

**VOLTAGE ACTIVATED CURRENTS IN HIPPOCAMPAL CA1  
NEURONES**

*by*

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### **Statement.**

The experiments described in Chapters 3 and 4 and some preliminary experiments for Chapter 6 were done in collaboration with Dr. Alasdair Gibb. All other experiments were designed and performed by the candidate.

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## Abstract

The aim of the work described in this thesis was to examine the ionic conductances underlying repetitive firing in hippocampal CA1 neurones. To this end we started out examining voltage activated currents using a single electrode voltage clamp in hippocampal slices. Under conditions where the sodium currents were blocked, several inward and outward currents were identified in these neurones. A transient outward potassium current, active at potentials more depolarized to  $-60\text{mV}$  was characterized and identified as the A-current. In these neurones it was found that the A-current was blocked by extracellular application of cadmium. Two separate calcium currents were also identified. A low threshold (approximately  $-60\text{mV}$ ), transient current and a higher threshold (approximately  $-40\text{mV}$ ) sustained current. It is suggested that the sustained calcium current may be localised preferentially in the dendrites.

In order to study neurones in the slice preparation, it is necessary to use high resistance ( $>20\text{M}\Omega$ ) microelectrodes. This limits the current passing capacity of the voltage clamp and thus seriously compromises the ability to clamp fast conductance changes. In addition, the extended geometry of the neurones precludes a good space clamp. For these reasons, it proved impossible to either study any of the fast conductance changes or to obtain any quantitative data on the recordable conductances.

In order to achieve better voltage clamp conditions a dissociated slice technique was developed. In this method neurones are acutely isolated from the CA1 region of adult guinea pig hippocampi following mild enzymatic treatment. Neurones obtained by this procedure had lost most of their dendritic tree, could be shown to be isopotential and were ideal candidates for voltage clamp.

Dissociated neurones were voltage clamped in the whole cell configuration of the patch clamp technique using a discontinuous single electrode voltage clamp. In cells perfused with fluoride solutions three voltage activated currents were identified and characterized. These were a fast transient sodium current, a fast activating noninactivating sodium current and a rapidly activating, slowly inactivating potassium current.



The fast sodium conductance activated at potentials positive to  $-50\text{mV}$ , and reached a peak conductance at  $0\text{mV}$ . This current was shown to underlie the rising phase of action potentials in these cells. Assuming a sodium channel conductance of  $17\text{pS}$ , it was calculated there are about 8 sodium channels per  $\mu^2$  of somatic membrane in hippocampal CA1 neurones.

The noninactivating sodium conductance activated at potentials positive to  $-80\text{mV}$  and reached a peak around  $-10\text{mV}$ . Peak conductance of this current was calculated to be about 2% that of the fast sodium current. It is argued that this current will play a pivotal role in controlling the firing characteristics of these cells at membrane potentials around threshold.

The outward potassium current is shown to be analagous to the delayed rectifier found in many other preparations. It activated at potentials positive to  $-50\text{mV}$  and reached a peak conductance around  $+40\text{mV}$ . This current has a peak current density comparable to that of the sodium current. This coupled with its rapid activation suggests that it may play a role in repolarization of the action potential.

I dedicate this thesis to my parents without whose foresight it would not have been possible.

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### 7. Discussion.

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## **Chapter 1**

### **Voltage Gated Ion Channels in Vertebrate Central Neurones.**

## Introduction.

One of the main goals of neuroscience is the functional explanation of the activity of the central nervous system. For a long time it was assumed that the complex electrophysiological properties of various parts of the central nervous system were a product of synaptic interactions between neurones. When recordings were first made from neurones, however, it became evident that many of the electrophysiological responses observed were intrinsic electrical properties of the neurones themselves. It is now clear that these electrical properties are the result of the actions of a number of voltage gated ion channels acting in concert with the passive membrane properties of the nerve membrane. Thus, in order to be able to describe the function of systems of neurones it is essential to have an understanding of the activity of single neurones.

A functional description of neuronal activity requires several pieces of information: We need to know the ionic specificity and voltage dependence of the different ionic conductances present in these neurones; the factors, such as neurotransmitters, that modulate their properties and lastly we need to know their spatial location over the surface of the neurones concerned. While much of this information is known for some vertebrate and invertebrate neurones, our understanding of mammalian neurones is relatively poor. The reasons for this are that firstly, mammalian neurones are small and have a complex structure. Secondly, these neurones are fragile and are buried within the mass of the brain making them poor candidates for analysis of their membrane conductances. With the development of the *in vitro* brain slice technique and single electrode voltage clamp many of these problems are now being overcome.

The initial experiments described in this thesis were done with the aim of trying to characterise some of the conductances underlying the firing properties of hippocampal CA1 pyramidal neurones. Using a single electrode voltage clamp, we were able to study some of the many conductances present in these cells in hippocampal slices. The results from this work are presented in Chapter 2. During the course of this work it became clear that with a single high resistance electrode at the soma, it was only possible to adequately voltage clamp these neurones over a limited voltage range. Additionally, it proved impossible to clamp the large and rapidly activating conductances such as the sodium current and the calcium current. To try and achieve better voltage clamp conditions, we began to look at acutely dissociated neurones as an alternate

system in which to study these conductances. Some of the properties of these dissociated cells are described in Chapters 3,4,5 and 6.

This chapter is a literature review of the the types and properties of voltage gated ion channels present in nerve membranes, with an emphasis on vertebrate central neurones.

### **Historical Background.**

The study of excitable cells dates from the seventeenth century when it was first appreciated that nervous activity may be electrical in nature. By the end of the 18th century Galvani and von Humbolt had concluded that the activity of nerve and muscle was due to 'animal electricity' and by 1850 Helmholtz had measured the conduction velocity of the nervous impulse. In the first half of the 19th century du Bois-Raymond first demonstrated that nerve and muscle could produce their own electricity (see Brazier, 1959).

It was not until 1902, however, that the membrane concept was advanced by Julius Bernstein. Bernstein postulated that the membrane of the nerve or muscle fibre at rest was selectively permeable to potassium ions and led to an accumulation of positive charge on the outside of the membrane causing a negative internal potential. Furthermore, he proposed that the action potential was a self propagating depolarization of this membrane caused by a nonselective permeability increase to all ions (Bernstein, 1902,1912). At about the same time, Hermann (1905a,b) proposed the core-conductor or cable theory for the passive spread of potentials in nerve. This model suggested that axons were like submarine cables with a conducting core surrounded by an insulator. The potentials generated by nerve and muscle fibres were small and fast and were not accurately measured until the introduction of electronic amplifiers and the cathode ray oscilloscope by Erlanger and Gasser in the early 1920s (Gasser and Erlanger, 1922; Erlanger and Gasser, 1937). One of the first people to use this new technology was E. D. Adrian who had previously deduced from experiments on whole nerves that nervous activity was an all or none phenomenon (Adrian, 1913). Using the new amplifiers he was able to demonstrate this directly in single nerve fibres (Adrian and Forbes, 1922).

The rediscovery by J. Z. Young (Young, 1936) of the giant axons in squids began the next phase of nerve physiology. These large fibres allowed membrane and action potentials to be recorded intracellularly and the membrane theory could be directly tested. One of the first pieces

of evidence in support of Bernstein's theory was obtained by Cole and Curtis (1939) who showed that the nerve impulse was associated with an increase in electrical conductivity of the nerve membrane (with no change in the capacitance). Intracellular recordings also showed that the resting membrane potential was indeed negative on the internal side of the membrane with respect to the outside. An unexpected observation was that the peak of the action potential was approximately +40mV and not near 0mV as expected from the ideas of Bernstein (Cole and Curtis, 1939; Hodgkin and Huxley, 1939, 1945). In order to explain these observations it was proposed by Hodgkin and Katz (1949) that the upstroke of the action potential was caused by a selective permeability increase to sodium ions. Definitive proof for this idea was obtained by Hodgkin and Huxley in the following years by direct measurement of the sodium current generating the action potential.

### **The Hodgkin-Huxley Axon.**

By 1951 it was clear that the rising phase of the action potential was due to an influx of sodium ions. In addition, Keynes (1951a,b) had shown that during activity of nerve, there was a net efflux of potassium ions. The prevailing idea was that the upstroke of the action potential was caused by a regenerative increase in the sodium permeability - the Hodgkin cycle and repolarization due to a later efflux of potassium ions (Hodgkin, 1951). These ideas were proven, and given a sound theoretical basis with the development of the voltage clamp technique by Marmont (1949), Cole (1949) and later Hodgkin, Huxley and Katz (1949, 1952).

In a now classic series of experiments (Hodgkin, Huxley & Katz, 1952; Hodgkin and Huxley, 1952a,b,c), Hodgkin and Huxley laid the foundations for all subsequent thinking about nervous activity for the next thirty years. They established that upon depolarization of the nerve fibre, there is a rapid, transient influx of sodium ions followed by a delayed efflux of potassium. They measured the kinetics of the two ionic currents and were able to describe them in terms of quantitative kinetic equations. Furthermore, they demonstrated that combining their results with cable theory they were able to correctly predict the shape of the space clamped and propagated action potential (Hodgkin and Huxley, 1952d).



### **The Nature of the Permeability Change.**

It had been shown by Hodgkin and Huxley that the sodium and potassium currents in squid axon could be separated by substitution of the sodium ions in the extracellular solution with a nonpermeant species. With sodium absent, the ionic current consisted solely of the potassium current. The sodium current could then be obtained by subtracting the potassium current from the total. It was not clear, however, whether the two ions (sodium and potassium) moved via the same pathway or whether they had separate pathways. The discovery that tetrodotoxin (TTX), the poison purified from the Japanese puffer fish, could selectively block sodium movements in lobster giant axons (Narahashi et al., 1964), while leaving the potassium conductance unaffected, provided the first direct evidence for the existence of separate pathways for the two ions. Another important drug that was discovered was tetraethylammonium (TEA) which was found to selectively block potassium channels (Armstrong and Binstock, 1965). It is now clear that upon depolarization, sodium and potassium ions move down their concentration gradients through separate pathways (for reviews see Hille 1970, Armstrong, 1975). The structures which control these selective increases in permeability have come to be called ion channels.

### **Channels are Membrane Proteins.**

The availability of toxins which have a high specificity and a high affinity for the sodium channel (eg. tetrodotoxin, saxitoxin, see Narahashi, 1974) has allowed the isolation and purification of the membrane components responsible for the sodium permeability change. It has been found that sodium channels are membrane spanning proteins with a molecular weight of about 316kD (for reviews see Catterall, 1982, 1986). These proteins form ion selective pores across the nerve membrane and can adopt one of two conformational states - open or closed. The currents flowing through the individual channels can now be recorded and the properties of the single channel protein analysed (eg. Sakmann and Neher, 1983). More recently, the sodium channels from eel electrophorus (Noda et al., 1984) and rat brain (Noda et al., 1986a) have been sequenced and cloned and shown to form functional sodium channels when placed in an appropriate expression system (Noda et al., 1986b; Stümer et al., 1987).

## Properties of Voltage Activated Sodium and Potassium Channels.

During a step depolarization of the squid axon, the sodium conductance rises rapidly then decays during the step. These processes are called activation and inactivation respectively. Activation is a rapid process during which sodium channels open with a sigmoidal time course when the membrane potential is stepped to potentials more positive than about  $-50\text{mV}$ . During a depolarization, inactivation is a slower process which closes sodium channels. Both activation and inactivation are dependent upon the membrane potential, becoming faster at more depolarized potentials. Once sodium channels inactivate, the membrane potential must be returned to a hyperpolarized level before they can be reactivated.

With few exceptions, sodium channels with properties very similar to the sodium channels in squid axon have been found to underlie the action potential in all excitable cells that have been examined. They have a fast activation, a slower inactivation and are blocked by micromolar concentrations of TTX. Properties of voltage gated sodium channels in neuronal cell bodies will be described in detail in Chapters 4 and 6.

Potassium channels of the squid also activate in a voltage sensitive manner. Their time course of activation is however slower than for sodium channels. In addition, the potassium channels do not inactivate during short depolarizations. Because these potassium channels open with a delay and pass outward current they are also often called delayed rectifiers. With prolonged depolarizations, these channels do inactivate but only very slowly. Unlike the sodium channel, there appears to be great diversity in the pharmacological properties of voltage activated potassium channels between preparations. The only consistent features that distinguish potassium channels in different preparations seem to be that (1) They are invariably blocked by TEA ions and (2) They are always blocked by caesium ions (Adelman and Senft, 1966; Bezanilla and Armstrong, 1972). Many different types of potassium channels have now been found in other preparations, some of these will be discussed below.

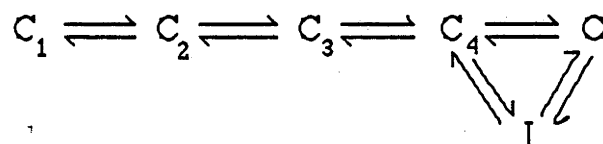
## Models and Gating currents.

The voltage sensitivity of sodium and potassium currents was explained by Hodgkin and Huxley by charged particles which move due to the influence of the membrane potential and "gate" the two currents. These charged particles can be thought of as dipoles on the channel

proteins, moving in the electric field of the membrane, to gate the channel between open and closed conformations. If this is the case, then, as Hodgkin and Huxley pointed out there should be a small current associated with the movement of these charges and this current should be detectable in voltage clamp experiments. This current has now been measured by several groups and come to be called the "gating current" (Armstrong and Bezanilla, 1973,1974; Keynes and Rojas, 1974; Bezanilla, 1985). The gating current associated with sodium channel opening has been well characterised (see Armstrong, 1981; Keynes, 1983), however, the gating current associated with the potassium channel has been more difficult to find (Armstrong and Gilly, 1980) and its properties have not been very well described.

In the classic Hodgkin-Huxley model activation and inactivation are seen as independant processes, both of which are instantaneously responsive to membrane potential. We would thus predict that inactivation would also have a charge movement associated with it. A charge movement associated with the inactivation parameter has not been found. The lack of an obvious inactivation gating current and data from single channel experiments have led some investigators to propose theories of sodium channel function in which inactivation is directly coupled to activation (Aldrich, Corey and Stevens, 1983; Armstrong, 1981).

Activity of sodium and potassium channels can now described in terms of a kinetic equation, with the different kinetic states representing open and closed conformations of the channel. Based on voltage clamp (eg. Armstrong, 1981) and single channel data (Horn and Vandenberg, 1984, 1986) the currently accepted model for the sodium channel can be represented by the following scheme:



Where  $C_1$  to  $C_4$  are different closed states, O the open state and I the inactive state of the sodium channel. Similar schemes, with multiple closed states, have also been postulated for the potassium channel (Bezanilla, 1985).

## **Electrical Activity in Neurones.**

The development of the intracellular microelectrode by Graham, Gerard and Ling and later by Hodgkin and Nastuk (see Frank, 1986 for references) allowed the sodium theory to be extended first to muscle (Nastuk and Hodgkin, 1950) and later by Eccles and others to nerve cells (Eccles, 1957). It very quickly became obvious that neurones were capable of producing much more complex activity than axons or muscle. For instance, neurones can exhibit a variety of slow oscillatory firing patterns and long lasting aftereffects of activity not seen in axons (for examples see Berridge, Rapp and Treherne, 1979; Llinas, 1985). We now understand that these complex phenomena are produced by the combined action of many different ionic conductances (for reviews see Adams, Smith and Thompson, 1980; Adams and Galvan, 1986).

## **Other Types of Channels.**

Shortly after the description of the voltage activated sodium and potassium current in squid axon, currents carried by other ions were found in different preparations. We now know that there are several types of calcium channels, many different types of potassium channels and several types of chloride channels in vertebrate and invertebrate neurones (eg. see Schwindt and Crill, 1983; Hille, 1984; Adams and Galvan, 1986). Like the sodium channel, these channels are also membrane proteins (eg. see Flockerzi et al., 1986; Takahashi and Catterall, 1987; chapter 7) and open and close in response to changes in membrane potential. All these different channel systems act together to control the overall electrical behavior of neurones.

## **Calcium Channels.**

It was not long after the sodium theory of action potential generation was established that Fatt and Katz (1953) found that the large muscle fibres of the crab legs had only very small graded action potentials. When they replaced the sodium ions in the extracellular solution with choline, the action potentials became larger and regenerative. This was the first demonstration of excitation without sodium ions. Fatt and Katz also showed that replacing sodium with TEA or other quaternary ammonium ions was even more effective than choline in turning the depolarizing response into a powerful propagated action potential. Subsequently, Fatt and Ginsborg (1958) went on to demonstrate that the action potential in the crab muscle was carried

by calcium ions. Later, Hagiwara and Naka (1964), using the giant muscle fibres from barnacles, measured calcium ion fluxes during action potentials and provided the final proof of the calcium hypothesis.

Although not realized by the early workers, we now understand why it is necessary to use solutions containing quaternary ammonium derivatives in order to be able to record regenerative calcium action potentials - these agents block potassium currents which otherwise mask the calcium response by providing an antagonistic, repolarizing current to the calcium current.

Calcium currents under voltage clamp were first measured by Reuter (1967) in cardiac muscle, Geduldig and Gruener (1970) in *Aplysia* neurones and Keynes et. al., (1975) and Hagiwara, Fukuda and Eaton (1974) in barnacle muscle fibres. Subsequently, calcium currents have been found in almost all cell types that have been examined from *Paramecium* to mammalian central neurones (for reviews see Reuter, 1973; Hagiwara and Byerley, 1981; Hagiwara, 1983; Tsien, 1983; Hille, 1984). However, because of the concomitant activation of outward potassium currents, these early descriptions of calcium current were necessarily qualitative. Pharmacological blockade of potassium currents with TEA or other drugs was never complete.

Quantitative description of the calcium current had to await the development of the intracellular perfusion technique (Kostyuk, Krishtal and Pidoplichko, 1975; Lee, Akaike and Brown, 1978). With these methods, intracellular potassium could be completely replaced with an impermeant cation such as caesium and calcium currents measured in isolation (Kostyuk and Krishtal, 1977; Akaike, Lee and Brown, 1978). Because these techniques relied on relatively large cells, all the initial quantitative measurements were made on invertebrate neurones (Kostyuk, 1980; Hagiwara and Byerley, 1981; Tsien, 1983). It was found that activation of calcium currents could be described by equations similar to the formulation for sodium, however inactivation appeared to be different from preparation to preparation.

### **Calcium Channels in Vertebrate Neurones.**

In the vertebrate central nervous system, sodium independent action potentials were first demonstrated by Koketsu et al. (1959) in the spinal ganglion cells of frogs. Subsequently, calcium action potentials have been recorded from frog (Barrett and Barrett, 1976) and rat

(Schwindt and Crill, 1980; Fulton and Walton, 1981) motoneurones, hippocampal CA1 pyramidal cells (Schwartzkroin and Slawsky, 1977) and many other areas of the central nervous system (Llinas, 1984). Calcium currents under voltage clamp have been recorded from hippocampal pyramidal neurones (Johnston, Hablitz and Wilson, 1980; Brown and Griffith, 1983b) and cortical neurones (Stafström et al., 1985) in slices, and from cat motoneurones *in vivo* (Schwindt and Crill, 1980a,b).

When calcium action potentials from the central nervous system were closely examined the results suggested that there was more than one type of calcium channel present in these neurones (Llinas and Yarom, 1981a,b; Jahnsen and Llinas, 1984a,b; Jahnsen, 1986a,b; Murase and Randić, 1983). One of these channels activated at hyperpolarized potentials, gave rise to a 'low threshold spike', and was inactivated by holding the cell at depolarized potentials. The other calcium channel activated at more depolarized potentials and gave rise to the 'high threshold spike' (Llinas and Yarom, 1981a,b; Jahnsen and Llinas, 1984a,b).

Voltage clamp studies of neurones and cells of neuronal origin have confirmed the presence of multiple types of calcium channels in these cells. However, because of the difficulties in separating out the calcium current from potassium currents these studies have been mostly been restricted to cells which can be easily isolated (eg. dorsal root ganglion cells) or cultured cells (eg. neuroblastoma cells). These cells can be internally perfused so allowing for separation of the calcium current as described above. Two types of calcium channels have been reported in rat and chick dorsal root ganglion cells (Carbone and Lux, 1984; Nowycky, Fox and Tsien, 1985; Fedulova, Kostyuk and Veselovsky, 1985) neuroblastoma cells (Fishman and Spector, 1981; Narahashi, Tsunoo and Yoshii, 1987), pituitary cells (Matteson and Armstrong, 1986), cultured hippocampal neurones (Yarri, Hamon and Lux, 1987), tractus solitarius neurones (Kunze, 1987) and CA1 pyramidal cells in hippocampal slices (Halliwell, 1983). These studies confirm the action potential data. There appears to be one calcium current which is only active at hyperpolarized potentials. This current is an inactivating current and is completely inactive at potentials more depolarized than  $-20\text{mV}$ . The high threshold current activates at potentials positive to  $-10\text{mV}$  and does not inactivate. The two types of channels can be separated on kinetic and pharmacological grounds (Nowycky et al., 1985; Matteson and Armstrong, 1986; Carbone

and Lux, 1987; Narahashi et al., 1987). Tsien and colleagues have called the low threshold channel the 'T' channel and the high threshold one the 'L' channel. It is possible that these different calcium channels carry out quite different functions in neurones. For example, it has been suggested that the low threshold, or 'T' calcium channel is mainly responsible for controlling repetitive firing, bursting and subthreshold behavior, whereas the high threshold or 'L' channel is responsible for injecting calcium into the cell for modulation of metabolic functions (Miller, 1987). In chick dorsal root ganglion neurones, a third type of calcium current designated the 'N' channel has also been described (Nowycky, Fox and Tsien, 1985). N-channels require strong depolarizations for activation (unlike the T channels) and they inactivate (unlike L channels). These channels have been suggested to be involved in transmitter release (Miller, 1987)

### Potassium Channels.

We now know that neuronal membranes contain a variety of potassium conductances which can be differentiated on kinetic, voltage dependence and pharmacological grounds (Adams, Smith and Thompson, 1980; Hille, 1984; Adams and Galvan, 1986). In hippocampal pyramidal cells, for example, there are thought to be six different potassium conductances. These conductances are designated  $I_A$ ,  $I_K$ ,  $I_C$ ,  $I_{AHP}$ ,  $I_M$  and  $I_Q$ . The A-current ( $I_A$ ) is a transient outward current which was first recorded by Hagiwara, Kusano and Saita (1961) in *Onchidium* nerve cells and subsequently characterised by Connor and Stevens (1971b) and Neher (1971).  $I_K$  is the classical delayed rectifier of Hodgkin and Huxley and is discussed in detail in Chapter 6.  $I_C$  and  $I_{AHP}$  are two late outward currents which are activated by an increase in intracellular calcium (Meech, 1978).  $I_M$  is a slow outward current activated by depolarization and distinguished from other outward currents by its sensitivity to muscarinic agonists.  $I_Q$  is a voltage activated current analogous to the anomalous rectifier seen in many other preparations.

### The A-Current.

The A-current ( $I_A$ ) has been recorded from almost all neurones that have been examined (Rowawski, 1985). In the mammalian nervous system, transient outward currents under voltage clamp were first seen in cat motoneurones (Schwindt and Crill, 1980a). Definitive A-currents

were first characterised in dorsal root ganglion neurones (Kostyuk et al., 1981) and in hippocampal CA3 neurones (Gustafsson et al., 1982; Zbicz and Weight, 1985) and have been shown in rat sympathetic neurones (Belluzzi, Sachhi and Wanke, 1984a; Galvan and Sedlmeir, 1985), locus coeruleus (Williams et al., 1984) dorsal raphe (Aghajanian, 1985) and cultured hippocampal and spinal neurones (Segal, Rogawski and Barker, 1984). In all preparations,  $I_A$  activates rapidly on depolarization to potentials more depolarized than  $-50\text{mV}$  and then inactivates with an exponential time course over several hundred milliseconds. It is blocked by millimolar concentrations of 4-aminopyridine (4-AP). In molluscan neurones, it has been modelled by equations similar to that developed for the sodium current (Connor and Stevens, 1971b; Neher, 1971). The A-current displays a steep steady-state inactivation and is only active at potentials more negative than  $-50\text{mV}$ . These characteristics - its transient nature, a steep steady-state inactivation and sensitivity to 4-AP are the distinguishing features of  $I_A$ . The A-current has been suggested to play a role in allowing neurones to fire in the low frequency range (Connor and Stevens, 1971c; Cassell, Clark and McLachlan, 1986). Additionally, because of its rapid activation kinetics this current has also recently been implicated in repolarization of the action potential (Belluzzi et al, 1985a; Størm, 1987; Lancaster and Nicoll, 1987) and in modulating the time course of synaptic potentials (Cassell and McLachlan, 1986). The properties of  $I_A$  in hippocampal CA1 neurones are described in chapter 2.

### Calcium Activated Potassium Currents.

Changes in potassium conductance in response to a rise in intracellular calcium were first seen in red blood cells (for review see Schwarz and Passow, 1983). In neurones, calcium activated potassium conductances were first demonstrated in *Aplysia* (Meech and Strömwater, 1970; Meech, 1974) and in cat cortical (Godfraind et al., 1971) and spinal motoneurones (Krnjević and Lisiewicz, 1972). These currents have since been described in many different excitable cells (Meech, 1978). It is now recognised that both membrane depolarization and physiological levels of intracellular calcium ( $0.1 - 10\mu\text{M}$ ) are required for activation of this current (Barrett, Magleby and Pallotta, 1982), and it is blocked by low extracellular concentrations ( $5-20\text{mM}$ ) of TEA. The role of this current in neuronal activity, however, is just starting to be understood.



Action potentials in many excitable cells are followed by a prolonged afterhyperpolarization (AHP), which has been attributed to an increase in potassium conductance (Eccles, 1957). It is now well established that the AHP is produced by calcium activated potassium conductances (Hirst and Spence, 1973; Krnjević, Puil and Werman, 1978; Hotson and Prince, 1980; Schwartzkroin and Stafstrøm, 1980; Alger and Nicoll, 1984). A calcium activated potassium current has been demonstrated under voltage clamp in guinea pig hippocampal pyramidal neurones (Brown and Griffith, 1983b; Segal and Barker, 1986), rat sympathetic neurones (Galvan and Sedlmeir, 1984; Belluzzi, Sacchi and Wanke, 1984b) and olfactory neurones (Constanti and Sim, 1987) and designated  $I_{K(Ca)}$ . A large conductance calcium activated potassium channel has also been recorded at the single channel level in muscle (Pallotta, Magleby and Barrett, 1981), chromaffin cells (Marty, 1981) and sympathetic neurones (Gardner, 1985) and thought to be the channel carrying  $I_{K(Ca)}$ . This potassium current was initially thought to underlie the AHP. It has become evident however, some properties of  $I_{K(Ca)}$  are different to those of the current that was responsible for producing the AHP (eg. Madison and Nicoll, 1986a).

The first problem is that  $I_{K(Ca)}$  decays with a time constant of tens of milliseconds at membrane potentials where the AHP has a duration of seconds. Secondly, in hippocampal pyramidal cells, carbachol blocked the AHP (Cole and Nicoll, 1984) at concentrations at which it has been shown to not affect  $I_{K(Ca)}$  (Brown and Griffith, 1983a). Lastly, the large conductance calcium activated potassium channel (Marty, 1981; Pallotta, Magleby and Barrett, 1981) is blocked by some drugs and toxins which do not affect the AHP in the same cells and vicer versa (eg. Romey and Lazdunski, 1984).

Careful examination of the calcium activated potassium currents in hippocampal pyramidal cells and bullfrog ganglion cells has revealed that there is more than one component to  $I_{K(Ca)}$ . It is now becoming apparent that there is a slower component to the calcium activated potassium current with a decay time time constant of the order of seconds at the resting membrane potential. This current has been characterised in bullfrog sympathetic ganglion cells (Pennfether et al., 1985; Lancaster and Pennfether, 1987) and rat hippocampal pyramidal cells (Lancaster and Adams, 1986) and has been designated  $I_{AHP}$  by these authors. This current is found to be

relatively insensitive to TEA and is blocked by nanomolar concentrations of apamin (a venom from the bee), characteristics very similar to that of the AHP.  $I_{AHP}$  has therefore been suggested to underlie the prolonged afterhyperpolarization seen in these cells (Pennefather et al., 1985; Lancaster and Adams, 1986). A slow calcium activated potassium conductance with properties similar to  $I_{AHP}$  has also recently been reported in olfactory neurones (Constanti and Sim, 1987) and an anterior pituitary cell line (Ritchie, 1987). Furthermore, a small conductance calcium activated potassium channel with the above pharmacological properties has also been reported in cultured rat skeletal muscle and have been suggested to underlie the AHP in these cells (Blatz and Magleby, 1986). Thus it appears that  $I_{AHP}$  may be quite widespread in its phylogenetic distribution.

The calcium activated potassium current activates very rapidly on depolarization to potentials more positive than about  $-40\text{mV}$  and reaches a peak in 5 to 10ms (Brown and Griffith, 1983a, Belluzzi, Sacchi and Wanke, 1985b). On repolarization back to the resting potential it turns off exponentially over several hundred milliseconds. It has been suggested that the fast components of this current i.e.  $I_C$ , act to repolarize the action potential (Størm, 1987; Lancaster and Nicoll, 1987) whereas the slower components ie.  $I_{AHP}$ , acts as a 'brake' during repetitive activity to reduce the frequency of firing and may contribute to the phenomenon of accommodation (Madison and Nicoll, 1982, 1984; Adams and Galvan, 1986).

It is interesting that a third type of calcium activated potassium current has also been reported in some preparations. In bullfrog ganglion cells (MacDermott and Weight, 1982), rat sympathetic ganglion cells (Galvan and Sedlmeir, 1984) and guinea pig hippocampal pyramidal cells (Zbicz and Weight, 1985) a transient voltage and calcium activated potassium current has been reported. This current seems to have properties similar to the A-current, except the it is also blocked by calcium channel blockers. It has been suggested that this current may play a role in the repolarization phase of action potentials (MacDermott and Weight, 1982).

### The Q-Current.

Many biological membranes show the property of inward rectification at potentials more negative to the resting membrane potential, that is, the membrane conductance is higher for inward current than for outward current. This unusual property was first seen by Katz (1949) in

skeletal muscle and he called it anomalous rectification. The inward rectifier has the unusual property that the conductance is a function of the electrical driving force for potassium ( $V_m - E_K$ ) rather than the membrane potential *per se* (Hagiwara, 1983). Since the initial observations of Katz, this conductance has been examined in detail in muscle and marine eggs (see Hagiwara, 1983; Latorre and Miller, 1983; Hille, 1984). It has been shown that in these preparations it is relatively  $K^+$  specific and is blocked by  $Ba^{2+}$  and  $Cs^+$ . In neuronal membranes, the properties of the inward rectifier are not so uniform. Although in olfactory cortex (Constanti and Galvan, 1983) it appears to be very similar to the classical description, inward rectifiers observed in other central neurones are different to varying degrees (eg. see Mayer and Westbrook, 1983; Crepel and Penit-Soria, 1986). In hippocampal neurones, anomalous rectification was first observed by Hotson et al. (1979) under current clamp. Under voltage clamp a current similar to the anomalous rectifier has been reported by Halliwell and Adams (1982), however, unlike the classical description, this current was not blocked by barium ions and thus was called  $I_Q$  by them. At physiological levels of extracellular potassium,  $I_Q$  is only active at very hyperpolarized potentials ( $< -80mV$ ) and it is unclear what role it might play in the activity of these neurones. One suggestion is that it has a role in maintaining a pacemaking function under some circumstances (Halliwell and Adams, 1982; Crepel and Penit-Soria, 1986) or perhaps, as suggested by Hille (1984) (see also Mayer and Westbrook, 1983), its function is to conserve intracellular potassium during prolonged depolarizations.

### The M-Current.

The M-current ( $I_M$ ) is a voltage activated potassium current which was first discovered in bullfrog sympathetic ganglion neurones (Brown and Adams, 1980). It has since also been described in rat sympathetic neurones (Constanti and Brown, 1981), olfactory neurones (Constanti and Sim, 1987) and hippocampal pyramidal neurones (Halliwell and Adams, 1982). The M-current has relatively slow kinetics and is preferentially activated at membrane potentials between  $-50$  and  $-20mV$  (Brown and Adams, 1980; Adams, Brown and Constanti, 1982a; Halliwell and Adams, 1982). Due to its activation at membrane potentials close to the resting membrane potential, it has been suggested that  $I_M$  exerts a "strong potential-clamping effect on the behavior of these neurones at membrane potential subthreshold to excitation" (Adams, Brown

and Constanti, 1982a). In hippocampal pyramidal neurones,  $I_M$ , in conjunction with  $I_{AHP}$  has also been implicated in the control of repetitive firing, particularly in the phenomenon of accommodation (Cole and Nicoll, 1983, 1984; Madison and Nicoll, 1984).

$I_M$  was so named because it is reversibly blocked by muscarinic agonists (Brown and Adams, 1980; Adams, Brown and Constanti, 1982b; Halliwell and Adams, 1982). In bullfrog ganglion cells it is also blocked by stimulation of the muscarinic afferents to these neurones. Thus, synaptically mediated blockade of  $I_M$  has been suggested to underlie the slow cholinergic excitation that is seen in bullfrog ganglion (Adams, Brown and Constanti, 1982c) and rat ganglion neurones (Brown and Selyanko, 1985). Slow cholinergic potentials are also seen in hippocampal CA1 neurones (Cole and Nicoll, 1984), it remains to be seen whether these are also produced by block of  $I_M$ .

### Chloride Currents.

Several types of chloride channels have been described in vertebrate neurones. In dorsal root ganglion (Mayer, 1985), cultured rat spinal neurones (Owen, Barker and Segal, 1984, 1986) and cultured hippocampal neurones (unpublished observations quoted by Owen, Barker and Segal, 1986) there appears to be a calcium and voltage activated chloride conductance. This conductance has slow kinetics and is activated by depolarization to voltages more positive than  $-50\text{mV}$ . In dorsal root ganglion neurones these currents have been suggested to underlie the afterdepolarization that is seen following action potentials in these neurones (Mayer, 1985). It is not clear what role it plays in spinal and hippocampal cells.

A voltage activated chloride conductance, not sensitive to calcium, has also been characterized in hippocampal pyramidal cells in the slice preparation (Madison, Malenka and Nicoll, 1986). This current also has slow kinetics, however, unlike the calcium activated current, this conductance is active at the resting membrane potential and is switched off by membrane depolarization. Furthermore, it has been demonstrated to be reduced by application of phorbol esters (potent activators of protein kinase C). It has been suggested by Madison et al. (1986) that this current is located in the dendrites of these neurones. Modulation of this current would therefore be a powerful mechanism of altering the excitability of these neurones.

## Transmitter Modulation of Voltage Gated Ion Channels.

Until quite recently, ionic channels in nerve and muscle had been divided into two broad classes: Those that participate in the generation of action potentials (ie. the voltage-activated channels) and those that generate postsynaptic potentials in response to neurotransmitters (transmitter activated channels). Recent results suggest, however, that in many systems neurotransmitters can modulate the activity of voltage-activated channels. An example of a neurotransmitter that acts in this way is acetylcholine acting at neuronal muscarinic receptors (eg. the M-current described above). This ability of neurotransmitters to modulate the properties of voltage-dependent conductances is a new and important class of synaptic action and was first clearly described in the heart (Reuter, 1983). It is now becoming apparent that modulation of voltage gated conductances is a widespread phenomenon throughout the central nervous system (Siegelbaum and Tsien, 1983; Rogawski and Barker, 1985).

