophoretic current used to eject bicuculline methochloride and the percentage decrease in the level of presynaptic inhibition of compound EPSPs. The line gives the linear regression fit to the data and has a correlation coefficient of 0.70.

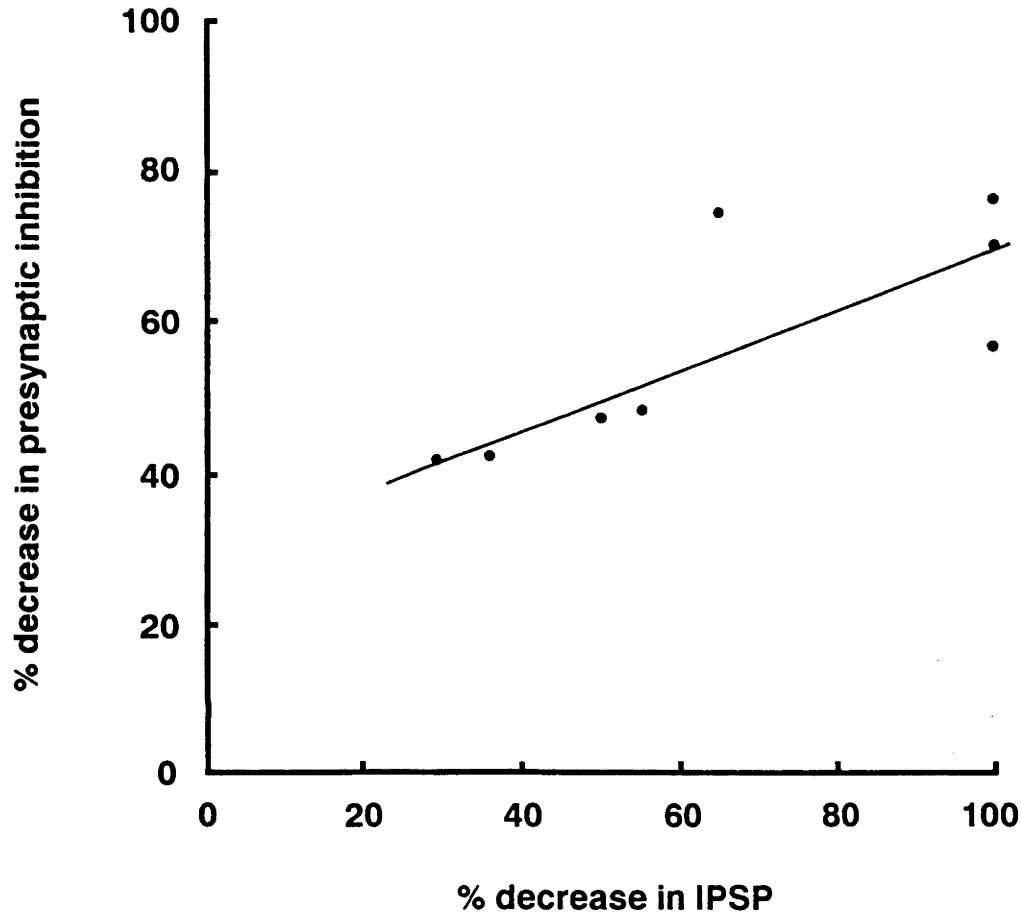


Fig. 4.9. The relationship between the percentage decrease in the IPSP, measured 20 to 30 ms after the conditioning PBSt stimulation, and the percentage decrease in the level of presynaptic inhibition of compound EPSPs during the application of bicuculline methochloride. The line gives the linear regression fit to the data and has a correlation coefficient of 0.78.

block presynaptic inhibition of EPSPs generated on distal regions of the motoneurone dendritic tree. The second possibility is that while presynaptic inhibition of Ia EPSPs appears to be mediated mainly by BMC sensitive $GABA_A$ receptors, a BMC insensitive receptor may also be involved.

To address the first possibility, the effect of the iontophoretic application of BMC was investigated on presynaptic inhibition of unitary EPSPs generated by synapses located near the soma. Presumably the local concentration of BMC around these synapses will be higher than at more distal synapses and so it would be expected that if presynaptic inhibition was generated *purely* through the activation of BMC sensitive $GABA_A$ receptors, then presynaptic inhibition of these near somatic, unitary EPSPs should be more easily blocked by the iontophoretic application of BMC .

Presynaptic inhibition of unitary EPSPs

Unitary EPSPs were evoked using the technique originally described by Honig, Collins & Mendell (1983) and described briefly in the Methods section. Once a recording from a single Ia afferent fibre from the MG muscle was established, brief (1 to 2 ms in duration, up to 10 nA in magnitude) depolarising current pulses were used to evoke an orthodromic action potentials in only that Ia afferent (Fig. 4.10*A*). Recordings were then made from MG motoneurones, and several hundred single sweeps of the membrane potential averaged to determine if the Ia afferent projected to that particular motoneurone (Fig. 4.10*B-E*). If a unitary EPSP was present, the rise time and half-width were measured to determine if they were consistent with a near somatic location (unitary EPSPs were sought which had unnormalised rise times of around 200 μs and half-widths of less than 2 ms; Jack *et al.* 1971).

Some examples of the effect of the conditioning PBSt stimulation on unitary EPSPs generated at different distances from the soma are shown in Fig. 4.11. As can be seen from Fig. 4.11 unitary EPSPs were decreased by different amounts by the conditioning stimulation (percentage decreases of unitary EPSPs ranged from

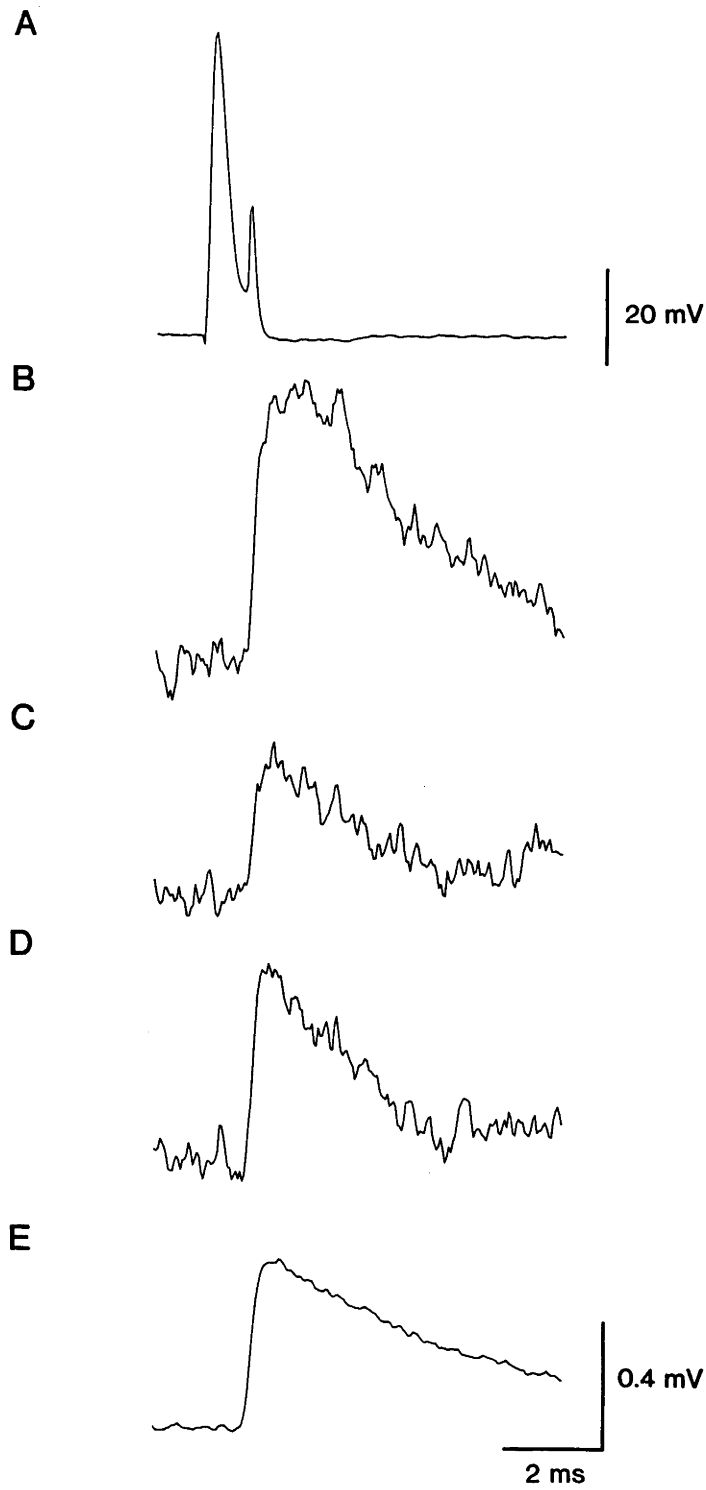


Fig. 4.10. Experimental protocol for recording unitary EPSPs. *A*, recording of an action potential evoked in a MG Ia afferent by a brief depolarising current pulse. *B-D*, single sweeps of the membrane potential of a MG motoneurone following the activation of the MG Ia afferent shown in *A*. *E*, the average unitary EPSP made up from 50 single sweeps like those shown in *B-D*.

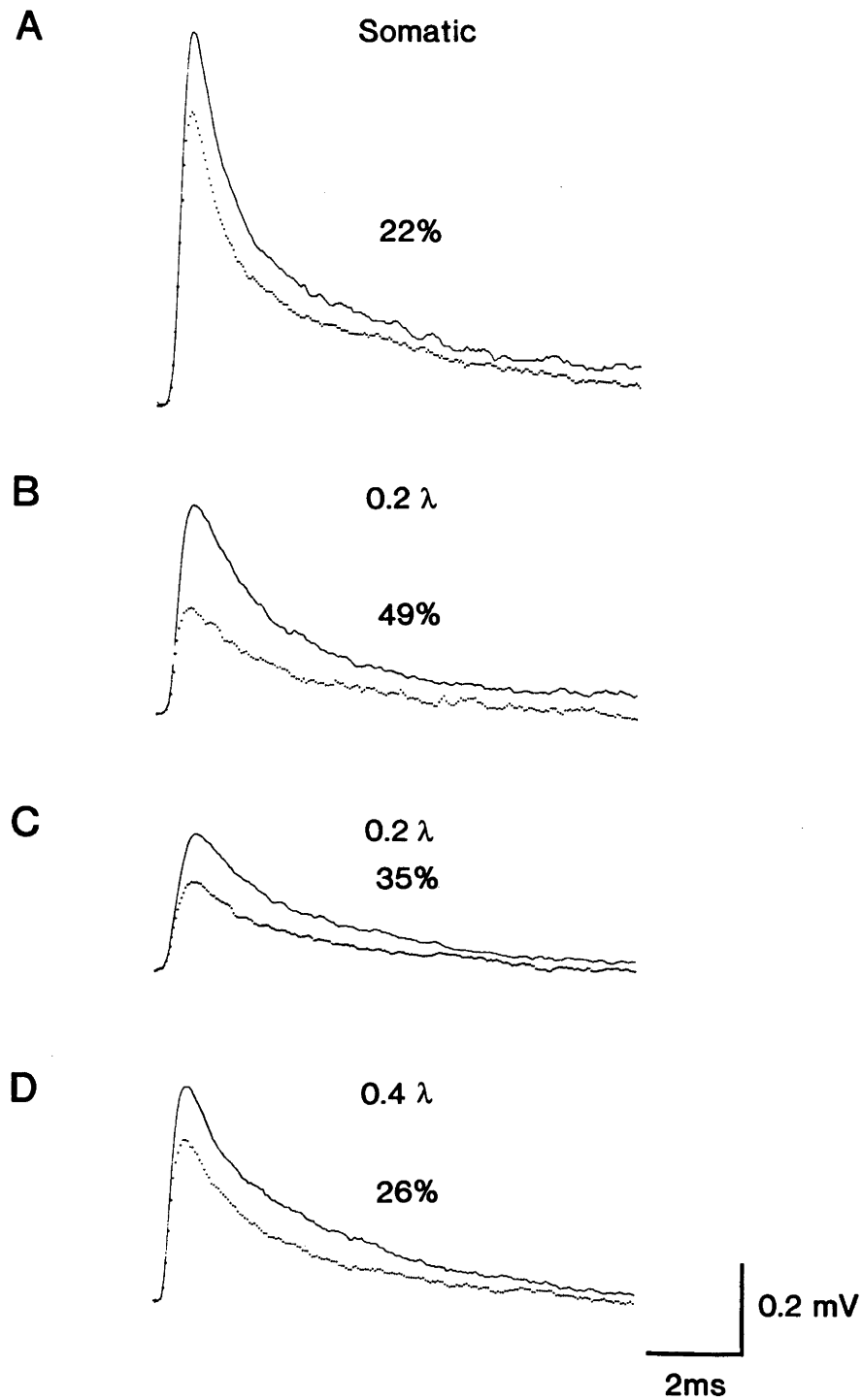


Fig. 4.11. Some examples of presynaptic inhibition of unitary EPSPs. *A*, unitary, somatic EPSP reduced in amplitude by 22% during the conditioning PBSt stimulation. *B,C*, two unitary EPSPs 0.2 λ from the soma reduced 49 and 35% respectively by the conditioning PBSt stimulation. *D*, a unitary EPSP 0.4 λ from the soma reduced by 26% during the conditioning PBSt stimulation.

4 to 55%). This is consistent with similar findings by Clements, Forsythe and Redman (1987), as is the finding that the amount of presynaptic inhibition of unitary EPSPs did not depend on their location, i.e. somatic EPSPs were inhibited by similar amounts to EPSPs generated at more distal locations. That the time course of the compound EPSP is unchanged during presynaptic inhibition (see above) also suggests a rather uniform inhibition of transmitter release from all Ia afferent synapses during presynaptic inhibition.

Effect of the $GABA_A$ antagonist bicuculline methochloride on presynaptic inhibition of near somatic unitary EPSPs

As expected, iontophoretic application of BMC also reduced the amount of presynaptic inhibition of unitary EPSPs generated near the soma. An example is shown in Fig. 4.12, where presynaptic inhibition of a unitary EPSP was reduced from 26 to 9% during the application of +100 nA of BMC for 5 minutes with recovery 30 minutes later; a reduction in presynaptic inhibition of 65%.

The effect of BMC was examined on presynaptic inhibition of six near somatic unitary Ia EPSPs. Five of these unitary EPSPs had normalised rise times and half-widths consistent with a location 0.2λ from the soma (Jack *et al.* 1971), the other unitary EPSP had a somatic location. Iontophoresis of BMC (100 to 200 nA) reduced presynaptic inhibition for these unitary EPSPs by between 47% to 85%. The same application of BMC reduced IPSPs recorded in these motoneurones by between 65 to 95%. Two of these unitary EPSP were lost during the application of BMC and so have not been included in further analysis (these EPSP showed reductions in presynaptic inhibition of 52 and 39%).

As with the compound Ia EPSPs the iontophoretic application of BMC did not completely abolish presynaptic inhibition of unitary Ia EPSPs located near the soma. This suggests that presynaptic inhibition may be mediated in part through a BMC insensitive receptor. As there is considerable evidence to suggest that the transmitter released during presynaptic inhibition is GABA, it seemed likely that GABA could also be acting at BMC insensitive $GABA_B$ receptors.

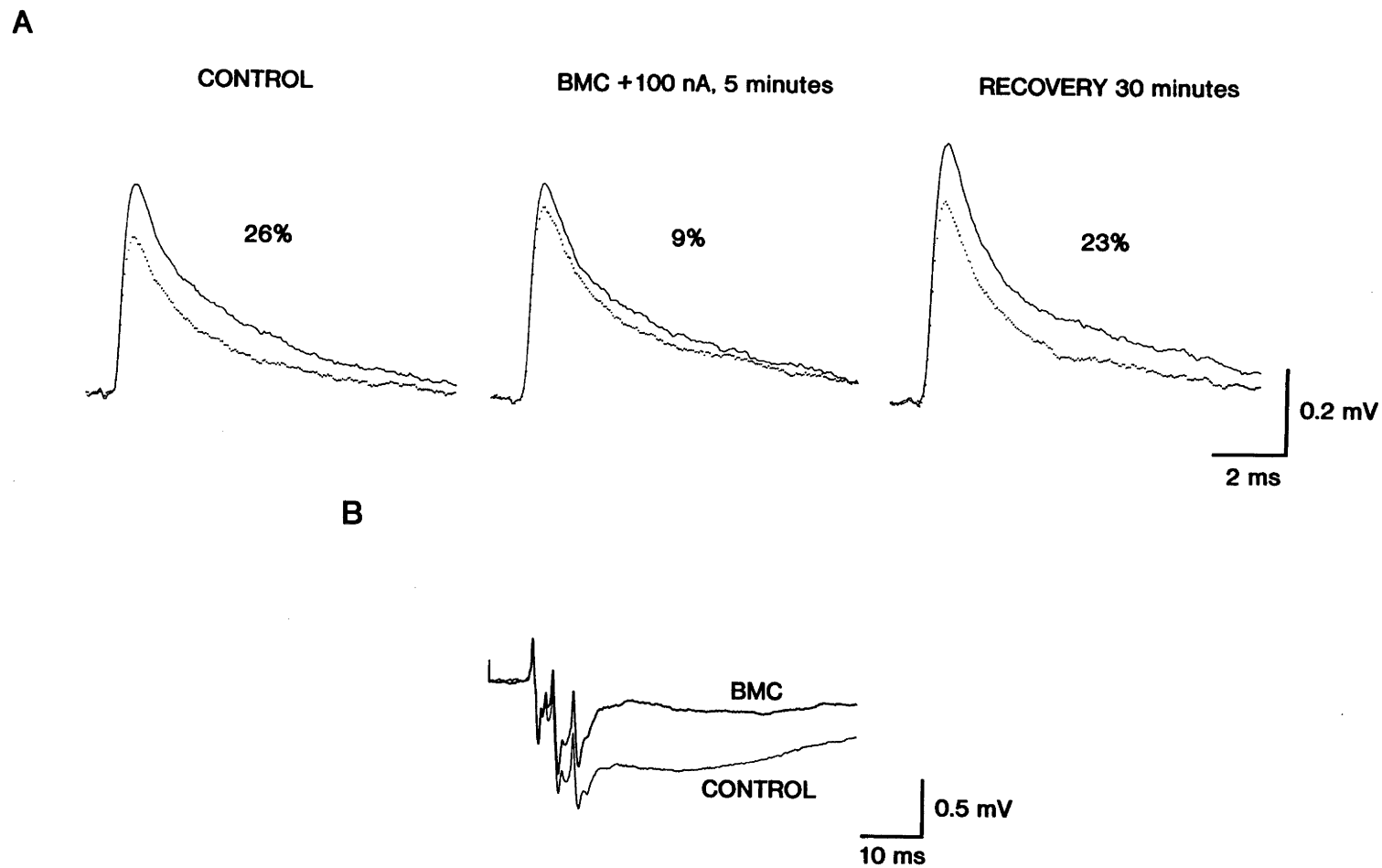


Fig. 4.12. Effect of bicuculline methochloride (BMC) on presynaptic inhibition of a near somatic unitary EPSP. *A*, the conditioning PBSt stimulation reduced the amplitude of the control EPSP by 26%. The application of BMC (+100 nA for 5 minutes) reduced the amount of presynaptic inhibition of this EPSP to 9% and there was recovery 30 minutes later. *B*, the late part of the IPSP evoked in this motoneurone by the conditioning PBSt stimulation was substantially reduced by the application of BMC.