Modulation of ion channels is one way in which synaptic activity can alter the firing characteristics of target neurones. For example, in hippocampal pyramidal cells noradrenaline, acting via a beta adrenergic receptor, leads to production of cAMP and closure of a calcium activated potassium channel (Madison and Nicoll, 1986a,b). This leads to a reduction of accommodation and the cell can now produce more spikes per stimulus than before the application of noradrenaline. Thus noradrenaline, acting on one set of receptors can alter the firing behavior of the whole neurones over a period of several seconds.

The action of transmitters that act by modulating voltage-gated channels differ from those of the classical neurotransmitters in two respects. Firstly, the time course of the transmitter action is very slow, lasting seconds to minutes compared to the brief action of the classical transmitters (eg. ACh at skeletal muscle synapses). Secondly, whereas the classical transmitters act by directly opening or closing ion channels, the modulators generally act via second messenger systems. Second messenger systems that have been implicated in transmitter modulation are  $\text{Ca}^{2+}$ , cAMP, inositoltrisphosphate ( $\text{IP}_3$ ) and G-proteins unrelated to cAMP or  $\text{IP}_3$  (Worley Baraban and Solomon., 1987; Dunlap, Holz and Rane, 1987). It has been suggested by Greengard and colleagues that this second messenger modulation of ion channels resembles the action of certain hormones on their target cells in that the binding of the transmitter to its receptor

sets off a chain of events leading ultimately to the phosphorylation of one or more substrate proteins (Nestler, Walaas and Greengard, 1984). Given that some voltage-activated ion channels have been shown to be substrates for protein kinases (Costa et al., 1982; Ewald et al., 1985; Curtis and Catterall, 1985; Ewald, Williams and Levitan, 1985), one attractive possibility is that the ultimate target for phosphorylation is the channel protein itself.

## **Chapter 2**

### **Some Voltage Activated Ionic Currents in Guinea Pig Hippocampal CA1 Neurones.**

## INTRODUCTION

Although a great deal is known about the mechanisms controlling repetitive firing in many vertebrate and invertebrate neurones, our understanding of the ionic basis of such activity in mammalian central neurones is relatively poor. While intracellular recording of voltage has provided much valuable information about the electrical properties of these cells (eg. Llinas and Sugimori, 1980a,b; Llinas and Yarom, 1981a,b; Jahnsen and Llinas, 1984a,b;), in order to obtain quantitative data on the ionic conductances underlying the firing properties of a particular neurone it is essential to be able to voltage clamp the cells of interest (Hodgkin and Huxley, 1952a,b,c; Connor and Stevens, 1971a,b,c).

In the past, voltage clamp techniques have been applied with great success to the large neurones of the invertebrate nervous system (Connor and Stevens, 1971a,b; Kostyuk and Krishtal, 1977; Adams and Gage, 1979). In the mammalian central nervous system, however, voltage clamp studies have been hampered because (a) It is difficult to obtain successful microelectrode impalements in small (20 - 50 $\mu$ m diameter) cells for extended periods of time, (b) it is difficult to block contaminating ionic currents and (c) an effective space clamp does not extend into the dendritic trees of these neurones.

With the introduction of the brain slice technique coupled with single electrode voltage clamp (Brenneke and Lindemann, 1974; Wilson and Goldner, 1975; Finkel and Redman, 1984) problems (a) and (b) can be resolved. Using brain slices, central neurones can be maintained *in vitro* and their ionic environment controlled. Because there is no movement due to respiration or heartbeat, stable impalements can be maintained for long periods of time. Using a single electrode voltage clamp it is possible to voltage clamp the neurones in these slices with minimal damage.

Although brain slices have now been studied from many regions of the mammalian brain (Dingledine, 1984), the hippocampus remains the most frequently studied region of the brain. The hippocampus has a simple neural architecture which is retained in the slices. The pyramidal cell layer is easily recognised in transverse slices and neurones in identified regions of the cell layer can be examined. In voltage clamp experiments, depolarization of hippocampal CA1 and



CA3 pyramidal cells have been shown to activate inward calcium (Brown and Griffith, 1983b) and sodium currents (French and Gage, 1985), and noninactivating calcium activated outward currents (Brown and Griffith, 1983a; Zbicz and Weight, 1985; Lancaster and Adams, 1986; Halliwell and Adams, 1982). Transient outward currents have been reported in CA3 pyramidal neurones (Gustafsson et al., 1982; Zbicz and Weight, 1985) and in hippocampal neurones in cell culture (Barker and Segal, 1984) and have been shown to be analagous to the A-current ( $I_A$ ) first described by Connor and Stevens (1971b) . In this Chapter the properties of a transient outward current in hippocampal CA1 neurones will be presented. In addition, evidence will be presented for the presence of two types of calcium currents in these cells.

## METHODS

Experiments were done on slices from hippocampi of adult Wistar rats or adult guinea pigs. No differences were found between the two species. Animals were killed by cervical dislocation and quickly decapitated. The brain was exposed by peeling back the skull using a pair of ronguers. The cerebellum and brainstem were cut off and the two hemispheres bisected with a scalpel blade. The two halves were then removed and placed in cold (approximately 4 °C), oxygenated Ringer and the hippocampus dissected free using plastic spoons. Transverse slices (400µm thick) were then cut using a hand operated tissue chopper. The slices were immediately transferred to a holding chamber containing oxygenated Ringer and allowed to recover for at least one hour before recording was attempted.

### Recording Techniques.

The standard Ringer consisted of (mM): NaCl 125, KCl 5.0, CaCl<sub>2</sub> 2.5, NaH<sub>2</sub>PO<sub>4</sub> 1.2, MgSO<sub>4</sub> 2.0, NaHCO<sub>3</sub> 25, glucose 10 which was continuously bubbled with a 95% O<sub>2</sub> - 5% CO<sub>2</sub> gas mixture, pH 7.2. In experiments in which cadmium was added to solutions to block calcium currents, the bicarbonate concentration was reduced to 13mM and NaH<sub>2</sub>PO<sub>4</sub> was omitted to avoid precipitation of divalent cations. 4-Aminopyridine (4-AP) and tetraethylammoniumBr (TEA) were added directly to the recording solution at the required concentration. Tetrodotoxin (TTX; 4 x 10<sup>-7</sup>M) was routinely added to all recording solutions in order to block sodium currents. All solutions were prepared from powders immediately before each experiment.

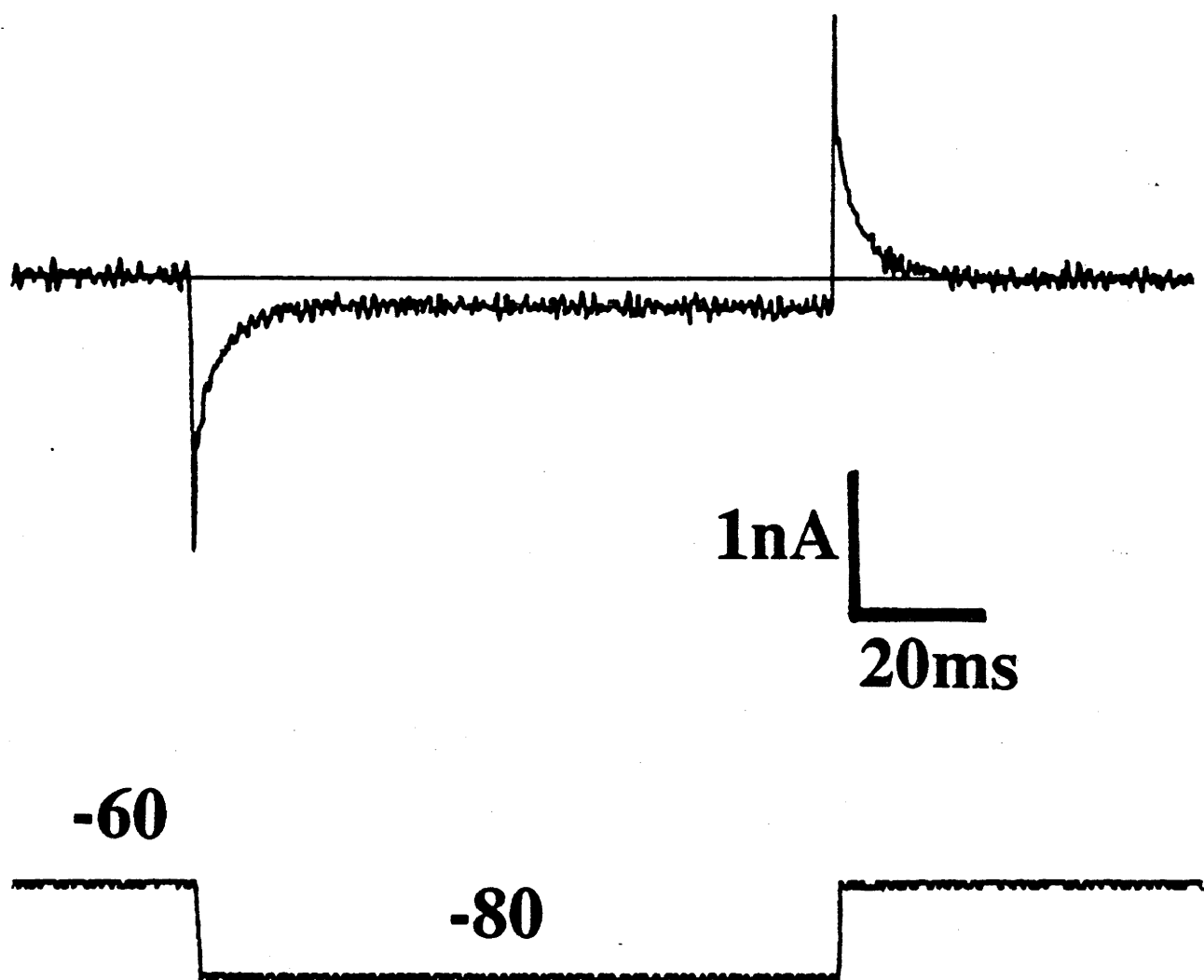
The recording chamber was similar in design to that described by Nicoll and Alger (1981). Slices were transferred from the holding chamber, one at a time, into the recording chamber as needed and kept partially submerged on a nylon mesh. The perfusion system consisted a Gilson pump which was used to both perfuse and suck the solution at a rate of 3mls/min. The dead time in changing solutions with this system was approximately 1 to 2 minutes. Slices were illuminated from below and visualised using a Wild Stereo dissecting microscope at a magnification of x50. The pyramidal cell layer was clearly visible and electrodes could be easily placed in the CA1 region using a micromanipulator (Narashige).

Microelectrodes were pulled from 1mm o.d. fibre filled glass (Clark Electromedical) using a Brown-Flaming electrode puller (PF-77) and were connected directly to the headstage of a single electrode voltage clamp amplifier (Axoclamp ASF-1, Axon Instruments) using a WPI electrode holder. Electrode resistance was monitored by injecting a small (0.2nA) current pulse across the electrode and measuring the voltage change. When filled with 3M KCl or 3M CsCl they had resistances between 40 and 70M $\Omega$ .

Potassium currents were studied with KCl filled electrodes in 66 CA1 neurones from 20 animals and calcium currents were studied with CsCl filled electrodes in 20 cells from 9 animals. The membrane potential of cells impaled with KCl electrodes ranged from -50 to -75mV with an average of  $-64 \pm 1$  mV ( $\pm$ SEM), and the average input resistance, measured by applying small (<20mV, 50ms) voltage steps from the resting potential of the cell, was  $39 \pm 3$  M $\Omega$ . In cells impaled with CsCl filled electrodes the membrane potential was more depolarized with an average resting potential of  $-44 \pm 3$  mV and the input resistance was higher at  $57 \pm 7$  M $\Omega$ . These differences are consistent with blockade of potassium channels with Cs ions.

### **Voltage Clamp.**

CA1 neurones were impaled using a stepping microdrive (Narashige) and voltage clamped using a single electrode voltage clamp amplifier (Axon Instruments ASF-1). The clamp was operated with a sampling frequency of 5 to 7 kHz and a 30% duty cycle. Membrane current was filtered at 1 kHz (4 Pole Bessel) and sampled with a PDP 11/23 computer at 2.5kHz. After obtaining a stable impalement, the clamp amplifier was switched into current clamp mode and, while monitoring the headstage of the input, the capacity neutralization was adjusted for optimal settling (Finkel and Redman, 1984; see also Fig 3.2). Only cells with resting potentials more negative than -50mV and input impedance > 20M $\Omega$  (KCl electrode) were used. With CsCl electrodes, cell with resting potentials as low as -30mV were used for further analysis. The clamp was then switched over to voltage clamp and the gain and phase adjusted until maximum gain could be obtained. The headstage voltage was continuously monitored during the course of an experiment to ensure that the voltage settled completely to baseline between samples (Finkel and Redman, 1984).



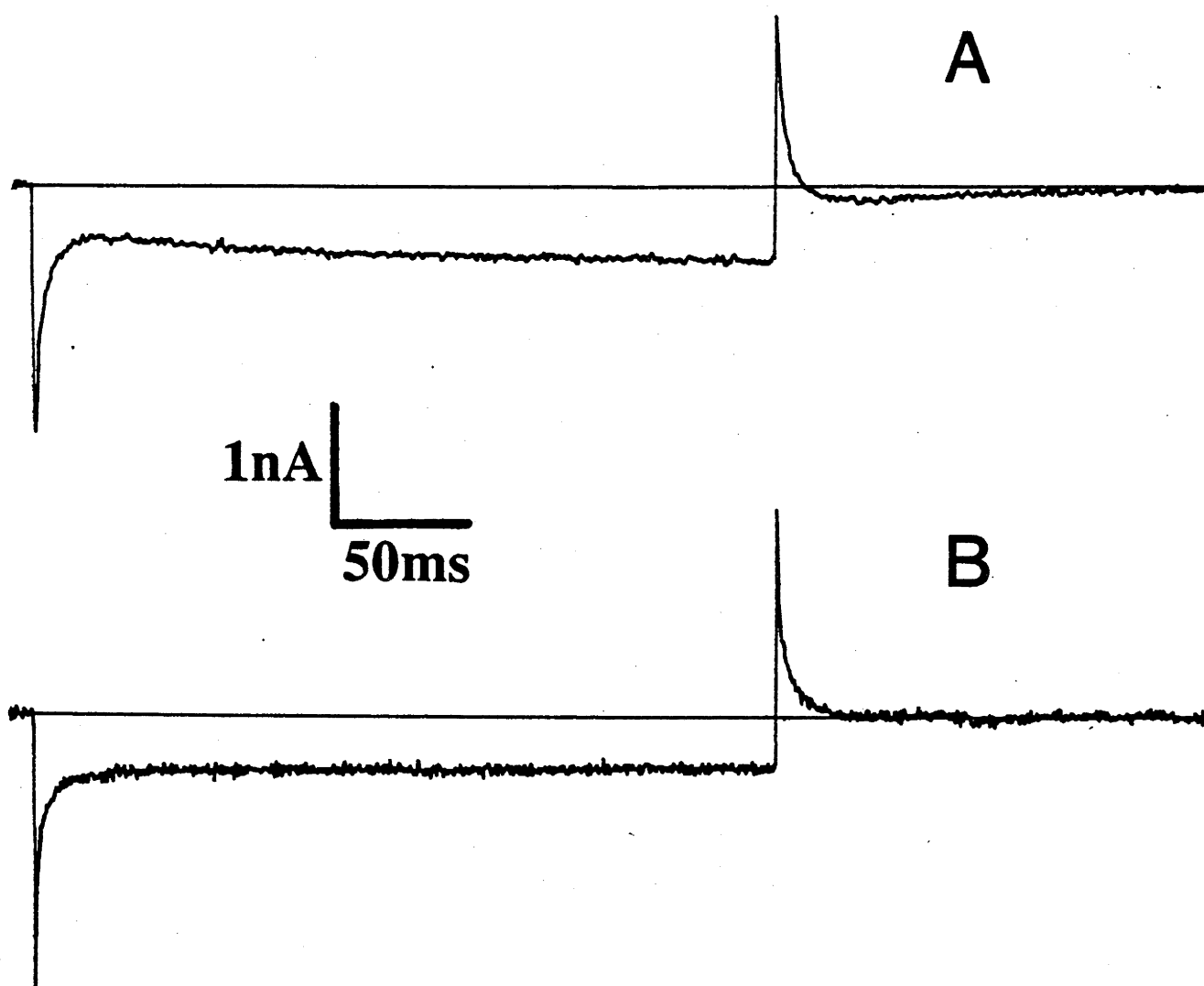
**Figure 2.1.** Dynamic characteristics of voltage clamp. Upper trace shows current generated in response to a 20mV hyperpolarizing voltage command from a holding potential of  $-60\text{mV}$  (lower trace). The capacity transient decays to within 95% of its final value within 10ms. Vertical calibration 1nA. Horizontal calibration, 20ms.

Using the single electrode voltage clamp, the electrode characteristics are the most important determinant of clamp speed (Johnston and Brown, 1983,1984; Finkel and Redman, 1984). In order to achieve the best possible clamp, electrodes with a relatively low resistance ( $<50\text{M}\Omega$ ) were preferentially used. In order to reduce the electrode capacitance, the fluid level over the surface of the slice was kept as low as possible. Clamp gains typically used ranged between 4 and  $10\text{nA/mV}$ . The response of the clamp for a  $20\text{mV}$  hyperpolarizing step is illustrated in Fig. 2.1. It can be seen that the membrane potential recorded at the soma reached the command potential within several milliseconds. The capacity transient on the current record, however, lasted for 10 to 15 ms due to the distributed capacitance of the dendritic tree (Rall, 1977). In this study, although the capacity and leak currents have been digitally subtracted out (see below), because of the slow capacity transient, quantitative measurements have only been made at time  $>10\text{ms}$  after the voltage step.

The problems of space clamping a cell with an extended structure such as a neurone have been extensively discussed in the literature (Halliwell and Adams, 1982; Johnston and Brown, 1983, 1984) and will not be addressed here. We feel that under the present recording conditions, we can expect the soma to be well clamped, however, clamp fidelity will be compromised by varying degrees over the rest of the dendritic tree. For this reason, very few kinetic measurements have been made on the recorded currents.

### Data Analysis.

Voltage clamp data was analysed off line on a PDP 11/44 computer. Capacity and leakage currents were digitally subtracted by scaling up and adding the current response to a  $20\text{mV}$  hyperpolarizing voltage step from the holding potential. The current response to a  $20\text{mV}$  hyperpolarizing voltage step often showed a voltage dependent component. An example of this is shown in Fig. 2.2A. The slow sag in the current is probably due to activation of a 'Q' current (Halliwell and Adams, 1982). In many cells, and particularly for short voltage pulses there no voltage dependence was seen in the current trace (eg. Figs. 2.1, 2.2B). In cases where there was a sag in the current, a single exponential was fitted to the first  $10\text{ms}$  of the capacity transient and leak and this calculated exponential was appropriately scaled and used as the subtraction pulse. In cases where there was no evidence of a voltage dependent component, the current response to



**Figure 2.2.** Currents generated by a 20mV hyperpolarizing voltage command in two cells from a holding potential of  $-70\text{mV}$ . A. shows a cell in which there was no voltage dependence in the current. B. shows a cell which had a significant voltage dependent component. Vertical calibration 0.5nA. Horizontal calibration, 2ms.

the hyperpolarizing voltage was directly scaled and used as the subtraction pulse. In some cells where no sag was seen, an exponential was also fitted to the hyperpolarizing current trace in order to compare the results of the subtractions using the different procedures. In all cases, apart from the amount of noise on the subtracted trace, there was no difference between the traces obtained by the two methods. Current peaks were measured by visually positioning a cursor on the peak of the current trace and averaging 5 to 10 data points around the cursor.

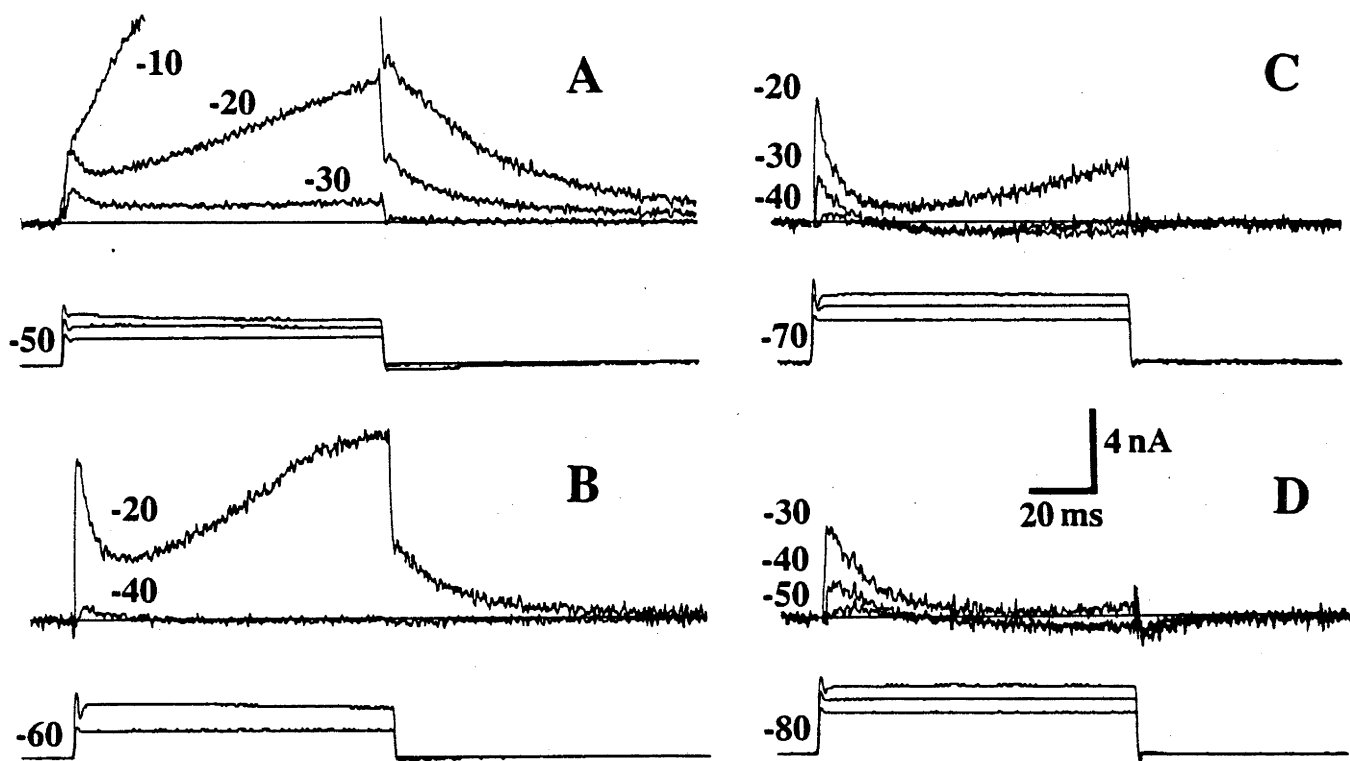
In order to slow down the voltage activated currents, all experiments were performed at 22 - 24 °C. Temperature was controlled by a Peltier element and measured by a small thermistor probe placed in the bath. All observations presented were observed on at least four different neurones and individual numbers are given when needed. Results are presented as mean  $\pm$  SEM. All drugs were obtained from Sigma.

## RESULTS

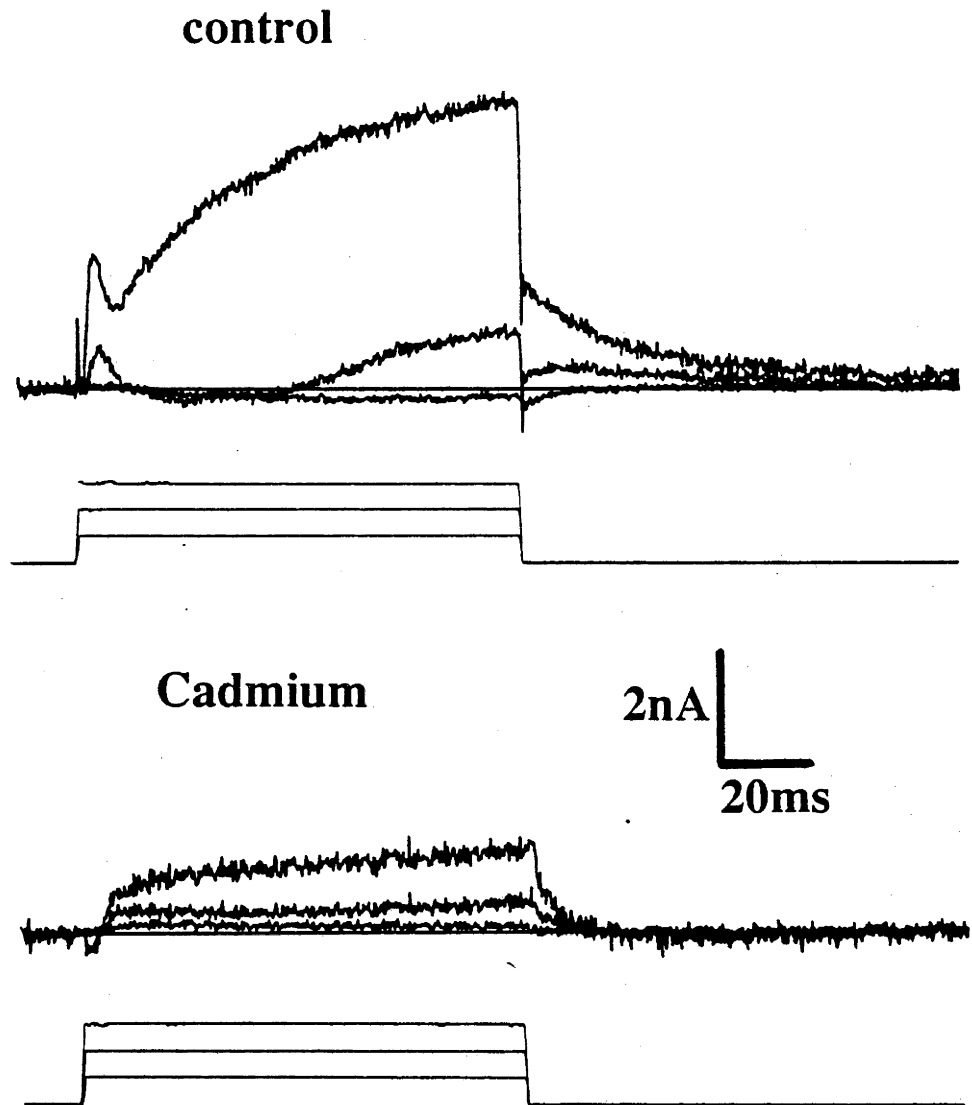
Figure 2.3 illustrates currents generated by depolarizing steps in a CA1 neurone voltage clamped at  $-50$  (A),  $-60$  (B),  $-70$  (C) and  $-80$  mV (D). The recording electrode contained 3M KCl, and capacity and leak currents have been subtracted as described in the methods. Focusing on the current generated at  $-20$  mV from a holding potential of  $-60$  mV, it is clear that depolarization elicits a complex current. There is initially a transient outward current, followed by an inward current after which there is a prolonged outward current. As the holding potential is changed there are marked changes in the currents that are generated. At  $-50$  mV, small depolarizing steps lead to a generation of a transient outward current which is followed by a sustained outward current. With increasing depolarization this outward current very rapidly increases in amplitude and in this cell saturated the clamp at a potential of  $-30$  mV. On repolarization to the holding potential large, slow outward tails are apparent. As the holding potential is made more negative, the transient outward current becomes more prominent, and, for the smaller depolarizations the sustained outward current is much smaller. At holding potentials of  $-70$  and  $-80$  mV, the sustained current is net inward for small depolarizations. It is clear from the voltage records that when large currents are generated (eg. steps to  $-20$  and  $-10$  in Fig. 2.3A) the membrane potential is not well clamped, however, for smaller currents the membrane potential is well clamped during the voltage command.

Sustained inward and outward currents have previously been reported in CA1 neurones by Brown and Griffith (1983a,b). These authors concluded that the inward current was a calcium current and the outward current was a calcium activated potassium current. The large sustained outward current seen in Fig. 2.3 is almost certainly the calcium dependent potassium current described by Brown and Griffith (1983a). The effects of 1mM cadmium, a calcium channel blocker (Hagiwara and Byerly, 1981) are illustrated in a different cell in Fig. 2.4. The cell was voltage clamped at  $-60$  mV and given depolarizing voltage commands to  $-40$ ,  $-20$  and  $0$  mV. For the smaller voltage steps it can be seen the net current is initially inward. Introduction of cadmium results in complete abolition of the inward current and the transient outward current. As expected for a calcium dependent current, the large sustained outward current is markedly





**Figure 2.3.** Currents generated by depolarizing voltage commands in one cell at holding potentials of -50, -60, -70 and -80 mV. For each holding potential current traces generated by depolarizations of 20, 30 and 40 mV from the holding potential. For the traces at a holding potential of -60 mV only traces for depolarization of 30 and 40 mV are shown. Lower traces in each panel are the voltage records. There is significant voltage escape for large currents. Vertical calibration 4 nA. Horizontal calibration, 20 ms.



**Figure 2.4.** Effects of 1mM cadmium on voltage activated currents. This cell was voltage clamped at  $-60\text{mV}$  and given depolarizing voltage commands to  $-40$ ,  $-30$  and  $-20\text{mV}$ . The currents recorded in the same 10 minutes after introduction of cadmium are shown in the lower traces. Cadmium blocks inward currents and some of the outward current. Vertical calibration 2nA. Horizontal calibration, 20ms.

reduced in the presence of cadmium. The residual outward currents in the presence of cadmium are probably a combination of the delayed rectifier (Hodgkin and Huxley, 1952a) and the M-current (Halliwell and Adams, 1982). The transient outward current and the inward current are the subject of this chapter. The sustained outward current is not examined further in this study.

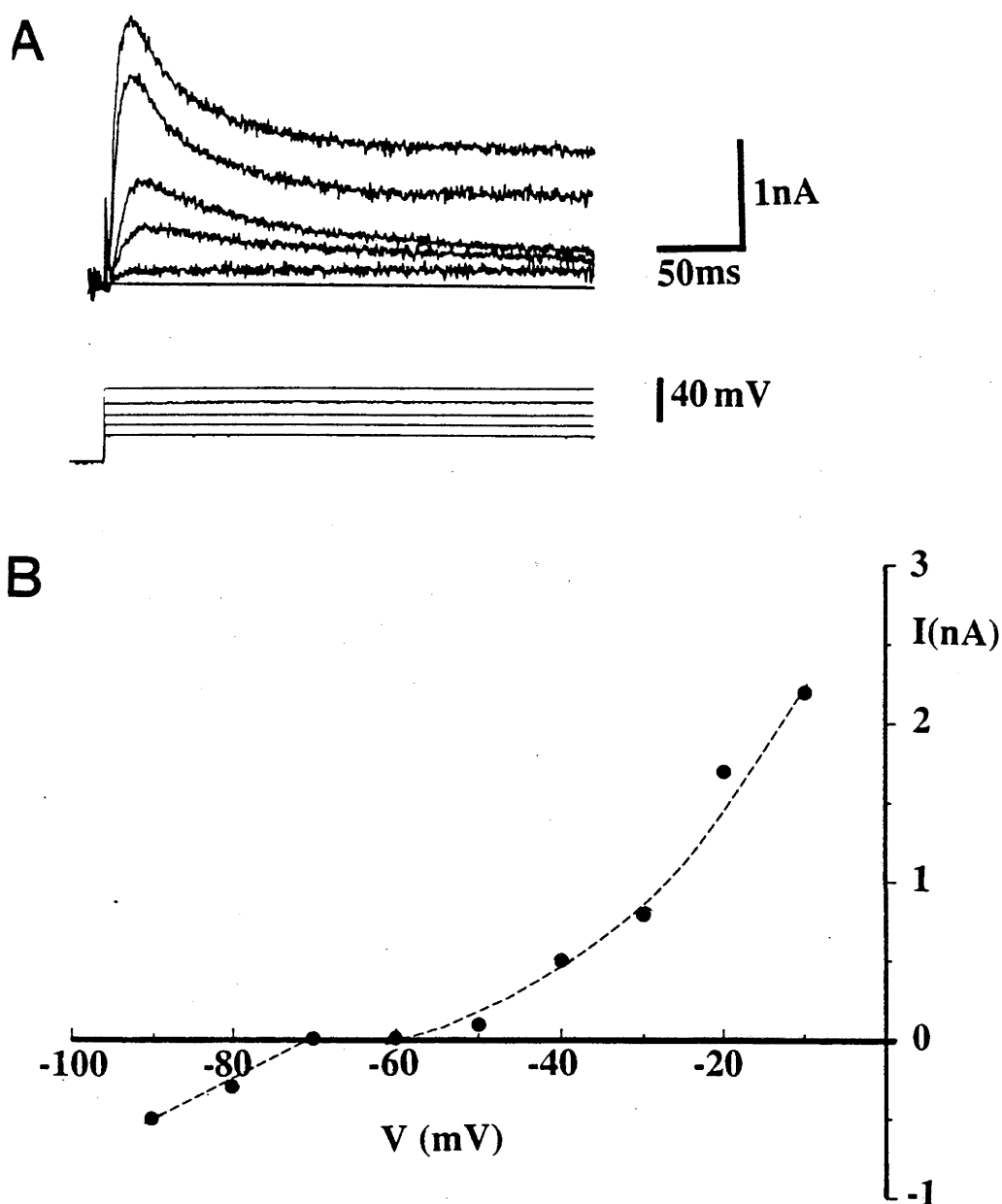
### Transient Outward Current.

The transient outward current could be recorded by voltage clamping cells at potentials more negative than  $-60\text{mV}$ . In some cells, the large calcium activated potassium current was not very prominent and the transient current could be recorded over a large range of potentials. An example of such a cell, voltage clamped at  $-70\text{mV}$ , is shown in Fig. 2.5A. It can be seen that depolarizing voltage steps elicited an outward current which rose to a peak in about  $15\text{ms}$  and then decayed to a steady state value. The inactivation is contaminated by calcium currents, but was found to be approximately exponential with a time constant of  $32 \pm 6\text{ms}$  at  $-30\text{mV}$  ( $n=6$ ). The peak current voltage relation for this outward current is shown in Fig. 2.5B. The current activates at around  $-60\text{mV}$  and increases with further depolarization. It was not possible to follow the current at potentials more positive to  $-10\text{mV}$  due to the limited current passing capacity of the recording electrode, however, it is clear that the current has not saturated at this potential.

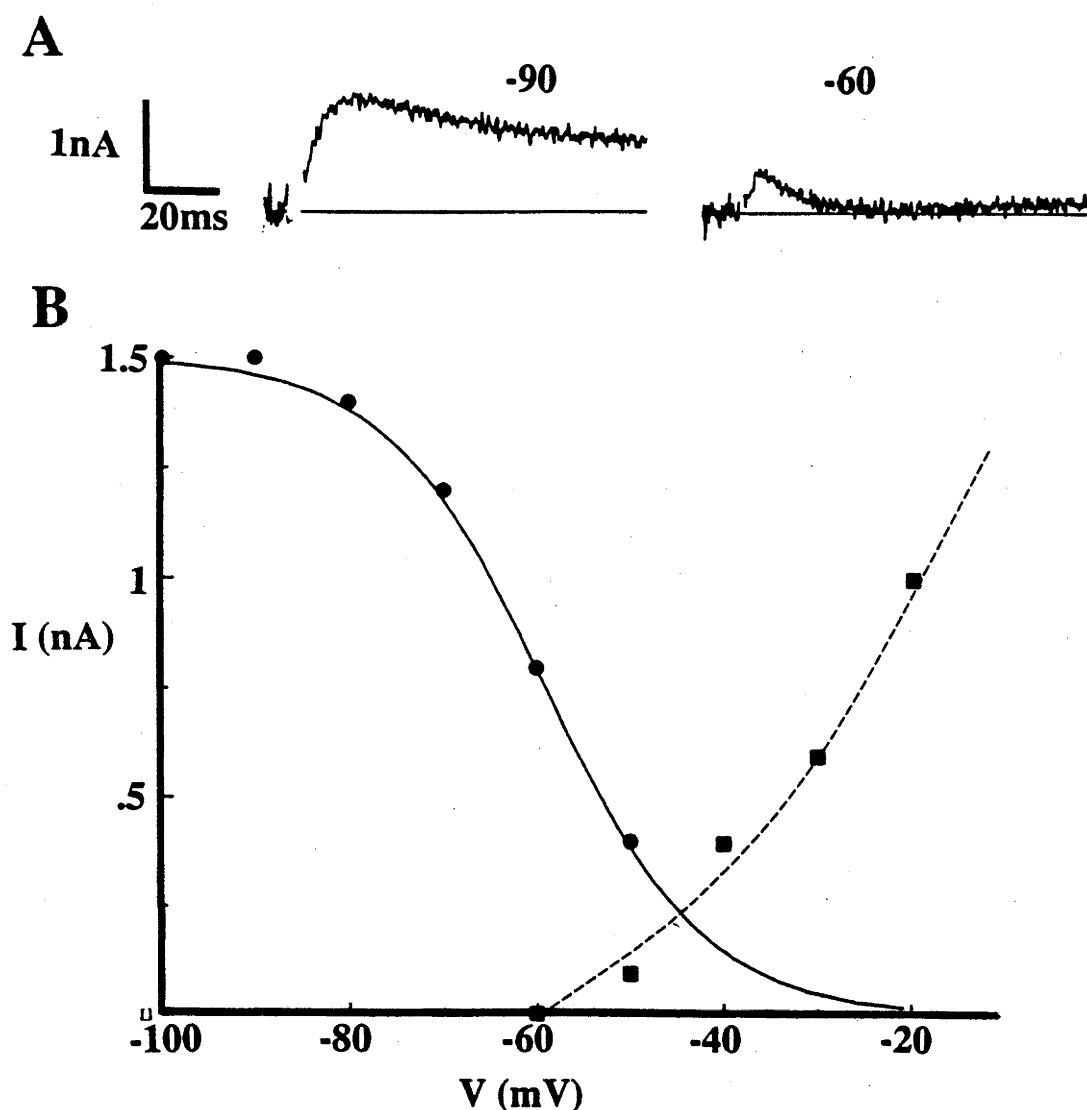
Steady state inactivation of the transient outward current was studied by voltage clamping the cell for  $>1\text{sec}$  at different potentials between  $-100$  and  $-50\text{mV}$  and then eliciting the current with a voltage step to  $-30\text{mV}$ . The current generated at holding potential of  $-90\text{mV}$  and  $-60\text{mV}$  in one cell are illustrated in Fig. 2.7A. It is clear that the current is much smaller at a holding potential of  $-60\text{mV}$  than at  $-90\text{mV}$ . Peak currents recorded over a range of potentials are plotted in Fig. 2.7B (circles). The solid line through the points is drawn according to the Boltzmann relation (Hodgkin and Huxley, 1952c):

$$I = I_{\max} / (1 + e^{(V - V')/k}) \quad \dots\dots\dots 2-1$$

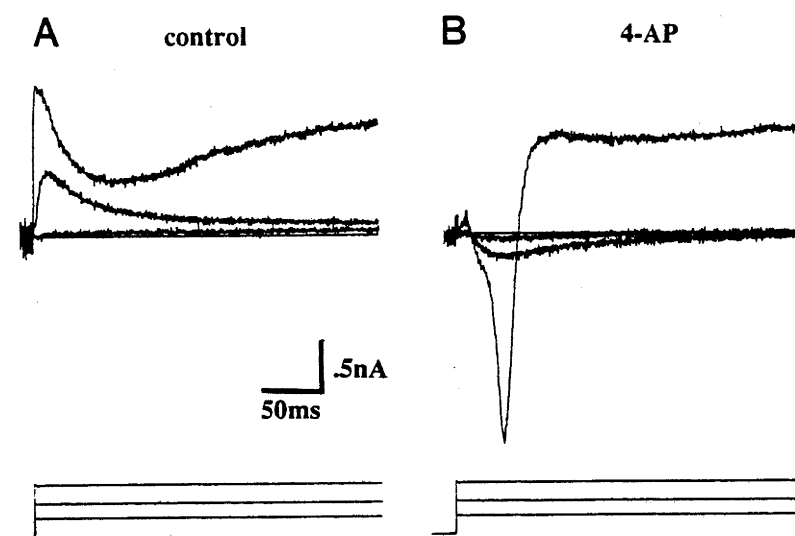
where  $I$  is the current at a membrane potential  $V$ ,  $I_{\max}$  is the maximum current,  $V'$  is the voltage at which  $I$  is  $I_{\max}/2$  and  $k$  is a constant. In the cell illustrated  $I_{\max}$  was  $1.5\text{nA}$  and  $V'$  and  $k$  were  $-59\text{mV}$  and  $8\text{mV}$  respectively. Average values of  $V'$  and  $k$  measured in four cells were  $-60 \pm 3\text{mV}$  and  $7.9 \pm 0.6\text{mV}$  respectively. The peak current-voltage relation, obtained with depolarizing



**Figure 2.5.** Transient outward current in hippocampal CA1 neurone. This cell was voltage clamped at  $-70\text{mV}$  and given depolarizing voltage commands from  $-90$  to  $-10\text{mV}$  in  $10\text{mV}$  increments. A. shows current traces (upper) generated in response to depolarizing commands (lower). Capacity and leak currents have been subtracted. Vertical calibration  $1\text{nA}$ . Horizontal calibration,  $50\text{ms}$ . B. Peak current voltage relation for the currents shown in (A).



**Figure 2.6.** Steady state inactivation of transient outward current. A. Outward currents generated by a depolarizing step to  $-30\text{mV}$  from holding potentials of  $-90$  and  $-60\text{mV}$  respectively. Vertical calibration  $1\text{nA}$ . Horizontal calibration,  $20\text{ms}$ . The peak currents for holding potentials between  $-100$  and  $-50\text{mV}$  are plotted as the closed circles in (B). The solid line is the line of best fit to equation 2-1 and gave a  $V'$  of  $-59\text{mV}$  and a  $k$  of  $8\text{mV}$ . Peak currents generated in the same cell from a holding potential of  $-70\text{mV}$  are also plotted for comparison (squares). The broken line was drawn by eye.



**Figure 2.7.** 4-Aminopyridine blocks the transient outward current. Traces on the left are currents generated in a cell voltage clamped at  $-70\text{mV}$  and given depolarizing steps to  $-50$ ,  $-30$  and  $-10\text{mV}$ . The traces on the right are from the same cell 10 minutes after perfusion with Ringer containing  $0.5\text{mM}$  4-AP. Vertical calibration  $0.5\text{nA}$ . Horizontal calibration,  $50\text{ms}$ .

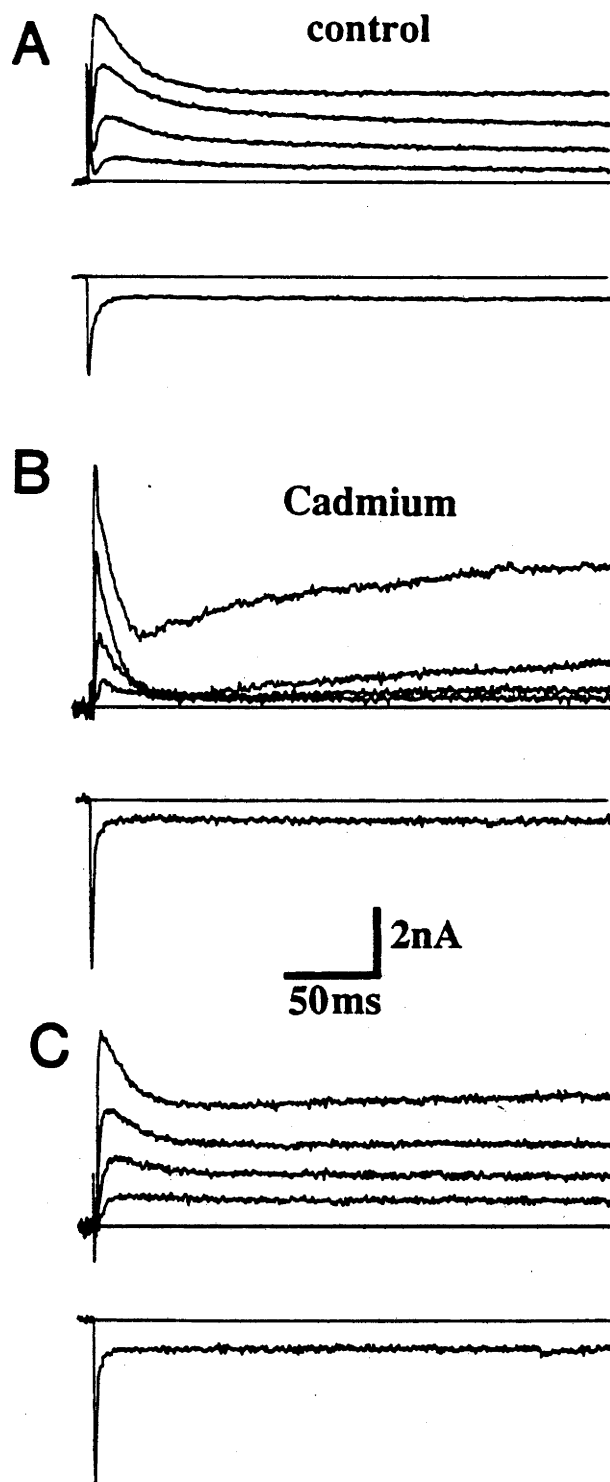
voltage steps from a holding potential of  $-70\text{mV}$  in the same cell is also shown for comparison in Fig. 5B (squares).

The effect of 4-AP on the transient outward current was examined in 5 cells and the results from one cell are illustrated in Fig. 2.6. This cell was voltage clamped at  $-70\text{mV}$  and given depolarizing commands to  $-50$ ,  $-30$  and  $-10\text{mV}$ . It can be seen in Fig. 2.6A that a typical transient current was elicited. At  $-10\text{mV}$ , a sustained outward current can also be seen. After perfusion of  $0.5\text{mM}$  4-AP, the same voltage commands now elicit a net inward current and at  $-10\text{mV}$  a 'spike' inward current (see below) can be seen. Surprisingly the amplitude of the calicum activated potassium current following the 'spike' has not been affected.

### Effects of Cadmium.

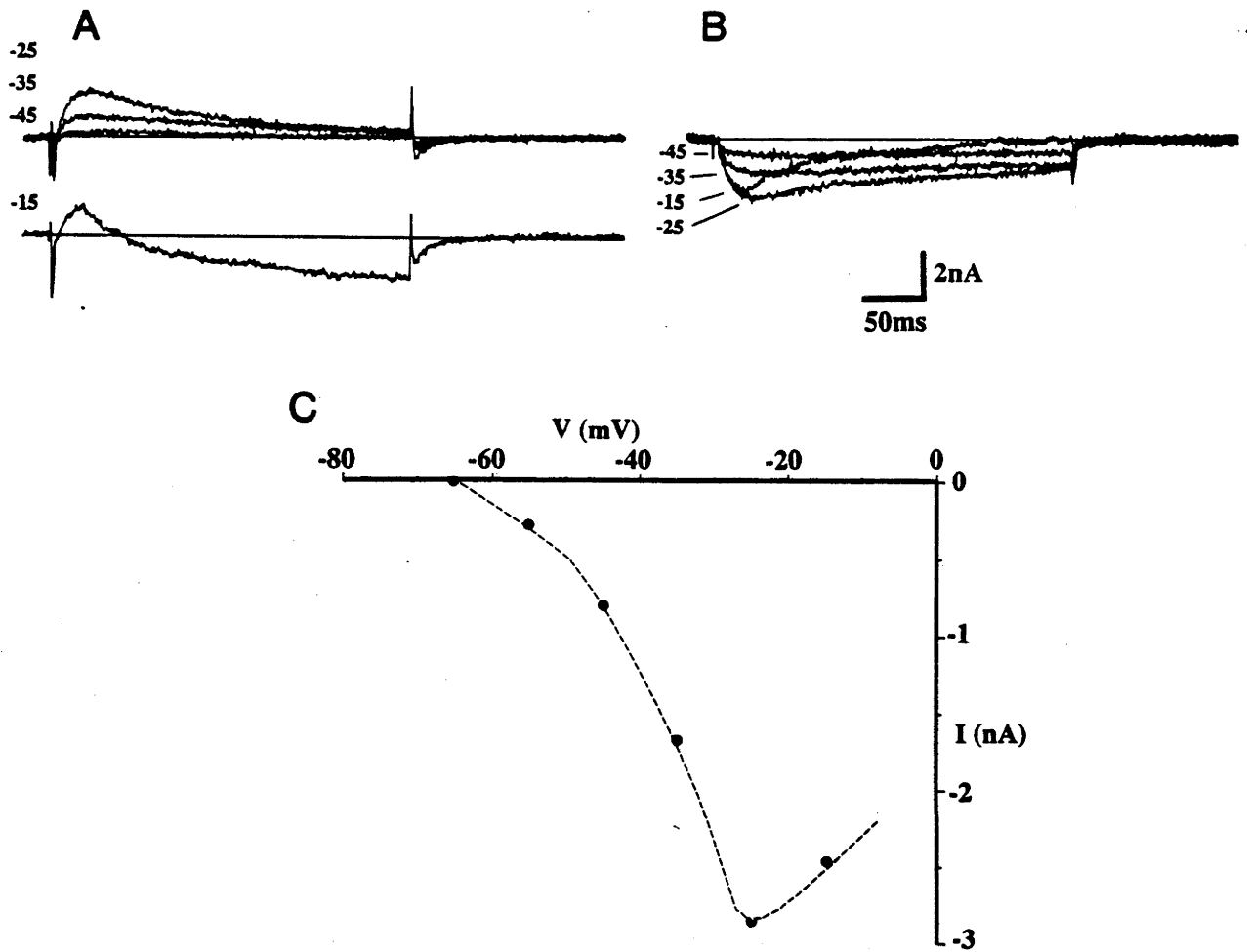
As shown in Fig. 2.4, cadmium blocked most of the outward current, and the TTX insensitive inward current in these neurones. These effects are more clearly illustrated in Fig. 2.8. In Fig. 2.8A are shown currents generated by voltage steps to  $-45$ ,  $-35$ ,  $-25$  and  $-15\text{mV}$  from a cell voltage clamped at  $-65\text{mV}$ . The lower trace is the current generated by a  $20\text{mV}$  hyperpolarizing command and monitors the input resistance of the cell. Each trace is the average of four repetitions at each potential. The currents shown in Fig. 2.8B are generated by a similar protocol 5 minutes after switching to a solution containing  $1\text{mM}$  cadmium (each current trace is a single sweep). The currents in the same cell 15 minutes after switching to a cadmium solution are shown in Fig. 2.8C. It can be seen that with time in the cadmium solution, there are complex effects on the ionic currents. These changes are more clearly shown in Fig. 2.9 where the cadmium sensitive currents (obtained by digital subtraction) are plotted. In Fig. 2.9A are shown traces obtained by subtracting the records in 2.8B from those in 2.8A. It can be seen that in this cell, perfusion of cadmium results in a block of an outward current. At  $-15\text{mV}$  a net inward current (probably a calcium current) is also seen. It is clear that the activation and inactivation kinetics of this current qualitatively resemble the transient outward current described above.

Once the outward current is blocked, subtraction of traces in Fig. 2.8C from those in Fig. 2.8B reveals the inward current shown in Fig. 2.9B. It is clear that depolarization leads to the generation of a slow transient inward current. The peak current voltage relation of this inward current is shown in Fig. 2.9C. The inward current activates around  $-60\text{mV}$  and rises to a peak



**Figure 2.8.** Effects of cadmium over time on voltage activated currents. Each set of traces show the currents generated by depolarising voltage commands to  $-45$ ,  $-35$ ,  $-25$  and  $-15$  mV from a holding potential of  $-65$  mV. The single trace below each set is the current generated by a 20 mV hyperpolarizing step. A, shows currents in control solution, B, 5 minutes after starting 1 mM cadmium and C, 15 minutes after starting cadmium. Vertical calibration 2 nA. Horizontal calibration, 50 ms.





**Figure 2.9.** Cadmium sensitive currents in CA1 pyramidal neurones. **A.** Currents obtained by subtraction of traces in Fig. 5B from those in Fig. 5A. a transient outward current has been blocked by cadmium. **B.** shows traces obtained by subtraction of traces in Fig. 5C from those in 5B. Vertical calibration 2nA. Horizontal calibration, 50ms. **C.** shows the peak I-V relation of the cadmium sensitive inward currents shown in (B).

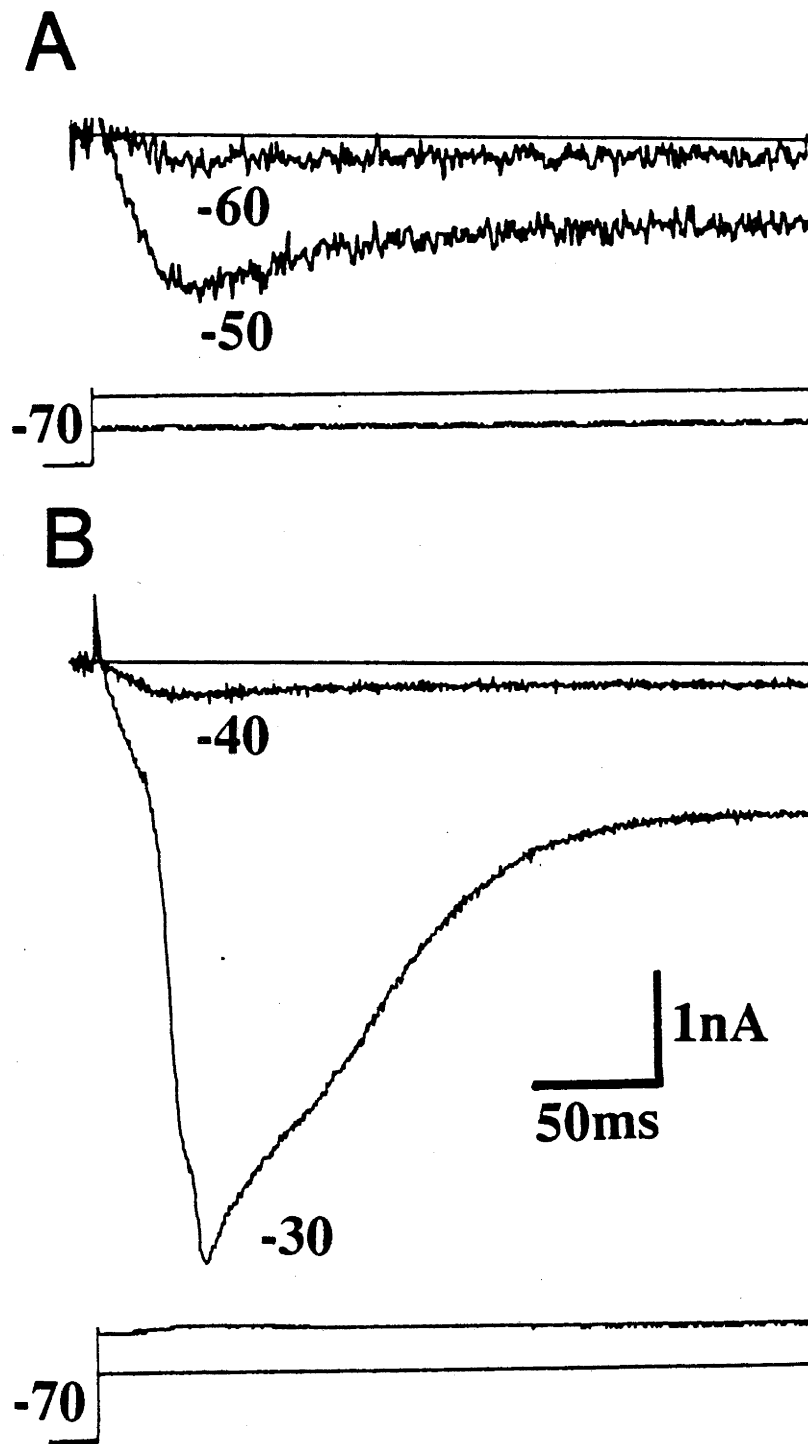
around  $-30\text{mV}$  and then becomes smaller. Thus cadmium blocks the TTX insensitive inward current and two outward currents in these cells. The inward current is probably the calcium current, the two outward currents are the sustained calcium activated potassium current and the transient outward current.

### Calcium Currents.

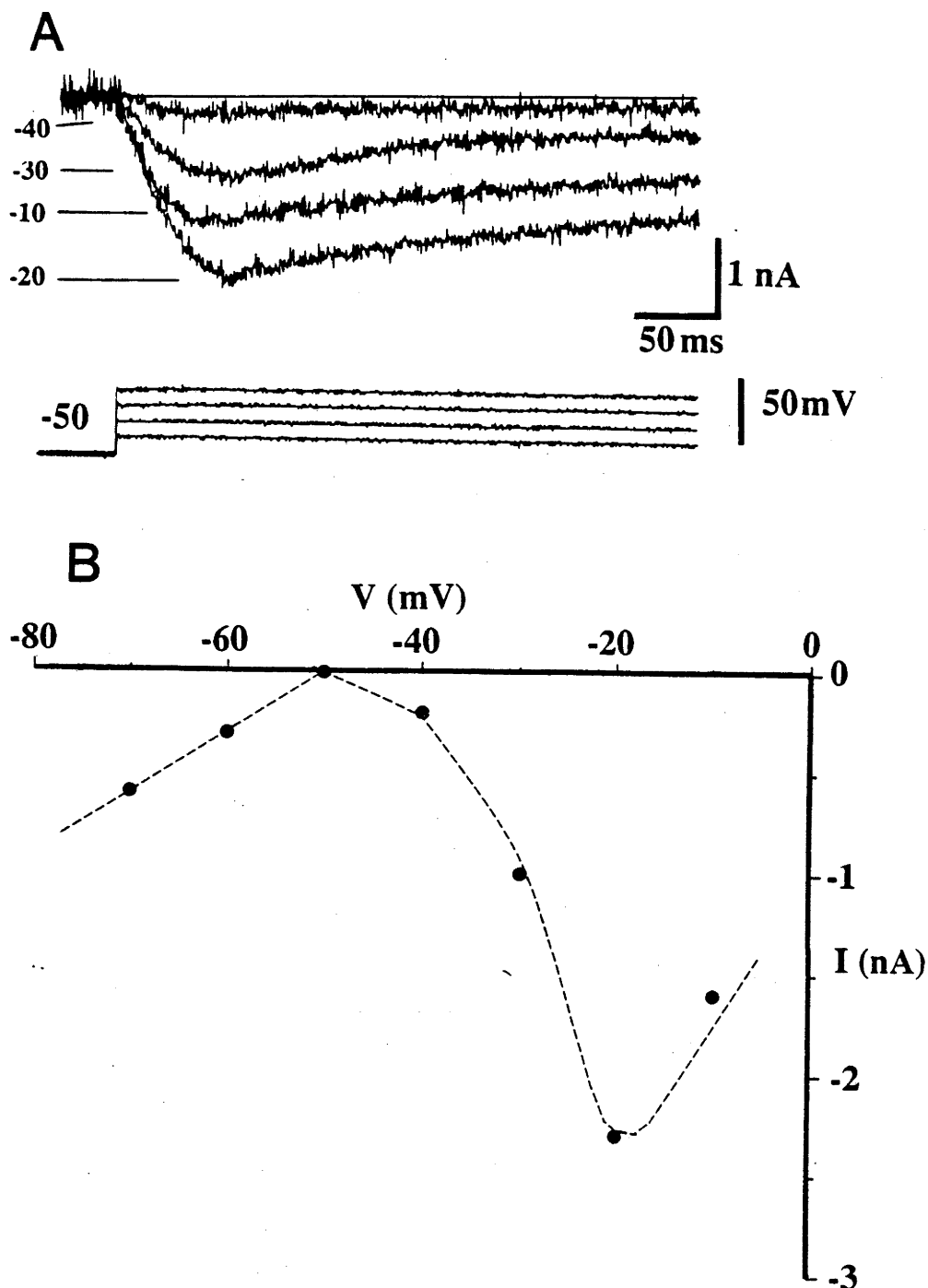
Calcium currents could be directly recorded after potassium currents were blocked. Potassium currents were blocked by inclusion of  $0.5\text{mM}$  4-AP and  $10\text{mM}$  TEA in the perfusing solution and by using electrodes filled with CsCl (Johnston, Hablitz and Wilson, 1980).

Calcium currents recorded under the above conditions in one cell voltage clamped at  $-70\text{mV}$  and given depolarizing commands to  $-60$  and  $-50\text{mV}$  are shown in Fig. 2.10A. It can be seen that depolarization elicits an inward current that peaks in about 10 ms and then inactivates with maintained depolarization. The recorded currents resemble the currents obtained by the subtraction procedure (Fig. 2.9B) and the inward currents seen in Fig. 2.6B). In all neurones which were voltage clamped at membrane potentials more negative than  $-60\text{mV}$ , small depolarizing steps led to activation of a transient calcium current as shown in Fig. 2.10A. Depolarizing commands to potentials more positive than about  $-40\text{mV}$  resulted in generation of an uncontrolled 'spike' current as shown in Fig. 2.10B. At the end of the 'spike' an apparently steady inward current can be seen.

In cells voltage clamped at  $-50\text{mV}$ , it was sometimes possible to control the calcium current over a wider range of membrane potentials. The results from one cell are shown in Fig. 2.11A. The cell was voltage clamped at  $-50\text{mV}$  and given depolarizing commands to  $-40$ ,  $-30$ ,  $-20$  and  $-10\text{mV}$ . As seen before, the current rises to a peak and then inactivates to a steady state. The peak current-voltage relation for this cells is shown in Fig. 2.11B. As shown in Fig. 2.9B the peak current occurs around  $-20\text{mV}$  and then decreases. Extrapolation of the current voltage relation gives a reversal potential of around  $+20\text{mV}$ . The calcium reversal potential in these neurones is expected to be much more positive than  $+20\text{mV}$ , suggesting that the reduction of the inward current is probably due to activation of incompletely blocked outward currents.



**Figure 2.10.** Calcium currents generated in two cells (A & B) voltage clamped at  $-70$  mV and given depolarizing commands to the indicated potentials. A transient current is seen for small depolarizations. Depolarizations beyond  $-40$  mV resulted in 'spike' currents. Vertical calibration  $1$  nA. Horizontal calibration,  $50$  ms.



**Figure 2.11.** Calcium currents recorded in a cell which did not show 'spike' currents. **A.** Currents (upper traces) generated in response to depolarizing voltage steps (lower traces) in a cell voltage clamped at  $-50\text{mV}$ . Vertical calibration  $1\text{nA}$ . Horizontal calibration,  $50\text{ms}$ . **B.** Peak I-V relation for the currents shown in (A).

## DISCUSSION

In this chapter, ionic currents generated by depolarizing voltage steps in hippocampal CA1 neurones have been described. When sodium currents are blocked with TTX, these currents consist of: A transient outward current; a sustained outward current and two calcium currents. The sustained outward current is a calcium activated potassium current previously described by Brown and Griffith (1983a) and will not be discussed here.

### Transient Outward Current.

The transient outward current activates at potentials more positive than  $-60\text{mV}$ , rises to a peak within 10-15ms and inactivates exponentially with maintained depolarization. The current shows steady state inactivation and is completely inactivated at potentials more positive than  $-50\text{mV}$ . The transient outward current is completely suppressed by application of  $0.5\text{mM}$  4-AP.

A transient current having these characteristics has been described in invertebrate (Connor and Stevens, 1971b; Neher, 1971; Adams, Smith and Thompson, 1980) and vertebrate (Adams, Brown and Constanti, 1982a; Belluzzi, Sacchi and Wanke, 1984a; Galvan and Sedlmeir, 1985) neurones (see Rogawski, 1985 for review). In the hippocampus, transient outward currents with similar properties have been reported in CA3 pyramidal neurones in slices (Gustafsson et al., 1982; Zbciz and Weight, 1985) and in cultured hippocampal neurones (Segal, Rogawski and Barker, 1984; Segal and Barker, 1984). This current has been shown to be carried by potassium ions and has been named the A-current ( $I_A$ ) by Connor and Stevens (1971b). In this study, although we have not directly shown the outward current to be carried by  $K^+$ , the current was blocked by low concentrations of a potassium channel blocker, 4-AP, and is very likely also a potassium current.

The time course and voltage dependent properties of  $I_A$  in CA1 neurones are qualitatively similar to that of the same current in CA3 neurones (Gustafsson et al., 1982; Zbciz and Weight, 1985) and cultured hippocampal neurones (Segal and Barker, 1984).  $I_A$  is active at potentials more positive to  $-60\text{mV}$  and undergoes steady state inactivation with half maximal inactivation at about  $-60\text{mV}$ . In CA1 neurones,  $I_A$  appears to activate within 10 to 15 ms then decays exponentially with a time constant of about 30 ms. This faster decay rate may partly be due to

concomitant activation of the transient calcium current. In CA3 neurones (Gustafsson et al., 1982; Zbicz and Weight, 1985), activation time constants of 10 - 25ms and inactivation time constants of 200 - 400ms have been measured.  $I_A$  in cultured hippocampal neurones (Segal and Barker, 1984), however, activated much faster (3-10ms) and decayed with a time constant of 10 to 30ms.  $I_A$  in rat sympathetic neurones has also been found to have a rapid activation (2-5ms; Galvan and Sedlmeir, 1984; Belluzzi, Sacchi and Wanke, 1984a). The reason for this difference in activation rates is not clear. A second, faster, transient outward current has been identified in bullfrog neurones (MacDermott and Weight, 1982) and in hippocampal CA3 neurones (Zbicz and Weight, 1985). In CA1 neurones, a fast transient outward current can be recorded in cells voltage clamped at potentials where  $I_A$  has been shown to be inactive (eg. Fig 2.3, -50 holding potential) and could be this current. It is possible that in different preparations there is different contribution of the fast transient current to the total outward current.

### Effects of Cadmium.

Cadmium, a known calcium channel blocker (Hagiwara and Byerly, 1981), has been reported to have variable effects on  $I_A$ . In cultured hippocampal neurones,  $Cd^{2+}$  has been shown to either increase or decrease  $I_A$  in different cells (Segal and Barker, 1984). In CA3 neurones, inorganic calcium channel blockers have been found to block a fast transient outward current but to not affect the slower A-current (Zbicz and Weight, 1985). In rat sympathetic neurones, two groups, using similar techniques found different effects of cadmium ions. Galvan and Sedlmeir (1984) reported that cadmium completely abolished  $I_A$  whereas Belluzzi, Sacchi and Wanke (1984a) found that cadmium did not effect  $I_A$ . In our hands, it appears that cadmium at a concentration of 1mM, blocked  $I_A$  in hippocampal CA1 neurones. It is difficult to reconcile these different observations with a single explanation. What is clear is that in vertebrate neurones at least some component of the transient outward current appears to be calcium sensitive. Perhaps the contribution of this calcium sensitive current varies between different preparations. Calcium channel blockers have also been found to directly affect the delayed rectifier potassium channel in squid axon (Armstrong and Lopez-Barneo, 1987) and in hippocampal CA1 neurones (Chapter 6) thus it is possible that some of the effects on the A-current could be direct effects of cadmium on the potassium channel.

(Bossu, Feltz and Thomann, 1985; Kunze, 1987). Recently, two components of calcium current have also been reported in cultured hippocampal neurones (Yaari et al., 1987). These authors also found that the transient component activated at potentials more positive than  $-60\text{mV}$  whereas the sustained component activated at potentials above  $-40\text{mV}$ .

When potassium currents were blocked with TEA, 4-AP and intracellular caesium we found that it was difficult to study calcium currents at potentials more positive to about  $-40\text{mV}$ . Depolarization to these potentials usually resulted in the appearance of an uncontrolled 'spike' current (eg. Fig. 2.10). This phenomenon was especially pronounced if the holding potential was more negative than  $-50\text{mV}$ . Llinas and others (Llinas and Sugimori, 1980b; Llinas and Yarom, 1981b; Jahnsen and Llinas, 1984b; Llinas, 1984; Mulle, Madariga and Deschênes, 1986) have suggested that a high threshold, noninactivating calcium current is located in the dendritic tree of central neurones. Moreover, in developing sympathetic ganglion cells such 'spike' currents can only be seen in neurones which have developed an extensive dendritic tree (Hirst and McLachlan, 1986). It may be that the 'spike' currents in CA1 neurones are also generated at sites remote from the soma where it is not possible to adequately clamp them with a single electrode at the soma. Similar conclusions have also been reached by Stafstrøm et al. (1985) for cat neocortical neurones in vitro.

In conclusion, we have shown that hippocampal CA1 neurones possess a variety of voltage activated ionic conductances that can be recorded at the soma. We have characterised a transient outward current which has properties analagous to the A-current. We have also shown that a transient calcium current can be recorded at the soma. Given the observation that only small sustained inward currents can be recorded at the soma, and strong depolarizations elicit 'spike' currents, it is possible, that as suggested for other neurones (Llinas and Sugimori, 1981b; Llinas and Yarom, 1982b), the physical location of the two types of calcium channels in hippocampal CA1 neurones is different with the high threshold channels preferentially localised in the dendrites.

## **Chapter 3**

### **Properties of Neurones Acutely Dissociated From Adult Guinea Pig Hippocampus.**



## INTRODUCTION

In the last chapter the properties of some voltage activated currents that can be recorded at the soma of CA1 pyramidal neurones in hippocampal slices were presented. It is clear from these results that using the single electrode voltage clamp in the slice preparation only relatively slow conductances can be studied and these over a limited voltage range. The study of Johnston and Brown (1984) in hippocampal CA3 neurones suggests that even if we use a  $20\text{M}\Omega$  electrode, and the conductance is located on the soma, a point clamp at the soma with a will only accurately follow conductance changes of frequencies less than about 300 Hz. Thus it is not possible to voltage clamp any fast conductance changes, especially if they are electrically distant from the soma. With currently available technology there are two ways to solve these problems.