The possibility that $GABA_B$ receptors are involved in mediating presynaptic inhibition has been suggested by many authors since the discovery by Dunlap and Fischbach (1978) that GABA could decrease the calcium component of action potentials recorded in the cell bodies of dorsal root ganglion cells grown in culture. This effect of GABA was not blocked by BMC and could be mimicked by the $GABA_B$ agonist baclofen (Dunlap, 1981*a,b*). If the same phenomenon occurs in the nerve terminals of these cells then this would be an effective way of deceasing transmitter release. This idea was supported by the finding that baclofen and GABA, acting through a BMC insensitive mechanism, could decrease the K^+-evoked release of noradrenaline, dopamine and serotonin from mammalian brain slices (Bowery *et al.* 1980).

To investigate the possibility that $GABA_B$ receptors are involved in mediating presynaptic inhibition the $GABA_B$ receptor antagonist 2-OH-saclofen was used (Kerr *et al.* 1988; Curtis *et al.* 1988). However, before investigating the effect of 2-OH-saclofen on presynaptic inhibition, it was felt necessary to first establish that 2-OH-saclofen could antagonize $GABA_B$ receptors. Therefore the ability of 2-OH-saclofen to antagonize the effect of the $GABA_B$ agonist (−)-baclofen was examined.

Effect of the $GABA_B$ agonist (−)-baclofen on compound EPSPs

When applied iontophoretically baclofen reduced the amplitude of Ia EPSPs. This was in agreement with previous findings using systemic injections of baclofen (Pierau & Zimmermann, 1973; Lev-Tov, Meyers & Burke, 1988; Edwards *et al.* 1989) and with the findings of Fox, Krnjevic, Morris, Puil and Werman (1978) using the iontophoretic application of baclofen. An example of this is shown in Fig. 4.13. Here, the peak amplitude of a compound EPSP was reduced by 18% by the application of baclofen (+20 nA for 1 minute). The decrease in amplitude of compound EPSPs occurred rapidly and usually recovered within 3 to 5 minutes (Fig. 4.13*B*).

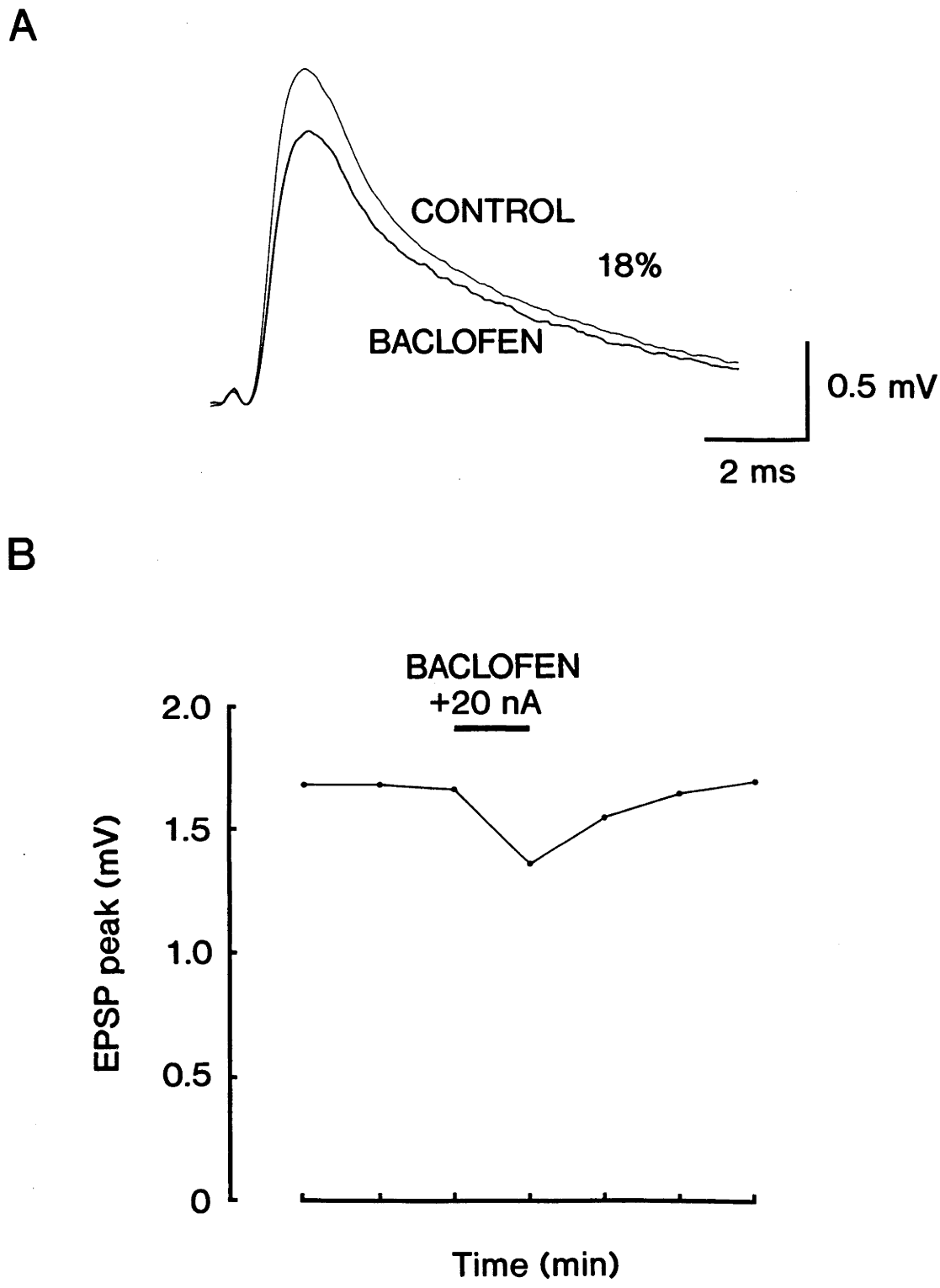


Fig. 4.13. Effect of (–)-baclofen on compound EPSPs. *A*, the application of baclofen (+20 nA for 1 minute) reduced the amplitude of a compound EPSP by 18%. The control response is the average of that recorded before and after recovery from baclofen. *B*, the time course of this effect of baclofen on the amplitude of the EPSP.

The iontophoretic application of baclofen in the experiments described in this study caused no detectable change in the membrane potential, input resistance or membrane time constant of MG motoneurones. An example of the effect of baclofen on the input resistance of a motoneurone is shown in Fig. 4.14*A*. Here, despite a large decrease in the peak amplitude of the compound EPSP by almost 50%, the input resistance measured by the voltage response to a 30 ms, -1 nA current pulse was unchanged. In five motoneurones where this was examined the average input resistance measured before and after recovery from baclofen was 2.5 ± 0.4 MΩ (±S.E.M., $n=5$), compared to 2.5 ± 0.3 MΩ (±S.E.M., $n=5$) during the application of baclofen. The same application of baclofen decreased Ia EPSPs recorded in these motoneurones by between 17 and 48%. The membrane time constant of these motoneurones was also unchanged during the application of baclofen. An example of this is shown in Fig. 4.14*B*. Here the membrane time constant, measured from the voltage responses shown in Fig 4.14*A*, was 8.7 ms before baclofen and 7.9 ms during the application of baclofen (this difference is within the bounds of experimental error for this type of measurement). The average membrane time constant measured before and after recovery from baclofen (5.3 ± 2.9 ms; ±S.E.M., $n=5$) was identical to that measured in motoneurones during the application of baclofen (5.3 ± 2.9 ms; ±S.E.M., $n=5$).

The lack of any effect of baclofen on the input resistance or membrane time constant of motoneurones is in agreement with previous findings by many authors (Pierau & Zimmermann, 1973; Lev-Tov *et al.* 1988; Peng & Frank, 1989*a*; Edwards *et al.* 1989, however see Fox *et al.* 1978; Wang & Dun, 1990) and consistent with the idea that baclofen, presumably via the activation of $GABA_B$ receptors, decreases the probability of transmitter release from primary afferent terminals (Peng & Frank, 1989*a*; Edwards *et al.* 1989).

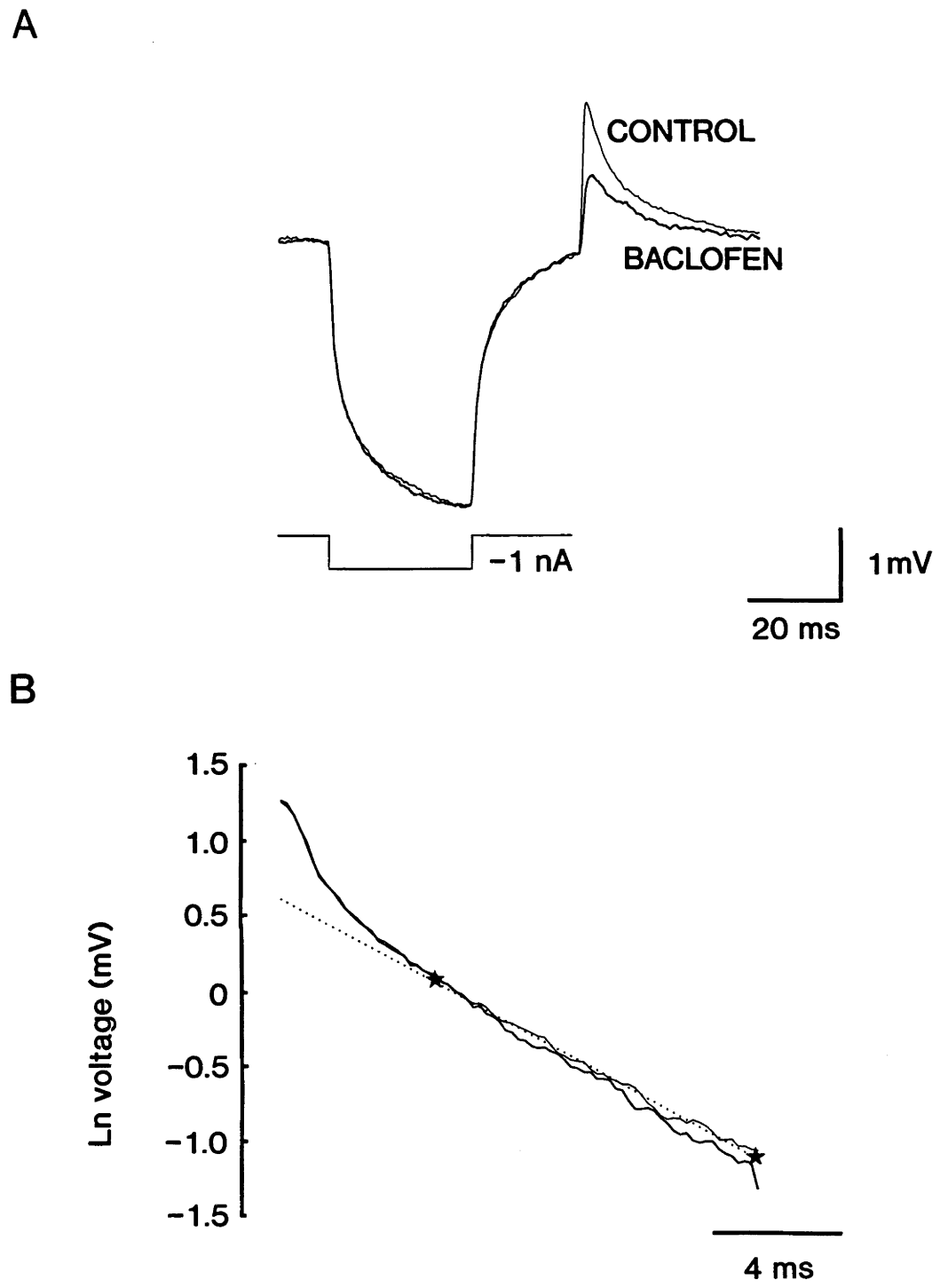


Fig. 4.14. Effect of (–)-baclofen on the input resistance and membrane time constant. *A*, response of a MG motoneurone to a 30 ms, -1 nA current pulse followed by a compound EPSP without (control) and during baclofen (+20 nA for 1 minute). The control response is the average of that recorded before and after recovery from baclofen. *B*, the final phase of the voltage responses shown in *A* have been displayed semilogarithmically and a linear regression line fitted to the control response between the two stars.

Antagonism by 2-OH-saclofen of the decrease in amplitude of compound EPSPs produced by (−)-baclofen

To establish that 2-OH-saclofen could block the activation of $GABA_B$ receptors, the ability of 2-OH-saclofen to reduce the decrease in amplitude of Ia EPSPs produced by baclofen was investigated. Antagonism by 2-OH-saclofen proved difficult to demonstrate, as often the iontophoretic application of 2-OH-saclofen had either no effect on the reduction in EPSP amplitude produced by the application of baclofen (from the adjacent barrel of the iontophoretic electrode) or 2-OH-saclofen *alone* reduced the amplitude of Ia EPSPs. An example of this is shown in Fig. 4.15*A*, where the application of 2-OH-saclofen (+250 nA for 2 minutes) reduced the peak amplitude of the EPSP by 27%. For reference, a reduction in the amplitude of the same EPSP by a comparable amount could be produced by the application of baclofen (+40 nA for 1 minute) from the adjacent barrel of the iontophoretic electrode (Fig. 4.15*B*). While the onset of the reduction in the amplitude of Ia EPSPs produced by 2-OH-saclofen occured rapidly (within less than 1 minute), the recovery from this effect was slow; EPSPs usually taking 5 to 10 minutes to return to the control amlitude. Taking into account the concentrations of baclofen and 2-OH-saclofen in the iontophoretic electrodes and the currents used to eject them, 2-OH-saclofen was considerably less potent than baclofen in reducing the amplitude of Ia EPSPs (approximately 50 times less potent in the example shown in Fig. 4.15, assuming equal ionisation of the two compounds).

2-OH-Saclofen reduced the amplitude of EPSPs without any change in the membrane potential or the membrane time constant, an action which appeared to be very similar to that of baclofen. This suggests that 2-OH-saclofen can, under certain circumstances, act as a weak $GABA_B$ agonist, as has been previously suggested by Curtis *et al.* (1988).

It was possible on some occasions to demonstrate antagonism by 2-OH-saclofen of the decrease in amplitude of Ia EPSPs produced by baclofen. An example of this is shown in Fig. 4.16. The control record shows that baclofen

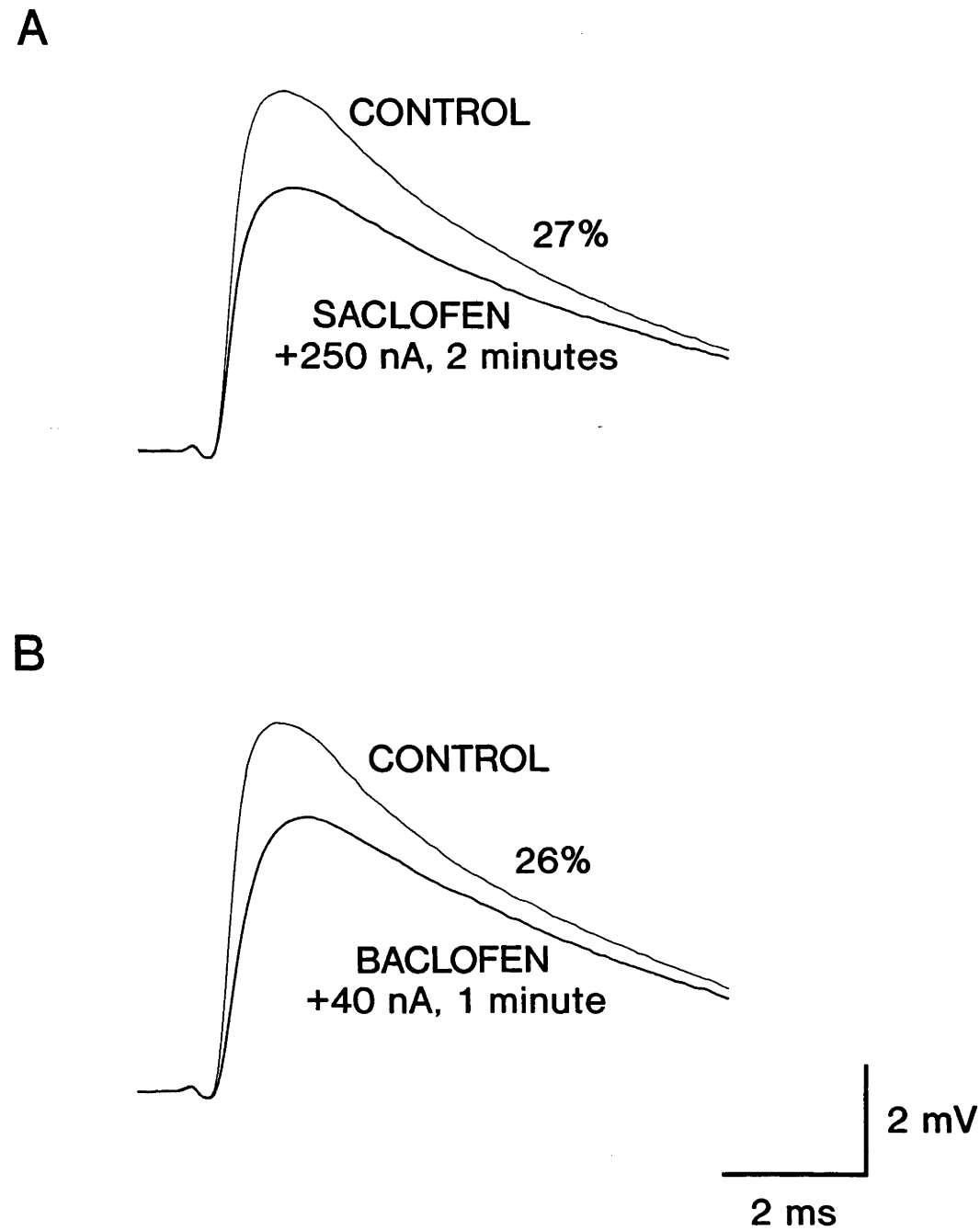


Fig. 4.15. Effect of 2-OH-saclofen on a compound EPSP. *A*, the application of saclofen (+250 nA for 2 minutes) reduced the amplitude of a compound EPSP by 27%. *B*, the application of (−)-baclofen (+20 nA for 1 minute) from the adjacent iontophoretic electrode reduced the amplitude of this EPSP by 26%.

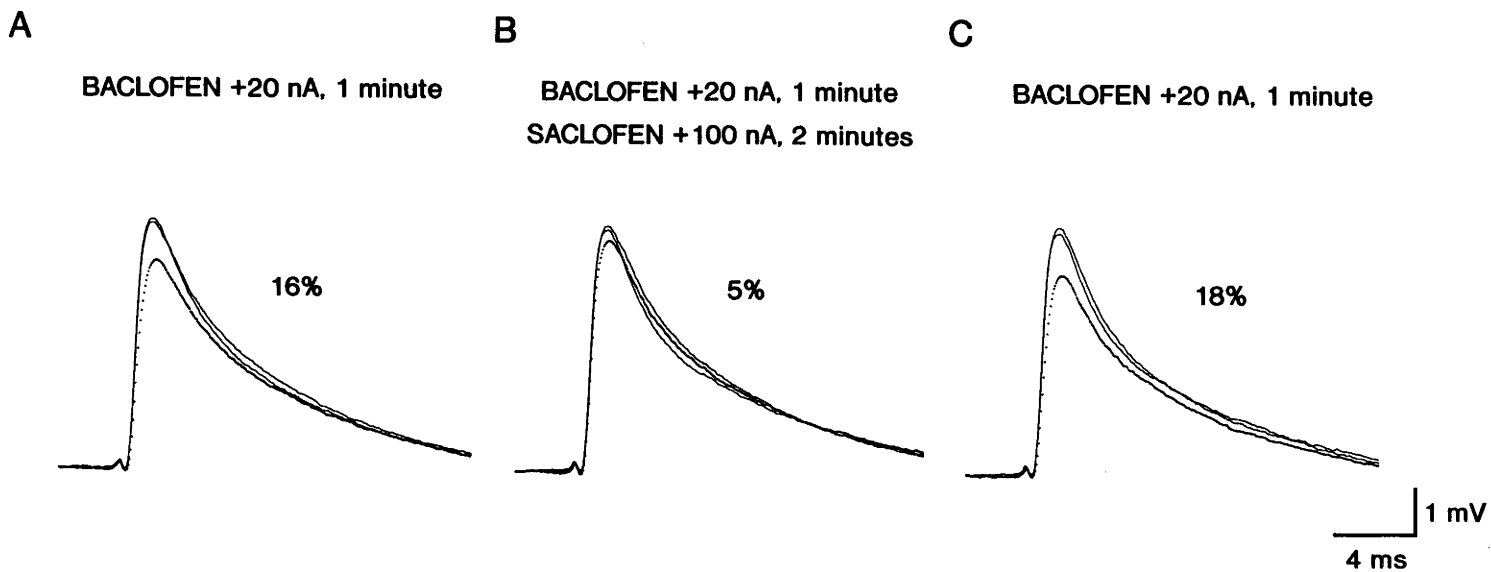


Fig. 4.16. Antagonism of the effect of (−)-baclofen by 2-OH-saclofen. *A*, baclofen (+20 nA for 1 minute) reduced the amplitude of this compound EPSP by 16% and this returned to the control amplitude in 3 minutes. *B*, in the presence of saclofen (+100 nA for 2 minutes), the same application of baclofen only reduced the amplitude of the EPSP by 5% and this returned to the control amplitude 6 minutes later where, in *C*, baclofen reduced the amplitude of the EPSP by 18%.

reduced the unconditioned EPSP by 16% with recovery within 3 minutes (Fig. 4.16*A*). During the application of 2-OH-saclofen (which caused little or no change in the size of the control EPSP), baclofen reduced the amplitude of the EPSP by only 5% (Fig. 4.16*B*). The EPSP returned to the control amplitude 6 minutes later, where the application of baclofen reduced the EPSP amplitude by 18% (Fig. 4.16*C*). This result is consistent with antagonism by 2-OH-saclofen of the decrease in amplitude of Ia EPSPs produced by baclofen. The time course of the antagonism between 2-OH-saclofen and the effect of baclofen is shown in Fig. 4.17. As baclofen presumably reduces the amplitude of Ia EPSPs by acting at presynaptic $GABA_B$ receptors on Ia afferent terminals these results suggest that 2-OH-saclofen can block the activation of presynaptic $GABA_B$ receptors on these terminals.

Effect of the $GABA_B$ antagonist 2-OH-saclofen on presynaptic inhibition of compound EPSPs

Once it was established that 2-OH-saclofen could reduce the effects of baclofen, the effect of 2-OH-saclofen was examined on presynaptic inhibition of the *same* Ia EPSP.

Examples of the effect of 2-OH-saclofen on presynaptic inhibition of two compound EPSPs are shown in Fig. 4.18. Presynaptic inhibition of the EPSP shown in Fig. 4.18*A* was not, or was only very slightly, reduced in the presence of 2-OH-saclofen. However, the amount of presynaptic inhibition of the EPSP shown in Fig. 4.18*B* was clearly reduced from 16 to 12% during the application of 2-OH-saclofen; a 25% decrease in presynaptic inhibition. Presynaptic inhibition of all five compound EPSPs examined was reduced to some extent by 2-OH-saclofen. The reduction in presynaptic inhibition produced by 2-OH-saclofen ranged from only 4% (Fig. 4.18*A*) to 25% (Fig. 4.18*B*), using iontophoretic currents of 100 to 200 nA (n.b. two cells did not show complete recovery back to the control level of presynaptic inhibition after the application of 2-OH-saclofen).

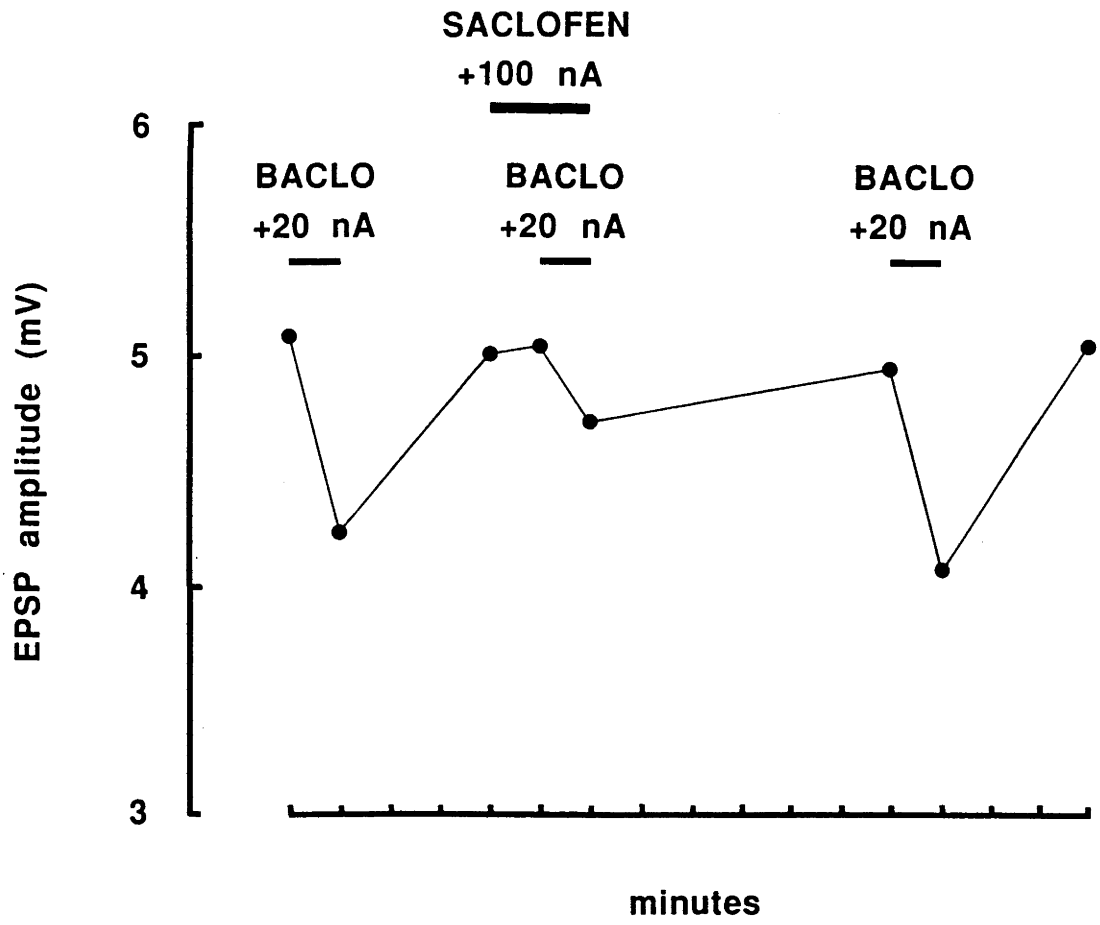


Fig. 4.17. Time course of antagonism by 2-OH-saclofen (+100 nA for 2 minutes) of the decrease by (−)-baclofen (Baclo; +20 nA for 1 minute) in amplitude of the compound EPSP shown in Fig. 4.16.

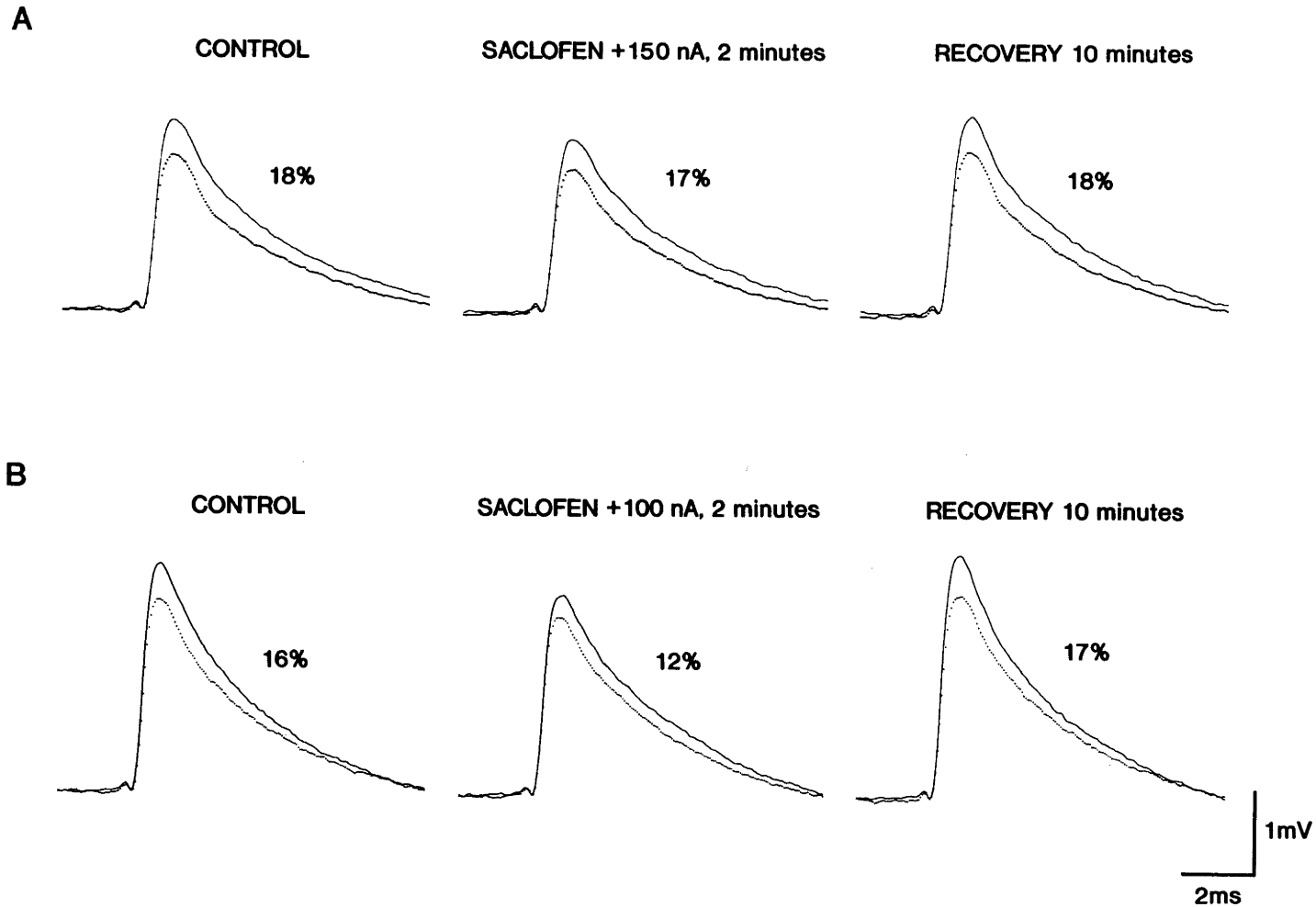


Fig. 4.18. Effect of 2-OH-saclofen on presynaptic inhibition of two compound EPSPs. *A*, the conditioning PBSt stimulation reduced the amplitude of the control EPSP by 18%. The application of saclofen (+150 nA for 2 minutes) had little or no effect on the amount of presynaptic inhibition of this EPSP. *B*, conditioning PBSt stimulation reduced the amplitude of the control EPSP by 16%. The application of saclofen (+100 nA for 2 minutes) reducing the amount of presynaptic inhibition to 12% with recovery 10 minutes later.

2-OH-saclofen also produced a slight decrease in the amplitude of unconditioned EPSPs by 1 to 13% (see Fig 4.18). This presumably occurred as a consequence of this compound acting as a weak $GABA_B$ agonist. As the reduction in presynaptic inhibition produced by 2-OH-saclofen was always associated with a decrease in the amplitude of the unconditioned EPSP, it was thought possible that this could be responsible for the decrease in presynaptic inhibition. To investigate this possibility the ability of baclofen to reduce presynaptic inhibition was examined.

Effect of the $GABA_B$ agonist (−)-baclofen on presynaptic inhibition of compound EPSPs

When applied iontophoretically using currents of between 20 or 40 nA for 1 to 2 minutes, baclofen reduced the amplitude of unconditioned EPSPs by between 16 and 60%. This reduction in amplitude of unconditioned EPSPs was usually not associated with any change in the amount of presynaptic inhibition or in the magnitude of the IPSP evoked in motoneurones by the conditioning stimulation. An example of this is shown in Fig. 4.19. Here, the application of baclofen (+40 nA for 2 minutes) reduced the unconditioned EPSP by 33% with no change in the amount of presynaptic inhibition.