The first and traditional method is to use cultured neurones. Neurones from embryos are taken and cultured for days to weeks and then used for electrophysiological studies (Nelson and Lieberman, 1981). Neurones grown in culture can be directly visualised and either patch clamped (Rogawski, 1986; Gardner, 1986) or voltage clamped with one or two microelectrodes (Moolenaar and Spector, 1979; Segal and Barker, 1984, 1986). Because the cells are exposed in a culture dish the external environment can also be readily controlled and drugs and chemicals applied. Unless one works with very immature cells, however, the problem of trying to clamp an extended structure however still remains (eg. see Segal and Barker, 1984, 1986).

The second alternative, and the one that has been used here, is to use neurones acutely dissociated from adult brain tissue (Numann and Wong, 1984; Huguenard and Alger, 1986; Kay and Wong, 1986). In this method tissue from adult animals is enzyme treated and then mechanically disrupted to produce isolated cells which can be used for electrophysiological studies. The dendrites and axon of the neurones are removed during the dissociation procedure producing cells which are ideal candidates for voltage clamp. These cells are easily accessible and it should be possible to voltage clamp them using whole cell recording techniques (Hamill et al., 1981). Using these recording techniques drugs can be applied to both the internal and external surface of the cell facilitating the separation of the ionic currents. In this and the

following two chapters some of the electrophysiological properties of neurones isolated from the CA1 region of adult guinea pig hippocampus are described.

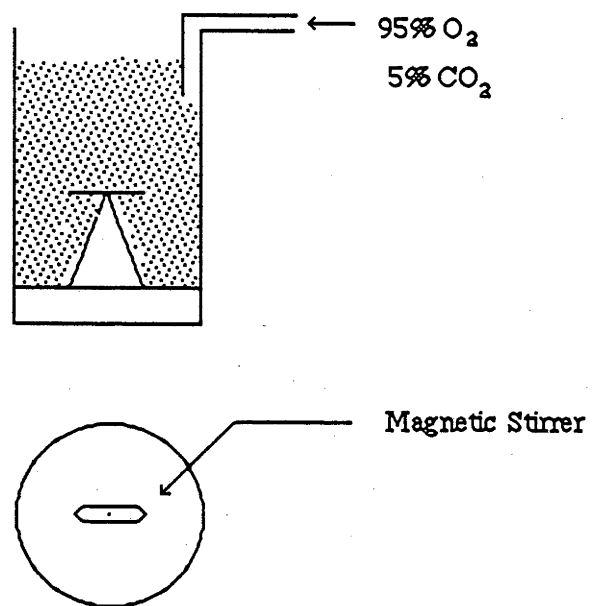


Figure 3.1. Stirring Chamber used for enzyme treatment. Chamber was constructed from perspex and has a capacity of 10mls. Magnetic stirrer was made from magnetised rubber.

## METHODS

### Hippocampal Slices.

All experiments were performed with cells dissociated from the hippocampus of 300-500g male or female adult guinea pigs. Guinea pigs were chosen in preference to rats because their brains (and the hippocampi) are larger which made handling the slices easier. Animals were killed by cervical dislocation and the brain quickly exposed. The brain was hemisected and dissected out into cold (4-8 °C) Ringer solution of composition (mM): NaCl, 125; KCl 5.0; NaHCO<sub>3</sub>, 25; MgSO<sub>4</sub>, 2; CaCl<sub>2</sub>, 2.5; NaHPO<sub>4</sub> 1.2; Glucose 11; pH 7.3 when bubbled with 95% O<sub>2</sub> / 5% CO<sub>2</sub>. Osmolality of the solution was 290 - 300mosmolar. The hemisected brain was glued to the stage of a Vibraslice (Campden Instruments) with cyanoacrylic glue (Supa Glue). Sections 600µm thick were then taken through the cerebral cortex and hippocampus to produce transverse hippocampal slices. Hippocampal slices were dissected free of the cortex and transferred to a dish of cold oxygenated Ringer. The CA1 region in each slice was isolated using fine iridectomy scissors. This piece of tissue was then further cut into two or three smaller pieces each approximately 1.5mm square. Dissociation was performed with a protocol very similar to that described by Numann and Wong(1984).

### Dissociated Cells.

Papain (Type III, Sigma, 24units/ml) and cysteine (5mM) (Sigma) were dissolved in 15ml of oxygenated Ringer. Pieces of hippocampus were placed in this solution and rapidly stirred in a specially constructed chamber (Fig. 3.1) at room temperature (22 - 24 °C). After about 60 minutes the pieces were taken out of the papain solution with a pasteur pipette and transferred to a holding beaker containing oxygenated Ringer and washed for a further 60 minutes. Individual pieces of tissue were removed and mechanically dissociated as needed by trituration with a fire polished pasteur pipette in 3 to 4 mls of Ringer. The dispersed cells and debris were decanted into 5cm plastic petri dishes, where after 5 to 10 minutes, the cells settled to the bottom. The petri dishes were mounted on the stage of an inverted phase contrast microscope (Olympus IM) and viewed at x400 magnification. Cell viability was aided by blowing a stream of moistened 95% O<sub>2</sub> / 5% CO<sub>2</sub> over the surface of the dish. Although the yield of cells was very variable, on

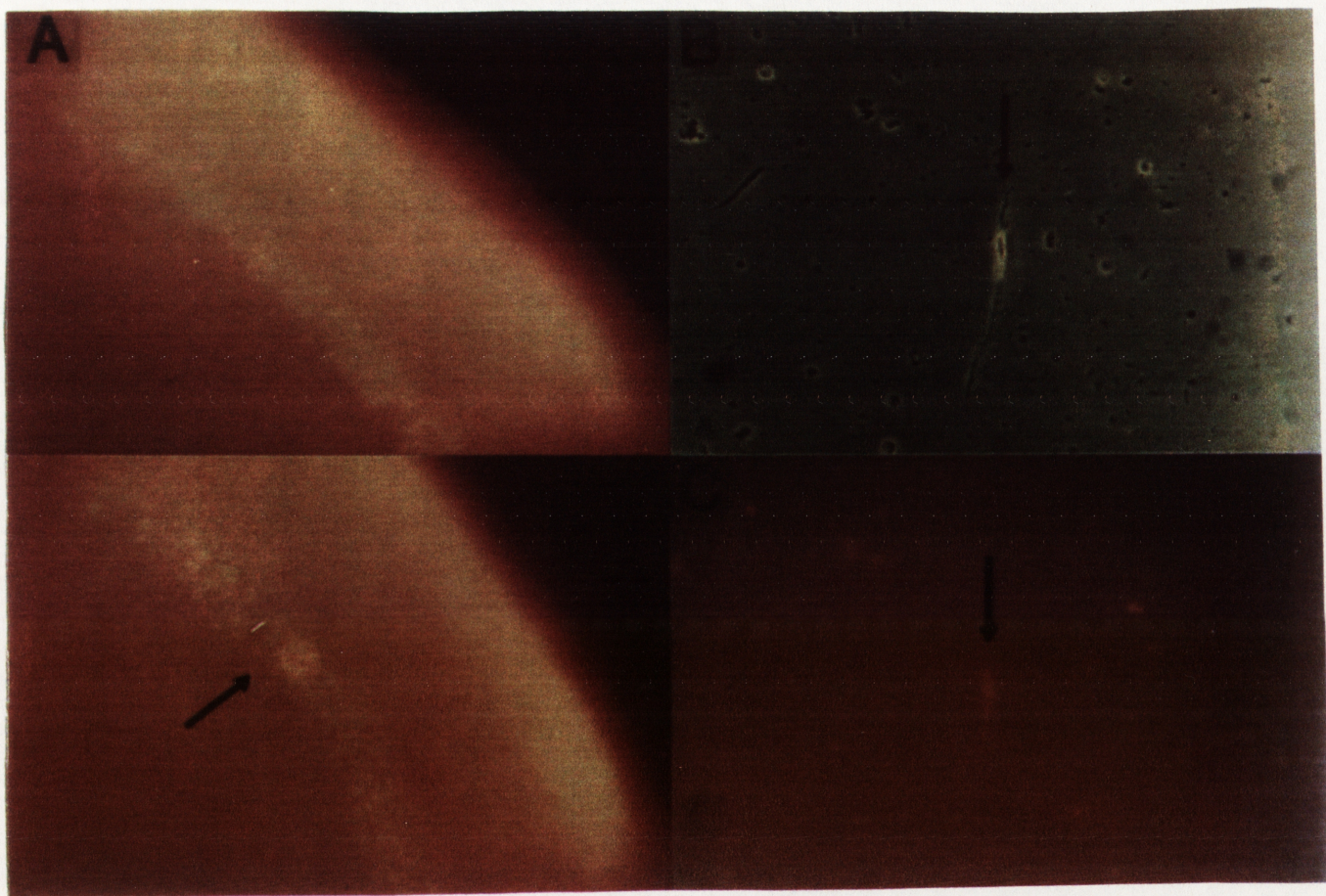
average, 10 to 20 healthy cells could be obtained from each piece of tissue. Some examples of the cells obtained from this procedure are illustrated in Plate 1.

It can be seen that many of the cells showed a pyramidal shape and retained some of their proximal dendritic stumps. Many cells however had no dendritic processes and were small and round. It was felt that these cells could either be rounded up pyramidal cells or they could be interneurons or glia. In an attempt to differentiate the types of cells, we tried to stain the cells with tetanus toxin, a known marker of neurons (for review see Mellanby and Green, 1981). Tetanus toxin (a gift from Mr. Ross Chrichton, National Biological Standards Laboratory) was conjugated with rhodamine B as described in Nairn (1964) and applied to the dissociated cells at a dilution of 1:100 for approximately one hour. The cells were then washed in normal Ringer and viewed through a red filter in a fluorescence microscope. Although the staining was not bright enough for us to use as a vital stain during electrophysiological recording, photographs of the cells with long time exposures clearly demonstrated the labelled cells. Plate 2A shows two examples of hippocampal slices which have been stained with rhodamine labelled tetanus toxin. Although there is a lot of background staining it is clear that the cells in the CA1 region are positively stained. A dissociated cell seen under phase contrast is shown in Plate 2B, in 2C the same cell is shown to be positively stained with tetanus toxin. Control experiments in which tetanus toxin was applied to hippocampal slices before conjugation with rhodamine did not fluoresce. We found that the large bipolar cells as well as the small round cells were stained positively by tetanus toxin, suggesting that they were both neuronal in origin.

### **Electrodes.**

Patch recording electrodes were made from borosilicate glass (haematocrit tubes) on a modified David Kopf electrode puller as described by Hamill et. al. (1981) and coated with Sylgard® to within 100µm of the tip to reduce electrode capacitance. Electrodes were held in an electrode holder (WPI) with a side arm allowing suction to be applied to the electrode interior. The electrode holder was plugged directly into the x1 head stage of an Axon 2A (Axon instruments) voltage clamp. In some experiments the x0.1 headstage was used in order to be able to inject currents smaller than 1nA.





**Plate 3.2.** Tetanus toxin stains cells obtained by dissociation. **A.** Upper and lower panel show two different hippocampal slices in which the pyramidal cell layer (arrow) is clearly stained by tetanus toxin conjugated with rhodamine (see text). **B.** A cell (arrow) obtained from dissociating CA1 region from slice in (A) seen under phase contrast optics. **C.** The same cell shown in B is now seen to fluoresce when viewed through a fluorescence objective. Some debris can also be seen to fluoresce.



Electrodes were filled with a solution containing (mM) : CsF or KF 140; NaCl 10; Hepes - CsOH or HEPES - KOH; 10; EGTA 11; pH 7.2. These electrodes had DC resistances of 1 to 3M $\Omega$ . High intracellular fluoride, as noted elsewhere (Fernandez, Fox and Krasne, 1984; Kay and Wong, 1986 ), greatly aided recording stability.

Ground electrodes were made by filling a haematocrit tube with agar/3M KCl solution, and were stored in 3M KCl. This electrode was then connected to the axoclamp with a WPI electrode holder.

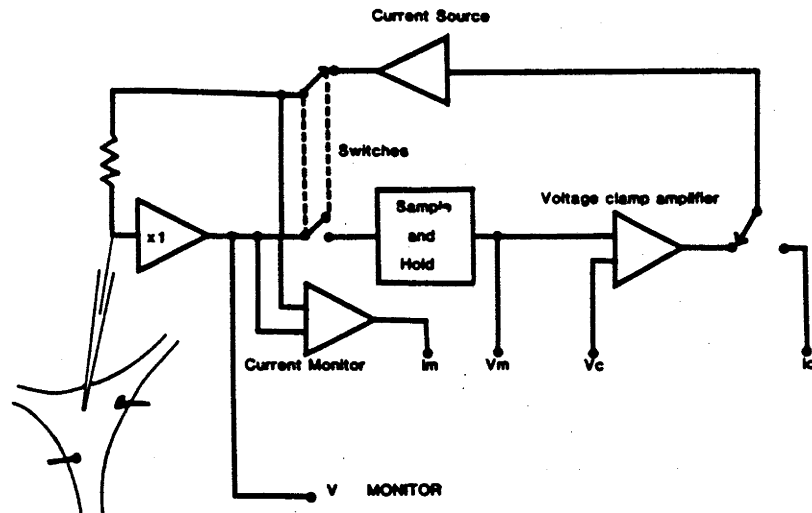
### Voltage Clamp.

Under visual control, the electrode tip was pressed against the surface of the cell. Electrode resistance was monitored by injecting 100pA of current across the electrode in bridge mode. Gentle suction applied to the electrode interior resulted in the formation of an electrode - cell membrane seal of several giga ohms resistance. Further suction and hyperpolarization of the patch resulted in membrane breakdown giving a low resistance access to the interior of the cell. With KF in the electrode, the membrane potential of the cell could be recorded when access to the cell was obtained. With CsF in the electrode, cell resting potential was 0mV, and access to the cell interior was accompanied by the appearance of a capacitive charging transient on the recorded potential. In both cases access to the cell was confirmed by eliciting action potentials by current injection. Once access had been obtained the capacity neutralization was adjusted as described in Chapter 2.

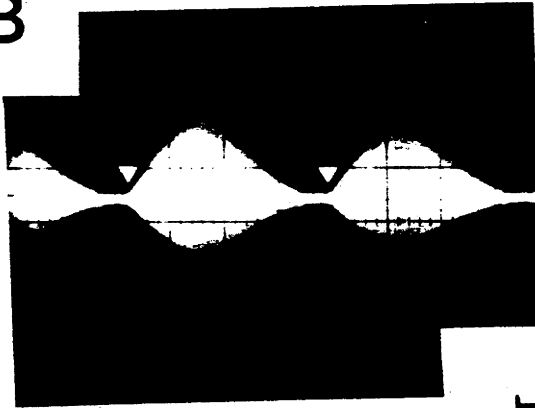
When using a switched current or voltage clamp, series resistance (eg see Hodgkin, Huxley and Katz, 1949) is not a source of error, however, we found it necessary to keep the electrode resistance low in order to achieve the rapid switching rate and high clamp gain necessary to adequately clamp the sodium currents (Chapter 4). In many cases, once the whole cell configuration was achieved the cell, attached to the end of the recording electrode, was raised to just below the surface of the bathing solution in order to reduce the coupling capacitance of the electrode to the bath (Finkel and Redman, 1984).

Typically, recordings were made with the voltage clamp cycling at between 20 and 40kHz and with a gain setting of 50 to 100 nA/mV. The headstage of the clamp was continuously

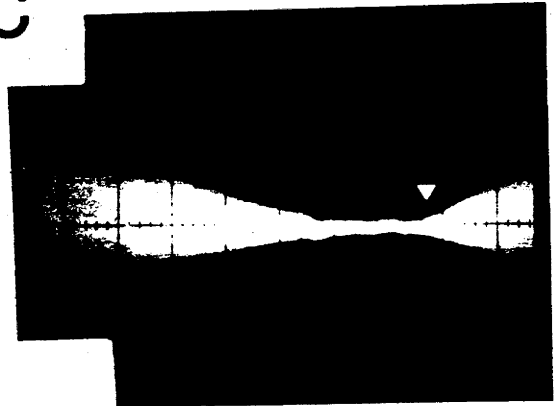
A



B

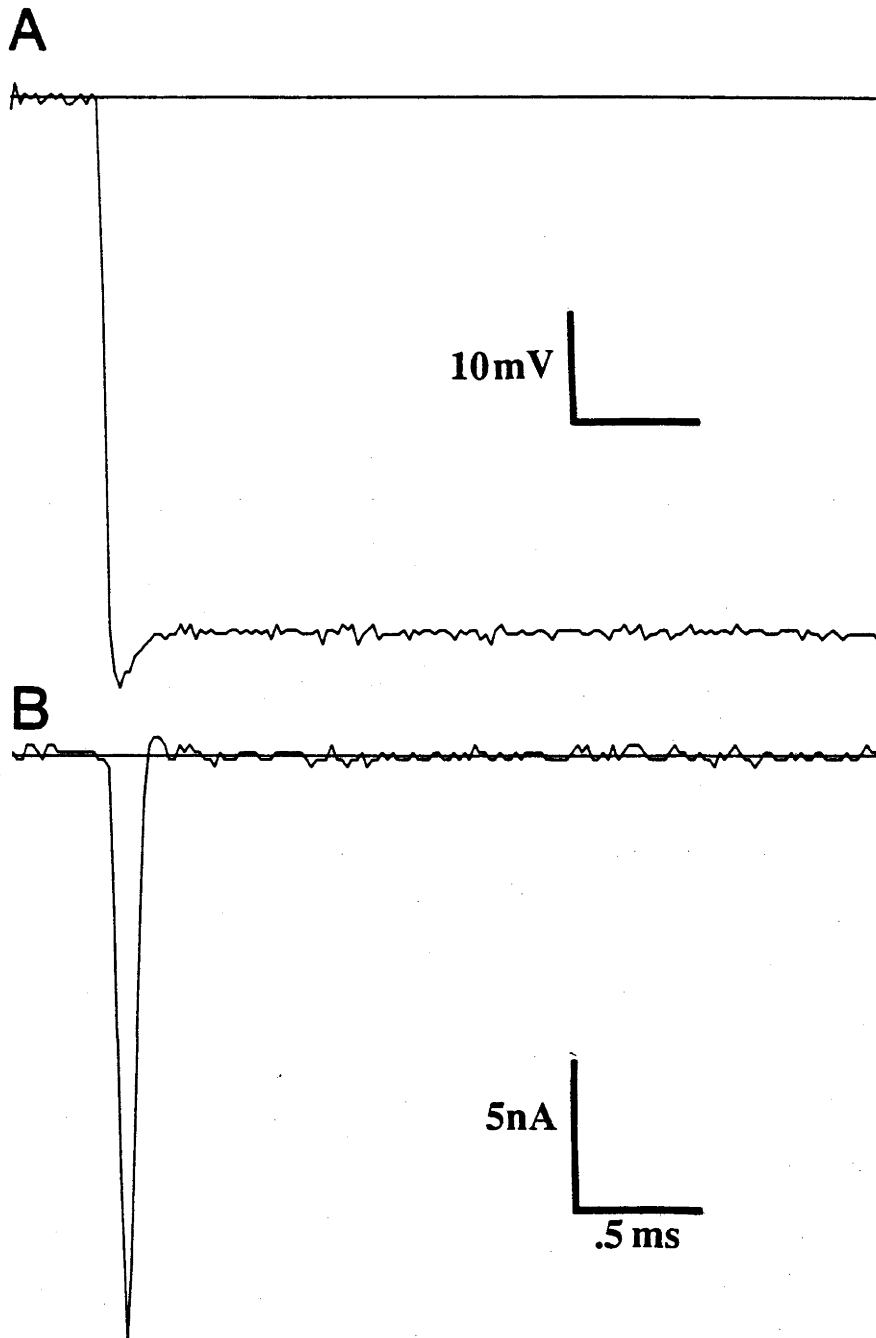


C



**Figure 3.2.** Single electrode voltage clamp (SEVC). A. Block diagram of SEVC circuit. B and C show low and high gain traces to monitor head stage output with a cell voltage clamped at the resting membrane potential. The voltage can be seen to settle to the base line before it is sampled by the calmp circuitry (white arrows). Vertical calibration, arbitrary voltage scale. Horizontal calibration,  $5\mu\text{s}$ .





**Figure 3.3.** Dynamic response of voltage clamp under optimal recording conditions. The clamp switching rate was 50kHz and total clamp gain was 100nA/mV. A. shows actual membrane potential in response to a 50mV hyperpolarizing command from a holding potential of  $-100\text{mV}$ . B. Current generated by voltage step shown in A. Vertical calibrations 10mV for A and 0.5nA for B. Horizontal calibration, 0.5ms. Both traces have been filtered at 6kHz.

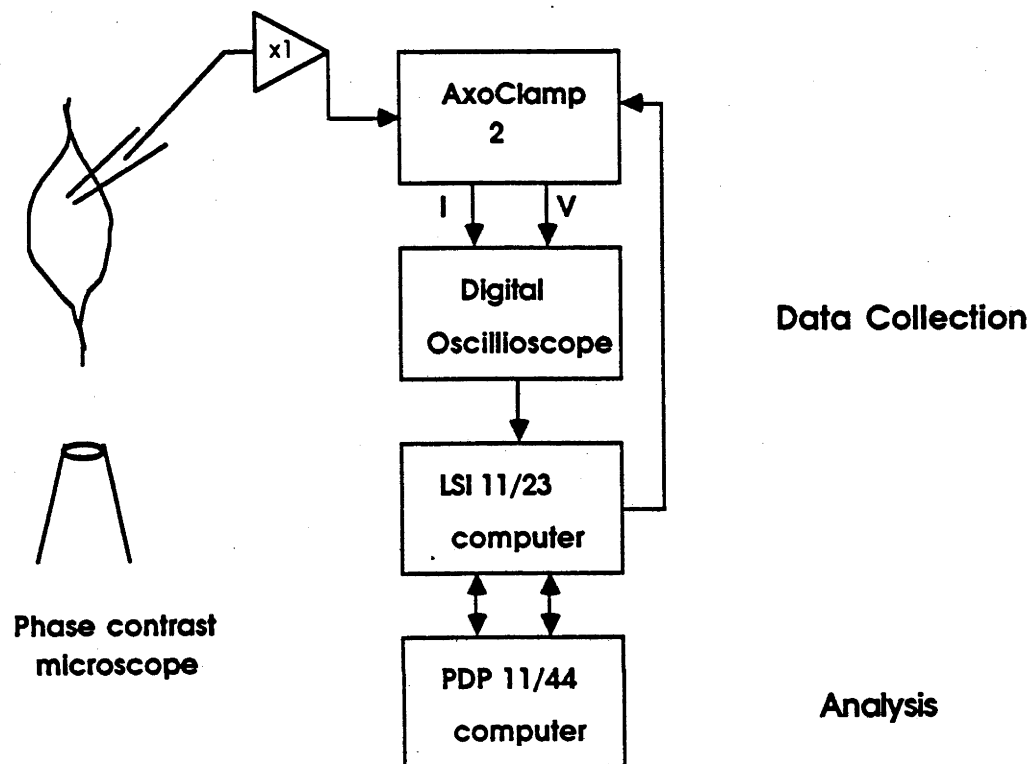
monitored to ensure that the voltage across the electrode settled completely to baseline before the membrane potential was sampled (Finkel and Redman, 1984, see Fig. 3.2). Under optimum conditions the clamp switched at 50kHz with a gain of 100nA/mV. The dynamic response of the voltage clamp under optimal recording conditions is shown in Figure 3.3. A CA1 neurone has been voltage clamped at -100mV and a 50mV hyperpolarizing voltage step applied. The current response is shown in the lower part of the Figure. It can be seen that the membrane potential and current settled to their final values within 400 $\mu$ s.

### Data Analysis.

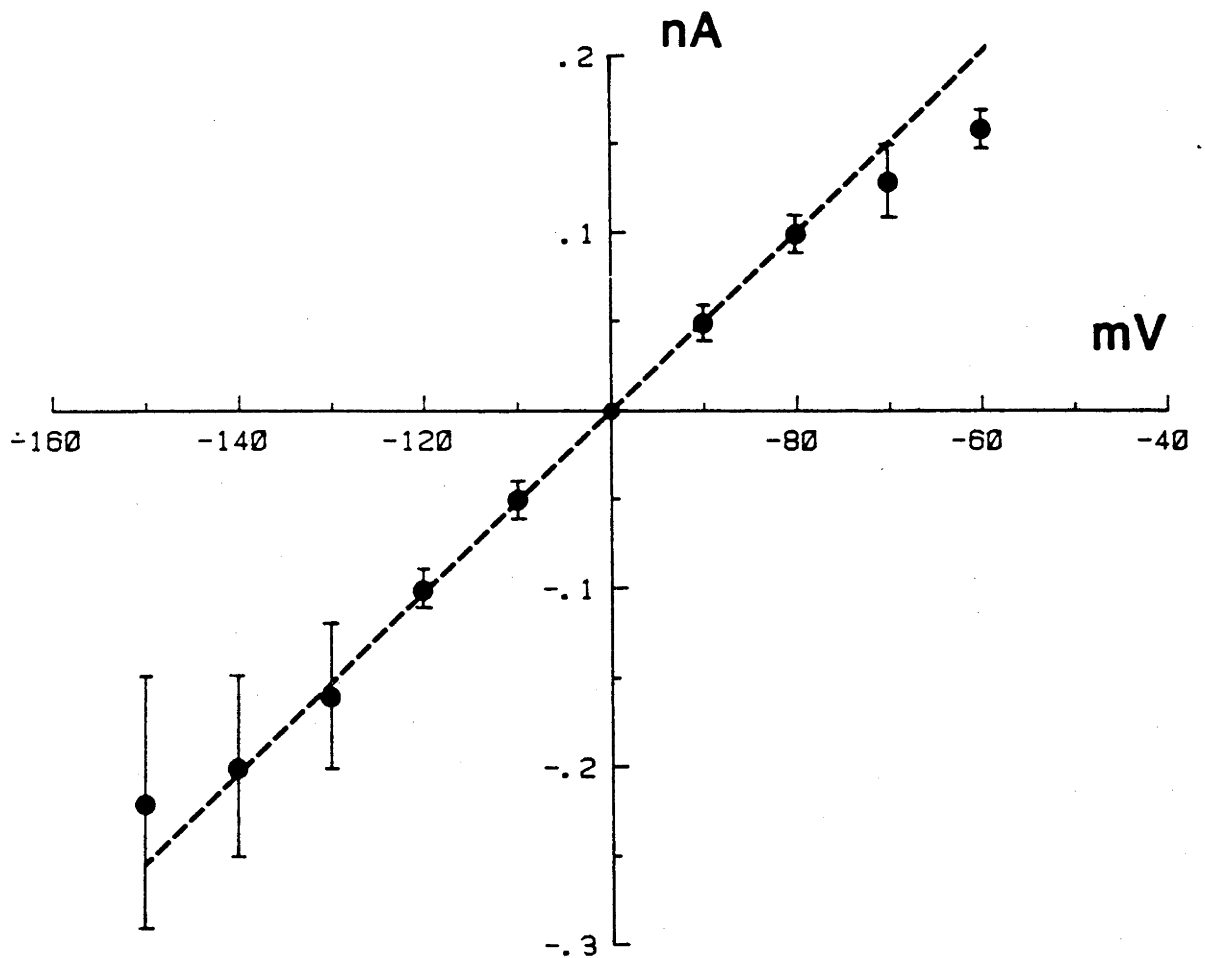
A schematic diagram of the experimental setup is shown in Figure 3.4. Voltage clamp command generation and data sampling was performed on line by a PDP 11/23 computer. Voltage and current traces were filtered at 2 or 3kHz (4 pole Bessel, -3dB) and sampled at 10 kHz. In later experiments, data acquisition was performed with a Tektronics 5211A digital oscilloscope at rates of up to 200kHz while filtering the data at 4KHz (Bessel, -3dB). The data was sampled with a resolution of 10 bits and transferred to the computer via a general purpose interface bus (GPIB).

Currents were analyzed off line on a PDP 11/44 computer (Digital Equipment). For each trace, peak amplitude was measured from the average of 5 data points around a cursor positioned at the required position. Curve fitting was done using a modified Levenberg-Morrison-Marquardt (LMM) algorithm (Osborne, 1976) which was implemented in FORTRAN by A. J. Miller (1981).

All experiments were done at room temperature, 22 - 24  $^{\circ}$ C. All results are given as mean  $\pm$  SEM.



**Figure 3.4.** Block diagram of experimental setup for voltage clamp experiments on dissociated neurones.



**Figure 3.5.** Passive current-voltage relation of dissociated hippocampal neurones. Currents were generated by voltage steps from  $-100\text{mV}$  to voltages shown on the abscissa. Data points are average values ( $\pm 1$  SEM) from 6 cells of similar size. The dotted line is the linear regression line of best fit for the points between  $-120\text{mV}$  and  $-80\text{mV}$ , and gave an input impedance of  $200\text{M}\Omega$ .

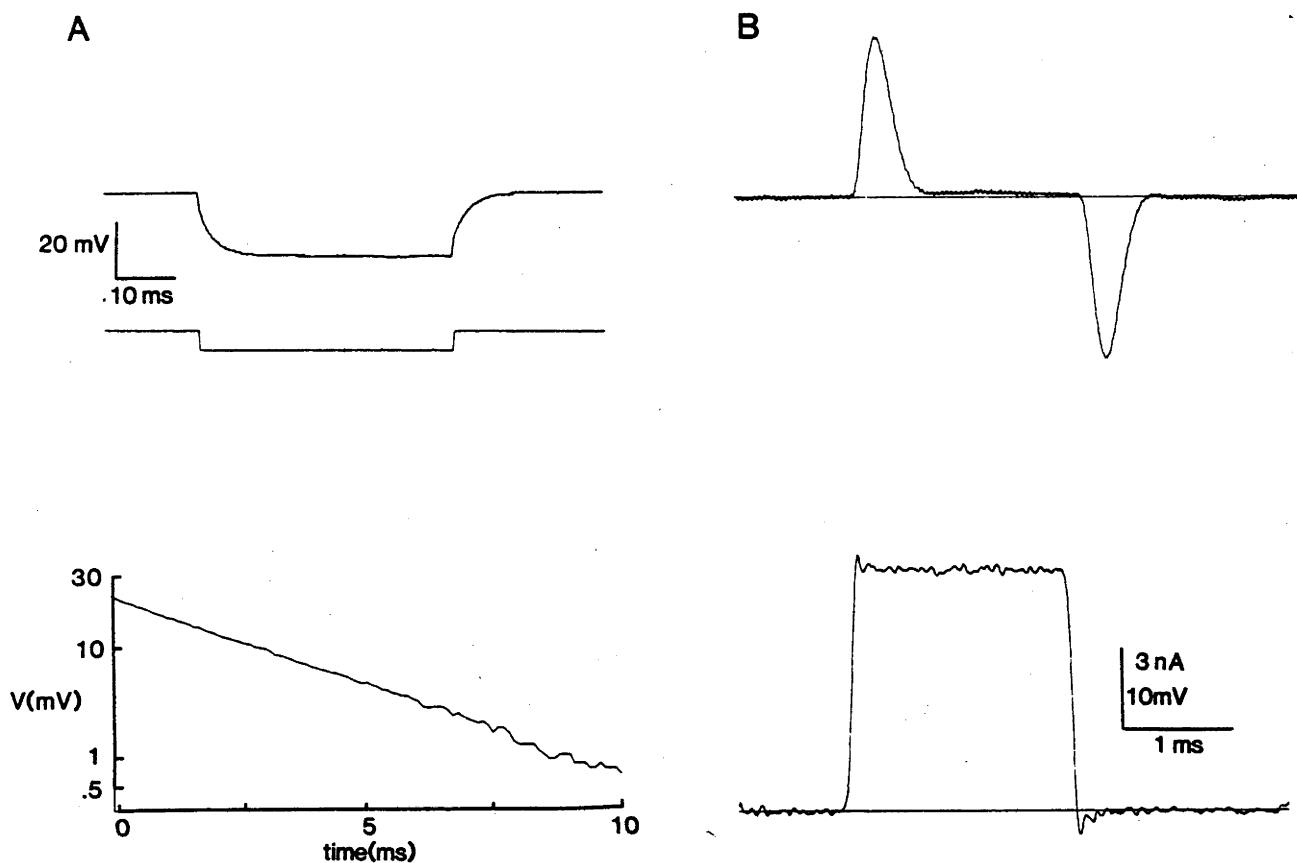
## RESULTS

The CA1 region of the hippocampus contains a wide variety of cells, the largest cells being the pyramidal neurones (Cajal, 1968). Cells obtained by dissociation displayed a wide variation in size and morphology (Plate 1). Healthy cells were recognised by their shiny appearance under phase contrast optics (Gray and Johnston, 1985; Numann and Wong, 1984; Kay and Wong, 1986; Huguenard and Alger, 1986). In many cases the cells retained some of their proximal dendrites and occasionally a myelinated axon could also be recognised. Most of the larger cells had a bipolar somatic shape which suggests that they were probably pyramidal cells (Cajal, 1968; Kay and Wong, 1986). In this study we have not discriminated between cells of different morphologies.

### Cell Passive Properties.

When perfused with KF solution, dissociated neurones had resting membrane potentials of  $-20$  to  $-60$  mV, the average being  $-40 \pm 2$  mV ( $n = 28$ ). In order to characterise the passive electrical properties of these cells it was first necessary to establish a voltage range in which the cell current-voltage relation was linear. We found that this was the case between membrane potentials of  $-130$  and  $-80$  mV. This is illustrated in Fig. 3.5 where the current response to small voltage steps from  $-100$  mV are plotted against membrane potential for 7 cells of similar size. It is clear that the membrane current-voltage relation is linear between  $-130$  and  $-80$  mV. At membrane potentials more positive to  $-80$  these neurones show an anomalous rectification as observed by Hotson et al. (1979). In the hyperpolarizing direction the membrane also rectifies, probably due to activation of a Q-current (Halliwell and Adams, 1982).

Membrane properties were measured in current clamp and in voltage clamp with the membrane potential clamped at  $-80$  or  $-100$  mV. In current clamp, small (10 - 100 pA) hyperpolarizing current pulses were applied from a holding potential of  $-80$  mV, and the resulting potential change measured. One such charging curve in response to 100 pA of current injection is shown in Figure 3.6A. A semilogarithmic plot of the first 10 ms after the onset of the current pulse is shown in the lower part of Fig. 3.6A. It can be seen that the potential rises as a single



**Figure 3.6.** Passive membrane properties of dissociated hippocampal neurones. **A.** Voltage response to a rectangular current of 100pA. Vertical calibration 20mV. Horizontal calibration, 10ms. The lower panel shows a semilogarithmic plot of the decay of the voltage transient. **B.** Current (upper panel, filtered at 1kHz) generated in a voltage clamped neurone by a 30mV depolarizing pulse (lower panel) from a holding potential of  $-100\text{mV}$ . Vertical calibration 3nA and 10mV. Horizontal calibration, 1ms.

exponential, indicating that the cell was isopotential (Rall, 1977). For larger current pulses the charging curve was biexponential probably due to turn on of the inward rectifier potassium channel (Adrian et. al., 1970b). Membrane time constants ( $\tau_m$ ) were obtained by fitting the potential change with a single exponential using a nonlinear least squares algorithm as described in the methods. Time constants had values of 1.2 to 10ms with a mean of  $5.0 \pm 0.8$  ms ( $n = 16$ ). The input resistance ( $R_o$ ) of the cells with KF in the perfusing solution was between 100 and 1000M $\Omega$  depending on the size of the cell, the average being  $445 \pm 85$  M $\Omega$  ( $n=13$ ).