The percentage change in the amplitude of the unconditioned EPSP during the application of baclofen has been plotted against the percentage change in the amount of presynaptic inhibition in Fig. 4.20. This figure shows a weak correlation between the percentage change in the amplitude of the unconditioned EPSP and the percentage change in the amount of presynaptic inhibition (correlation coefficient of 0.53, slope 0.37), however, for reductions in the amplitude of unconditioned EPSPs of less than 20% there appeared to be no significant effect of baclofen on presynaptic inhibition. As the maximum reduction in the amplitude of the unconditioned EPSP during the application of 2-OH-saclofen was 13%, this would suggest that the decrease in presynaptic inhibition of Ia EPSPs observed in

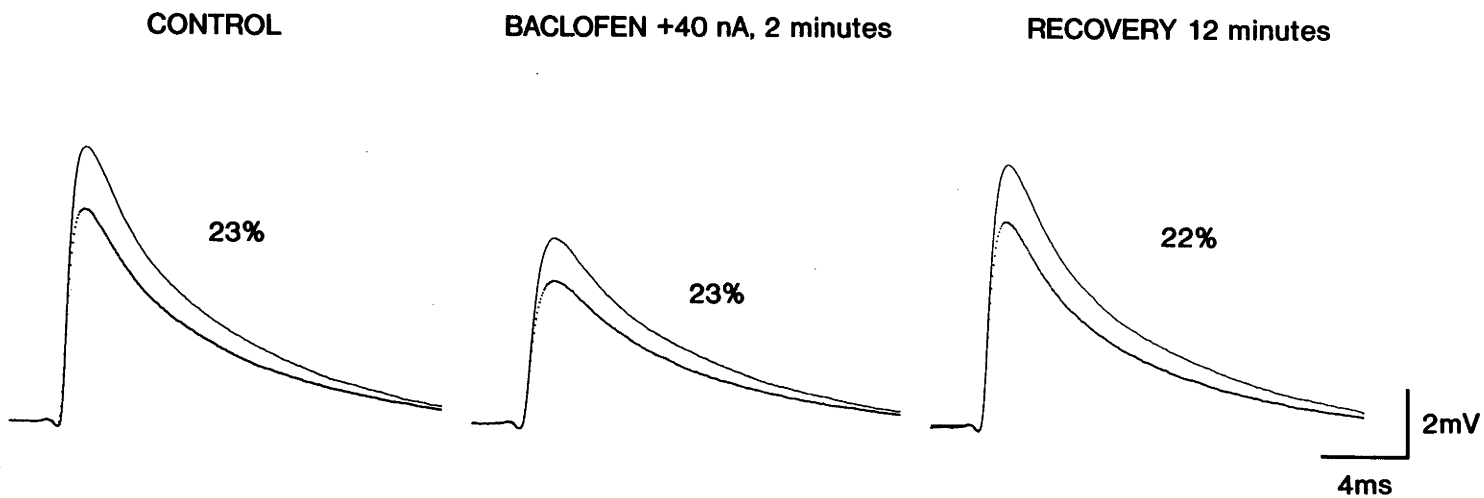


Fig. 4.19. Effect of (−)-baclofen on presynaptic inhibition of a compound EPSP. The conditioning PBSt stimulation reduced the amplitude of the control EPSP by 23%. The application of baclofen (+40 nA for 2 minutes) reduced the amplitude of the unconditioned EPSP by 33%, however had no effect on the level of presynaptic inhibition. The amplitude of the unconditioned EPSP returned back to the control level 12 minutes later.

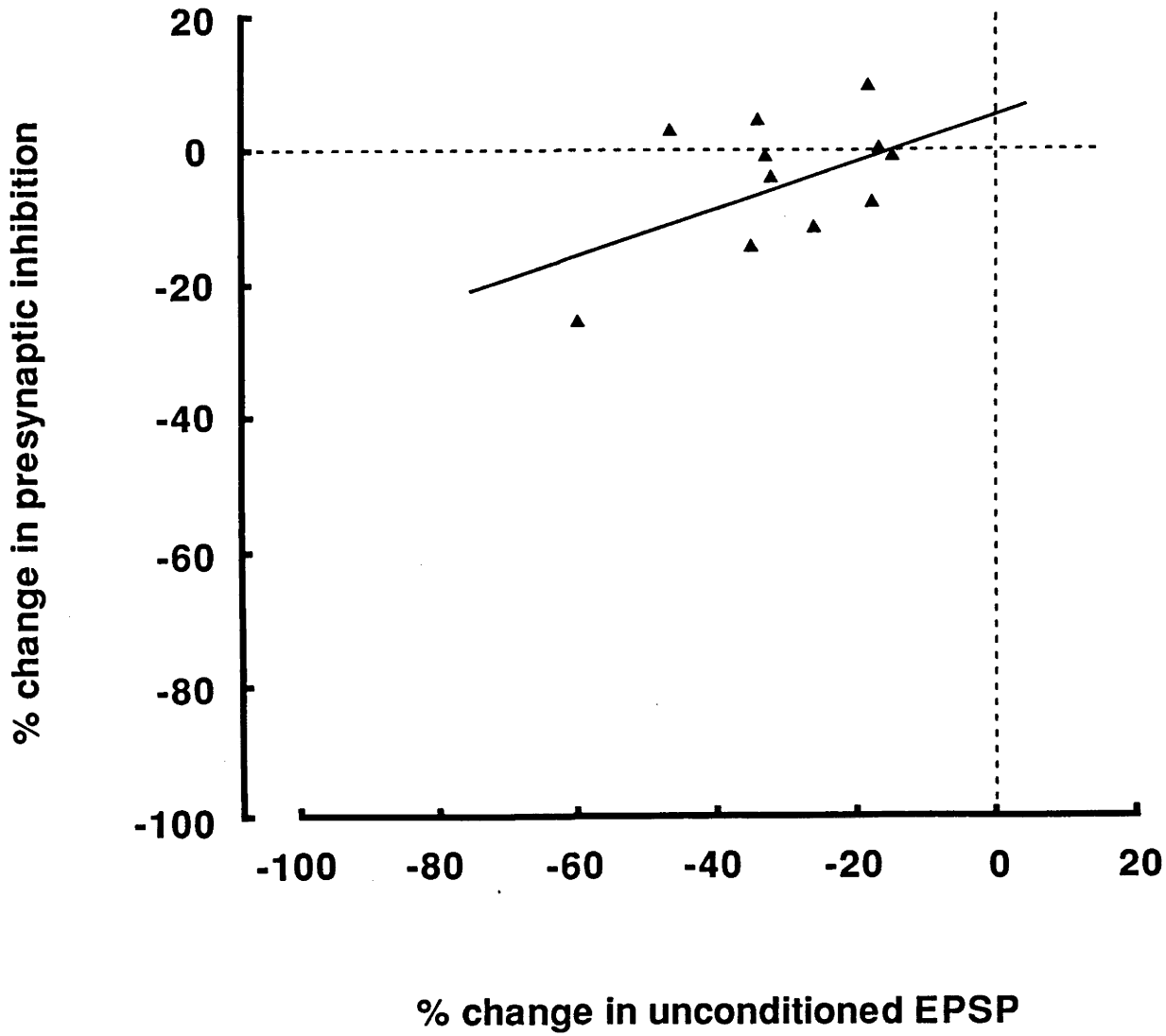


Fig. 4.20. Relationship between the percentage change in the unconditioned EPSP produced by the application of (−)-baclofen and the percentage change in the amount of presynaptic inhibition. The linear regression fit has a correlation coefficient of 0.53 and a slope of 0.37.

the presence of 2-OH-saclofen was not due to the *activation* of $GABA_B$ receptors.

Combined effects of bicuculline methochloride, 2-OH-saclofen and (−)-baclofen on presynaptic inhibition of Ia EPSPs

The pooled data for the effects of BMC, 2-OH-saclofen and baclofen have been combined and are shown in Fig. 4.21. As in Fig. 4.20, the percentage change in the amplitude of the unconditioned EPSP has been plotted against the percentage change in the amount of presynaptic inhibition. The BMC data include the results obtained from both compound (filled circles) and unitary (open circles) EPSPs. This figure shows that BMC, a $GABA_A$ antagonist, often caused a slight increase in the amplitude of the unconditioned EPSP and a large decrease in the amount of presynaptic inhibition of Ia EPSPs by 42 to 85%. 2-OH-saclofen, a $GABA_B$ antagonist (open squares), caused a slight decrease in the amplitude of unconditioned EPSPs and a small reduction in the amount of presynaptic inhibition of Ia EPSPs by 4 to 25%. In contrast, baclofen, a $GABA_B$ agonist (filled triangles), caused a large decrease in the amplitude of the unconditioned EPSP, but was relatively ineffective in reducing presynaptic inhibition.

Discussion

Presynaptic inhibition of Ia EPSPs was markedly reduced during the iontophoretic application of BMC. This is consistent with earlier findings which had shown that picrotoxin and bicuculline (both $GABA_A$ antagonists) reduced both prolonged inhibition and dorsal root potentials (Eccles *et al.* 1963*a*; Curtis *et al.* 1971) and suggests that at this synapse presynaptic inhibition is mediated primarily through the activation of $GABA_A$ receptors. The small reduction in presynaptic inhibition produced by 2-OH-saclofen suggests that $GABA_B$ receptors may also play a minor role in presynaptic inhibition of Ia EPSPs.

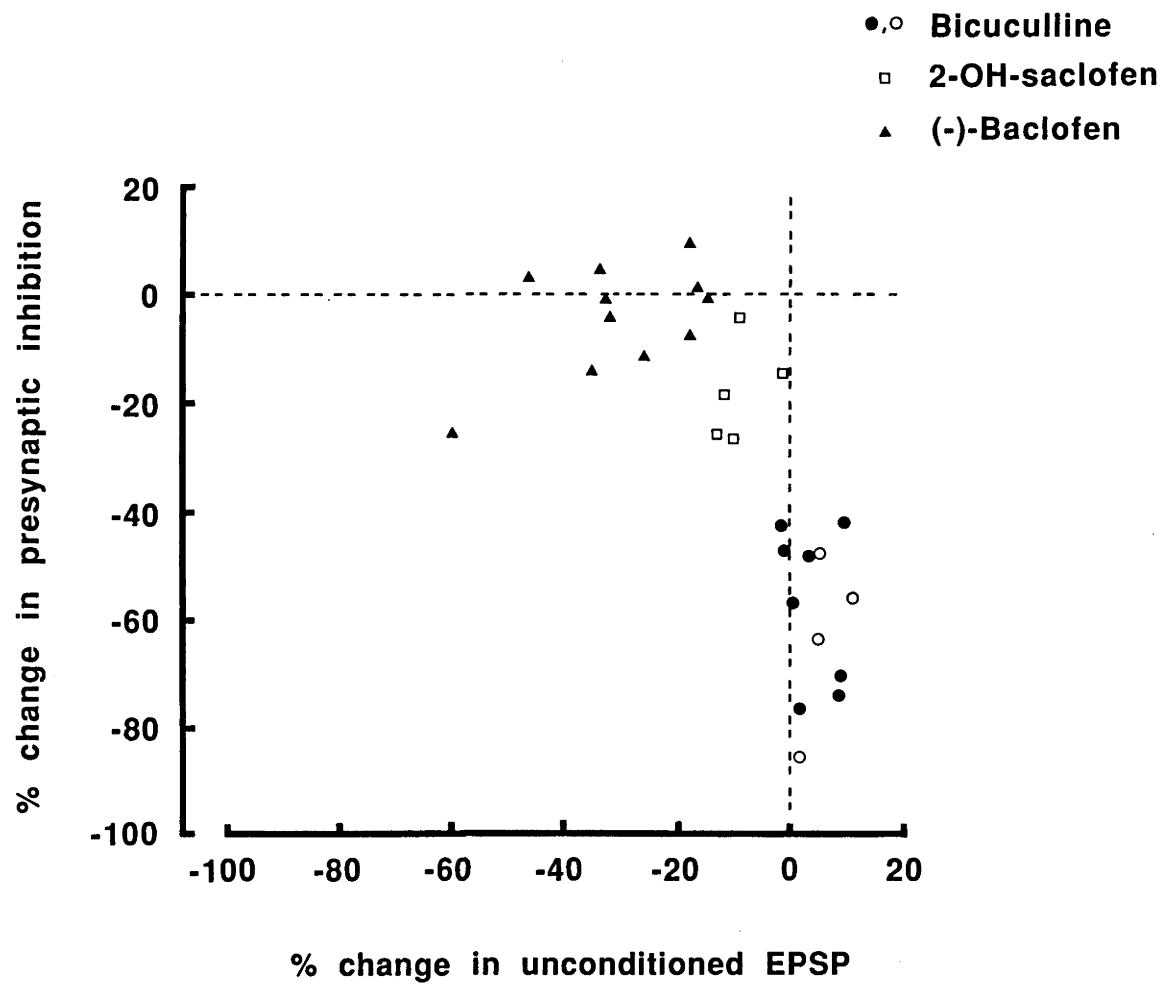


Fig. 4.21. Combined data from experiments using bicuculline methochloride, 2-OH-saclofen and (−)-baclofen. The percentage change in the unconditioned EPSP is plotted against the percentage change in the amount of presynaptic inhibition. The bicuculline methochloride data include the results from both compound (filled circles) and unitary (open circles) EPSPs. The results from the experiments with saclofen (open squares) and baclofen (filled triangles) only include results from compound EPSPs.

The effect of 2-OH-saclofen on presynaptic inhibition was complicated by its ability to reduce the unconditioned EPSP. This action of 2-OH-saclofen was very similar to that of baclofen and suggested that 2-OH-saclofen can, under certain circumstances, act as a partial agonist at $GABA_B$ receptors. However, recent *in vitro* studies, using concentrations of 2-OH-saclofen of up to 500 μM, have not observed any effect of 2-OH-saclofen on the amplitude of EPSPs in CA1 neurones of the hippocampus (Randall, Schofield, Davies & Collingridge, 1990; Harrison, Lovinger, Lambert, Teyler, Prager, Ong & Kerr, 1990). As the only other study to apply 2-OH-saclofen iontophoretically also reported a weak baclofen like action of 2-OH-saclofen (Curtis *et al.* 1988), the reduction in the amplitude of Ia EPSPs by 2-OH-saclofen may occur as a consequence of the iontophoretic application of 2-OH-saclofen. High local concentrations of 2-OH-saclofen might be expected to occur close to the tip of the iontophorectic electrode during iontophoresis. If at high concentrations 2-OH-saclofen can act as a partial $GABA_B$ agonist this may explain the effects of 2-OH-saclofen observed in the present study.

Experiments designed to investigate the effect of activation of $GABA_B$ receptors on presynaptic inhibition, using the $GABA_B$ agonist baclofen, suggested that activation of $GABA_B$ receptors did not account for the small reduction in presynaptic inhibition observed in the presence of 2-OH-saclofen. This supports the suggestion that, to some extent, $GABA_B$ receptors are activated during presynaptic inhibition of Ia EPSPs.

It is also possible that 2-OH-saclofen could have decreased presynaptic inhibition by blocking the action of GABA at $GABA_A$ receptors. A recent report has cautioned that high concentrations of 2-OH-saclofen will displace the binding of muscimol, a $GABA_A$ receptor agonist (Al-Dahan, Jalilian Tehrani & Thalmann, 1990). However, this finding is difficult to interpret as muscimol has previously been shown to also have some affinity for $GABA_B$ receptors (Hill & Bowery, 1981; Bowery, Hill & Hudson, 1983). Therefore, the displacement of muscimol binding by 2-OH-saclofen may simply reflect 2-OH-saclofen's ability to displace the binding of muscimol to $GABA_B$ receptors. To establish if 2-OH-saclofen can

antagonize binding to $GABA_A$ receptors it would be necessary to establish if 2-OH-saclofen can displace the binding of a specific $GABA_A$ receptor agonist which has no affinity for the $GABA_B$ receptor, such as piperidine-4-sulphonic acid (Bowery *et al.* 1983). Previous studies have shown that 2-OH-saclofen, in concentrations necessary to antagonize $GABA_B$ receptors, has little or no effect on the activation of $GABA_A$ receptors (Kerr *et al.* 1988; Curtis *et al.* 1988).

The only evidence available from the present study to suggest that 2-OH-saclofen did not antagonize $GABA_A$ receptors comes from the observation that 2-OH-saclofen had little or no effect on the IPSP evoked in MG motoneurones by the conditioning PBSt stimulation. The late part of this IPSP could occasionally be completely abolished by BMC and so is presumably mediated exclusively by the activation of $GABA_A$ receptors. The lack of an effect of 2-OH-saclofen on this IPSP suggests that 2-OH-saclofen does not antagonize $GABA_A$ receptors, at least postsynaptically.

Pharmacology of postsynaptic inhibition

The early part of the IPSP evoked in motoneurones by the conditioning PBSt stimulation could be reduced by the iontophoretic application of strychnine. This would suggest that the early part of this IPSP is mediated by inhibitory glycinergic interneurones which activate postsynaptic glycine receptors on motoneurones and presumably lead to an increase in chloride conductance (Bormann *et al.* 1987). The application of BMC reduced, and occasionally completely abolished, the late part of this IPSP. This suggests that this BMC-sensitive IPSP is mediated by another interneuronal pathway with last order GABAergic interneurones which activate postsynaptic $GABA_A$ receptors and presumably also cause an increase in chloride conductance in motoneurones (Bormann *et al.* 1987). As this GABAergic IPSP could occasionally be completely abolished by BMC, it is presumably mediated exclusively through the activation of $GABA_A$ receptors. There was no suggestion of a BMC insensitive component to

this IPSP, as has been observed to follow $GABA_A$ mediated IPSPs in the hippocampus (Newberry & Nicoll, 1984; Dutar & Nicoll, 1988*a*).

The presence of axo-axonic synapses on Ia afferent terminals which are also presynaptic to motoneurones (Fyffe & Light, 1984) raises the possibility that axo-axonic synapses can mediate both presynaptic and postsynaptic inhibition. As the transmitter released at these synapses is thought to be GABA, the BMC sensitive IPSP evoked in MG motoneurones by the conditioning PBSt stimulation is probably produced partly by the release of GABA from these 'triadic' axo-axonic synapses.

Effects of bicuculline methochloride on the unconditioned EPSP

As can be seen from Fig. 4.21, application of BMC often caused a slight increase in the amplitude of the unconditioned EPSP. This presumably reflects the removal of background $GABA_A$ mediated presynaptic inhibition of these EPSPs caused by the spontaneous release of GABA from axo-axonic synapses. The greatest increase in peak amplitude was 10%, with about half the EPSPs showing no increase in amplitude in the presence of BMC. This suggests that in the spinal cord of the barbiturate anaesthetized cat background levels of tonic presynaptic inhibition are low.

BMC also caused a slight increase in the half-width and final time constant of decay of EPSPs. This probably occurred due to a small increase in the membrane time constant of motoneurones. An increase in the membrane time constant can be most easily explained by the removal of tonic $GABA_A$ mediated postsynaptic inhibition by BMC, presumably from the release of GABA by spontaneously active GABAergic inhibitory interneurones. Apart from this slight increase in the membrane time constant there was no indication of any direct effect of BMC on the resting conductance of motoneurones, as has been suggested by some authors (Krnjevic, Puil & Werman, 1977; Chase, Soja & Morales, 1989).

Mechanisms of presynaptic inhibition

If presynaptic inhibition of Ia EPSPs is mediated primarily through the activation of $GABA_A$ receptors, as the present study would suggest, how does activation of $GABA_A$ receptors cause a decrease in the probability of transmitter release?

$GABA_A$ receptors are thought to form part of a receptor/channel protein which when activated by GABA leads to an increase in chloride conductance (Scholfield *et al.* 1987; Bormann *et al.* 1987). The application of GABA to the cell bodies of primary afferent fibres (Nishi *et al.* 1974; Feltz & Raminsky, 1974; Gallagher *et al.* 1978) and to their terminals (Gmelin & Creletti,1976; Sastry, 1979*a*; Curtis & Lodge, 1982) has been shown to cause an increase in membrane conductance and depolarisation. Both of these effects are bicuculline-sensitive and are thought to occur due to an increase in chloride conductance following the activation of $GABA_A$ receptors. Similarly, the conditioning stimulation used to evoke presynaptic inhibition also causes a depolarisation of primary afferent fibres (Eccles *et al.* 1961*a*) and terminals (Wall, 1958; Eccles *et al.* 1962; Gmelin & Creletti,1976; Curtis & Lodge, 1982). This depolarisation is also blocked by bicuculline (Curtis *et al.* 1971; Curtis & Lodge, 1982) and is thought to be associated with an increase in the membrane conductance (Curtis & Lodge, 1982; Padjen & Hashiguchi, 1983; Curtis *et al.* 1986). Presumably, the depolarisation and increase in membrane conductance of primary afferent fibres which occurs in conjunction with presynaptic inhibition also results from an increase in chloride conductance following the activation of $GABA_A$ receptors on primary afferent terminals.

It has been suggested that during presynaptic inhibition the depolarisation of primary afferent terminals leads to a decrease in transmitter release (Eccles *et al.* 1961*a*; Eccles, 1964; Schmidt, 1971; Lev-Tov *et al.* 1983). As mentioned in Chapter one this depolarisation could cause inactivation of voltage dependent calcium or sodium channels. While it is possible that this could cause a decrease in transmitter release, it seems more likely that during presynaptic inhibition the

increase in the membrane conductance of primary afferent terminals plays a more important role than the depolarisation *per se* (Deschenes, Feltz & Lamour, 1976; Ryall, 1978; Nicoll & Alger, 1979; Levy, 1980; Padjen & Hashiguchi, 1983; Segev, 1990).

This increase in membrane conductance could shunt the presynaptic action potential, decreasing its amplitude and/or duration. This would lead to reduced activation of voltage dependent calcium channels, reduced calcium influx and hence decreased transmitter release. Some experimental evidence for this comes from the findings that action potentials recorded intra-axonally near Ia afferent terminals and field potentials which are thought to reflect action potential invasion into Ia afferent terminals are both reduced during presynaptic inhibition (Eccles *et al.* 1963*b*; Sypert *et al.* 1980). In addition, the presynaptic decrease in transmitter release produced by the $GABA_A$ agonist muscimol is also associated with a decrease in the amplitude of the field potential thought to reflect action potential invasion into Ia afferent terminals (Peng & Frank, 1989*b*).

Presynaptic actions of (−)-baclofen on Ia afferent terminals

Baclofen caused a marked reduction in the amplitude of Ia EPSPs without any effect on the membrane potential, input resistance or the membrane time constant of motoneurones. This was in agreement with previous findings and consistent with the idea that baclofen, presumably through the activation of $GABA_B$ receptors, causes a decrease in the probability of transmitter release from Ia afferent terminals (Pierau & Zimmermann, 1973; Lev-Tov *et al.* 1988; Peng & Frank, 1989*a*; Edwards *et al.* 1989).

However, how baclofen leads to a decrease in transmitter release is unknown. As baclofen can cause an increase in a potassium conductance in many central neurones (Newberry & Nicoll, 1985; Inoue *et al.* 1985; Gahwiler & Brown, 1985), it is possible that baclofen could reduce transmitter release by causing an increase in the potassium conductance of primary afferent terminals. This could shunt the presynaptic action potential, reducing calcium influx through voltage

activated calcium channels and decrease transmitter release. There are two reasons why this probably does not happen. Firstly, such an effect should decrease the size of the field potential thought to reflect action potential invasion into Ia afferent terminals. Baclofen has, however, been shown to cause a marked reduction in transmitter release from Ia afferent fibres without any effect on this field potential (Lev-Tov *et al.* 1988; Peng & Frank, 1989*a*). Secondly, activation of a potassium conductance would be expected to hyperpolarize Ia afferent terminals and so should cause a decrease in the excitability of these terminals. However, baclofen has no direct effect on the excitability of Ia afferent terminals (Curtis *et al.* 1981; Curtis *et al.* 1986).

Baclofen has also been shown to decrease a voltage dependent calcium conductance in the cell bodies of dorsal root ganglion cells (Dunlap & Fischbach, 1978; Dunlap, 1981*a*,*b*; Deisz & Lux, 1985; Dolphin & Scott, 1986; Robertson & Taylor, 1986). Such an effect at the terminals of Ia afferents would reduce calcium influx and decrease transmitter release. This would probably not have any detectable effect on the field potential reflecting invasion of Ia afferent terminals or on the resting excitability of these terminals. Some experimental evidence for such an action of baclofen on primary afferent terminals comes from the finding that a small calcium component of the presynaptic action potential in Ia afferent terminals (Sastry, 1979*b*) is thought to be reduced by baclofen (Curtis, 1990), an effect blocked by the $GABA_B$ antagonist 2-OH-saclofen.

A recent report suggests that baclofen can shift the voltage dependence of inactivation of a transient potassium current, recorded in the cell bodies of cultured hippocampal neurones, to more depolarised potentials (Saint *et al.* 1990). This could also be responsible for the decrease in transmitter release produced by baclofen. Such an effect at primary afferent terminals could reduce the duration of the presynaptic action potential, leading to reduced calcium influx and decreased transmitter release. This effect of baclofen would probably also cause no detectable change in the field potential reflecting invasion of Ia afferent terminals

or the resting excitability of these terminals. At present, however, there is no experimental evidence available to suggest that this occurs at Ia afferent synapses.

Antagonism of presynaptic $GABA_B$ receptors

Early reports using the then new $GABA_B$ receptor antagonist phaclofen (Kerr *et al.* 1987) found that phaclofen could selectively antagonize $GABA_B$ receptor mediated responses produced by either baclofen or GABA, in the presence of BMC (Kerr *et al.* 1987, Dutar & Nicoll, 1988*a*). However, many presynaptic effects of baclofen in the CNS have been found to be insensitive to phaclofen (Dutar & Nicoll, 1988*b*; Wang & Dun, 1990; Harrison, 1990). This lead to the suggestion that there may be pharmacological differences between pre- and postsynaptic $GABA_B$ receptors in the mammalian CNS (Dutar & Nicoll, 1988*b*). However, recently the more potent $GABA_B$ receptor antagonist 2-OH-saclofen (Kerr *et al.* 1988; Curtis *et al.* 1988) has been shown to antagonize the presynaptic effects of baclofen in the hippocampus (Davies *et al.* 1990; Randall *et al.* 1990; Harrison *et al*, 1990). The results from the present study show that in the mammalian spinal cord the presynaptic action of baclofen can also be antagonized by 2-OH-saclofen.

Effect of (−)-baclofen on presynaptic inhibition

In contrast to the ease with which baclofen reduced transmitter release from Ia afferent terminals, it was relatively ineffective in reducing presynaptic inhibition. The interpretation of this result is complicated, as both baclofen and presynaptic inhibition are thought to effect calcium influx into Ia afferent terminals. As the relationship between calcium influx and transmitter release is thought to be highly non-linear (Dodge & Rahamimoff, 1967; Augustine *et al.* 1985), it is extremely difficult to predict the effect baclofen will have on presynatic inhibition.

A simplistic interpretation would be that the slight decrease in presynaptic inhibition observed during the application of baclofen is due to a reduction in

transmitter release at axo-axonic synapses. Consistent with this idea baclofen is thought to reduce transmitter release at axo-axonic synapses as primary afferent depolarisation of single Ia afferent terminals has been shown to be reduced by the iontophoretic application baclofen, without any effect on the sensitivity of these terminals to $GABA_A$ agonists (Curtis *et al.* 1981; Curtis *et al.* 1986).

The finding that only small reductions in presynaptic inhibition were observed following large reductions in the amplitude of the unconditioned EPSP suggests that $GABA_B$ receptors on axo-axonic terminals are less effective in reducing transmitter release than similar receptors on Ia afferent terminals. This is consistent with previous reports that in the spinal cord transmitter release from the terminals of interneurones and descending spinal projections is less sensitive to baclofen than transmitter release from the terminals of primary afferent fibres (Pierau & Zimmermann, 1973; Kato, Waldmann & Murakami, 1978; Davies, 1981; Curtis *et al.* 1981; Curtis & Malik, 1985; Edwards *et al.* 1989; Rudomin, Jimenez & Enriquez, 1989).

Conclusion

The main findings from this study suggest that at the Ia afferent/motoneurone synapse presynaptic inhibition is mediated primarily through the activation of $GABA_A$ receptors. The activation of $GABA_B$ receptors appears to play only a minor role in presynaptic inhibition at this synapse. This contrasts with the relative ease with which baclofen, presumably via the activation of $GABA_B$ receptors, can reduce transmitter release from Ia afferent terminals and suggests that the receptors that are activated by baclofen are predominantly extrasynaptic.

Chapter five:

PAIRED-PULSE FACILITATION IS ENHANCED DURING PRESYNAPTIC INHIBITION OF Ia EPSPS

Introduction

Paired-pulse facilitation (PPF) is a short-term form of synaptic plasticity in which the response to the second of two paired stimuli is facilitated relative to the first. The facilitation of the response to the second stimulus is thought to occur due to a transient increase in the probability of transmitter release (Castillo & Katz, 1954; Dudel & Kuffler, 1961*a*; Kuno, 1964; Zucker, 1973; Hirst, Redman & Wong, 1981; Lin & Faber, 1988), which has been attributed to the presence of residual calcium within the presynaptic nerve terminal following action potential invasion and calcium influx during the first stimulus (Katz & Miledi, 1965, 1968; Charlton, Smith & Zucker, 1982; Dudel, 1990).

This form of potentiation has proved a valuable tool for the elucidation of the mechanisms underlying synaptic transmission, particularly at the neuromuscular junction (Castillo & Katz, 1954; Katz & Miledi, 1968; Mallart & Martin, 1968; Parnas, Dudel & Parnas, 1982; Dudel, 1990; Parnas & Segel, 1989). Many studies have shown that changes which directly effect calcium influx into nerve terminals alter PPF. Decreasing the probability of transmitter release by lowering the extracellular calcium concentration or raising the extracellular magnesium concentration either unmasks or enhances PPF, whereas increasing the probability of transmitter release by raising the extracellular calcium concentration reduces PPF (Lundberg & Quilisch, 1953; Castillo & Katz, 1954; Takeuchi, 1958; Thies, 1965; Rahamimoff, 1968; Mallart & Martin, 1968; McNaughton, 1980; Parnas *et al.* 1982; Harris & Cotman, 1983; Dudel, 1989; Muller & Lynch, 1989; Trombley & Westbrook, 1990).

The aim of the present study was to investigate the effect of presynaptic inhibition on PPF of Ia EPSPs in the mammalian spinal cord *in vivo*. The results indicate that PPF is enhanced during presynaptic inhibition. This increase in PPF is analogous to that seen *in vitro* at many other synapses under conditions that would be expected to reduce calcium influx and supports the idea that presynaptic inhibition Ia EPSPs is associated with a reduction in calcium influx into Ia afferent terminals.

Methods

Initial surgery was performed on adult cats as described in Chapter two. All experiments were preformed on cats anaesthetised with sodium pentobarbitone. As sodium pentobarbitone is known to enhance presynaptic inhibition (Eccles *et al.* 1963*a*), supplementary doses were only given after a recording session with a particular cell.

The hindlimb was dissected as follows. The nerves to posterior biceps and semitendinosus (PBSt) in the left hindlimb were separated from surrounding tissue, cut distally and mounted on a stimulating electrode. Usually the most medial branch of posterior biceps, which usually runs together with a branch of anterior biceps, was not used. The nerves to medial gastrocnemius (MG) and lateral gastrocnemius-soleus (LGS) were also carefully freed from surrounding tissue, cut distally and mounted on stimulating electrodes. Ventral roots S1, L7 and L6 were cut and the S1 and L7 ventral roots were mounted on a stimulation electrode.

Recording

Intracellular recordings were made from antidromically identified motoneurones in the L7/S1 spinal segment using conventional glass microelectrodes filled with 2M KCH_3SO_4 (resting membrane potentials greater than -60 mV, spike height greater than 70 mV). Monosynaptic Ia excitatory

postsynaptic potentials (EPSPs) were evoked in these motoneurones by stimulation of either the MG or LGS muscle nerves using a stimulus intensity supermaximal for activation of Ia afferents (usually 2 to 3 x group I threshold). The use of supermaximal stimuli ensured that the second stimulus, evoked briefly after the first, was of sufficient strength to activate all available Ia afferent fibres. To reduce problems associated with nonlinear summation of EPSPs only small Ia EPSPs were used (2 to 4 mV in amplitude).

PPF was evoked by recording the response to two paired stimuli separated by intervals of 2 to 10 ms. The amount of potentiation was expressed as the percentage increase in the peak amplitude of the second EPSP relative to the first EPSP. The protocol for evoking PPF is shown in Fig. 5.1. Single and paired EPSPs were evoked on alternate sweeps at 1 Hz, stored in separate buffers in a microcomputer and averaged. Offline, the response to the first stimulus alone (Fig. 5.1*B*) was digitally subtracted from that containing the response to the paired stimuli (Fig. 5.1*A*), leaving the response to the second stimulus alone (Fig. 5.1*C*). The peak amplitude of the first and second EPSPs were determined and the increase in the amplitude of the second EPSP relative to the first expressed as a percentage. The individual EPSPs evoked by the first and second stimuli during PPF (Fig. 5.1*B*,*C*) have been plotted together in Fig. 5.1*D* and show that in this example the amplitude of second EPSP was increased by 13% relative to the first.