In voltage clamp, the membrane potential was clamped to -100mV and small voltage steps (<30mV) applied in depolarizing and hyperpolarizing directions. In Fig. 3.6B, the current response to a 30mV depolarizing step is illustrated (this current record has been filtered at 1KHz in order to slow down the capacity transient). The membrane potential during this step is shown in the lower part of the Figure.  $R_o$  can be calculated as the slope of the straight line fitted to the current voltage relation obtained from a number of such steps as illustrated in Fig. 3.5.  $R_o$  measured by this method gave similar values to those obtained with current clamp.

Input capacitance ( $C_o$ ) was calculated either by applying the formula

$$\tau = R_o C_o \quad \dots\dots\dots 3-1$$

to the measured values of  $R_o$  and  $\tau$ , or by using the formula

$$C_o = Q/\Delta V \quad \dots\dots\dots 3-2$$

where Q is the total charge transferred during a voltage step of  $\Delta V$ . Q is measured by integrating the area under the capacity transient shown in Figure 3.6B. These methods gave values for  $C_o$  of between 5 and 50 pF, depending on the size of the cell. Average cell capacitance was  $25 \pm 3$  pF ( $n = 44$ ). By estimating the area of the cell from direct measurement of cell length and diameter, and assuming the cells have a circular cross section we were able to calculate the specific membrane capacitance ( $C_m$ ) and resistance ( $R_m$ ).

The specific membrane capacitance ranged from 0.5 to 1.5  $\mu\text{F}.\text{cm}^{-2}$ , with a mean value of  $1.0 \pm 0.2$   $\mu\text{F}.\text{cm}^{-2}$  ( $n = 6$ ) and the specific membrane resistance ranged from 3560 to 10817  $\Omega.\text{cm}^2$ , with a mean of  $5776 \pm 1400$   $\Omega.\text{cm}^2$  ( $n = 7$ ).

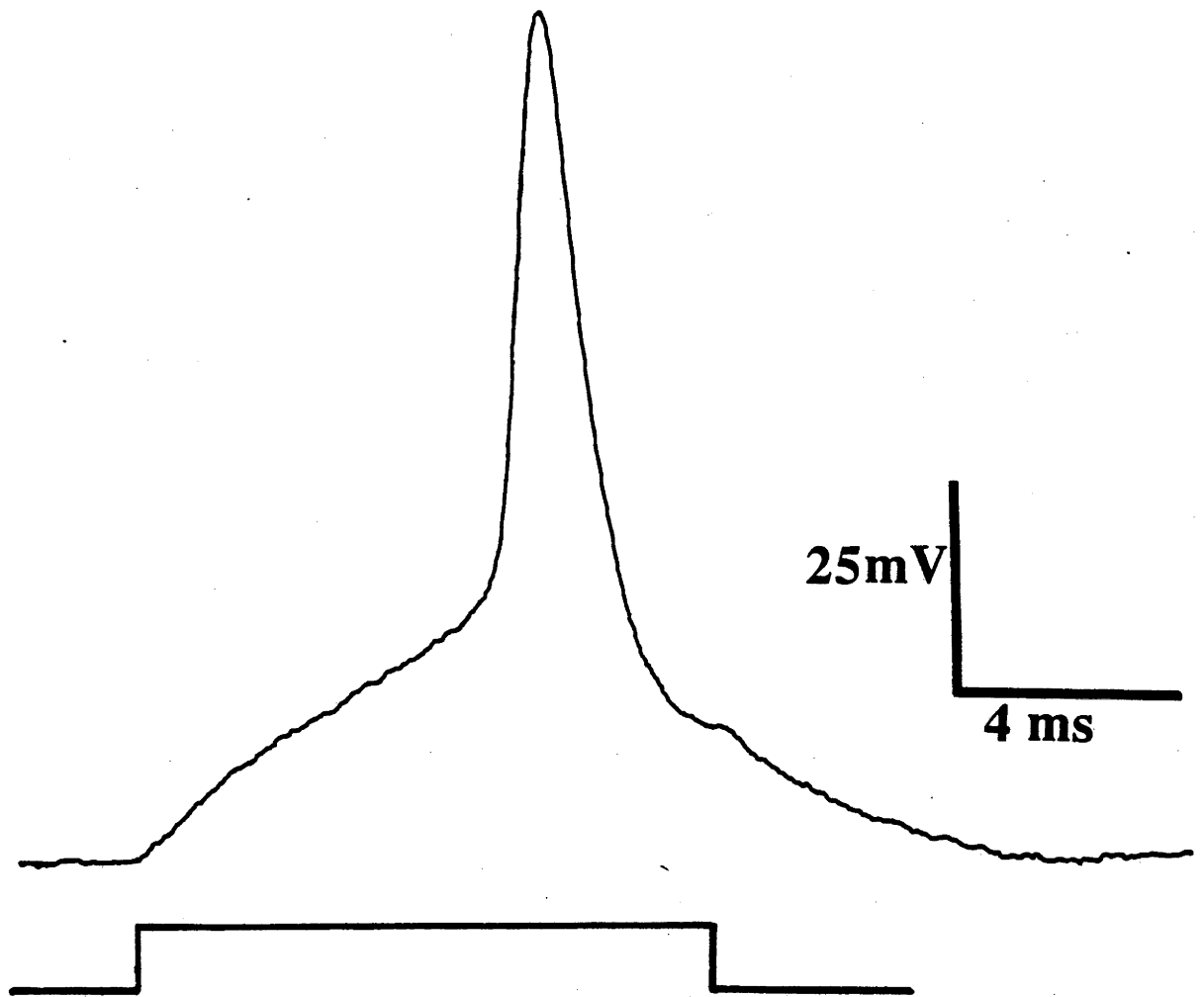
## Action Potentials.

Action potentials occurred spontaneously and could also be elicited by injection of depolarizing current. An action potential produced by direct injection of 0.1nA of current from a holding potential of -70mV is shown in Figure 3.7. Calcium currents have been blocked with 1mM Cd in the external solution. Threshold for action potential generation was -50mV. The action potential has an amplitude of 100 mV and was 2ms in duration. Anode break action potentials could also be elicited by injecting hyperpolarizing current pulses and an example is shown in Figure 3.8. With cells current clamped at -80mV, action potential amplitudes ranged between 90 and 110mV and with an average of  $103 \pm 3$  mV ( $n=15$ ) and were completely suppressed by application of  $10^{-6}$ M tetrodotoxin (TTX) as illustrated in Figure 3.9. It can be seen that even with a larger current injection (0.2nA) there is no regenerative activity in the presence of TTX. The maximum rate of rise of the action potentials ranged from 600 to 1000  $Vs^{-1}$ .

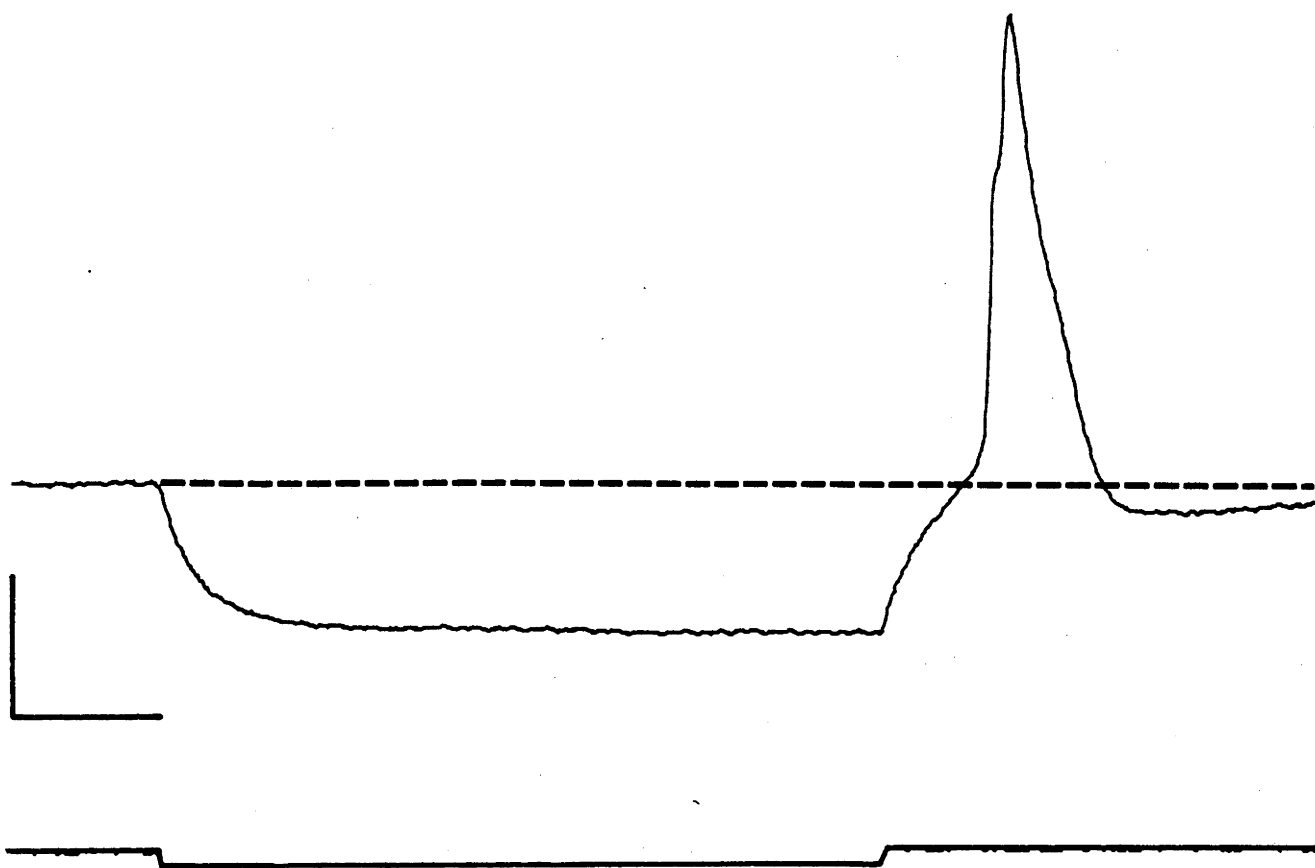
When calcium currents were not blocked action potential duration was very variable and generally had a plateau during the repolarizing phase as has been seen in axons (Tasaki and Hagiwara, 1957) and CA1 neurones in hippocampal slices (Størm, 1987; Lancaster and Nicoll, 1987) when potassium currents have been blocked. Spontaneous action potentials, and trains of action potentials elicited by current injection in normal Ringer are shown in Fig. 3.10A, and at a faster time base in Figs. 3.10B-F. In each case, the neurone was current clamped at -80 mV and a small (0.1 - 0.5nA) depolarizing current injected.

In response to depolarizing currents, some neurones fired repetitively (eg Fig. 3.10A and 3.10D & E). In other cells there was a prolonged plateau potential which repolarized spontaneously after a varying period of time (Fig. 3.10G). This behavior was more pronounced in cells perfused with CsF. In some cells this "flipping" between depolarized and hyperpolarized states occurred spontaneously. This behavior was not investigated further in our study.

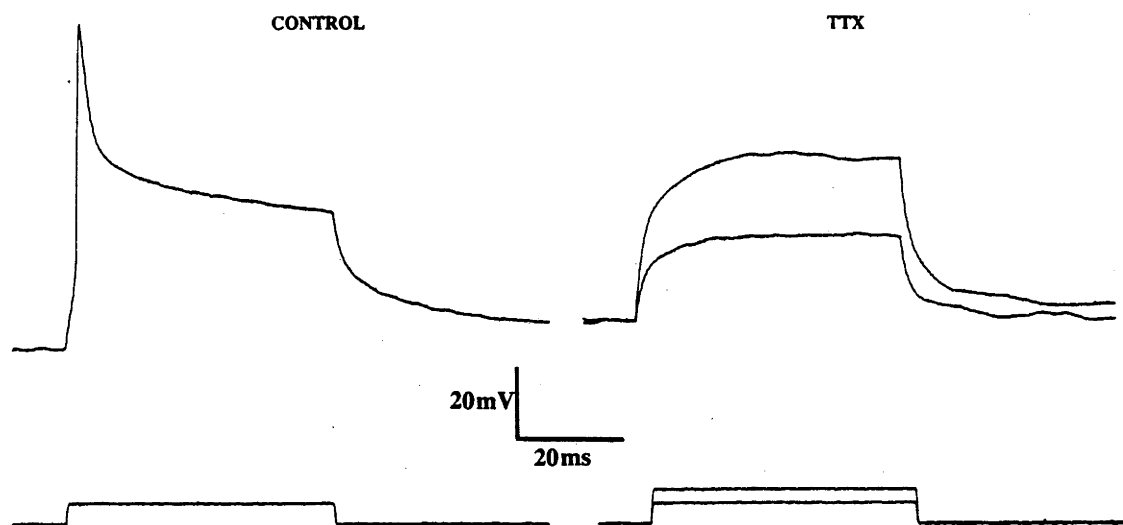




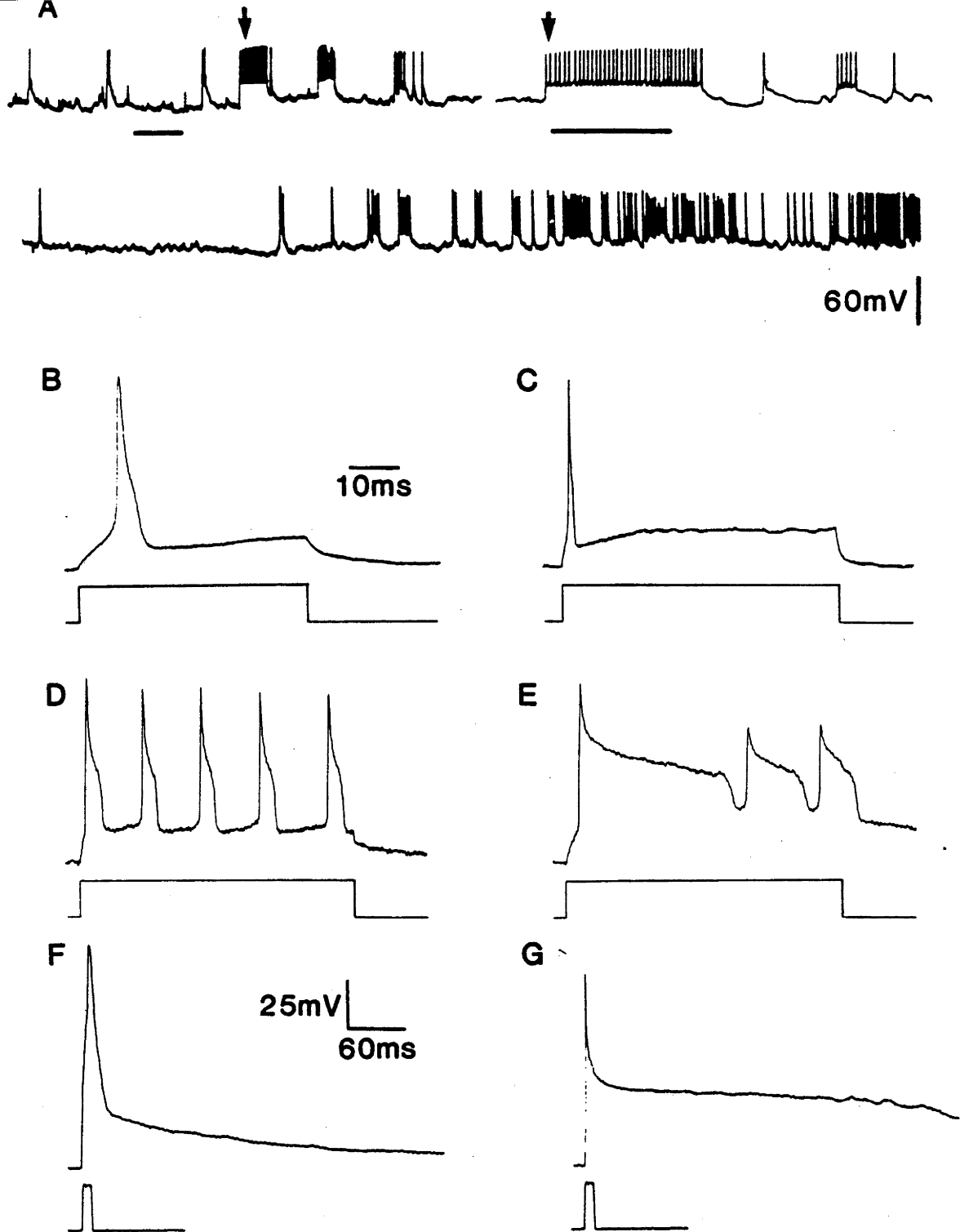
**Figure 3.7.** Action potential in dissociated hippocampal CA1 neurone recorded with a pipette containing KF. Action potential shown was generated by injection of a 200pA current from a holding potential of  $-80\text{mV}$ . Calcium currents have been blocked with  $1\text{mM Cd}^{2+}$  in the external solution. Vertical calibration 25mV. Horizontal calibration, 4ms.



**Figure 3.8.** Anode break action potential generated by injection of a 100pA hyperpolarizing current from a holding potential of -60mV. Vertical calibration 30mV. Horizontal calibration, 10ms.



**Figure 3.9.** Tetrodotoxin blocks action potentials. Action potential generated in control Ringer by injection of 100pA of current from a holding potential of  $-80\text{mV}$ . Addition of TTX ( $1\text{ }\mu\text{M}$ ) abolishes all regenerative activity (panel on right). In the presence of TTX, injection of 200pA of current (larger trace on right) still does not produce any regenerative activity.



**Figure 3.10.** Action potentials recorded in cells perfused with KF and normal Ringer in the external solution. **A.** Spontaneous action potentials recorded on a Hewlett Packard chart recorder. A depolarizing current (100pA) was injected at times marked by arrows. In the top trace, the time base has been expanded during the second segment: horizontal scale bars represent 1second. Vertical calibration, 60mV. **B-G.** Action potentials recorded from different cells in response to depolarizing currents shown in the lower trace of each record. Note the time calibration for B is 10ms whereas the time calibration for C, D, E, F and G denotes 60ms. Vertical calibration, 25mV.

## DISCUSSION

The results presented in this chapter show that neurones from the guinea pig hippocampus can be isolated by acute dissociation. These neurones are reasonably healthy and can be used for electrophysiological studies. The dendritic trees of the neurones are removed during the dissociation procedure leaving small cells which can be shown to be isopotential and thus are ideal for voltage clamp. These cells have stable resting membrane potentials and can fire action potentials both spontaneously and in response to injected current pulses. The membrane properties of these cells, though not exactly the same as those measured from neurones in slices, can be accounted for if the dissociation procedure is taken into account.

Passive properties of dissociated hippocampal CA1 neurones reported in this chapter are somewhat different to those measured in the slice, and those reported by Huguenard and Alger (1986) in acutely dissociated hippocampal cells. These differences are compared in Table 3.1. In our hands, the resting membrane potential of dissociated cells was in the range  $-20$  to  $-60$  mV. Both in slices (chapter 2), and in cells obtained by Huguenard and Alger, the resting potential was in the range  $-40$  to  $-70$  mV. We believe that this difference can be explained by the presence of a nonspecific leak in our dissociated cells of the order of  $1\text{ G}\Omega$ . This leak could be at a point where a dendrite was torn off during dissociation or it could be leakage around the seal of the electrode. The presence of a leak would also explain the small value of the membrane time constant measured by us.

Time constants measured in dissociated neurones were 3-5 ms which are much smaller than the time constants measured from CA1 neurones in slices (10-20 ms; Brown, Fricke and Perkel, 1981; Turner, 1984, Table 3.1). After correcting for the leak these are 2-16 ms which are in the range of time constants measured in slices.

The input resistance of our dissociated cells was in the range  $100$ – $1000\text{ M}\Omega$  which is an order of magnitude larger than those reported from slice experiments (Brown, Fricke and Perkel, 1981; Turner, 1984). The high input impedance can be attributed to several factors. Firstly, the dissociation procedure removes the extensive dendritic tree and synaptic input present in these neurones in the slice preparation (Turner and Schwartzkroin, 1980). Secondly, the presence of

**TABLE 3.1. Comparison of passive properties of hippocampal pyramidal cells.**

| preparation                   | $V_m$<br>(mV) | $\tau$<br>(ms) | $R_o$<br>(M $\Omega$ ) | $R_m$<br>(k $\Omega$ .cm <sup>2</sup> ) | $C_m$<br>( $\mu$ F.cm <sup>-2</sup> ) |
|-------------------------------|---------------|----------------|------------------------|-----------------------------------------|---------------------------------------|
| Slice <sup>1</sup>            | -65           | 10-20          | 30-60                  | 10                                      | 2-4                                   |
| Dissociated Cell <sup>2</sup> | -60           | 20-36          | 200-1200               | -                                       | -                                     |
| Dissociated Cell <sup>3</sup> | -40           | 1.2-10         | 200-1000               | 5.8                                     | 1.0                                   |
|                               | -60           | 2-16*          | 360-1600*              | 10.7*                                   |                                       |

<sup>1</sup>Brown et al., 1981; Turner, 1984

<sup>2</sup>Huguenard and Alger, 1986

<sup>3</sup>This report \* corrected for leak

fluoride and a high concentration of EGTA in the electrode filling solution produces a very low intracellular calcium concentration so that any resting calcium activated potassium conductance is inactive (see also discussion in chapter 6). Lastly, the leak resistance around an intracellular microelectrode, estimated to be as low as  $20\text{M}\Omega$  (Hodgkin and Nakajima, 1972; Lassen and Rasmussen, 1977; Neher and Marty, 1984), much lower than the giga ohm seals we can obtain with the patch electrode.

Specific membrane capacitance measurements calculated by estimating the area of the neurones by direct observation gave a value equal to the expected  $1\mu\text{Fcm}^{-2}$  (Cole, 1968; Jack, Noble and Tsien, 1975). The close agreement of the measured specific membrane capacitance with the theoretically expected value suggests that there are very few infoldings in the membrane of cells obtained by dissociation. This result is consistent with the smooth surface membrane seen in electron microscopic examination of dissociated hippocampal neurones (Kay and Wong, 1986).

Action potentials in the presence of calcium channel blockers had amplitudes of 104 mV and were about 2ms in duration (Fig 3.7). This is about twice as long as action potentials seen in the slice preparation and is probably due to a combination of the presence of the calcium activated potassium currents in neurones in slices (Størm, 1987; Lancaster and Nicoll, 1987; see discussion chap. 6) and the lower temperatures used in this study. When calcium channel blockers were not present in the extracellular solution, there was often a distinct hump in the decay phase of the action potential, probably due to activation of a small calcium current as has been suggested for TEA injected motoneurons (Schwindt and Crill, 1980a,b; also cf. Fig 5 in Størm, 1987). With the high input impedance of the cells a current of only 100pA would be sufficient to account for this hump.

In many cases there was a prolonged plateau during the decay of the action potential. This plateau was more pronounced in the presence of  $\text{Cs}^+$  in the intracellular solution (see Fig 3.11). These phenomena were not studied in any great detail but it is possible that in the absence of significant potassium currents, the small calcium current (Chapter 4) or the noninactivating sodium current (Chapter 5) could cause the pronounced plateaus.

## **Chapter 4**

### **The Sodium Current Underlying the Action Potential In Acutely Dissociated Guinea Pig Hippocampal CA1 Neurones.**



## INTRODUCTION

The voltage activated sodium currents which underlie the ionic basis of action potential generation have been extensively studied in a wide variety of excitable cell types over the past thirty years. It is now known that sodium currents in such diverse preparations as the squid giant axon, mammalian and amphibian peripheral nerve, mammalian and amphibian skeletal muscle and cardiac muscle have qualitatively similar properties (for a recent review see Hille, 1984). Due to the problems of effectively voltage clamping rapid conductance changes in small cells with extensive dendritic processes, sodium currents in mammalian central neurones have been recorded in only a few instances (Araki and Terzuolo, 1962; Barrett and Crill, 1980). Activation of this conductance generates the upstroke of the action potential in these cells and hence characterising this conductance is an essential element in understanding central neurone activity.

In the last chapter it was shown that neurones can be acutely isolated from the guinea pig hippocampus. These cells have stable resting membrane potentials and can fire action potentials and trains of action potentials. These neurones have lost most of their dendrites and are isopotential. In this chapter the sodium currents underlying these action potentials is characterised. It will be shown that sodium currents in guinea pig hippocampal CA1 neurones are in some respects different from those recorded from squid axon (Hodgkin and Huxley, 1952b) and cat motoneurones (Barrett and Crill, 1980).

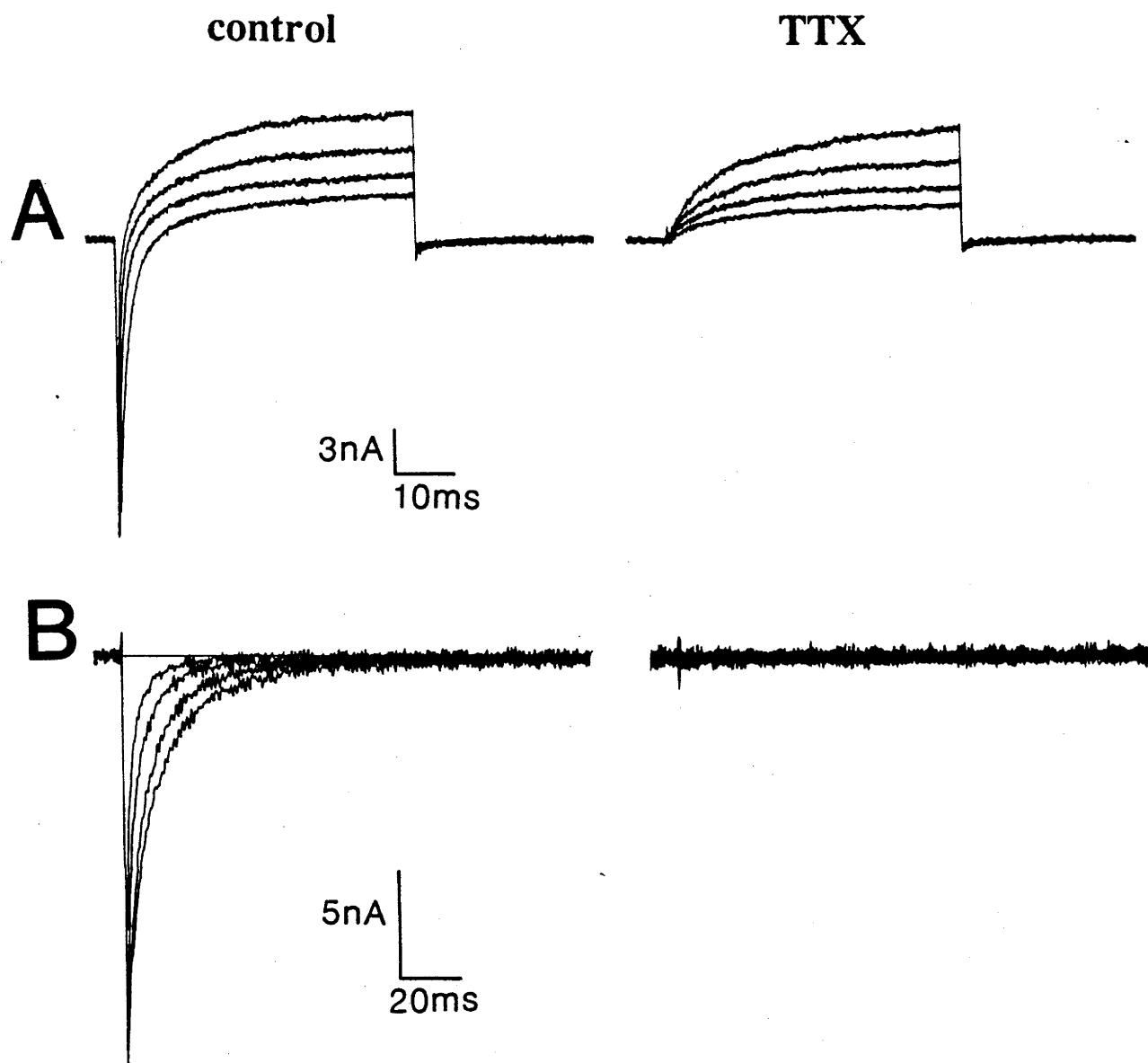
## METHODS

Details of preparation of neurones and voltage clamp are the same as described in the last chapter. The pipette solution consisted of (mM) CsF 130, KCl 10, HEPES 10, EGTA 10, titrated to a pH of 7.2 with CsOH. In some experiments, CsF was replaced with KF and KOH used to balance the pH. Osmolality of intracellular solution was 290 - 300 mosmolar. The bathing solution consisted of (mM): NaCl, 125; KCl 5.0; NaHCO<sub>3</sub>, 25; MgSO<sub>4</sub>, 2; CaCl<sub>2</sub>, 2.5; NaH<sub>2</sub>PO<sub>4</sub> 1.2; Glucose 11; pH 7.3 when bubbled with 95% O<sub>2</sub> / 5% CO<sub>2</sub>. When the sodium concentration was varied choline chloride was substituted for NaCl in the required concentration.

Leakage and capacitive currents were subtracted by scaling and adding the currents obtained from small (<20mV) hyperpolarising steps to the test pulse. In later experiments, leak and capacity subtractions pulses were subtracted by delivering a series of small (<30mV) hyperpolarizing pulses which were added together on line until the sum of the pulses was equal to the depolarizing pulse.

Curve fitting was performed using the Levenberg-Marquardt algorithm. Cell areas were estimated by integrating the area under the capacity transient and assuming a specific membrane capacitance of 1 $\mu$ F.cm<sup>-2</sup>. Most experiments were done at 22 - 24 °C. In some experiments in which the effects of temperature were examined, the bath temperature was controlled with a Peltier element mounted on the microscope stage. The temperature of the dish was monitored by a small thermocouple element placed in the dish containing the cells.

All results are presented as  $\pm$  SEM.



**Figure 4.1.** TTX blocks inward currents. The currents shown were generated by positive voltage pulses from a holding potential of  $-100\text{mV}$ , before and after exposure to TTX ( $1\mu\text{M}$ ). **A.** The perfusing solution was KF. Currents were generated by  $50\text{ms}$  voltage steps to  $-10$ ,  $0$ ,  $+10$  and  $+20\text{mV}$ . TTX abolished the inward current leaving only outward currents (right panel). Vertical calibration,  $3\text{nA}$ . Horizontal calibration,  $10\text{ms}$ . **B.** When the perfusing solution was CsF, there was no outward current. Currents shown were generated by  $100\text{ms}$  voltage steps to  $-30$ ,  $-20$ ,  $-10$  and  $0\text{mV}$ . In the presence of TTX, the inward current disappeared (right panel). Vertical calibration,  $5\text{nA}$ . Horizontal calibration,  $20\text{ms}$ .

## RESULTS

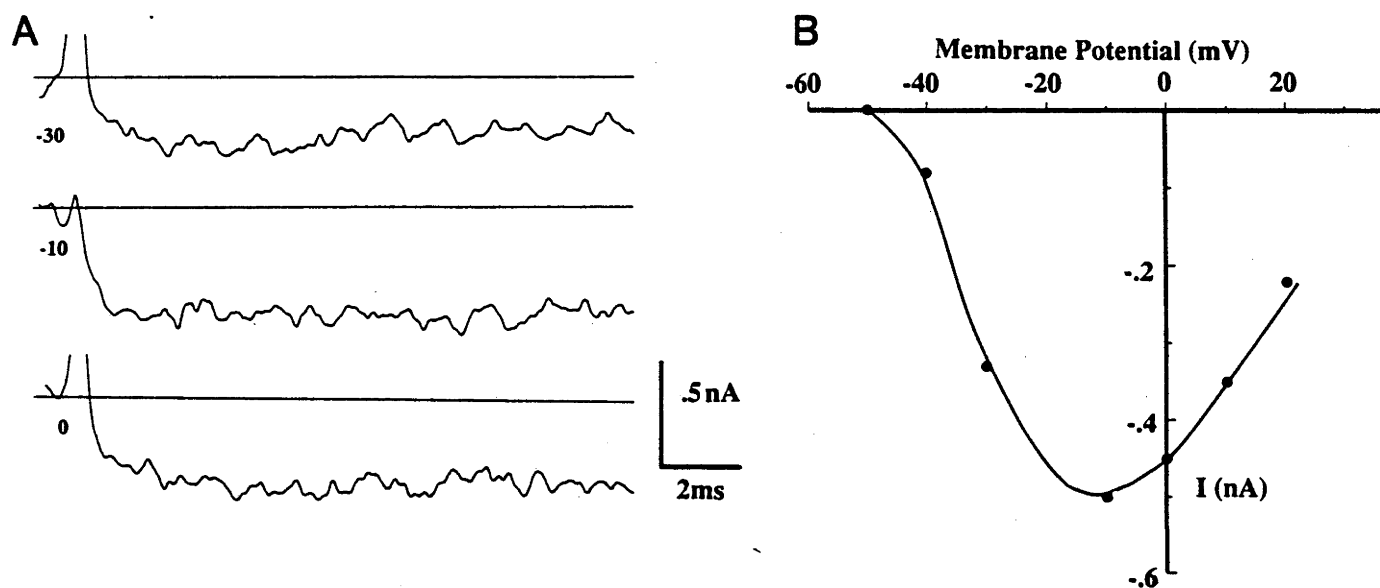
### Voltage Clamped Currents.

With potassium as the main cation in the recording pipette, currents evoked by depolarizing steps from a holding potential of  $-100\text{mV}$  consisted of a transient inward current followed by a sustained outward current (Fig. 4.1A). The inward current could be completely blocked by application of  $1\mu\text{M}$  TTX (Fig. 4.1A) and its amplitude was dependent upon the internal and external sodium concentration (data not shown). The outward current could be suppressed by replacing the KF in the internal solution with CsF (Fig. 4.1B, different cell) and has properties similar to the delayed rectifier present in many cell types (see Chapter 6). When TTX is again added no voltage activated currents are apparent (Fig. 4.1B).