Presynaptic inhibition of EPSPs was evoked by prior conditioning stimulation of the PBSt muscle nerve. This conditioning PBSt stimulation was composed of a train of 2 to 14 stimuli evoked at 300 Hz, 2 x group I threshold, initiated 50 ms prior to Ia EPSPs and repeated at one second intervals (see Chapter four, Fig. 4.1 and 4.2). By changing the number of stimuli applied to the PBSt muscle nerve it was possible to vary the amount of presynaptic inhibition of a particular Ia EPSP (Eccles *et al.* 1961*a*). Alternate records of conditioned and unconditioned EPSPs were stored in separate buffers in a microcomputer and averaged. As the conditioned EPSP was often superimposed on the repolarising

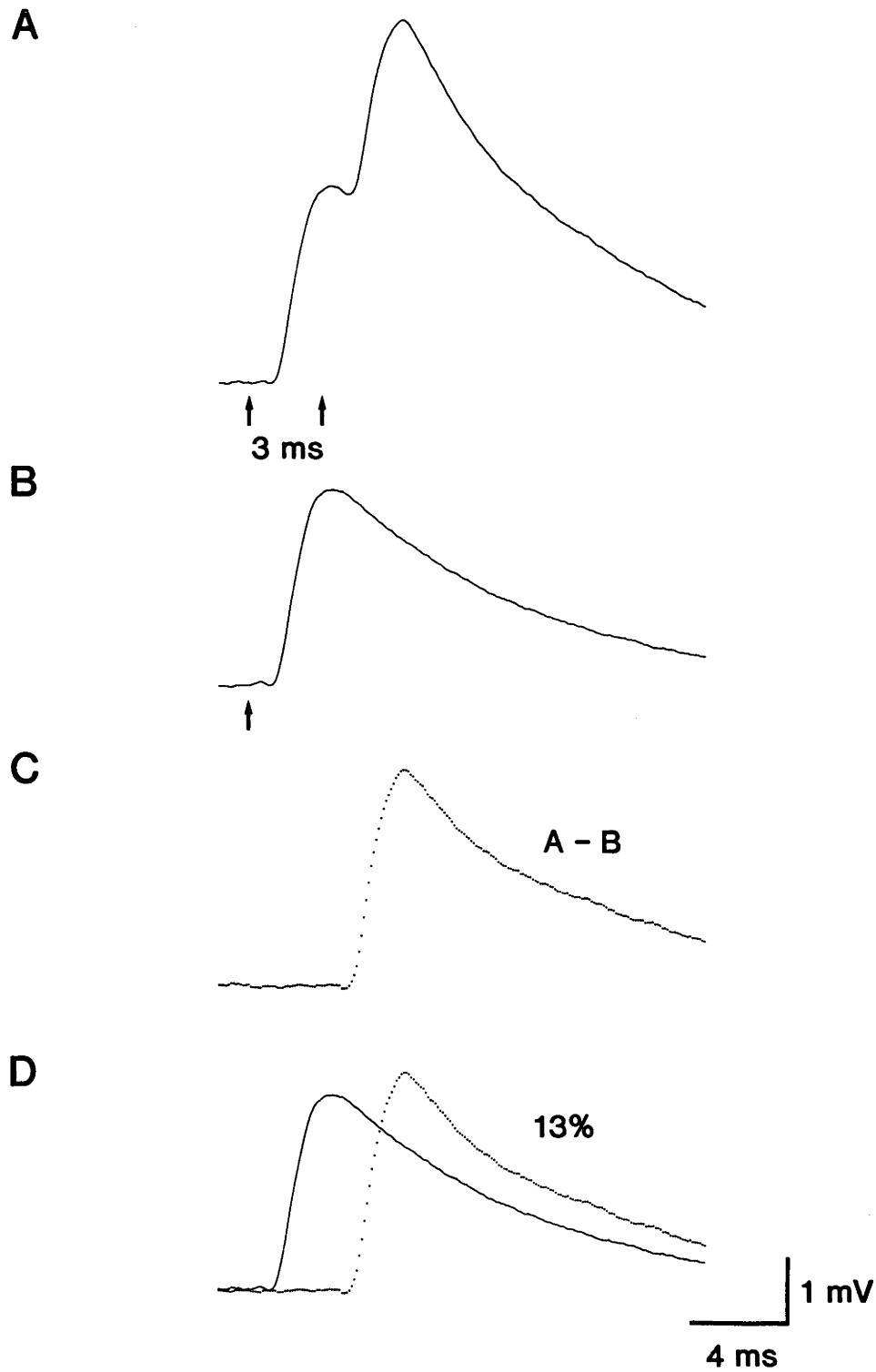


Fig. 5.1. Protocol used to evoke paired-pulse facilitation. *A*, the response in a MG motoneurone to paired stimuli (3 ms interval) of the MG muscle nerve. *B*, the response in the same motoneurone to a single stimulus to the MG muscle nerve. *C*, the response to the second stimulus alone, obtained following digital subtraction of trace *B* from trace *A*. *D*, trace *B* and *C* shown together. The second EPSP was potentiated in amplitude by 13% relative to the first.

phase of an inhibitory postsynaptic potential (IPSP), a linear, sloping baseline was used in an attempt to overcome the distortion of the conditioned EPSP caused by the repolarising IPSP. The amount of presynaptic inhibition is expressed as the percentage decrease in the peak amplitude of Ia EPSPs produced by the conditioning PBSt stimulation.

The effect of presynaptic inhibition on PPF was determined by recording the response to alternate single and paired stimuli evoked 50 ms after the initiation of the conditioning PBSt stimulation. The response to the first stimulus was then subtracted from the response during the paired stimuli. The peak amplitude of this second EPSP during presynaptic inhibition was then compared to the amplitude of the first EPSP during presynaptic inhibition (see Results).

Results

Paired-pulse facilitation

When a second EPSP was evoked briefly after the first EPSP its amplitude was often increased. An example of this is shown in Fig. 5.1. Here, the amplitude of the second EPSP evoked by stimulation of the MG muscle nerve 3 ms after the first stimulus was potentiated, in this case by 13%. Potentiation of the second EPSP was not always observed (see also Hirst *et al.* 1981) and ranged from zero to 22 %, using stimulus intervals between the first and second EPSPs of 2 to 4 ms. The average percentage increase in the amplitude of the second EPSP during PPF was 11 ± 2 % (±S.E.M., $n=10$). This average value for the amount of PPF of compound Ia EPSPs is very similar to that observed by Hirst, Redman and Wong (1981) during PPF of single fibre Ia EPSPs.

The second EPSP usually decayed faster than the first (Fig. 5.1). The most likely explanation for this is that during PPF the supermaximal stimulus used activates a polysynaptic inhibitory pathway which increases the rate of decay of the response to the paired stimulus. If this inhibition was active during the peak of the second EPSP it could decrease the amplitude of this EPSP, decreasing the amount

of PPF. The average amount of PPF of the compound EPSPs in the present study was not, however, significantly different from that found under similar experimental conditions using single fibre EPSPs (Hirst *et al.* 1981). As single fibre EPSPs would not be expected to cause activation of polysynaptic inhibitory pathways, this suggests that polysynaptic inhibition probably has little effect on the amount of PPF of the peak amplitude of compound Ia EPSPs .

The amount of potentiation of the second EPSP during PPF was dependent on the interval between the first and the second EPSPs. An example of the effect of changing the delay between the first and second EPSPs on the percentage increase in the peak amplitude of the second EPSP is shown in Fig. 5.2. In agreement with previous work on PPF at the Ia afferent/motoneurone synapse by Curtis and Eccles (1960), as the delay between the first and second EPSPs was increased the amount of PPF of the second EPSP decreased, the maximum amount of PPF occurring for separations between the first and second EPSPs of only a few milliseconds.

Paired-pulse facilitation and presynaptic inhibition

The main observation from the present study was that during presynaptic inhibition PPF was enhanced. An example of this is shown in Fig. 5.3. In the absence of any presynaptic inhibition the second EPSP, evoked at a delay of 2 ms, was increased in amplitude by 11% (Fig. 5.3*A*). During presynaptic inhibition, which caused a 24% decrease in the amplitude of the first EPSP, the percentage increase in the amplitude of the second EPSP was 33% (Fig. 5.3*B*). Increasing the amount of presynaptic inhibition by increasing the number of conditioning stimuli applied to the PBSt muscle nerve caused an even greater increase in the amount of PPF of the second EPSP. Following a 44% decrease in the first EPSP during presynaptic inhibition the percentage increase in the second EPSP was 55% (Fig. 5.3*C*).

The pooled data on the effect of different amounts of presynaptic inhibition on the percentage increase in the second EPSP is shown in Fig. 5.4. Fig. 5.4*A*

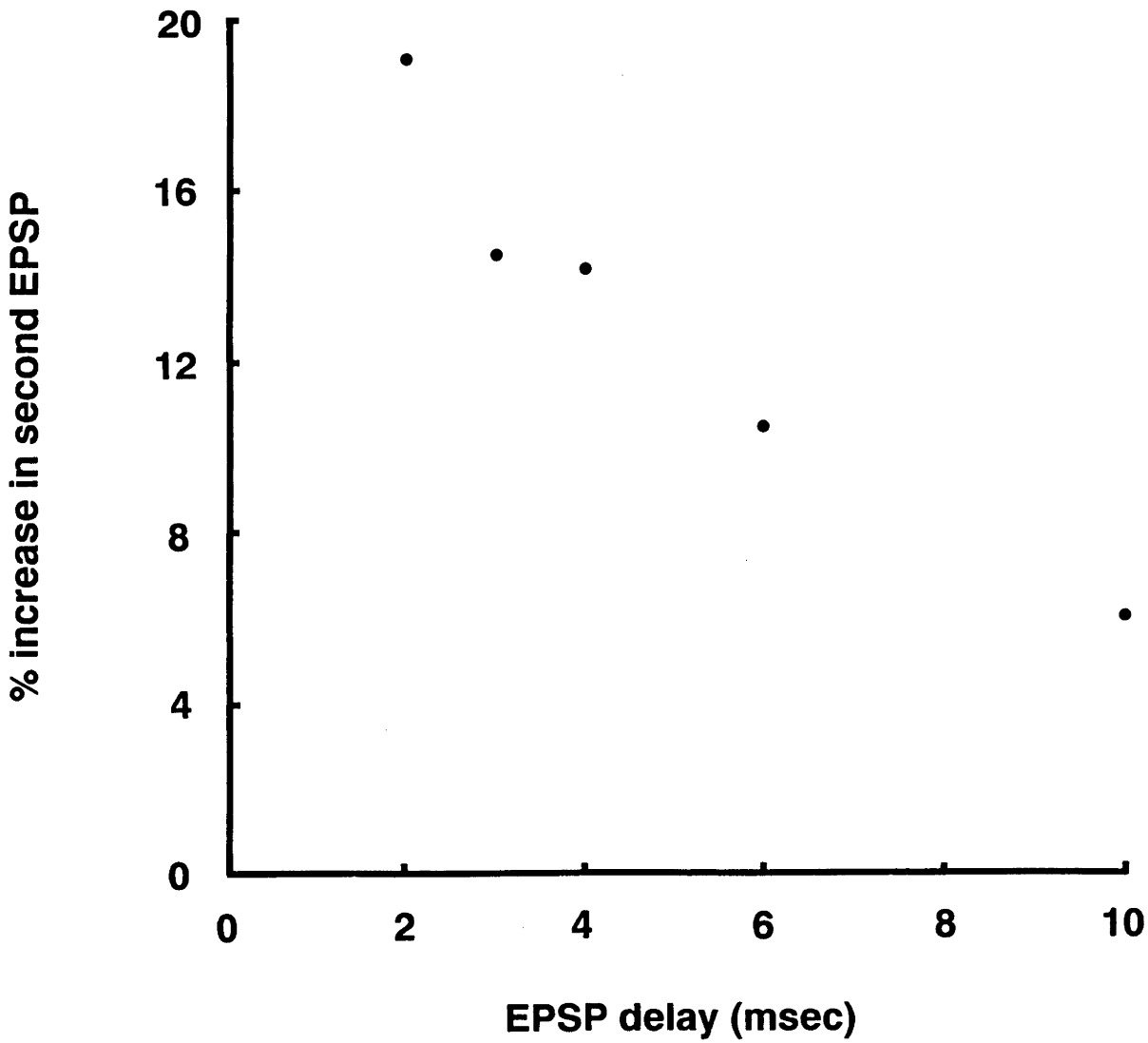


Fig. 5.2. The effect of increasing the delay between the first and second EPSPs on the percentage increase in the second EPSP during paired-pulse facilitation.

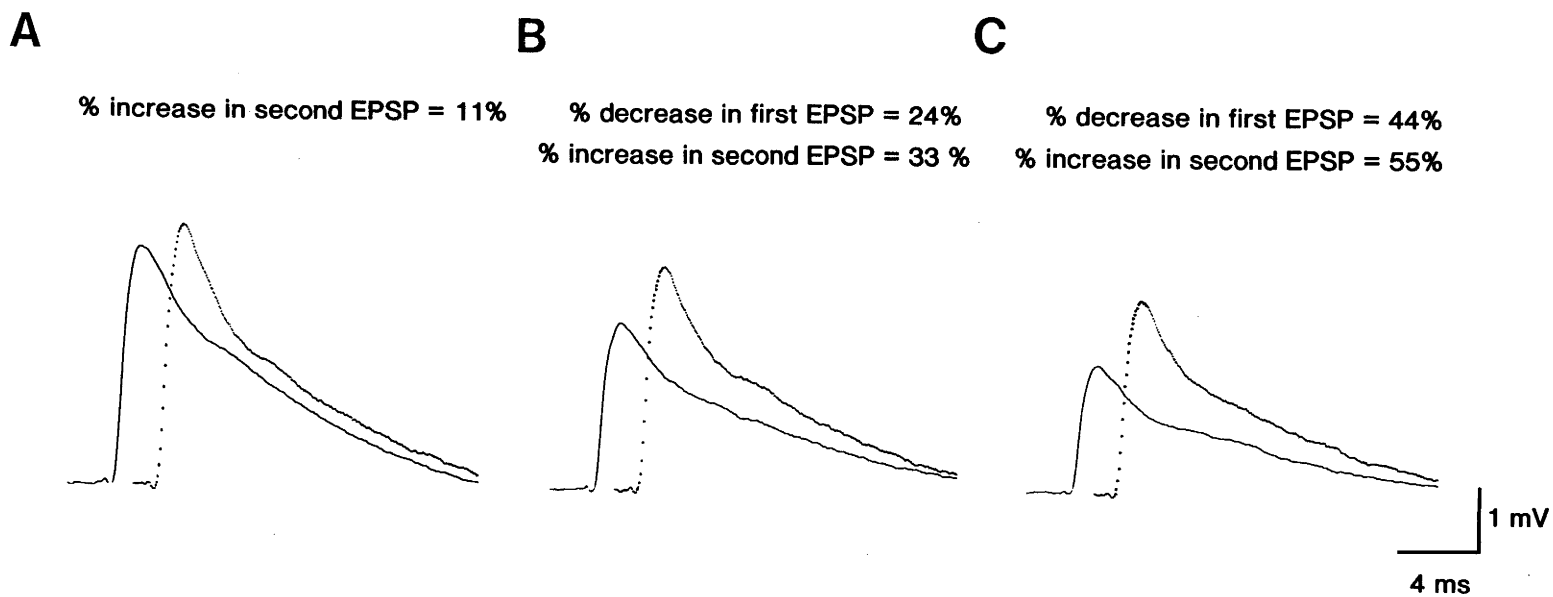


Fig. 5.3. The effect of presynaptic inhibition on paired-pulse facilitation. *A*, in the absence of presynaptic inhibition (control) the second EPSP is increase by 11% relative to the first. *B*, during presynaptic inhibition a 24% decrease in the peak amplitude of the first EPSP is associated with an increase in the second EPSP of 33%. *C*, a further decrease in the peak amplitude of the first EPSP by 44% during presynaptic inhibition is associated with an even larger increase in the second EPSP by 55%.

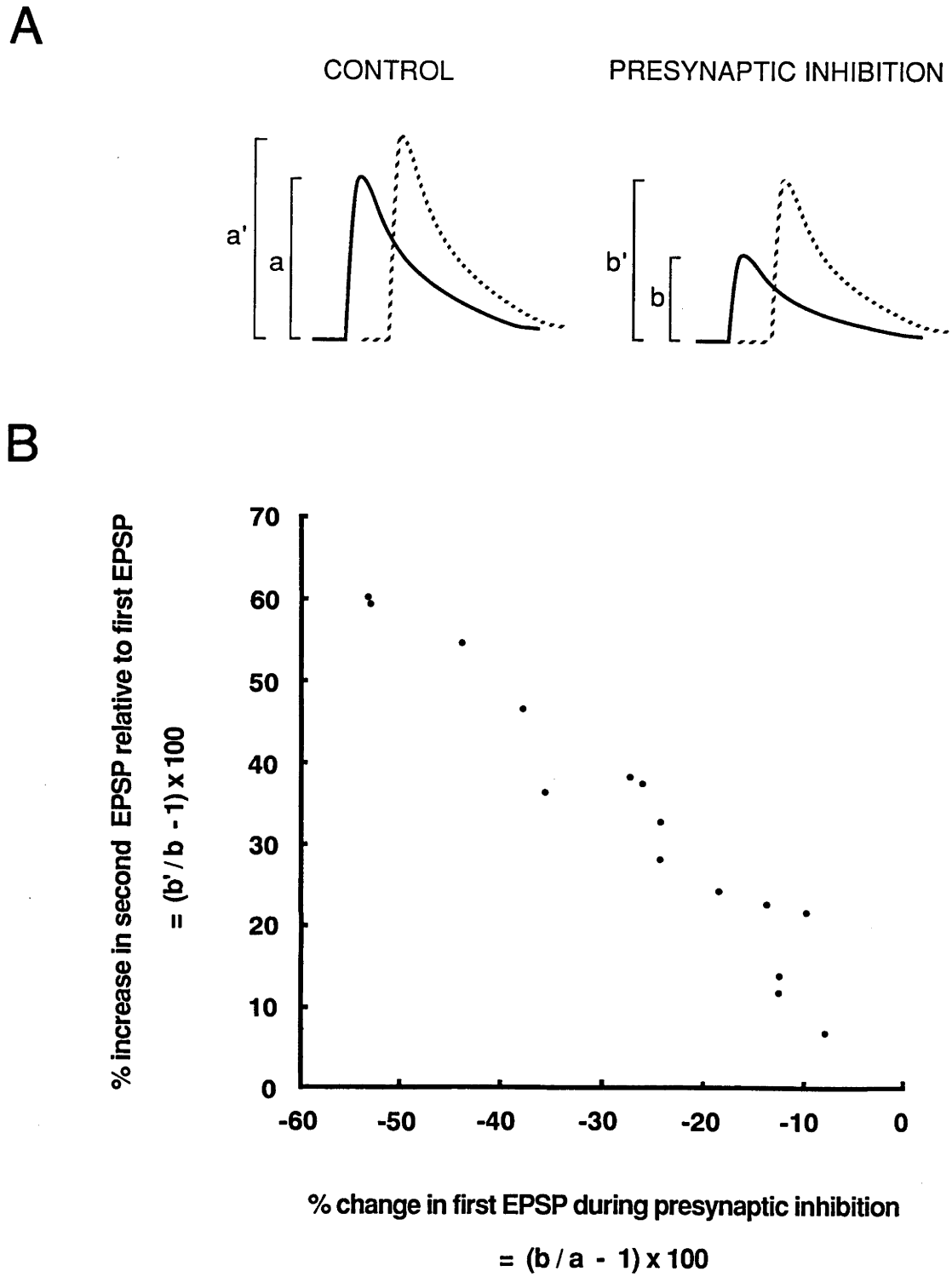


Fig. 5.4. The effect of presynaptic inhibition on paired-pulse facilitation (PPF). *A*, a schematic diagram of PPF in the absence (control) and during presynaptic inhibition. The amplitudes of the first and second EPSPs are a and a′ in the absence of presynaptic inhibition b and b′ during presynaptic inhibition. *B*, the relationship between the percentage change in the first EPSP during presynaptic inhibition, (b/a - 1) x 100, and the percentage increase in the second EPSP relative to the first during PPF, (b′/b - 1) x 100.

shows a schematic diagram of PPF in the absence (control) and during presynaptic inhibition. The percentage change in the amplitude of the first EPSP during presynaptic inhibition is given by:

$$(b/a - 1) \times 100 \qquad \ldots\ldots 1$$

The percentage increase in the second EPSP relative to the first during PPF is given by:

$$(b'/b - 1) \times 100 \qquad \ldots\ldots 2$$

Fig. 5.4*B* plots the relationship between equation 1 and equation 2. Fig. 5.4*B* clearly shows that as the amplitude of the first EPSP is decreased during presynaptic inhibition the amount of PPF is increased, i.e. there is an inverse relationship between the size of the first EPSP during presynaptic inhibition and the amount of PPF.

It was also of interest to investigate how effective presynaptic inhibition was during PPF. This is shown in Fig. 5.5, where the relationship between the percentage decrease in the first EPSP during presynaptic inhibition has been plotted against the percentage decrease in the second EPSP during presynaptic inhibition. Fig. 5.5*A* again shows a schematic diagram of PPF in the absence (control) and during presynaptic inhibition. The percentage decrease in the first EPSP during presynaptic inhibition is given by:

$$(1 - b/a) \times 100 \qquad \ldots\ldots 3$$

The percentage decrease in the second EPSP during presynaptic inhibition is given by:

$$(1 - b'/a') \times 100 \qquad \ldots\ldots 4$$

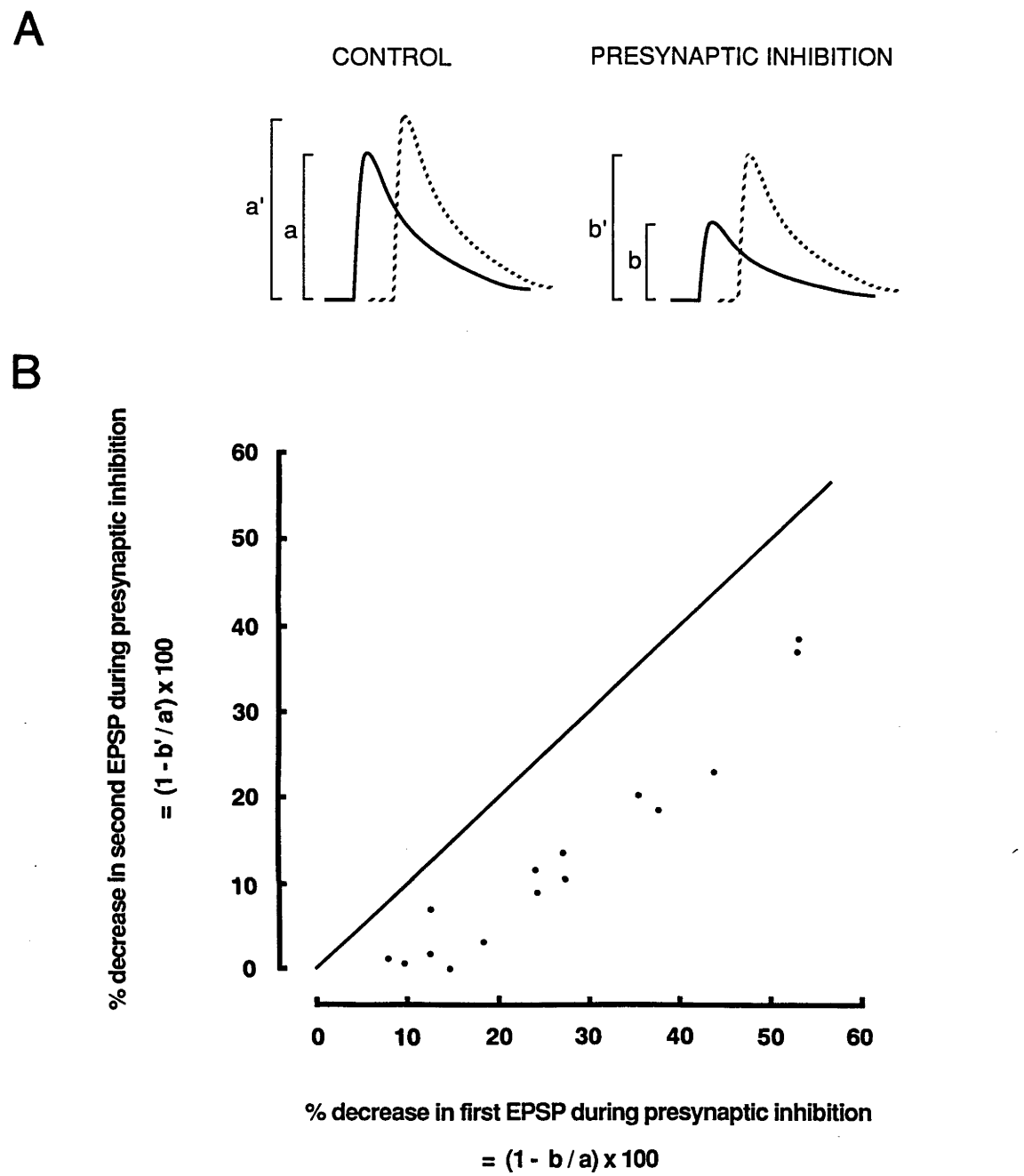


Fig. 5.5. The effectiveness of presynaptic inhibition during paired-pulse facilitation (PPF). *A*, a schematic diagram of PPF in the absence (control) and during presynaptic inhibition. The amplitudes of the first and second EPSPs are a and a′ in the absence of presynaptic inhibition b and b′ during presynaptic inhibition. *B*, the relationship between the percentage decrease in the first EPSP during presynaptic inhibition, (1 - b/a) x 100, and the percentage decrease in the second EPSP during presynaptic inhibition, (1 - b′/a′) x 100. The diagonal line has a slope of 1.

Fig. 5.5*B* plots the relationship between equation 3 and equation 4. If there was no difference in the ability of presynaptic inhibition to decrease the first and second EPSPs during PPF, then the points should lie on or close to the diagonal line which has a slope of 1. The finding that all the points lie below this line shows that presynaptic inhibition is less effective in reducing the potentiated second EPSP during PPF.

Discussion

The main finding from this study is that PPF is enhanced during presynaptic inhibition of compound Ia EPSPs. The interaction between presynaptic inhibition and PPF suggests that both presynaptic inhibition and PPF are acting at a common site. As PPF is thought to act presynaptically to increase transmitter release (Castillo & Katz, 1954; Dudel & Kuffler, 1961*a*; Kuno, 1964; Zucker, 1973; Hirst *et al.* 1981; Lin & Faber, 1988) this would suggest that presynaptic inhibition is also acting presynaptically to modify transmitter release, as has been suggested by many previous authors (Frank & Fuortes, 1957; Eccles *et al.* 1961*a*; Kuno, 1964; Eide *et al.* 1968; Clements *et al.* 1987; McCrea *et al.* 1990). In addition, as the amount of PPF at many synapses has been shown to be altered by changes that directly affect calcium influx into presynaptic terminals (Lundberg & Quilisch, 1953; Castillo & Katz, 1954; Takeuchi, 1958; Thies, 1965; Rahamimoff, 1968; Mallart & Martin, 1968; McNaughton, 1980; Parnas *et al.* 1982; Harris & Cotman, 1983; Dudel, 1989; Muller & Lynch, 1989; Trombley & Westbrook, 1990), the interaction between presynaptic inhibition and PPF at the Ia afferent/motoneurone synapse suggests that presynaptic inhibition may also act to modulate calcium influx.

Other possible explanations

It is possible that the increase in PPF observed during presynaptic inhibition does not involve an interaction between the mechanisms underlying PPF

and presynaptic inhibition. For example, if nonlinear summation of the second EPSP occurs during PPF, then it could be argued that a decrease in the amplitude of the first EPSP during presynaptic inhibition may reduce the amount of nonlinear summation of the second EPSP. This would result in an increase in PPF during presynaptic inhibition. However, the amount of nonlinear summation of the second EPSP is probably only small, as the voltage change that occurs in the dendrites during the first EPSP will decay rapidly back to the resting level in only a few milliseconds (i.e. its time course will be much briefer than recorded at the soma), making it impossible for nonlinear summation of the second EPSP to occur unless the second EPSP is evoked almost similtaneously with the first EPSP (Ianslek & Redman, 1973). In addition, previous experimental evidence has shown that nonlinear summation of different compound Ia EPSPs occurs infrequently (Burke, 1968). On the occassions when nonlinear summation was observed Burke (1968) found that it could be removed by seperating the two compound Ia EPSPs by only a few milliseconds. A recent theoretical study also suggests that nonlinear summation will have only a small effect on the amplitude of compound Ia EPSPs (Segev, Fleshman & Burke, 1990).

Another possible explanation for the increase in PPF during presynaptic inhibition is that it could be due to the activation of polysynaptic pathways. If during presynaptic inhibition, polysynaptic excitatory input to the motoneurone was enhanced or inhibitory input reduced, this could lead to a greater increase in the second EPSP.

An increase in the activation of a polysynaptic excitatory pathway during presynaptic inhibition seems unlikely as the afferent input to such a pathway would be expected to be *reduced* during presynaptic inhibition. A reduction in polysynaptic inhibition of the second EPSP during presynaptic inhibition is possible. The second EPSP evoked during PPF usually decayed faster than the first, which suggests that some polysynaptic inhibition occured during the decay phase of the second EPSP. It is conceivable that during presynaptic inhibition this

polysynaptic inhibition could be reduced due to reduced afferent input and this could lead to an increase in PPF during presynaptic inhibition.

However, the time course of the second EPSP during presynaptic inhibition was not significantly different from the time course of the second EPSP in the absence of presynaptic inhibition. The 10 to 90% rise time and duration at half peak amplitude (half-width) of the second EPSP in the absence of presynaptic inhibition was 0.57 ± 0.05 ms and 3.8 ± 0.32 ms (±S.E.M., $n=10$) respectively, compared to 0.55 ± 0.02 ms and 3.9 ± 0.27 ms (±S.E.M., $n=15$) during presynaptic inhibition (percentage decrease in first EPSP ranged from 8 to 53%). As the half-width of the second EPSP should be particularly sensitive to the changes in the level of polysynaptic excitation or inhibition, this finding suggests that presynaptic inhibition does not significantly change the level of polysynaptic input to motoneurones during the second EPSP.

Comparisons with paired-pulse facilitation in low calcium or high magnesium

PPF is enhanced at many synapses *in vitro*, when the probability of transmitter release is lowered by lowering the extracellular calcium concentration or raising the extracellular magnesium concentration (Lundberg & Quilisch, 1953; Thies, 1965; Rahamimoff, 1968; Mallart & Martin, 1968; McNaughton, 1980; Parnas *et al.* 1982; Harris & Cotman, 1983; Dudel, 1989; Muller & Lynch, 1989; Trombley & Westbrook, 1990). Under these conditions calcium influx into presynaptic nerve terminals would be expected to be reduced. By analogy, the increase in PPF observed in the present study during presynaptic inhibition suggests firstly, that presynaptic inhibition is associated with a decrease in the probability of transmitter release and secondly, that presynaptic inhibition of Ia EPSPs is acting to reduce calcium influx into Ia afferent terminals. Quantal analysis of synaptic transmission during presynaptic inhibition also suggests that presynaptic inhibition is associated with a decrease in the probability of transmitter release (Kuno, 1964; Clements *et al.* 1987).

How does a decrease in the probability of transmitter release lead to an increase in paired-pulse facilitation?

It is thought that during PPF two opposing factors act to modulate transmitter release during the second response; residual calcium in the presynaptic nerve terminal (Katz & Miledi, 1965, 1968), leading to an increase in the probability of transmitter release and 'synaptic depression' (Castillo & Katz, 1954; Takeuchi, 1958; Thies, 1965; Mallart & Martin, 1968; Kusano & Landau, 1975; Zucker, 1989), leading to a decrease in the probability of transmitter release. As the amount of synaptic depression of the second response directly correlates with the amount of transmitter released during the first response, synaptic depression has been attributed to depletion of the 'immediately avaliable store of releasable transmitter' (Thies, 1965; Mallart & Martin, 1968; Kusano & Landau, 1975; Zucker, 1989). An increase in PPF could then occur if the probability of transmitter release is lowered as this would reduce synaptic depression, allowing increased potentiation of the second EPSP due to the presence of residual calcium. Evidence for synaptic depression at the Ia afferent/motoneurone synapse has been observed previously (Curtis & Eccles, 1960; Kuno, 1964).

A formal representation of the effect of residual calcium and synaptic depression on paired-pulse facilitation

If transmitter release at different release sites at the Ia afferent/motoneurone synapse occurs independently (Edwards *et al.* 1976*a,b*), then for a population of n release sites with an average probability of transmitter release p, the average number of release sites that release transmitter will be np and the average number which do not release will be $n(1 - p)$. At Ia afferent synapses the value of p is thought to be different at different release sites (Jack, Redman & Wong, 1981; Walmsley, Edwards & Tracey, 1988). An average value of p for all release sites can, however, be obtained if a large number of release sites are sampled over many trials. At the Ia afferent/motoneurone synapse this average value of p will be directly related to the amplitude of the compound Ia

EPSP, assuming that there is linear summation of quantal events and that the quantal size recorded at the soma is the same for all release sites (Jack *et al.* 1981).

The probability of release on the second trial will depend on the previous history of transmitter release. Following transmitter release on the first trial, the probability of transmitter release on the second trial will be increased due to the presence of residual calcium and decreased due to depletion of the releasable store of transmitter (synaptic depression). If these two factors are independent (Mallart & Martin, 1968), then the probability of transmitter release on the second trial will be determined by the combined effects of residual calcium and synaptic depression. If there is no transmitter release on the first trial, then the probability of transmitter release on the second trial will be determined only by the increased probability of transmitter release due to residual calcium. This is shown schematically in Fig. 5.6. At some time t after the first trial curve A gives the increased probability of transmitter release on the second trial due to residual calcium, while curve B gives the decreased probability of transmitter release on the second trial due to synaptic depression. The curve labelled $A + B$ gives the combined change in the probability of transmitter release due to residual calcium and synaptic depression. The probability of release on the first trial is p. If there is release on the first trial then the probability of release on the second trial is given by p', where $p' = p + \Delta p'$. If there is no release on the first trial then the probability of release on the second trial is given by p'', where $p'' = p + \Delta p''$.

The values of p' and p'' will be different for each release site. However, if a large number of release sites are sampled over many trials an average value of p' and p'' can be obtained. The value of p' will depend on the magnitude of the effects of synaptic depression and residual calcium on the probability of transmitter release on the second trial and may be greater than or less than the probability of transmitter release on the first trial, p. In reality, the duration of the synaptic depression far exceeds the increase in the probability of release due to residual calcium (Thies, 1965; Mallart & Martin, 1968; Kusano & Landau, 1975).