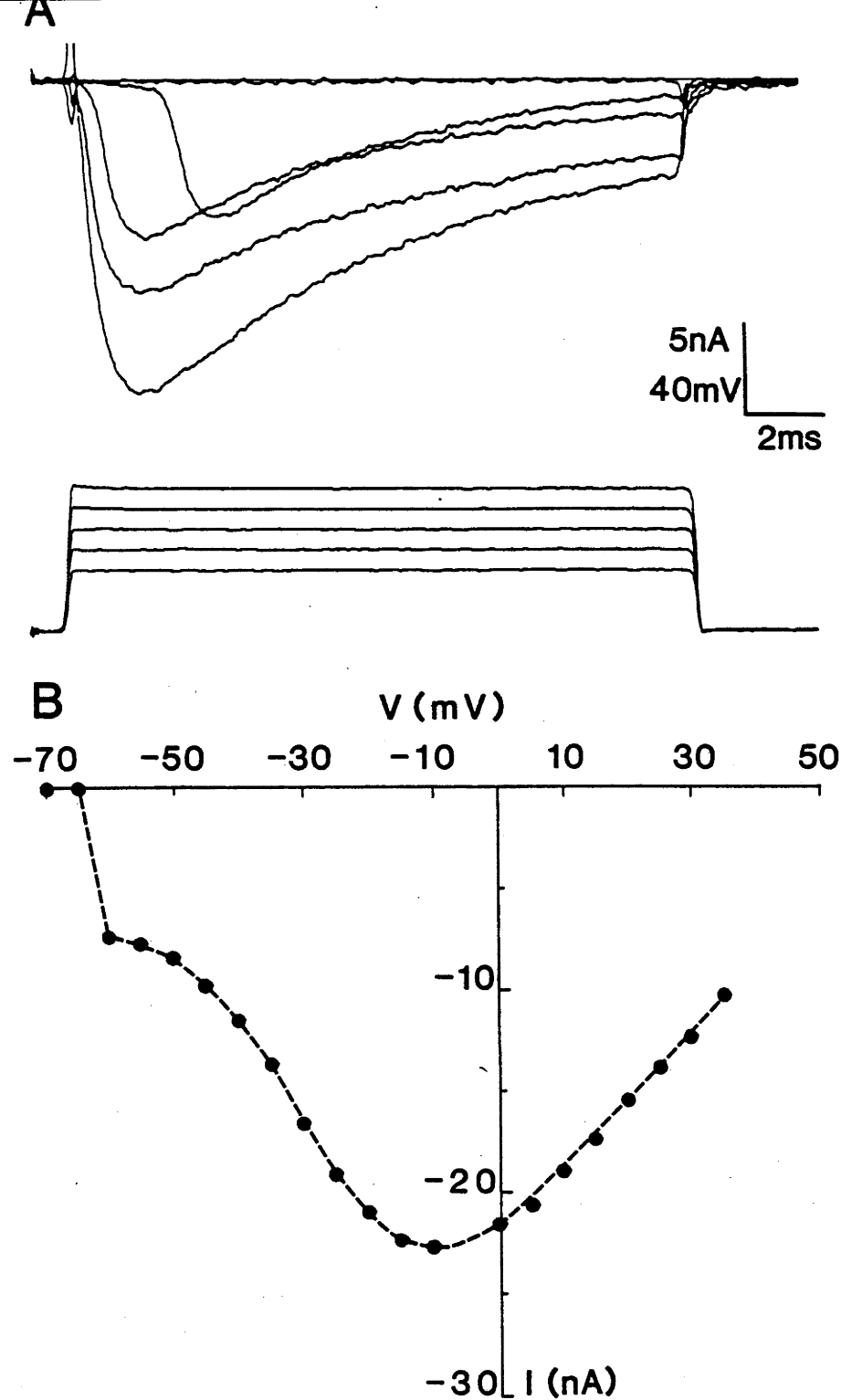
In many cases, a small residual net inward current, presumed to be a calcium current, still remained in the presence of concentrations of tetrodotoxin as high as  $1\mu\text{M}$ . The TTX insensitive current was usually very small, the largest current that was ever seen had a peak amplitude of  $0.5\text{nA}$  and is illustrated in Figure 4.2A. The steady state current-voltage relation for this current is shown in Fig 4.2B. This current-voltage relation (Fig. 4.2B) is similar to the current-voltage relation for a calcium current reported by Kay and Wong (1986) in dissociated hippocampal neurones under comparable conditions and to the current-voltage relation of the sustained calcium current in these neurones in hippocampal slices (Brown and Griffith, 1983a; chapter 2). It was concluded that the large, TTX sensitive, inward current was a sodium current and that it could be accurately described in the presence of the TTX-insensitive current because the latter was so much smaller (see also Kay and Wong, 1986). No attempt was made to characterise the TTX insensitive current. All subsequent observations on the sodium current were made with the recording pipette containing CsF.

### Sodium Currents.

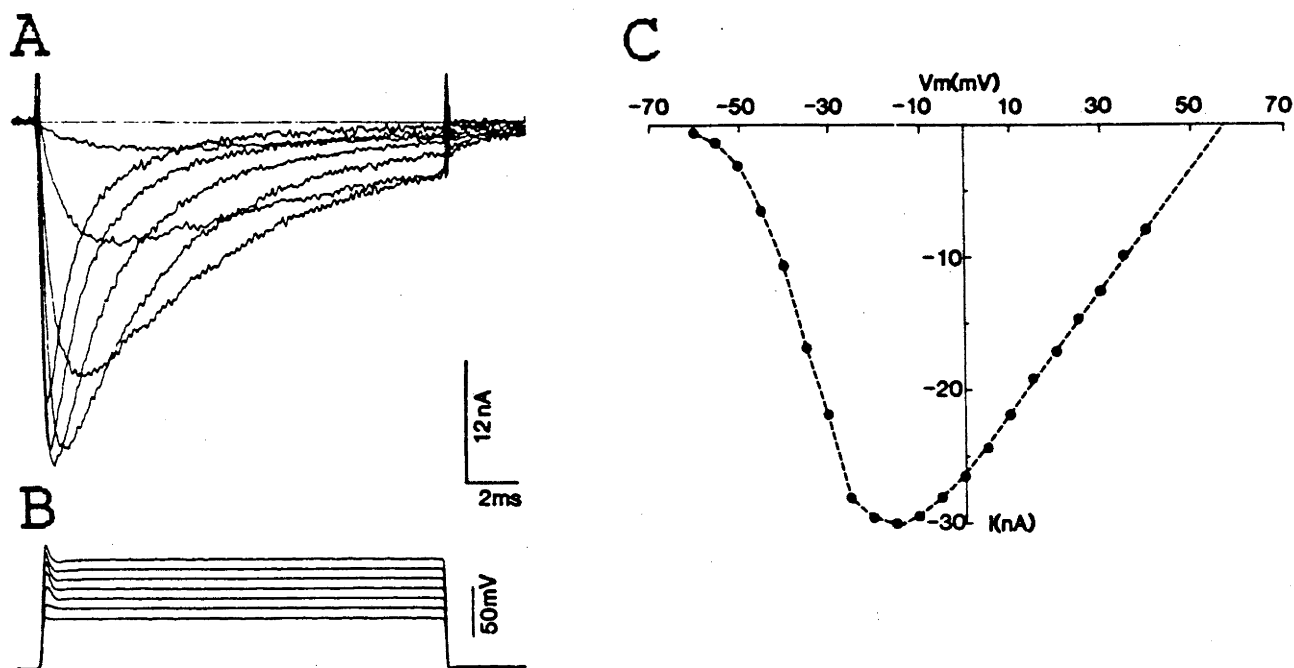
When cells were voltage clamped at  $-100\text{mV}$  and depolarizing voltage steps applied, a frequently observed phenomenon, seen with small depolarizing steps was the appearance of an inward current following a delay of 2-3ms after the onset of the voltage step (Fig. 4.3A). In many cells this current appeared to be well clamped on inspection of the voltage trace (eg. lower



**Figure 4.2.** TTX insensitive inward current. **A.** The currents shown were generated by positive voltage pulses from a holding potential of  $-100\text{mV}$  to  $-30$ ,  $-10$  and  $0\text{mV}$ . Sodium currents have been blocked with  $1\mu\text{M}$  TTX. Vertical calibration  $0.5\text{nA}$ . Horizontal calibration,  $2\text{ms}$ . **B.** Steady state current-voltage (I-V) relation for currents shown in (A).



**Figure 4.3.** "Breakthrough" sodium currents. **A.** Inward currents (upper traces) generated by voltage steps (lower traces) to between  $-60$  and  $-30\text{ mV}$  from a holding potential of  $-100\text{ mV}$ . Vertical calibration  $5\text{ nA}$  and  $40\text{ mV}$ . Horizontal calibration,  $2\text{ ms}$ . **B.** The peak I-V curve for the currents shown in (A) contains an obvious discontinuity at  $-60\text{ mV}$ .



**Figure 4.4.** Inward currents generated in a voltage clamped cell with an electrode containing CsF. **A.** Sodium currents generated by potential steps from a holding potential of  $-100\text{mV}$  to potentials between  $-50$  and  $+10\text{mV}$  (**B**,  $10\text{mV}$  increments). Vertical calibrations in **A** and **B**,  $12\text{nA}$  and  $50\text{mV}$ . Horizontal calibration,  $2\text{ms}$ . **C.** I-V curve from the same cell as in **A**. The extrapolated null potential was  $+58\text{mV}$ .

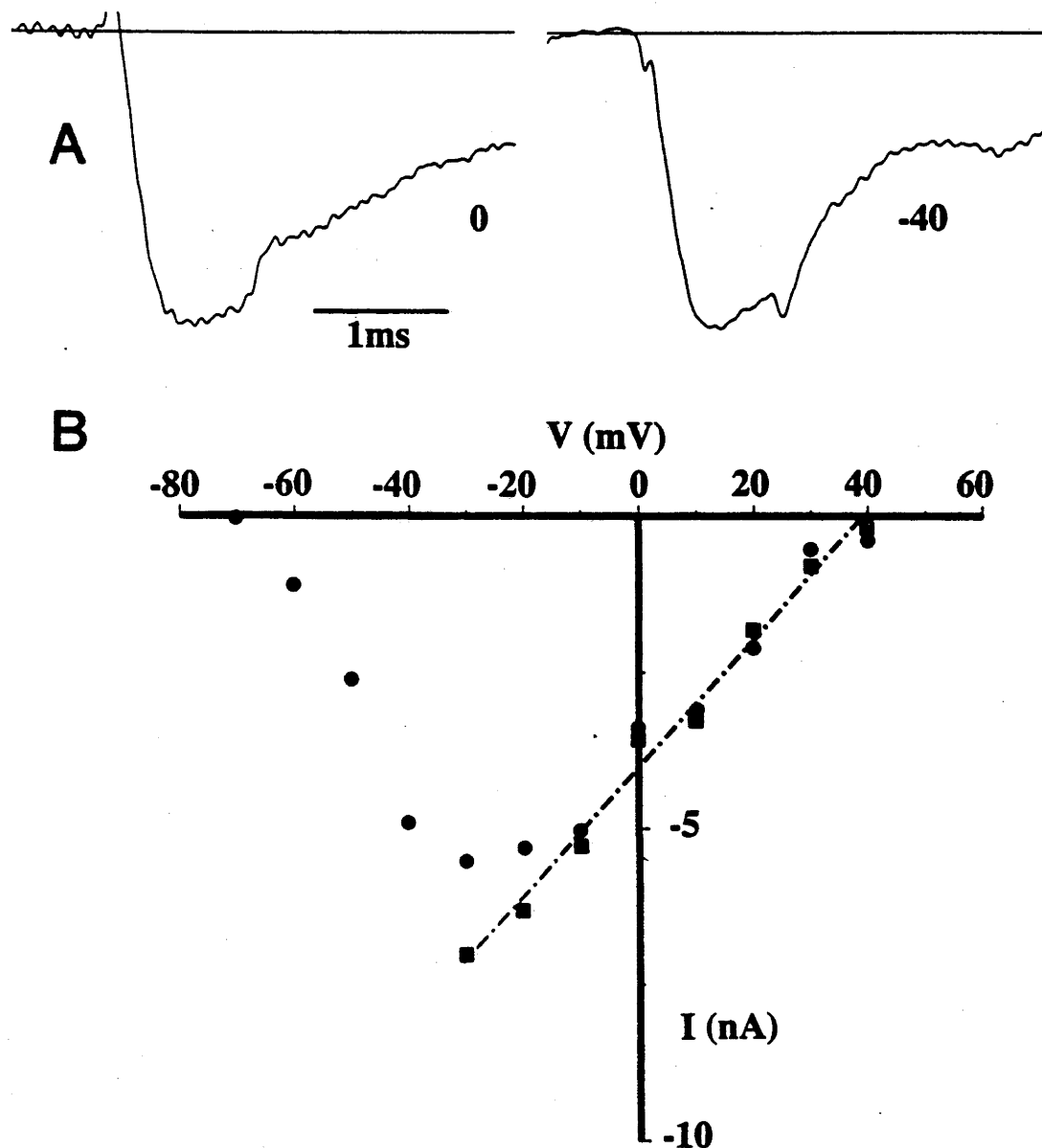
half Fig 4.3A), whereas in others there appeared to be significant voltage escape associated with the peak of the current. These 'breakthrough' currents resulted in discontinuity in the peak current-voltage relationship (Fig. 4.3B) as has been noted elsewhere (Brown, Lee and Powell 1981a; Barrett and Crill, 1980). Such currents were commonly seen in cells which had long processes ( $> 50\mu\text{m}$ ) still attached. It was concluded that these 'breakthrough' currents are generated in inadequately clamped patches of membrane, perhaps in the initial segment (Barrett and Crill, 1980), on the dendrites (eg. Hirst and McLachlan, 1986) or in the axon (eg. Taylor, 1986). Cells displaying 'breakthrough' currents were not used in further analysis.

A typical family of sodium currents are shown in Fig. 4.4A. At depolarized potentials ( $> +20\text{mV}$ ), sodium current activation and inactivation became too fast to be accurately resolved from the capacity current and are not shown. The peak current-voltage relation for this cell is shown in Fig. 4.4C. It can be seen that the current-voltage relationship rises smoothly, peaks at about  $-20\text{mV}$  and then becomes smaller as the step potential approaches the sodium equilibrium potential. The extrapolated reversal potential was  $+58\text{mV}$ . Taking into account the  $4\text{mV}$  junction potential at the electrode tip, this is close to the calculated Nernst potential for sodium ions of  $+68\text{mV}$ .

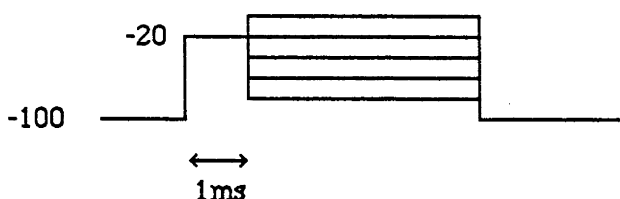
The sodium reversal potential can be shifted to a less positive potential by altering the concentration of  $\text{Na}^+$  either inside the cell or in the bathing medium. Reversal of sodium current could then be obtained at membrane potentials where the kinetics of the current allowed the peak to be resolved. Figure 4.5B (circles) shows the peak current-voltage relation for a cell bathed in  $50\text{mM}$  external sodium. The currents reversed at  $+38\text{mV}$  which is close to the Nernst potential of  $+41\text{mV}$ .

Instantaneous current-voltage relations were constructed using a two step voltage pulse paradigm as shown below (Hodgkin and Huxley, 1952c). The cell was voltage clamped at  $-100\text{mV}$ , then stepped to  $-20\text{mV}$  for  $1\text{ms}$  to activate the sodium current, the membrane potential was then stepped to various potentials as shown.





**Figure 4.5.** Instantaneous sodium currents in an external solution containing 50mM  $\text{Na}^+$ . **A.** Two instantaneous currents generated by repolarization to  $-40\text{mV}$  and  $0\text{mV}$  in a cell voltage clamped at  $-100\text{mV}$  and given a 1ms activating pulse to  $-20\text{mV}$  (see text for details). Horizontal calibration, 1ms. Vertical calibration not shown. **B.** The circles show the peak sodium currents in this cell generated by positive voltage steps from a holding potential of  $-100\text{mV}$ . The squares show the peak amplitude of the instantaneous currents over a range of voltages. The null potential obtained from the linear regression line was  $+38\text{mV}$ .



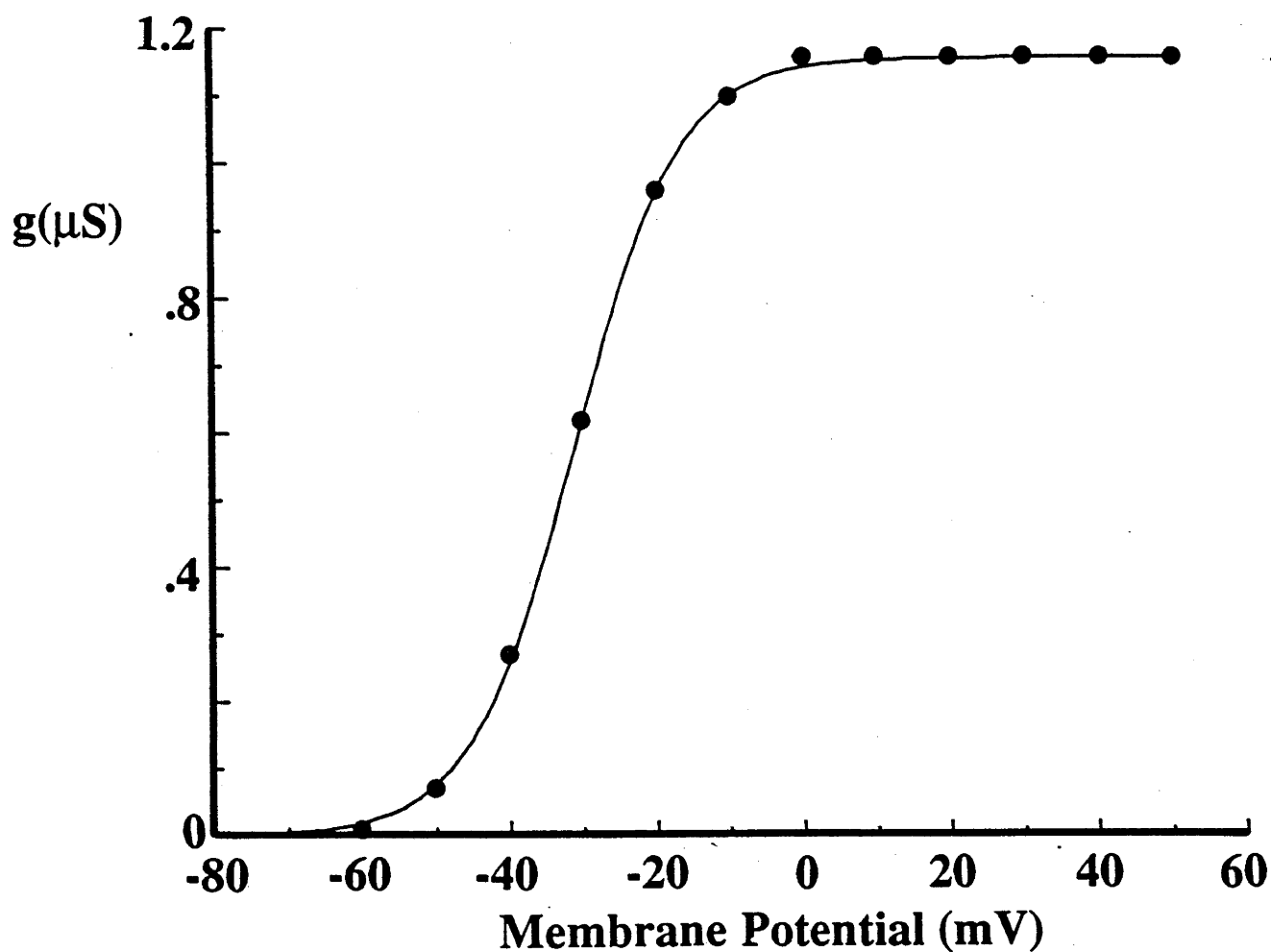
We found that the clamp was unable to follow the large instantaneous currents generated in cells bathed in normal Ringer. Instantaneous current - voltage relation were therefore measured with the sodium in the external solution reduced to 50mM. Instantaneous currents measured in one cell at -40mV and 0mV are shown in Figure 4.5A. The peak current (circles) and the instantaneous current (squares) measured in the same cell are shown in Fig. 4.5B. The instantaneous current-voltage relation is linear over the voltage range -30 to +40 indicating that the channel acts as a simple ohmic resistor. At potentials more hyperpolarized than -30mV, the sodium tail current inactivates too rapidly for accurate measurement. The broken line is the line of best fit estimated by a linear least squares regression algorithm and gave a reversal potential of +38mV. The reversal potential obtained from extrapolation of the peak current-voltage relation in this cell was also +38mV.

### Conductance-Voltage Relation.

Because the instantaneous current varied linearly with voltage it is possible to calculate the sodium conductance from the peak current voltage plot using the equation

$$g_{Na} = I_{Na} / (V - E_{Na}) \dots\dots\dots 4-1$$

Where  $I_{Na}$  is the peak current,  $g_{Na}$  the peak conductance,  $E_{Na}$  the sodium reversal potential and  $V$  the membrane potential (Hodgkin and Huxley, 1952b). In cells where an adequate instantaneous current-voltage relation could not be obtained the reversal potential was estimated from extrapolation of the peak current voltage relation. The conductance thus obtained was related to



**Figure 4.6.** Peak sodium conductance ( $g$ ) as a function of voltage. The circles show conductance calculated according to equation 4-1 ( $E_{\text{Na}} = +65\text{mV}$ ) from the peak amplitude of currents recorded at  $22^\circ\text{C}$ . The solid line shows the best fit of equation 4-2 (see text).  $V'$  and  $k$  were  $-31\text{mV}$  and  $7.1\text{mV}$  respectively.

voltage in a sigmoidal fashion with a threshold around -55mV and a peak conductance at 0mV. The peak conductance-voltage curve for the cell shown in Fig 4.4 is illustrated in Fig 4.6. The solid line is the line of best fit using a Boltzmann function:

$$g_{Na} = g_{max} / (1 + e^{(V'-V)/k}) \quad \dots\dots\dots 4-2$$

where  $g_{Na}$  is the sodium conductance at voltage  $V$ ,  $V'$  is the voltage at which  $g_{Na}$  is half of  $g_{max}$  and  $k$  is a constant that describes the slope of the Boltzmann relation. For the data illustrated in Fig 4.6,  $g_{max}$  was 1.2  $\mu$ S,  $V'$  was -31mV and the slope was 7.1mV. Average values measured from 8 cells gave a half maximal activation of  $-38.7 \pm 3.3$ mV and a slope of  $6.6 \pm 0.3$ mV.

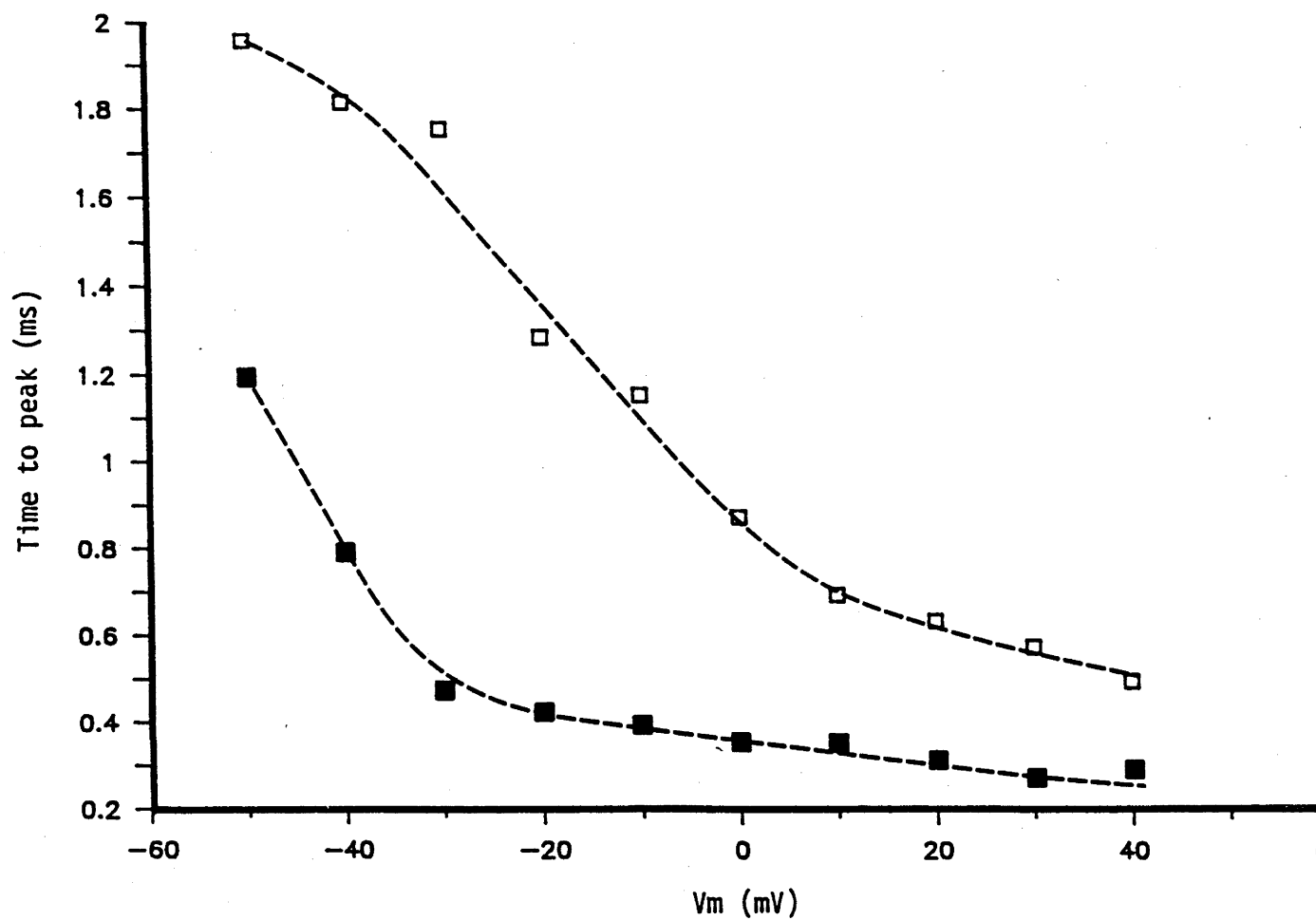
No attempt was made to construct conductance-voltage curves by correcting for 'inactivation' of channels because the decay of the currents could not always be fitted with a single exponential (see below).

### Time Course of Activation.

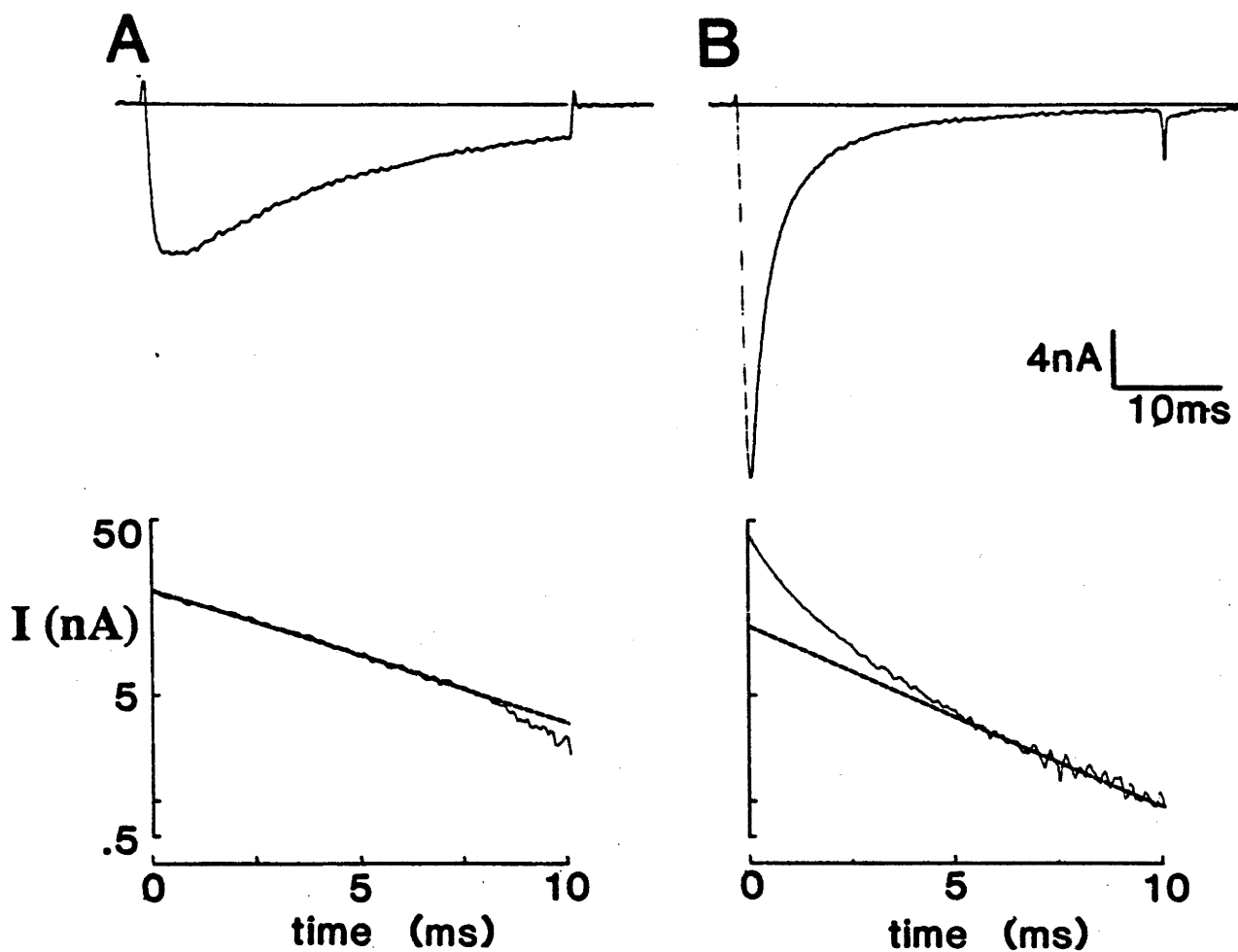
The rate of sodium activation was measured from the time to peak of the sodium current at each potential. The times to peak for the cell shown in Figure 4.4 are plotted against step potential in Fig 4.7 (solid squares). It can be seen that as the membrane is stepped to more depolarized potentials the sodium current reached peak amplitude more quickly. The time to peak saturated at a membrane potential of about 0mV. This saturation probably reflects the inability of the clamp to follow faster events because as shown by the open squares in Fig 4.7, when the activation of the sodium current is slowed by lowering the temperature to 18°C, no saturation is seen.

### Time Course of Inactivation.

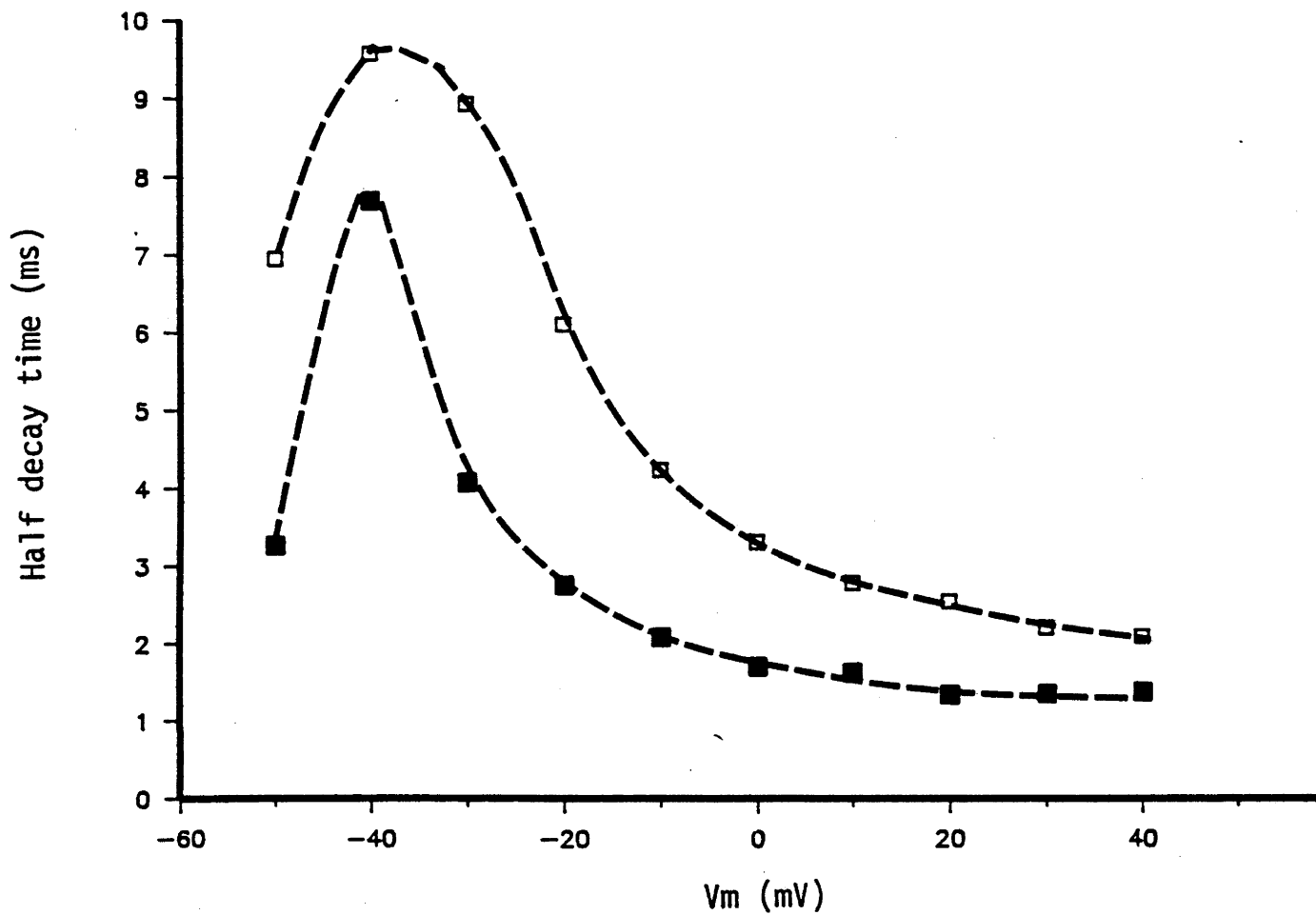
Inactivation followed a single exponential at potentials up to -20mV. At more depolarized potentials, however, the decay often displayed a second slower component. Currents from one cell in which this was particularly evident are shown in Fig. 4.8. In Fig. 4.8A is shown a sodium current elicited by a depolarization to -40mV, a semilogarithmic plot of the decay phase



**Figure 4.7.** Times to peak of sodium currents recorded at different potentials in a cell at 22 °C (solid squares) and 18 °C (open squares). Lines were drawn by eye.



**Figure 4.8.** Decay of sodium currents generated by pulses to  $-40\text{mV}$  (A) and  $0\text{mV}$  (B) from a holding potential of  $-100\text{mV}$ . Upper traces show the currents recorded and the graphs below contain semilogarithmic plots of their decays. The decay does not follow a single exponential at  $0\text{mV}$ . Vertical calibration  $4\text{nA}$ . Horizontal calibration,  $10\text{ms}$ .