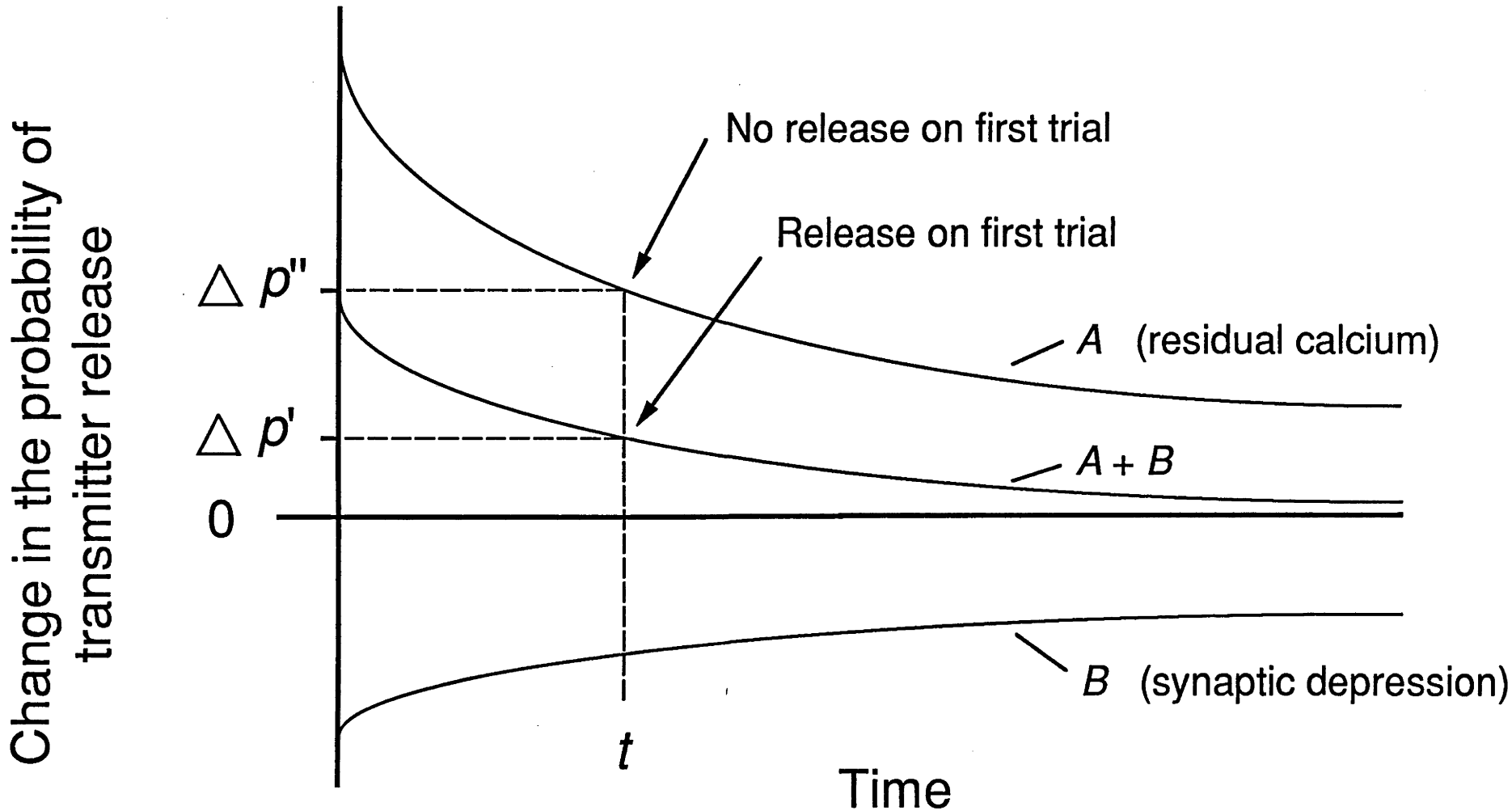


Fig. 5.6. A schematic diagram of the change in the probability of transmitter release after the first trial. Curve *A* and curve *B* give the increase and decrease in the probability of release due to residual calcium and synaptic depression. Curve *A* + *B* gives the net effect of these two factors on the probability of release. The change in the probability of transmitter release at time *t* after the first trial is $\Delta p'$ if there is release on the first trial and $\Delta p''$ if there is no release on the first trial.

An expression can now be derived describing the amount of facilitation for different values of p. If the average number of release sites that release transmitter on the first trial is np and the average number of release sites that do not release transmitter on the first trial is $n(1 - p)$, then the average number of release sites that release transmitter on the second trial will be:

$$npp' + n(1-p)p''$$

Assuming the quantal size recorded at the soma is the same for all release sites and that there is no nonlinear summation of quantal events, the amount of facilitation (F) of the second EPSP can be expressed as the ratio of the number of release sites that release transmitter during the second EPSP compared to the number of release sites that release transmitter during the first EPSP. i.e.

$$F = [\, npp' + n(1-p)p'' \,] / np$$

$$\Rightarrow F = [\, pp' + p'' - pp'' \,] / p$$

$$\Rightarrow F = (p'' / p) + p' - p''$$

This equation has been graphed in Fig. 5.7 and predicts that the amount of facilitation (F) of the second EPSP will be inversely related to the probability of transmitter release (p). This type of relationship is very similar to that found experimentally by others as the probability of transmitter release is altered over a large range by changing the extracellular calcium or magnesium concentrations (see Rahamimoff, 1968; Mallart & Martin, 1968; Parnas *et al.* 1982; Dudel, 1989). It is also similar to the relationship between the amount of facilitation of the second EPSP and the amount of presynaptic inhibition of the first EPSP shown in Fig. 5.4. As the amplitude of the first EPSP was decreased during presynaptic inhibition the amount of facilitation of the second EPSP increased. However, the

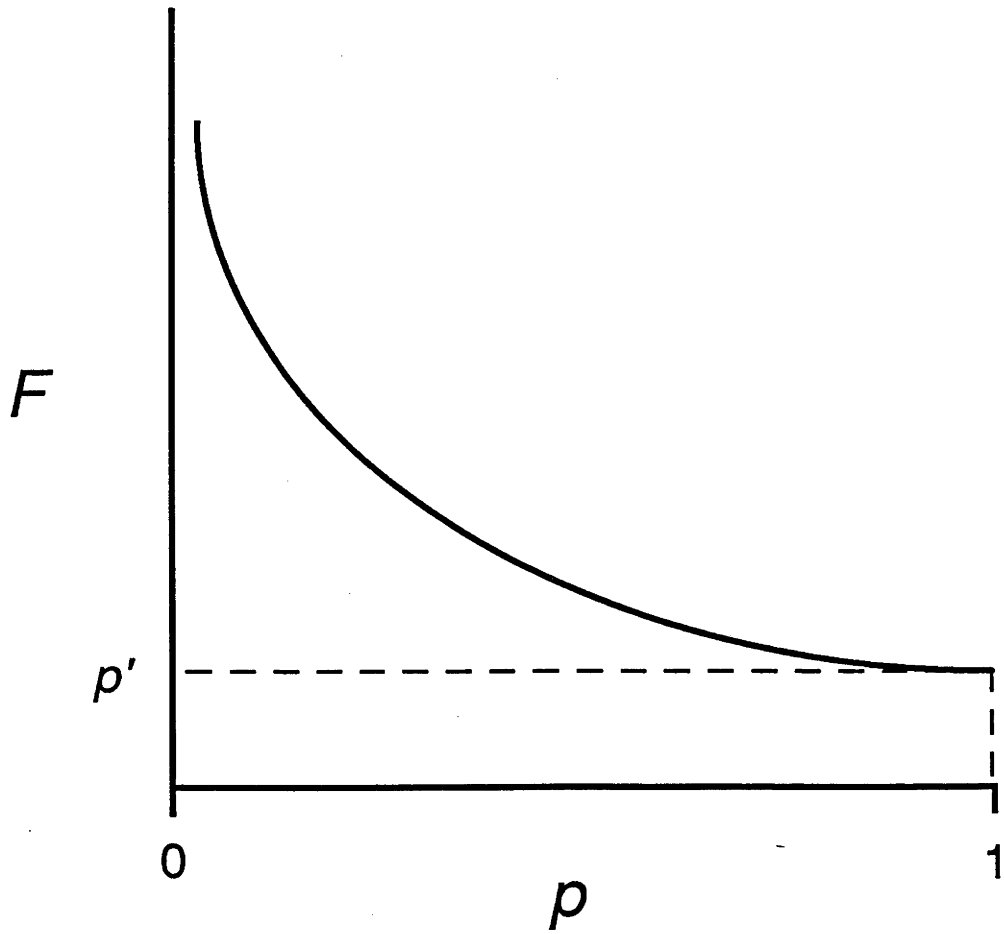


Fig. 5.7. A schematic grapical representation of the relationship:

$$F = (p''/p) + p' - p''$$

$$0 < p < 1;\ \text{at}\ p = 1, F = p'$$

relationship between the amount of facilitation and the amount of presynaptic inhibition shown in Fig. 5.4 appeared to be linear rather than hyperbolic, as predicted by the above equation. There are a number of possible explanations for this apparent discrepancy. Firstly, the range of possible p's examined in the present study may be quite small. Even a ~50% reduction in the first EPSP, suggesting a 50% reduction in p (however, see below), will represent only a small absolute change in p if the resting probability of transmitter release is low, as is thought to be the case at the Ia afferent/motoneurone synapse (Clamann, Mathis & Luscher, 1989). Secondly, as the amount of presynaptic inhibition was increased, by increasing the number of conditioning stimuli applied to the PBSt muscle nerve, its is possible that some of the observed inhibition in the first EPSP occurs due to postsynaptic rather than presynaptic inhibition. This would lead to an over-estimation of the decrease in p for a given amount of facilitation, converting the hyperbolic relationship shown in Fig. 5.7 into one that more closely resembles a straight line, as seen in Fig. 5.4.

The effectiveness of presynaptic inhibition during paired-pulse facilitation

The present study has also shown that during PPF presynaptic inhibition of the second EPSP is reduced (Fig. 5.5). This finding, which could have functional implications during high frequency activation of Ia afferents, is contrary to a related finding by Lev-Tov, Fleshman and Burke (1983) who found that during posttetanic potentiation, presynaptic inhibition was *enhanced*. At the Ia afferent/motoneurone synapse posttetanic potentiation and PPF are thought to involve similar mechanisms (Hirst *et al.* 1981). This makes it difficult to reconcile the findings of Lev-Tov, Fleshman and Burke with the results from the present study. One possibility is that during posttetanic potentiation the observed enhancement in presynaptic inhibition is mediated via a mechanism unrelated to the direct effect posttetanic potentiation has on the probability of transmitter release. As presynaptic inhibition of Ia afferent synaptic transmission can be evoked by prior activation of the same Ia afferent fibres (Decandia, Provini &

Taborikova, 1966, 1967), perhaps the high frequency train used by Lev-Tov, Fleshman and Burke to evoke posttetanic potentiation (500 Hz for 20 seconds) also potentiates the pathways mediating presynaptic inhibition.

Conclusion

The main findings from this study are three fold. Firstly, the increase in PPF during presynaptic inhibition provides further evidence that presynaptic inhibition is associated with a reduction in the probability of transmitter release. Secondly, presynaptic inhibition was found to be less effective in reducing the amplitude of the second EPSP during PPF and thirdly, by analogy with the increase in PPF seen at other synapses under conditions that would be expected to reduce calcium influx, the increase in PPF observed during presynaptic inhibition supports the idea that presynaptic inhibition of Ia EPSPs is associated with a reduction in calcium influx into Ia afferent terminals.

Chapter six:

GENERAL DISCUSSION

This thesis has focused on some aspects of presynaptic and postsynaptic inhibition in the mammalian CNS. These two types of inhibition will shape excitation in different ways. Postsynaptic inhibition at the soma will act to inhibit all excitatory inputs to a neurone, whereas presynaptic inhibition will selectively inhibit only those excitatory inputs which have axo-axonic synapses on their terminals.

The cell bodies of all excitatory neurones within the CNS receive postsynaptic inhibition; only the excitatory inputs from dorsal root ganglion cells, which form part of the peripheral nervous system, do not receive postsynaptic inhibition. For this reason, it is perhaps not surprising that axo-axonic synapses are located exclusively on the nerve terminals of these cells. In the absence of the ability of the CNS to selectively inhibit the monosynaptic excitatory input from primary afferent fibres via postsynaptic inhibition, nature has devised an ingenious method to inhibit this input presynaptically.

In the mammalian spinal cord postsynaptic inhibition is mediated primarily by the neurotransmitter glycine, whereas presynaptic inhibition, at least of Ia afferent fibres, is mediated by the neurotransmitter GABA. In order to more fully understand postsynaptic inhibition a detailed study was made of glycinergic postsynaptic inhibition in lumbar spinal motoneurones. The results from this study were described in Chapter three. The main findings were 1) that the time course of the inhibitory synaptic current underlying glycinergic postsynaptic inhibition was brief, with a rise time of ~0.4 ms and decay time constant of ~0.8 ms at resting membrane potentials (37°C), and 2) that the rate of decay of glycinergic IPSCs was slowed by depolarisation. The time constant of decay of IPSCs was found to

increase *e*-fold for a 91 mV depolarisation. This later result was interpreted as a voltage dependence of the glycine channel open time.

The voltage dependence of the decay of glycinergic inhibitory synaptic currents, together with the direct effect of the membrane potential on the peak inhibitory current, will act to enhance the strength of postsynaptic inhibition as the membrane potential of the postsynaptic cell approaches threshold.

The pharmacology of presynaptic inhibition of transmitter release from the terminals of Ia afferent fibres was described in Chapter four. Of particular interest was the role of $GABA_A$ and $GABA_B$ receptors in presynaptic inhibition. Specific pharmacological blockers were applied locally by iontophoresis. The main finding from this study was that presynaptic inhibition of transmitter release at the Ia afferent/motoneurone synapse is mediated primarily through the activation of $GABA_A$ receptors.

Activation of $GABA_A$ receptors during presynaptic inhibition will result in an increase in Cl^- conductance of Ia afferent terminals. This increase in membrane conductance would be expected to shunt the presynaptic action potential in Ia afferent nerve terminals, decreasing its amplitude and/or duration. A smaller presynaptic action potential would open fewer voltage activated calcium channels, which would result in reduced calcium influx and decreased transmitter release.

There are other possibilities. It has been known for some time that presynaptic inhibition occurs in conjunction with depolarisation of primary afferent fibres. This depolarisation, which presumably results from the efflux of Cl^- ions following the activation of $GABA_A$ receptors, may also contribute to presynaptic inhibition. As discussed in Chapter one this could occur via inactivation of voltage activated calcium or sodium channels. At present it is not known whether either of these mechanisms contribute to presynaptic inhibition.

An investigation on the interaction between paired-pulse facilitation and presynaptic inhibition of Ia EPSPs was described in Chapter five. The main finding from this study was that paired-pulse facilitation was enhanced during presynaptic inhibition of compound Ia EPSPs. This finding is analogous to that seen at other

synapses *in vitro*, when the probability of transmitter release is lowered by reducing the extracellular calcium concentration.

The results from this study provided further evidence that presynaptic inhibition is associated with a reduction in the probability of transmitter release. By analogy with the effects of reduced calcium influx on paired-pulse facilitation at other synapses, this study supported the idea that presynaptic inhibition is associated with a decrease in calcium influx into primary afferent terminals.

Suggestions for future experiments

The experiments on unitary glycinergic IPSCs were technically difficult. An alternative approach would be to use an *in vitro* preparation. The most attractive possibility would be to use whole cell recording techniques to record spontaneous glycinergic IPSCs from motoneurones in spinal cord slices (Edwards, Konnerth, Sakmann & Takahashi, 1989; Blanton, Lo Turco & Kriegstein, 1989; Takahashi, 1990). Pharmacological blockers could be used to isolate only those spontaneous events due to the release of glycine. To reduce problems due to spontaneous events which are generated at locations not adequately space clamped, TTX could be added to the external medium and hypertonic saline applied to the soma of motoneurones from an extracellular pipette. This would increase the frequency of spontaneous quantal events generated near the soma (Bekkers & Stevens, 1989).

A more difficult, but potentially rewarding experiment would be to record intracellularly from glycinergic interneurones in contact with motoneurones. Quantal analysis of IPSCs evoked in motoneurones following activation of single glycinergic interneurones would help to elucidate the junctional mechanisms operating between inhibitory interneurones and motoneurones in the spinal cord. Both cells could be filled with different fluorescent dyes and the morphology of the synaptic connections between them determined using confocal microscopy.

There are still many unanswered questions relating to presynaptic inhibition. While the results presented in Chapter four showed that at the Ia afferent/motoneurone synapse this inhibition was mediated primarily by $GABA_A$

receptors, it is possible that different receptors may be involved in mediating presynaptic inhibition at axo-axonic synapses on terminals of other primary afferents. A study of the pharmacology of presynaptic inhibition of cutaneous afferent fibres in the dorsal horn could prove interesting. It might be expected that the long term regulation of transmitter release, for example during suppression of tonic cutaneous input to the spinal cord, may be mediated via receptors coupled to second messenger systems. Perhaps $GABA_A$ receptors mediate presynaptic inhibition of Ia afferent transmission, while $GABA_B$ receptors mediate presynaptic inhibition of input from cutaneous afferents. In favour of such a proposal is the finding that the highest density of $GABA_B$ receptors in the spinal cord is on the terminals of primary afferents in the dorsal horn (Price *et al.* 1984; Price *et al.* 1987).

One of the major problems encountered in the pharmacological experiments on presynaptic inhibition described in Chapter four was that the application of drugs by iontophoresis resulted in nonuniform, unknown concentrations within the spinal cord. One way around this would be to bath apply known concentrations of drugs using an *in vitro* preparation. The difficulty here is to find an *in vitro* preparation of presynaptic inhibition. Possibly the hemisected neonatal rat spinal cord preparation with attached hind limb nerves could be used for this purpose.

As mentioned earlier it is unknown how activation of $GABA_A$ receptors leads to presynaptic inhibition. While presynaptic inhibition will undoubtably be mediated, at least in part, by an increase in conductance of Ia afferent terminals, the possibility that depolarisation of Ia afferent fibres also reduces transmitter release cannot be ruled out. One way to investigate the contribution of depolarisation of primary afferents to presynaptic inhibition would be to monitor presynaptic inhibition before and after the removal of this depolarisation by abolishing the gradient for Cl^- efflux from primary afferent terminals.

Using an *in vitro* preparation this could be achieved in two ways. Either by the application of compounds that inhibit active transport of Cl^- ions, causing

passive redistribution of Cl^- ions across the membrane of primary afferent fibres, or alternatively by raising the extracellular Cl^- concentration.

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