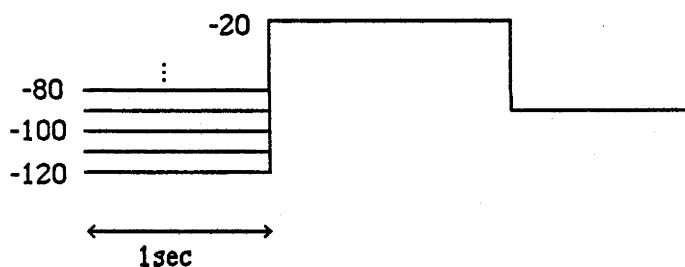


**Figure 4.9.** Half decay time (measured from the peak) of sodium currents recorded at different potentials in a cell at 22 °C (solid squares) and at 18 °C (open squares). Lines were drawn by eye.

is shown in the lower part of this Figure, and gives a straight line. The current at 0mV for the same cell, and its semilogarithmic plot are shown in Fig. 4.8B. Clearly, the current decay is more complex than a single exponential. Because of this nonexponential decay, inactivation was measured as the time for the sodium current to decay to half its peak value. Half decay time became smaller as the potentials became more positive. This is illustrated in Fig. 4.9 where the half decay times for the cell in Fig. 4.4 are plotted against membrane potential (filled squares). Inactivation was slowest at  $-40\text{mV}$  and became progressively faster with depolarization. The half decay times at a lower temperature ( $18^{\circ}\text{C}$ ) are also shown for comparison.

### Steady State Inactivation.

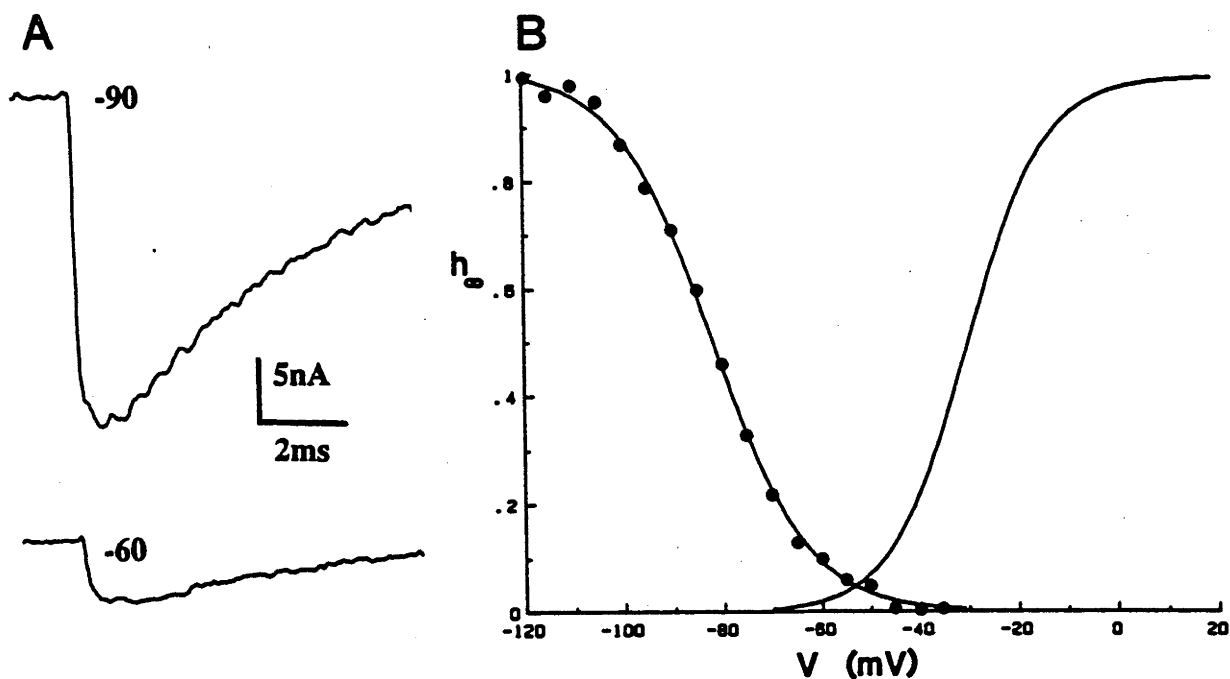
Steady state inactivation (Hodgkin and Huxley 1952c) was measured in one of two ways. Either the membrane potential was manually shifted to a new conditioning potential before a step to  $-20\text{mV}$ , or a 1sec conditioning potential was delivered immediately before the test step to  $-20\text{mV}$ :



Both methods gave identical results. Sodium currents recorded at  $-20\text{mV}$  from one cell at holding potentials of  $-90\text{mV}$  and  $-60\text{mV}$  are shown in Fig. 4.10A. The ratio ( $h_{\infty}$ ) of the current amplitude with the conditioning potential  $V$ , to that of the current with a conditioning potential of  $-120\text{mV}$  is plotted in Fig. 4.10B. The peak conductance-voltage curve from the same cell is also shown for comparison. The data could be fitted by an equation of the form

$$h_{\infty} = 1 / (1 + e^{(V-V')/k}) \quad \dots\dots\dots 4-3$$



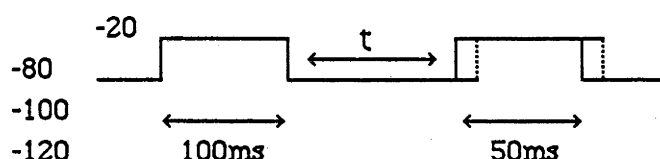


**Figure 4.10.** Steady state inactivation of sodium current. **A.** Sodium currents generated by a pulse to  $-20\text{mV}$  from 1sec conditioning potentials of  $-90\text{mV}$  (upper panel) and  $-60\text{mV}$  (lower panel). Vertical calibration  $5\text{nA}$ . Horizontal calibration,  $2\text{ms}$ . **B.** The amplitude of the sodium current following a conditioning to different potentials is expressed as a fraction of the sodium current generated following a conditioning step to  $-120\text{mV}$  (holding potential  $-100\text{mV}$ ). The solid line through the points is the line of best fit (equation 4-3) and gives a  $V'$  of  $-81.9\text{mV}$  and a  $k$  of  $9.6\text{mV}$ . Normalised conductance ( $g$ , solid line without data points) calculated from currents generated in the same cell by voltage steps to potentials shown on the abscissa from a holding potential of  $-100\text{mV}$  are shown for comparison.

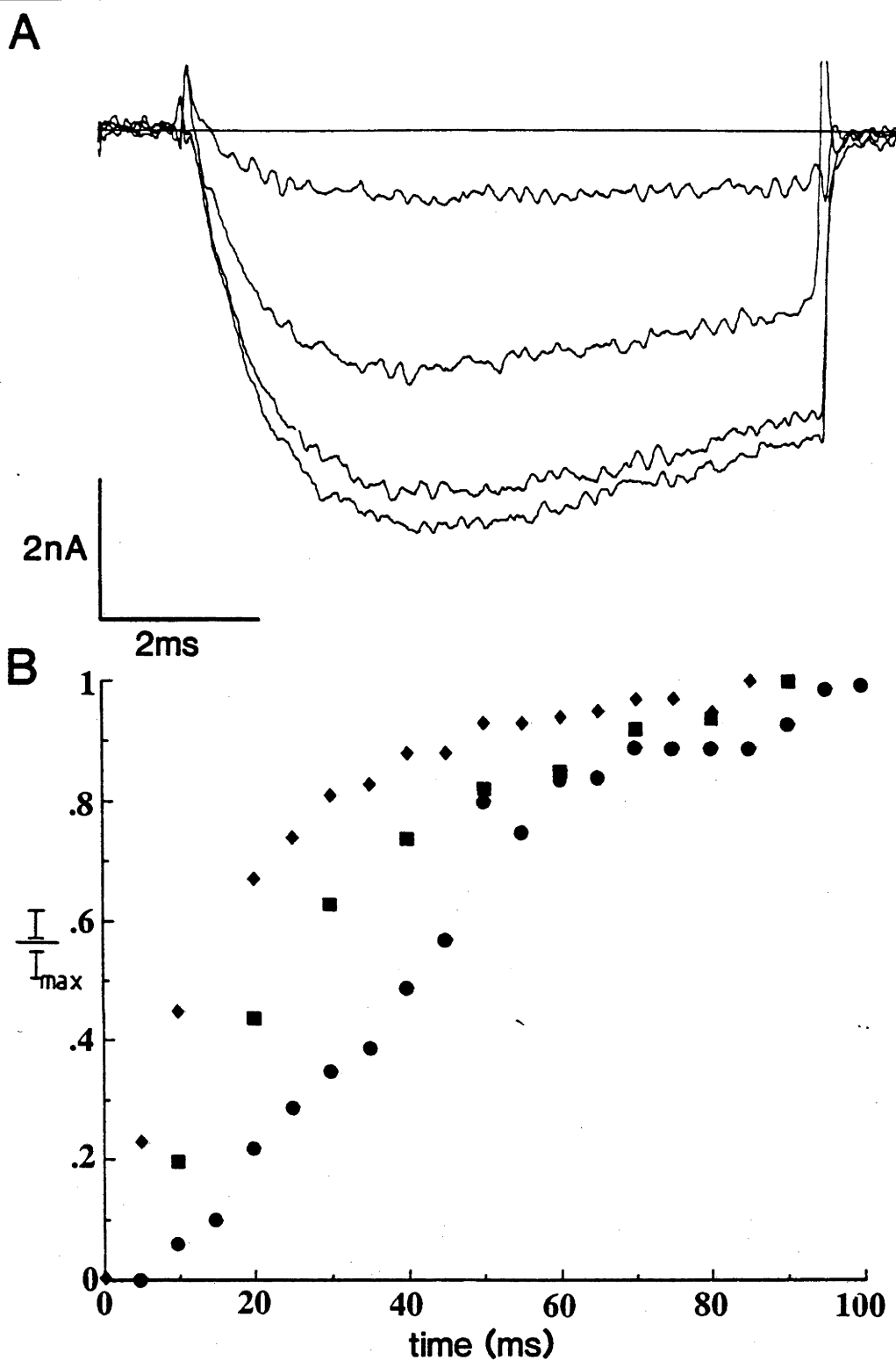
(Hodgkin and Huxley 1952c), where the half inactivation voltage ( $V'$ ) was  $-81.9\text{mV}$  and the slope factor ( $k$ ) was  $9.6\text{mV}$ . Average values from 8 cells were  $-74.9 \pm 2.0\text{mV}$  and  $7.7 \pm 1.0\text{mV}$  respectively. At  $-65\text{mV}$ , the resting potential of hippocampal pyramidal cells (Chapter 2; papers in Dingledine, 1984), the  $h_{\infty}$  value would be about 0.4.

### Recovery from Inactivation.

Measurements were also made of the time course of recovery from inactivation. This was done using the following pulse sequence.



The cell was voltage clamped at  $-100\text{mV}$  and a conditioning voltage pulse to  $-20\text{mV}$  was delivered for  $100\text{ms}$  after which a second pulse to  $-20\text{mV}$  was delivered for  $50\text{ms}$ . The time interval between the pulses ( $t$ , in the above diagram) was varied between 0 and  $200\text{ms}$ . A test pulse which immediately followed the conditioning pulse did not elicit any current, but as the interpulse interval was made longer the test pulse current became progressively larger until it was equal to the amplitude of the current produced by the prepulse. Fig. 4.11A shows the progressively larger current elicited by the test pulse for interpulse durations of 10, 30, 50 and  $70\text{ms}$ . Fig. 4.11B shows the ratio of the current amplitude with the prepulse, to the amplitude without the prepulse plotted against the interpulse duration for holding potentials of  $-80$ ,  $-100$  and  $-120\text{mV}$ . As expected from the multiexponential nature of the onset of inactivation recovery from inactivation follows a time course with at least two exponentials and was faster at more negative potentials.



**Figure 4.11.** Recovery from inactivation. **A.** Currents generated by a test pulse to  $-20\text{mV}$  at 10, 30, 50 and 70ms after a 100ms conditioning pulse to  $0\text{mV}$  (holding potential,  $-100\text{mV}$ ). Vertical calibration 2nA. Horizontal calibration, 2ms. **B.** Amplitude of sodium current after a conditioning depolarization, expressed as a fraction of the amplitude with no conditioning pulse ( $I/I_{\max}$ ), plotted against time after the conditioning pulse. The graph shows data obtained with holding potentials of  $-80\text{mV}$  (circles),  $-100$  (squares) and  $-120\text{mV}$  (diamonds).

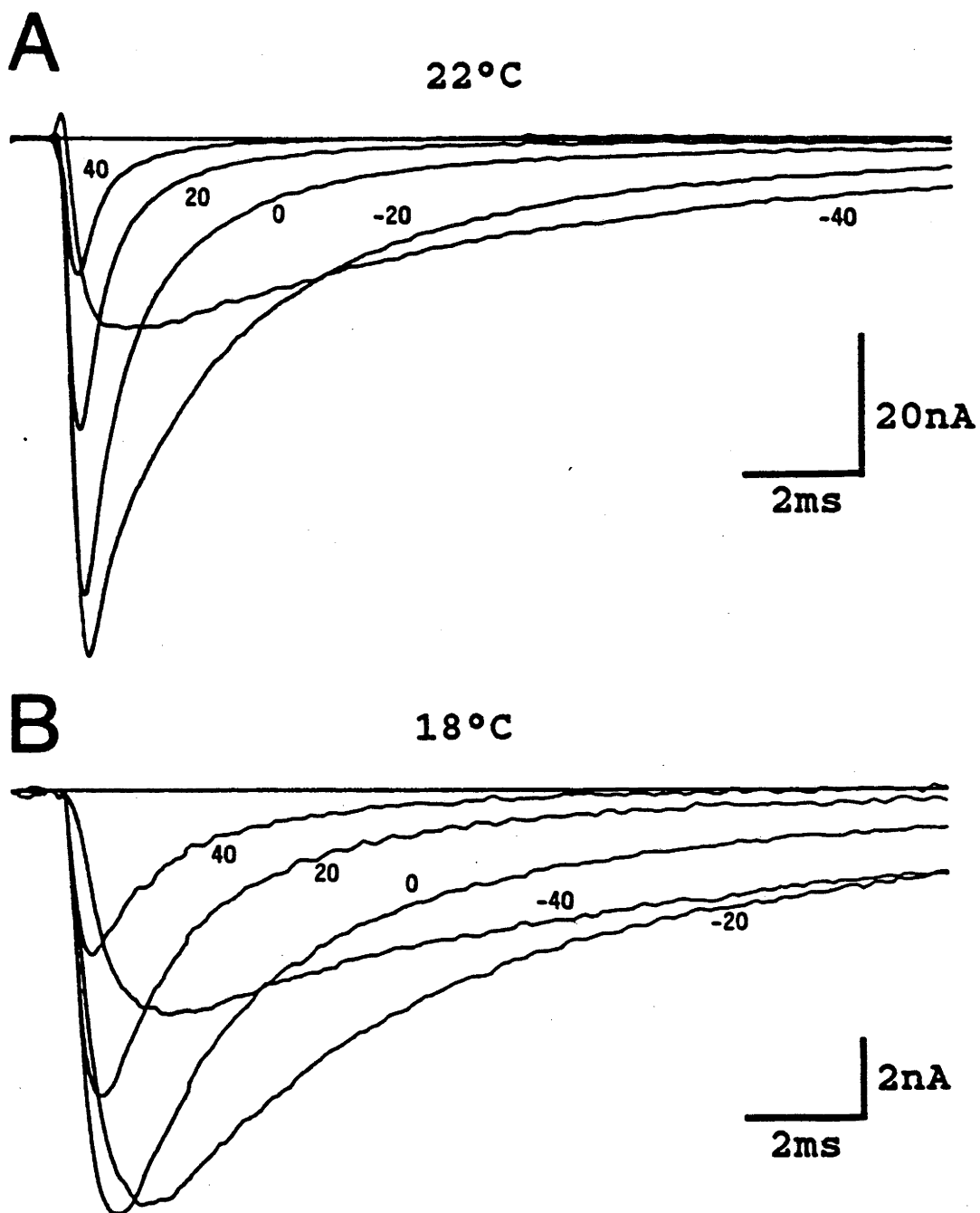
### Effect of Temperature.

In three cells the effect of temperature on sodium current kinetics was examined. As the temperature was lowered, the currents became smaller and slower as illustrated in Figure 4.12. The upper traces were recorded at 22°C and the lower traces at 18°C (note the difference in vertical calibrations). The decrease in peak amplitude was prominent in all three cells. The conductance voltage curves for two temperatures, 18°C and 22°C, in one cell are shown in Figure 4.13. It can be seen that peak conductance fell from 1.2  $\mu$ S at 22°C to 0.37  $\mu$ S at 18°C. The  $k$  value changed from 7.1 mV to 13 mV but  $V'$  was essentially unchanged. These effects were fully reversible on returning to the higher temperature.

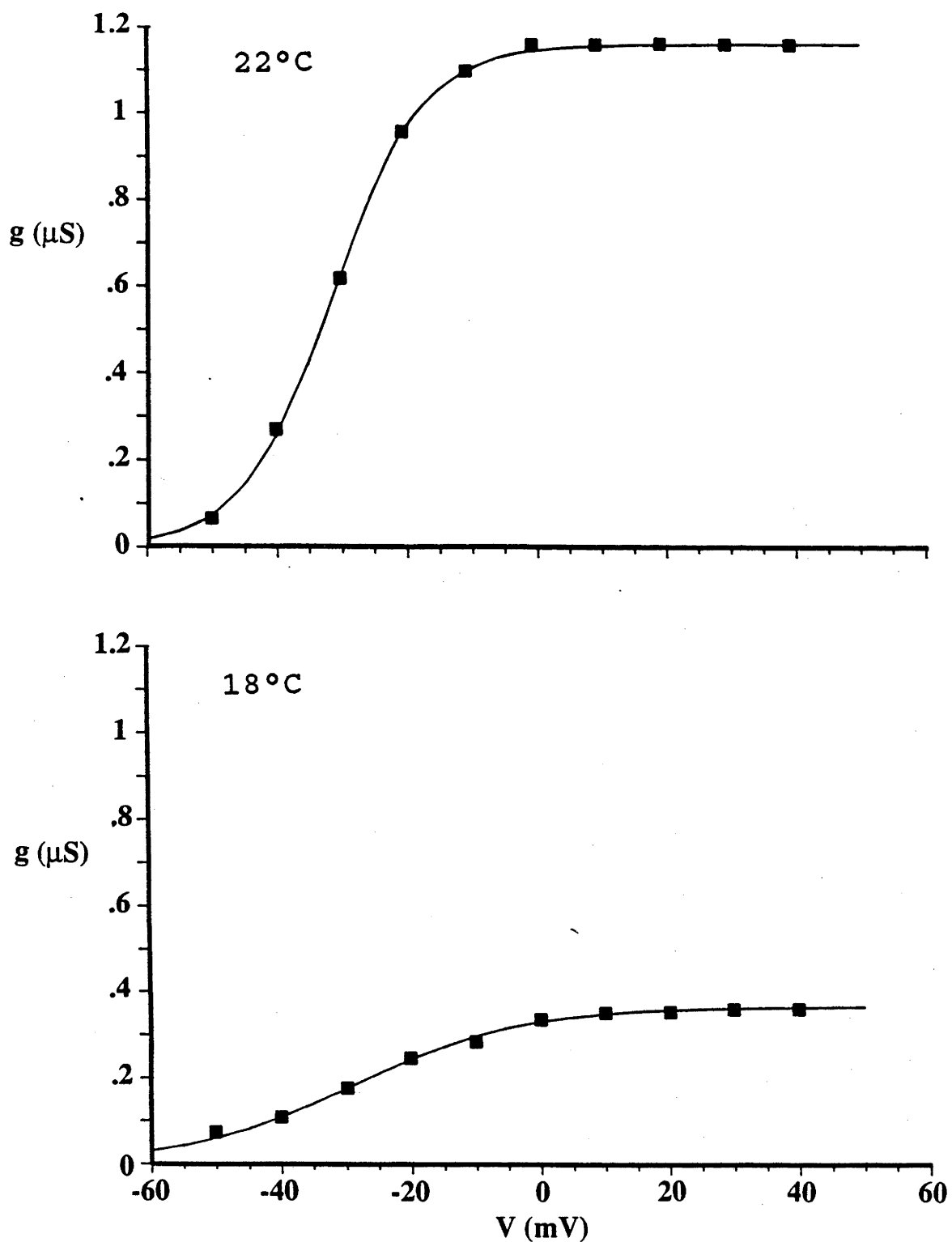
Peak current-voltage curves recorded in one of the cells over a range of temperatures are shown in Fig 4.14A. It can be seen that there was a larger fall in current amplitude between 20°C and 17.5°C than between 22.5°C and 20°C. Maximum current amplitude is plotted semilogarithmically against temperature in Fig 4.14B. The line through the points at 20°C to 26°C gave a  $Q_{10}$  of 1.5. The current amplitude at 17.5°C is less than expected from the line fitted to the points at the other three temperatures.

A similar phenomenon was seen with time to peak and half decay times, as illustrated in Figure 4.15. There appeared to be a steep change in both measures between 17.5 and 20°C. All three cells showed a clear break in the temperature dependence of current amplitude and time course in this temperature range.

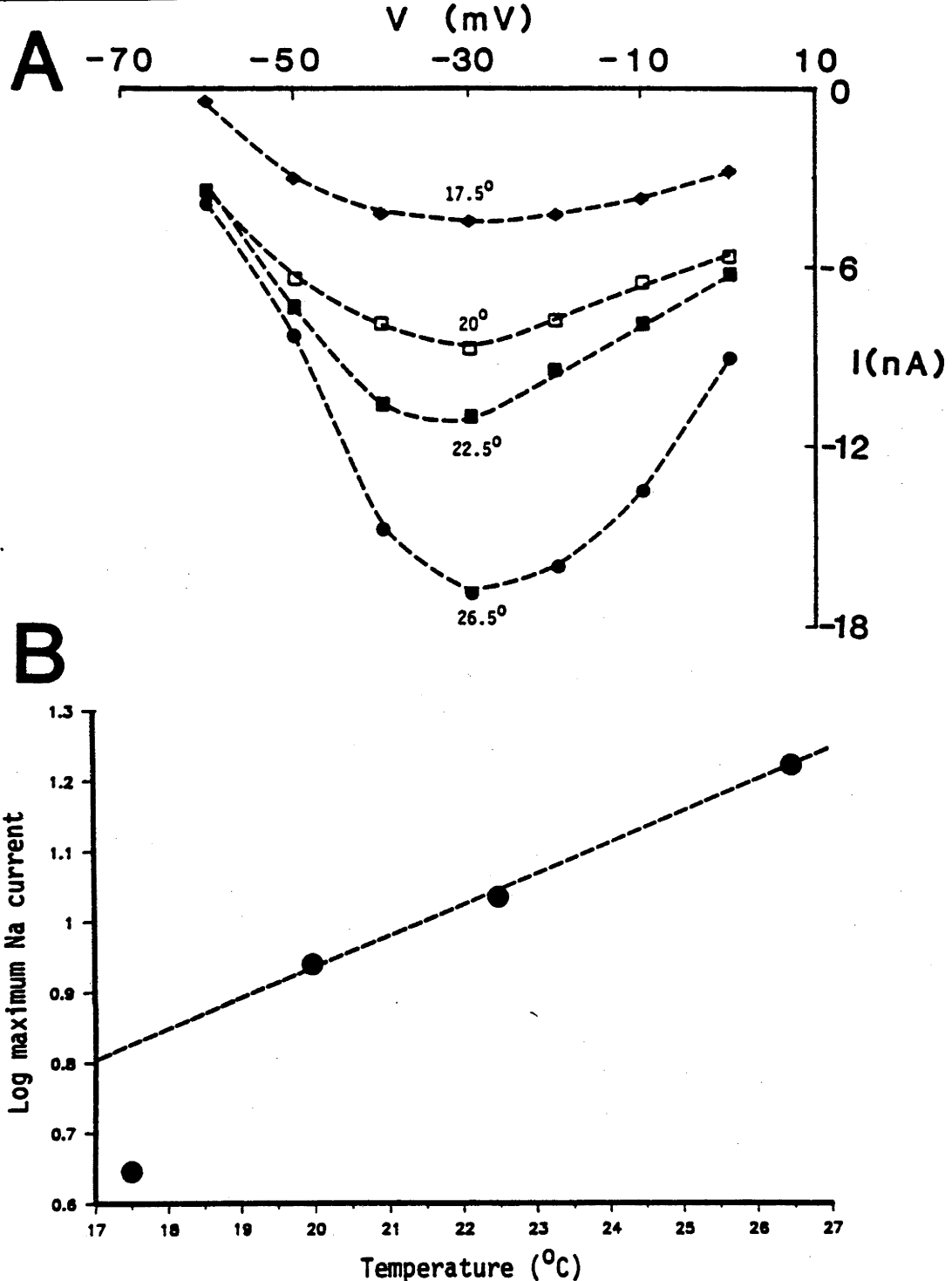
In one cell in which it was possible to do a full analysis it was found that as reported for rabbit (Chiu, Mrose and Ritchie, 1979) and rat (Schwarz, 1986) myelinated nerve, the  $h_{\infty}$  curve shifted to more positive potentials as the temperature was raised. Results from this cell are shown in Figure 4.16. It is clear that as the temperature is raised the curve shifts to more positive potentials.  $V'$  values for the two temperatures 23 and 20°C were -81.9 and -89.5 mV respectively. The slopes of the fitted Boltzmann functions were 9.9 and 9.7 mV respectively.



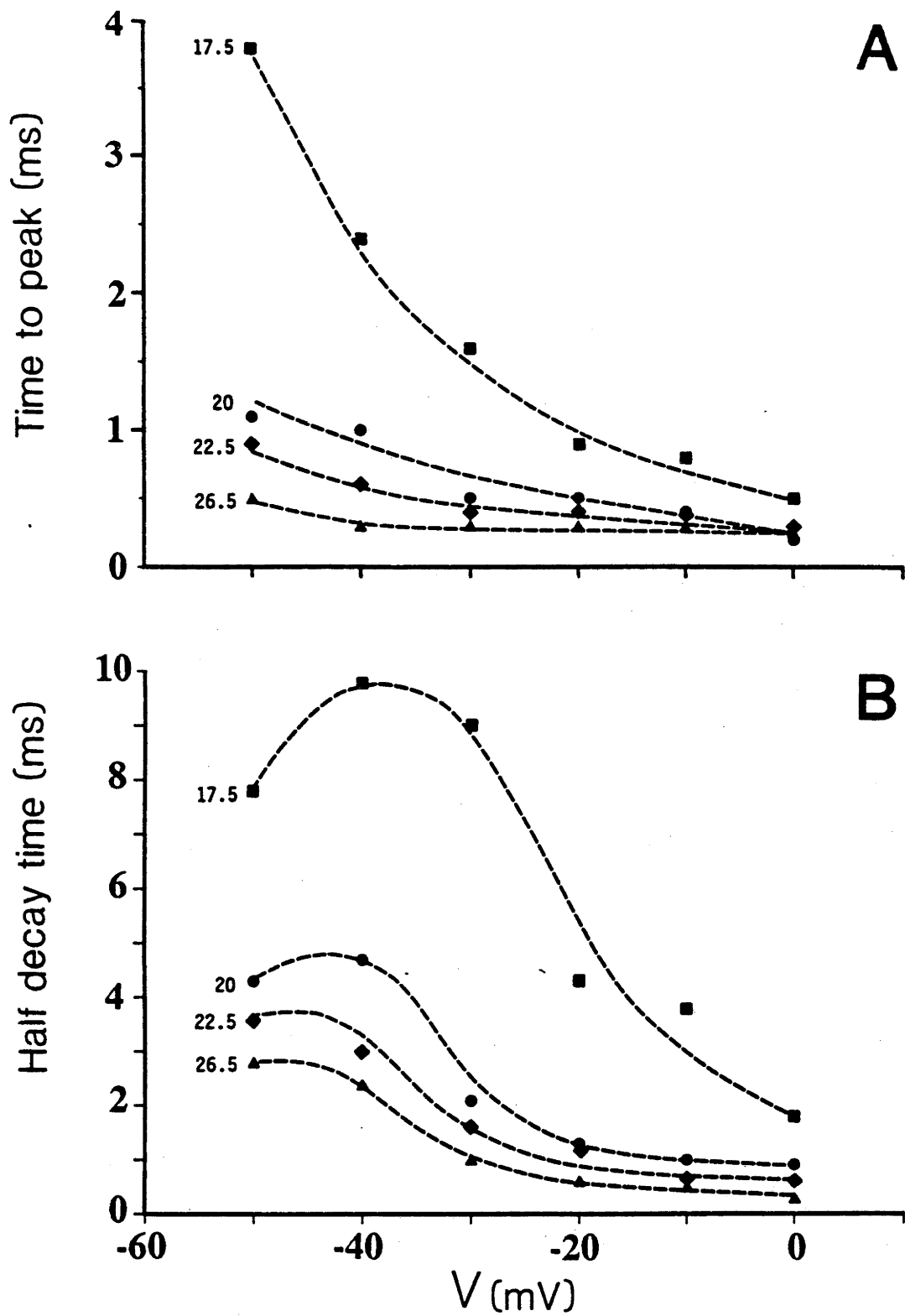
**Figure 4.12.** Effect of temperature on sodium currents. Sodium currents generated by pulses from a holding potential of  $-100\text{mV}$  to the potentials shown, recorded in the same cell at  $22^{\circ}\text{C}$  (A) and at  $18^{\circ}\text{C}$  (B). Note the difference in vertical calibrations in A (20nA) and B (2nA). horizontal calibration 2ms.



**Figure 4.13.** Effect of temperature on conductance-voltage relations. **A.** The squares show conductance calculated according to equation 4-1 ( $E_{Na} = +65\text{mV}$ ) from the peak amplitude of currents recorded at  $22^\circ\text{C}$ . The solid line shows the best fit of equation 4-2.  $V'$  and  $k$  were  $-31$  and  $7.1\text{mV}$  respectively. **B.** Conductance was calculated as in **A** from currents recorded at  $18^\circ\text{C}$ .  $V'$  and  $k$  values for the solid line were  $-29\text{mV}$  and  $13\text{mV}$  respectively.

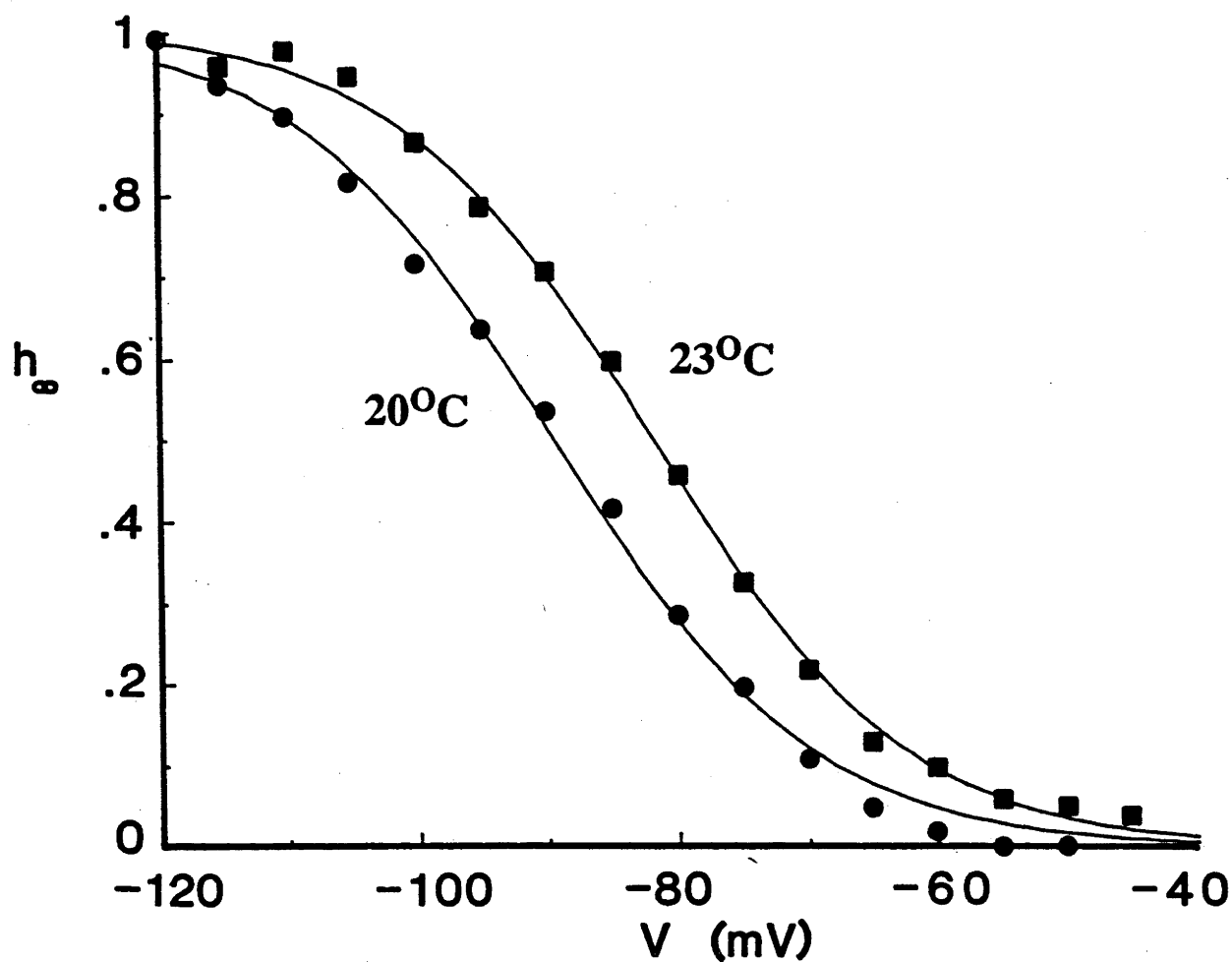


**Figure 4.14.** Effect of temperature on maximum sodium current. **A.** I-V curves (peak current) recorded at different temperatures in the same cell (from above down: 17.5  $^{\circ}$ C, diamonds; 20  $^{\circ}$ C, open squares; 22.5  $^{\circ}$ C, solid squares; 26.5  $^{\circ}$ C, circles). **B.** Semilogarithmic plot of maximum sodium current (nA) (measured at -20 to -40 mV with a holding potential of -100 mV) against temperature ( $^{\circ}$ C). The solid line, drawn by eye through the points from 20  $^{\circ}$ C to 27  $^{\circ}$ C gives a  $Q_{10}$  of 1.5.



**Figure 4.15.** Effect of temperature on the time course of sodium currents (17.5 °C, squares; 20 °C, circles; 22.5 °C, diamonds; 26.5 °C, triangles). **A.** Time to peak of sodium currents recorded at different temperatures in the same cell. **B.** Half decay times of the same sodium currents at different temperatures.



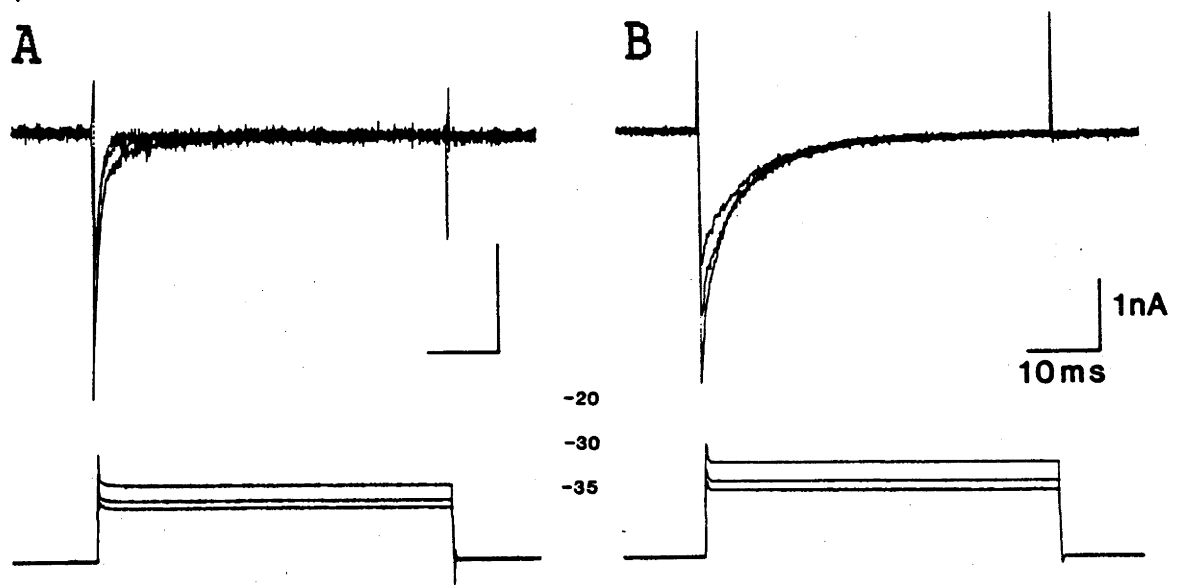


**Figure 4.16.** Effect of temperature on steady state inactivation. Steady state inactivation ratio ( $h_{\infty}$ ) measured as described in the text at 20 °C (circles) and 23 °C (squares). Solid lines were the lines of best fit to equation 4-3.  $V'$  and  $k$  values were  $-81.9\text{mV}$  and  $9.9\text{mV}$  and  $-89.5\text{mV}$  and  $9.7\text{mV}$  at 23 °C and 20 °C respectively.

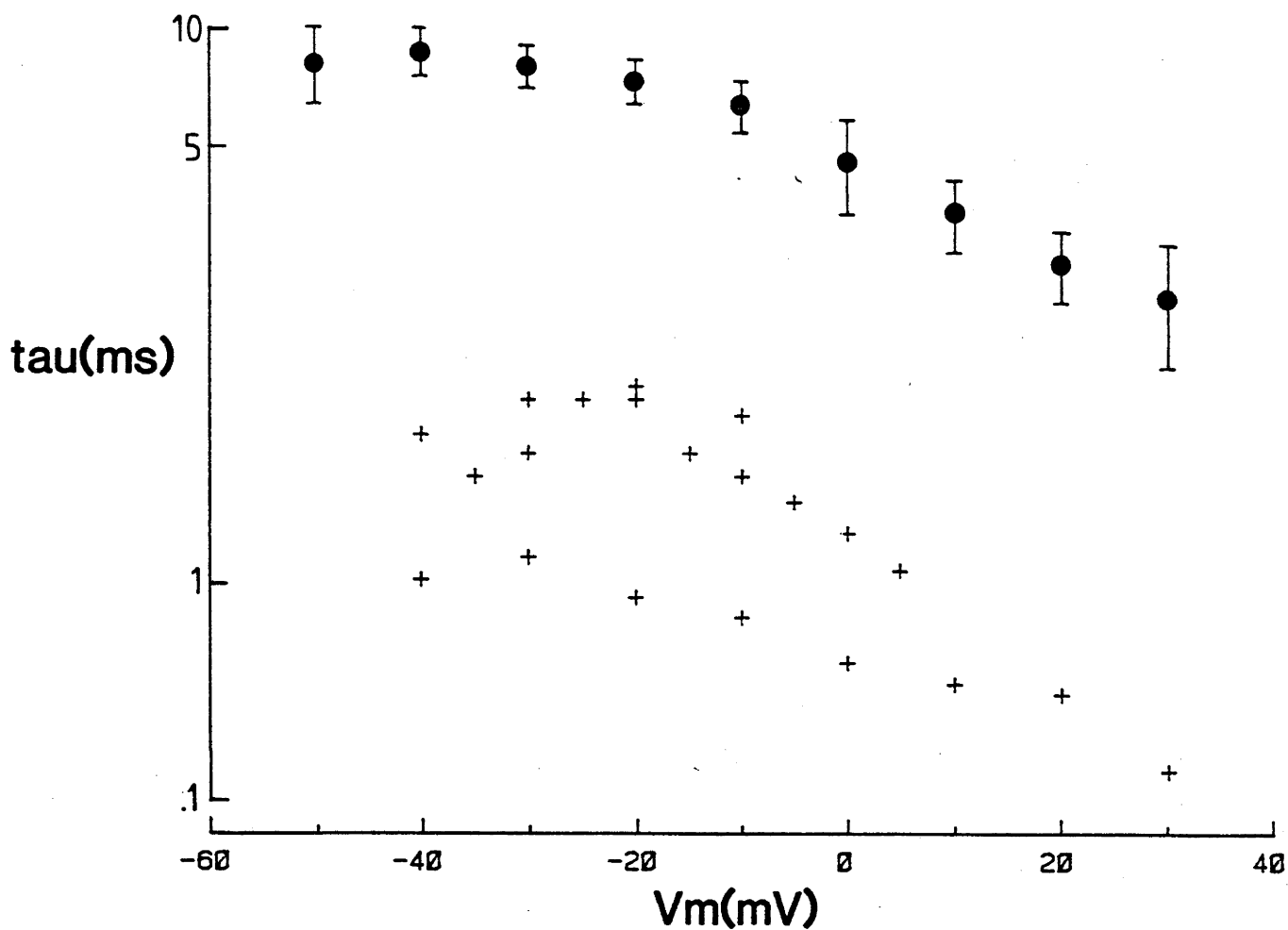
### Sodium Currents with Different Kinetics.

In 15 cells out of a total of 120 cells studied we found that the sodium current had different kinetics - both the activation and the inactivation of the sodium current in these cells was much faster. Figs. 4.17 and 4.19 illustrate the properties of one such fast cell in which we were able to do a complete analysis. In Fig. 4.17B are shown sodium currents which displayed the usual 'slow' kinetics as already described above. Fig. 4.17A illustrates the 'fast' sodium currents which are seen in some cells. It can be seen that the time course of the currents in Fig. 4.17A is much faster than those in 4.17B. The difference in the time course was quantified by fitting single exponential functions to decay phase of the currents. As shown in Fig. 4.8 the decay of the 'slow' sodium current at potentials above  $-20\text{mV}$  is not a single exponential, in these cases an exponential was fit to the initial (fast) decay of the current. Average time constants of decay measured in 11 'slow' cells are shown in Fig 4.18(circles). The time constants from 3 'fast' cells are shown by the crosses. It is clear that the 'fast' cells represent a different population of cells - distinct from the 'slow' ones. Half decay times from the same three cells are shown in Table 4.1. It is clear that the decay of sodium current in the 'fast' cells is almost an order of magnitude faster than in the 'slow' cells.

The peak current - voltage relation for a 'fast' sodium current is shown in Fig. 4.19A. The activation and steady state inactivation kinetics for this cell are shown in Fig. 4.19B. The half maximal value and slope values for a Boltzmann fit to the activation were  $-29\text{mV}$  and  $5.7\text{mV}$  respectively and the values for inactivation were  $-67.6\text{mV}$  and  $7.4\text{mV}$ . These curves are very similar to those for the more common sodium currents (see Fig. 4.10).



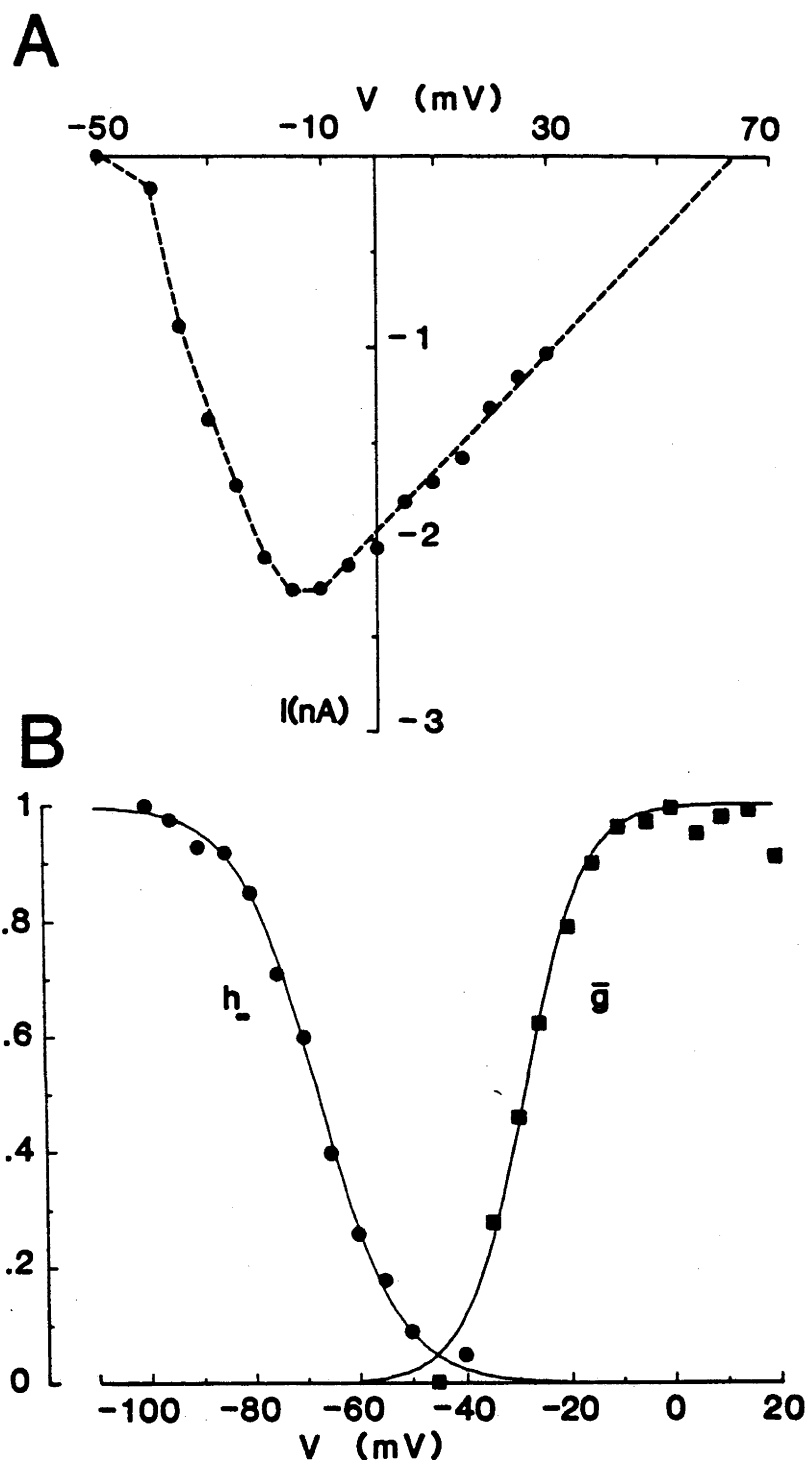
**Figure 4.17.** "Fast" sodium currents (A) compared with normal sodium currents recorded in a different cell (B). Upper traces show sodium currents generated by voltage steps (lower traces) to  $-35$ ,  $-30$  and  $-20$  mV from a holding potential of  $-100$  mV. Vertical calibration 1 nA. Horizontal calibration, 10 ms.



**Figure 4.18.** Time course of decay of "fast" sodium currents compared with normal sodium currents. Circles show average time constant of decay (measured as described in text;  $\pm$ SEM) measured in 9 cells. The crosses show individual measurements of time constant of decay from three cells which fast "fast" sodium current decay. All cells were voltage clamped at  $-100\text{mV}$  and given depolarizing commands to the potentials on the abscissa.

**TABLE 4.1. Time constant of decay for two populations of sodium channels.**

| V (mV) | Taus(ms)      |               |
|--------|---------------|---------------|
|        | 'slow'        | 'fast'        |
| -40    | $8.8 \pm 1.2$ | 0.9           |
| -20    | $7.4 \pm 0.9$ | $0.9 \pm 0.2$ |



**Figure 4.19.** Properties of the "fast" sodium current. **A.** An I-V curve showing the change in amplitude of the sodium current with voltage (holding potential  $-100\text{mV}$ ). **B.** A normalised conductance ( $\bar{g}$ ) - voltage curve (squares) and  $h_{\infty}$  curve (circles) recorded in the same cell. Solid lines through the data points are the Boltzmann curves of best fit (equations 4-2 and 4-3 in text).

## DISCUSSION

The data presented in this chapter demonstrate that neurones acutely isolated from the adult guinea pig hippocampus can be voltage clamped using a single electrode voltage clamp. Under voltage clamp, these cells display voltage dependent inward and outward currents. The inward current can be blocked with tetrodotoxin, decreased in amplitude when the extracellular sodium concentration was reduced and had a null potential close to the sodium equilibrium potential, typical characteristics of a sodium current. This current underlies the upstroke of the action potential in these neurones and has properties similar to the sodium current seen in many other preparations.

### Clamp Fidelity

The conditions of our experiments were specifically chosen to allow the application of a fast, high gain, voltage clamp to dissociated hippocampal neurones. Dissociation removes all synaptic input to the neurone and the majority of the dendritic tree. This yields an isopotential cell with high input impedance. A discontinuous single electrode voltage clamp using whole cell patch clamp electrodes provides near ideal conditions for clamping fast, large amplitude conductance changes in these cells. With a discontinuous voltage clamp, errors associated with series resistance between recording headstage and cell membrane (Hodgkin et al., 1952) are avoided (Finkel and Redman, 1984). The use of low resistance and low capacitance electrodes allows high clamp gains and switching between current injection and voltage recording at up to 50kHz. Although under optimal conditions the membrane potential settled to the command potential in under 400 $\mu$ s in these cells (see Fig 3.3) it was still necessary to work at relatively low temperatures (22-24  $^{\circ}$ C) in order to control membrane potential during the rising phase of some sodium currents. At these temperatures the clamp settled before the peak of the sodium current for potentials less than -10mV. However, at more depolarized potentials there would be some error in measurements of peak sodium currents. The fact that the extrapolated reversal potential from the peak current-voltage graphs is very close to the predicted Nernst potential for sodium (Figs. 4.4,4.5) makes us confident that this error is small.

## Contaminating Currents

Hippocampal neurones have been shown to possess a variety of voltage and calcium activated conductances all of which could potentially contaminate the sodium currents reported here. Each of these currents will be dealt with in turn.

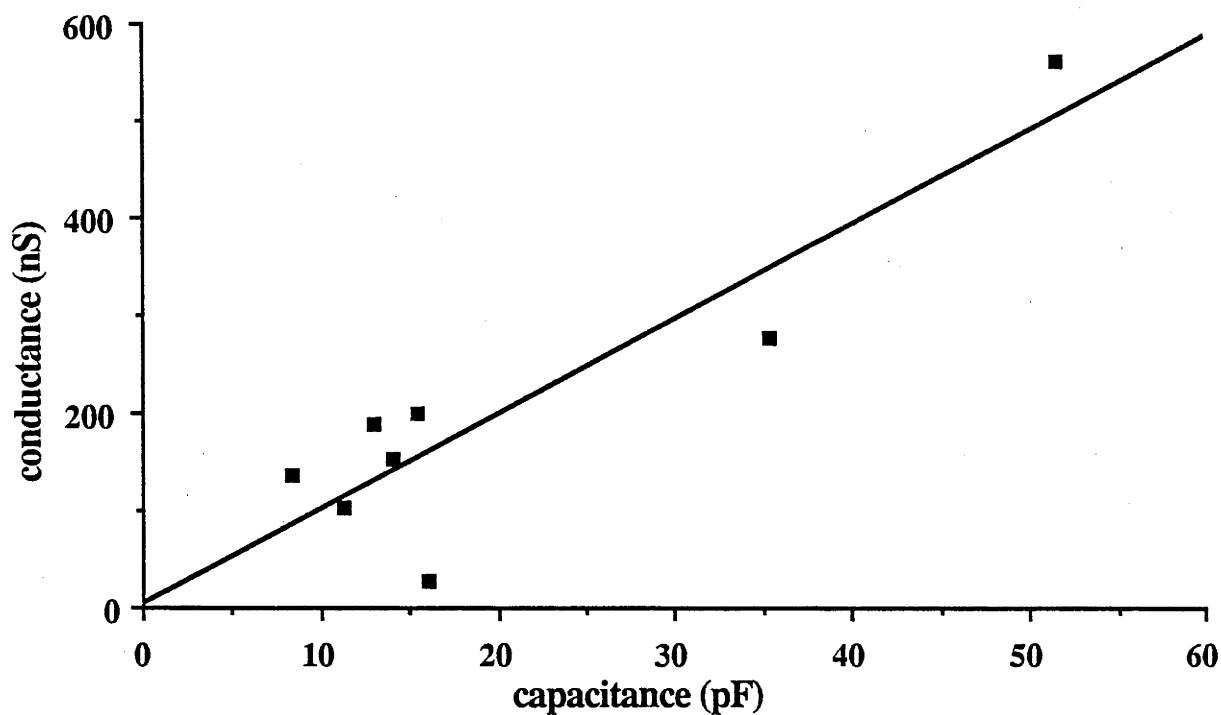
The outward current is completely blocked by application of caesium ions to the interior of the cell and will be discussed in Chapter 6.

A residual small and slower, TTX-insensitive inward current, probably a calcium current (Kay et al., 1986), was sometimes seen (Fig. 4.2) but was not examined further. When present, it would have made an insignificant contribution to the total inward current and was not apparent in many cells (eg. see Fig 4.1). It is interesting that calcium currents recorded from CA1 neurones in hippocampal slices (Johnston et al., 1980; Brown and Griffith, 1983b; Chapter 2), are much larger (1 - 3nA) than reported here. Calcium currents have been shown to be very labile (Kostyuk, 1984; Fenwick et al., 1982) and run down during intracellular perfusion, it is thus possible that in our isolated neurones they were 'washed out' during the dissociation procedure. Another possibility is that most of the calcium channels may be located on the dendrites (Llinas and Sugimori, 1980; Jahnsen, 1986; Chapter 2) and are thus removed during dissociation.

A slow non-inactivating, TTX-sensitive sodium current found in rat hippocampal CA1 neurons by French and Gage (1985) (see also Stafström et al., 1985) could also be demonstrated in dissociated neurons. This current, like the calcium current, was relatively very small (maximum amplitude < 0.6nA, see Chapter 5) and had little effect of the amplitude or time course for the larger sodium current.

A voltage activated chloride current has recently been described in CA1 pyramidal cells in hippocampal slices (Madison, Malenka and Nicoll, 1986). This current is tonically active at the resting membrane potential and is switched off by membrane depolarization. If this current were present in dissociated hippocampal neurones, we would expect to see an instantaneous outward step of current at the onset of depolarizing voltage steps. No such instantaneous currents were ever observed. Furthermore we would expect to see chloride current relaxations during long depolarizing steps as the chloride current switched off (Madison, Malenka and





**Figure 4.20.** Peak sodium conductance as a function of cell capacitance. Each data point was obtained from a different cell. The straight line is the linear regression line of best fit and gives the average cell sodium conductance as 15 nS/pF.

Nicoll, 1986). No such relaxations were ever observed (eg. see next two chapters). It is possible that the presence of fluoride as the major intracellular cation somehow blocked this current. A simpler explanation, however, is that as suggested by the above authors, the chloride current is indeed localized to the dendrites and therefore removed during the isolation procedure.

No evidence was found for the TTX insensitive sodium current seen in some preparations (Kostyuk, Veselosky and Tsyndrenko, 1981; Ikeda et. al., 1986).

### Comparison with Other Sodium Currents

The decay of sodium currents recorded in this study are generally of a slower time course than those recorded at room temperature from rabbit or rat node of Ranvier (Chiu et. al., 1979; Neumcke and Stampfli, 1982), bovine chromaffin cells (Fenwick, Marty and Neher, 1982), cultured chick dorsal root ganglion neurones (Carbone and Lux, 1986), GH<sub>3</sub> cells (Vandenberg and Horn, 1984) or cultured rat sympathetic neurons (Belluzzi and Sacchi, 1986). The time to peak and decay times measured from different preparations are compared in Table 4.2. The time course of activation is comparable to that in other preparations, however the decay of the currents is much slower as evidenced by the large value of the ratio  $\tau_h/\tau_p$ .

Peak sodium conductance was different from cell to cell and varied between 7.2 and 14.7 mS.cm<sup>-2</sup>. Assuming a single channel conductance of 18pS (Sigworth and Neher, 1980), suggests that there are between 5 and 10 sodium channels per  $\mu\text{m}^2$ . Another way to look at this data is to plot the peak sodium conductance against cell capacitance as has been done for 8 cells in Figure 4.20. The straight line through the points is a linear regression fit to the points and gives an average cell conductance of 15nS/pF. Again using the 18pS/channel this gives a figure of 8.5 channels/ $\mu\text{m}^2$ . This is similar to the sodium channel density on bovine chromaffin cells (Fenwick et. al., 1982) but about five times the number in GH<sub>3</sub> cells (Matteson and Armstrong, 1984; Dubinsky and Oxford, 1984). By comparison, there are in the order of a hundred channels per  $\mu\text{m}^2$  in neuroblastoma cells (Moolenaar and Spector, 1978), in frog and rat node of Ranvier and in the squid giant axon (for review see Hille, 1984). Densities of sodium channels in different preparations are compared in Table 4.3.

TABLE 4.2. Sodium current kinetics in different preparations.

| Cell                   | <sup>1</sup> t <sub>p</sub><br>(ms) | <sup>2</sup> τ <sub>h</sub><br>(ms) | τ <sub>h</sub> /t <sub>p</sub> | <sup>3</sup> V<br>(mV) | Temp.<br>(°C) |
|------------------------|-------------------------------------|-------------------------------------|--------------------------------|------------------------|---------------|
| Squid Axon             | 0.5                                 | 1.5                                 | 3                              | 0                      | 5             |
| Frog Node              | 0.15                                | 0.5                                 | 3.3                            | 0                      | 20            |
| Aplysia Neurone        | 5                                   | 18                                  | 3.6                            | -6                     | 13.5          |
| Mouse Neuroblastoma    | 0.5                                 | 0.5                                 | 1                              | -8                     | 20            |
| Bovine Chromaffin      | 0.7                                 | 1.2                                 | 1.7                            | 0                      | 20            |
| Rat Pituitary GH3      | 1.5                                 | 2.2                                 | 1.5                            | 0                      | 22            |
| Rat Pituitary GH3      | 0.7                                 | 1.1                                 | 1.6                            | +20                    | 22            |
| Chick DRG              | 1.1                                 | 1.5                                 | 1.4                            | 0                      | 12            |
| Rat Brain cDNA II      | 0.15                                | 0.5                                 | 3.3                            | 0                      | 15            |
| G. Pig Hippocampus CA1 | 0.3                                 | 2 <sup>a</sup>                      | 6.7                            | 0                      | 24            |

Table modified from Table 2 in Carbone and Lux (1986).

<sup>1</sup> Time to peak.

<sup>2</sup> Time constant of decay.

<sup>3</sup> Voltage at which measurements made.

<sup>a</sup> Time to half decay.

**TABLE 4.3. Sodium Current density in different preparations.**

| Cell                   | <sup>1</sup> I <sub>p</sub><br>(mA.cm <sup>-2</sup> ) | <sup>2</sup> E <sub>t</sub><br>(mV) | density<br>(channels/μm <sup>2</sup> ) | Temp.<br>(°C) |
|------------------------|-------------------------------------------------------|-------------------------------------|----------------------------------------|---------------|
| Squid Axon             | 1.3                                                   | -10                                 | 330                                    | 5             |
| Frog Node              | 13                                                    | -20                                 | -                                      | 20            |
| Frog Node              | -                                                     | -                                   | 1,900                                  | 13            |
| Frog Node              | -                                                     | -                                   | 400-920                                | 2-5           |
| Snail Neurone          | 0.5                                                   | -6                                  | -                                      | 13.5          |
| Mouse Neuroblastoma    | 0.4                                                   | -10                                 |                                        | 20            |
| Bovine Chromaffin      | 0.06                                                  | +10                                 | 1.5-10                                 | 21            |
| Rat Pituitary GH3      | 0.1                                                   | +12                                 | -                                      | 22            |
| Rat Pituitary GH3      | 0.2                                                   | 0                                   | -                                      | 22            |
| Rat DRG                | 0.07                                                  | -25                                 | -                                      | 20            |
| Guinea Pig DRG         | 2.5                                                   | 0                                   | -                                      | 20            |
| Chick DRG              | 0.5                                                   | +10                                 | -                                      | 25            |
| Chick DRG              | 0.2                                                   | -10                                 | 2-20                                   | 12            |
| G. Pig Hippocampus CA1 | 1.3                                                   | -20                                 | 5-10                                   | 24            |

Table modified from Table 3 Carbone and Lux (1986).

<sup>1</sup> Peak sodium current.

<sup>2</sup> Voltage at which peak current measured

With a membrane capacitance of  $1\mu\text{F}\cdot\text{cm}^{-2}$ , a sodium equilibrium potential of  $+70\text{mV}$ , a sodium channel density of 5 to 10 channels. $\mu\text{m}^{-2}$  and a peak inward current at  $-20\text{mV}$ , the maximum rate of rise of the membrane depolarization in these neurones would be 600 to  $1200\text{Vs}^{-1}$ . This figure is close to the measured rates of rise of action potentials of 600 to  $1000\text{Vs}^{-1}$  (Chapter 3) and indicates that the sodium current described here is responsible for the rising phase of the action potentials recorded in these cells.

Both activation and inactivation (Fig. 4.10) occurred at more negative potentials than has been reported for sodium channels in other mammalian neurons (Barrett and Crill, 1980; Matteson and Armstrong, 1983; Fenwick et al., 1982; Fernandez et al, 1984; Vandenberg and Horn, 1984). However, activation of mammalian brain sodium channels expressed in oocytes (Stümer et al., 1987) occurred at potentials similar to those reported here. Some of the differences in voltage dependence of activation and particularly inactivation may be associated with differences in temperature (Schwarz, 1986, and below). A further difference may be due to the introduction of fluoride ions into the cell interior.

Fernandez et al.(1984) have suggested that, with time, the presence of internal fluoride shifts the voltage dependence of sodium currents in the hyperpolarizing direction. This does not seem to happen in the squid giant axon (Adams and Oxford, 1983). Most cells in this study were recorded from for at least 10 to 15 minutes after obtaining access to the interior of the cell. Although we did not systematically look for a change in voltage dependence with time, no difference in voltage dependence was obvious between the start and end of an experiment.

In many preparations, particularly at the node of Ranvier of both frog and rat nerve(Chiu, 1977; Neumcke and Stampfi, 1982) and rat heart (Brown et. al., 1981b) sodium current inactivation follows a biexponential time course. In hippocampal neurones however, only one time constant was generally observed although in several cells and particularly at depolarized potentials, two time constants were evident (Fig 4.8). It is possible that this biexponential decay may be an artifact due to activation of the noninactivating sodium current (Chapter 5) or a calcium current. These possibilities, however, were not explored further in this study.

A very slow inactivation with a time course of minutes has recently been described in skeletal muscle (Simoncini and Stümer, 1987). If such a long term inactivation process was also

present in hippocampal neurones then the densities of channels calculated here would have been underestimated by a factor of about 80%. Because of the difficulty in voltage clamping dissociated neurones to very negative membrane potentials for long periods of time characterization of the slow steady state inactivation was not attempted.

The macroscopic and single channel properties of sodium channels expressed by injection of one rat brain sodium channel cDNA (sodium channel II, Noda et al 1986a,b) into *Xenopus* oocytes have recently been described (Stümer et al., 1987). These sodium channels had activation and inactivation kinetics faster than those reported here. For example at 0mV (15°C) the activation and inactivation time constants from the oocyte data were 0.15 and 0.5ms respectively. In dissociated hippocampal neurones the corresponding values at 18°C were 0.5 and 3ms respectively. The steady state activation curve was very similar to the peak conductance-voltage curve reported here however the steady state inactivation curve was shifted to more depolarized potentials by about 10mV. The reasons for these differences are not clear. Perhaps the properties of sodium channels are different when expressed in the oocytes. In the experiments described in this chapter the intracellular contents of the cell were replaced with an artificial solution. The kinetic properties of single sodium channels have been shown to be different in cell attached patches and 'ripped off patches' (Horn and Vandenberg, 1987). It may therefore be that the differences our macroscopic current properties are due to the replacement of the intracellular solution. Another possibility is that since three different sodium channel cDNA clones were isolated from rat brain (Noda et al, 1986a) the cDNA injected was coding for a channel different from the one examined here. Certainly, the inactivation kinetics described by Stümer et al. (1987) are more comparable to the 'fast' currents described here (see below). Lastly, it may be that the properties of sodium channels in guinea pig hippocampal neurones are different to the sodium channels cloned from rat brain cDNA.

### Effect of Temperature.

Previous studies of the temperature sensitivity of sodium currents have found that peak  $g_{Na}$  in both rabbit (Chiu, Mrose and Ritchie, 1979) and rat (Schwarz, 1986) myelinated nerve has a low  $Q_{10}$  at temperatures above 10 °C but the  $Q_{10}$  increases at lower temperatures. In contrast,

in frog nerve fibres the  $Q_{10}$  does not change with temperature (Chiu et. al., 1979). In hippocampal although, although the temperature range examined was not very large, the effect of temperature on peak  $g_{Na}$ , half decay time, and time to peak (Figs 4.13,4.14) appeared to be higher for temperatures below 20 °C.  $Q_{10}$  for peak  $g_{Na}$  above 20 °C was 1.5 (Fig 4.13). In one cell in which a complete analysis was done the  $h_{\infty}$  was found to shift to more depolarized potentials as the temperature was raised. A similar result was obtained by Schwarz (1986) in rat myelinated nerve.

### Cells with Different Sodium Channel Kinetics.

Despite the variety of cell morphologies obtained after dissociation, the sodium conductance of most cells has very similar properties. A few cells, however, displayed a sodium conductance of much more rapid kinetics . It has been suggested that injection of mRNA isolated from rat brain into *Xenopus* oocytes yields sodium channels which appear to have two populations with different decay time constants (Gundersen et al.,1983). In addition, there are at least two different kinds of sodium-channel specific mRNA in rat brain (Noda et. al., 1986). These observations may explain the two different types of channel kinetics observed in the dissociated cells.

Interneurones in the hippocampus and in the cortex have been found to have action potentials which have a faster time course than action potentials in pyramidal cells (Schwartzkroin and Mathers, 1978; McCormick et. al., 1985) it is thus possible that the underlying sodium current may also have faster kinetics than in pyramidal cells. By analogy, cells we have observed with fast conductance changes may be interneurones while cells with the slower conductance may be the pyramidal cells. Alternatively, recent evidence shows that glial cells, when in tissue culture, display a fast sodium conductance (Bevan et. al., 1985; Nowak, Ascher and Berwald-Netter, 1987; A. J. Gibb, unpublished observations) raising the possibility that the cells displaying fast sodium channels may be isolated glial cells.

In conclusion, the results of this chapter indicate that the sodium conductance present in the somatic membrane of hippocampal neurones does not differ greatly from that seen in other preparations. Description of the kinetics of this conductance is another step towards a complete

understanding of hippocampal neurone activity.



## **Chapter 5**

### **A Noninactivating Sodium Current in Acutely Dissociated Guinea Pig Hippocampal CA1 Neurones.**

## INTRODUCTION

Hippocampal pyramidal neurones display the property of "anomalous inward rectification" when depolarizing current injections shift the membrane potential into the subthreshold region. It has been reported that this rectification is abolished by TTX and that the current underlying the anomalous rectification is carried by sodium and or calcium ions (Hotson, Prince and Schwartzkroin, 1979, Connors and Prince 1982, Benardo, Masukawa and Prince, 1982).

Using the single electrode voltage clamp technique, a noninactivating, TTX sensitive sodium current has been demonstrated in rat CA1 pyramidal neurones in hippocampal slices (French and Gage, 1985). Because of the presence of large outward currents which cannot be easily eliminated in the slice preparation the presence of this current was detected by applying depolarizing voltage pulses before and after addition of TTX and the TTX sensitive component resolved by subtraction. This technique is very indirect and there are many uncertainties due possible changes in leakage, making it difficult to quantify the properties of the slow current.

The dissociated slice preparation (Numann and Wong, 1984; chapter 3) where the outward currents can be completely blocked by perfusing the cells with caesium solutions and leak conductances are minimal offers a unique opportunity to examine the properties of the persistent current in detail. In this chapter the properties of the persistent sodium current present in dissociated hippocampal cells will be described.

## METHODS

Details of preparation of cells and voltage clamp are the same as described previously (Chapter 3). The pipette solution consisted of (mM) CsF 130, KCl 10, HEPES 10, EGTA 10, titrated to a pH of 7.2 with CsOH. Osmolality of intracellular solution was 290 - 300 mosmolar. The bathing solution consisted of (mM): NaCl, 125; KCl 5.0; NaHCO<sub>3</sub>, 25; MgSO<sub>4</sub>, 2; CaCl<sub>2</sub>, 2.5; NaH<sub>2</sub>PO<sub>4</sub> 1.2; Glucose 11; pH 7.3 when bubbled with 95% O<sub>2</sub> / 5% CO<sub>2</sub>. In experiments where calcium channel blockers were added NaHCO<sub>3</sub> was replaced by NaTRIS and the NaHPO<sub>4</sub> was omitted. Cadmium was added at a concentration of 1mM. Cell areas were estimated as described in chapter 4. All experiments were done at room temperature 22 - 24 °C. All results are presented as  $\pm$  SEM.

## RESULTS

### Persistent Inward Currents.

Figure 5.1A shows currents generated in a cell voltage clamped at  $-100\text{mV}$  and given depolarizing voltage steps to  $-50$ ,  $-30$  and  $-10\text{mV}$ . Calcium currents were blocked by including  $1\text{mM}$  cadmium in the external solution. It can be seen that upon depolarization there is a large, rapidly activating and inactivating inward current, followed by a persistent inward current. The transient current was not clamped at  $-50\text{mV}$  and appears as a breakthrough spike. The fast current is the sodium current responsible for the upstroke of the action potential in these cells and has been characterised in the previous Chapter. The noninactivating current is shown at higher gain in Fig 5.1B (the peak of the fast current has been truncated). The activation characteristics of the persistent current cannot be fully resolved because of the presence of the fast current.

### Effect of Tetrodotoxin.

Fig 5.2A shows persistent inward currents recorded in another cell clamped at  $-100\text{mV}$  and given depolarizing steps to  $-60$  and  $-40\text{mV}$ . When  $1\mu\text{M}$  TTX was added to the external solution both the transient and the persistent current were completely blocked (Fig 5.2B).

### Current-Voltage Relations.

The persistent inward current recorded over a range of potentials is shown in Figure 5.3. From the record at  $-70\text{mV}$ , where the transient current is very small, it appears that at this potential the persistent current is fully activated within 5 to 10ms. It is clear that the persistent inward current is quite large even at depolarized potentials. The current voltage relation for the steady state current in this cell is shown in Figure 5.4A. It can be seen that the current activates around  $-80\text{mV}$  and reaches a peak amplitude around  $-30\text{mV}$ . The reversal potential obtained by extrapolation of the current-voltage relation was  $+60\text{mV}$ , close to the calculated equilibrium potential for sodium ions of  $+67\text{mV}$ .

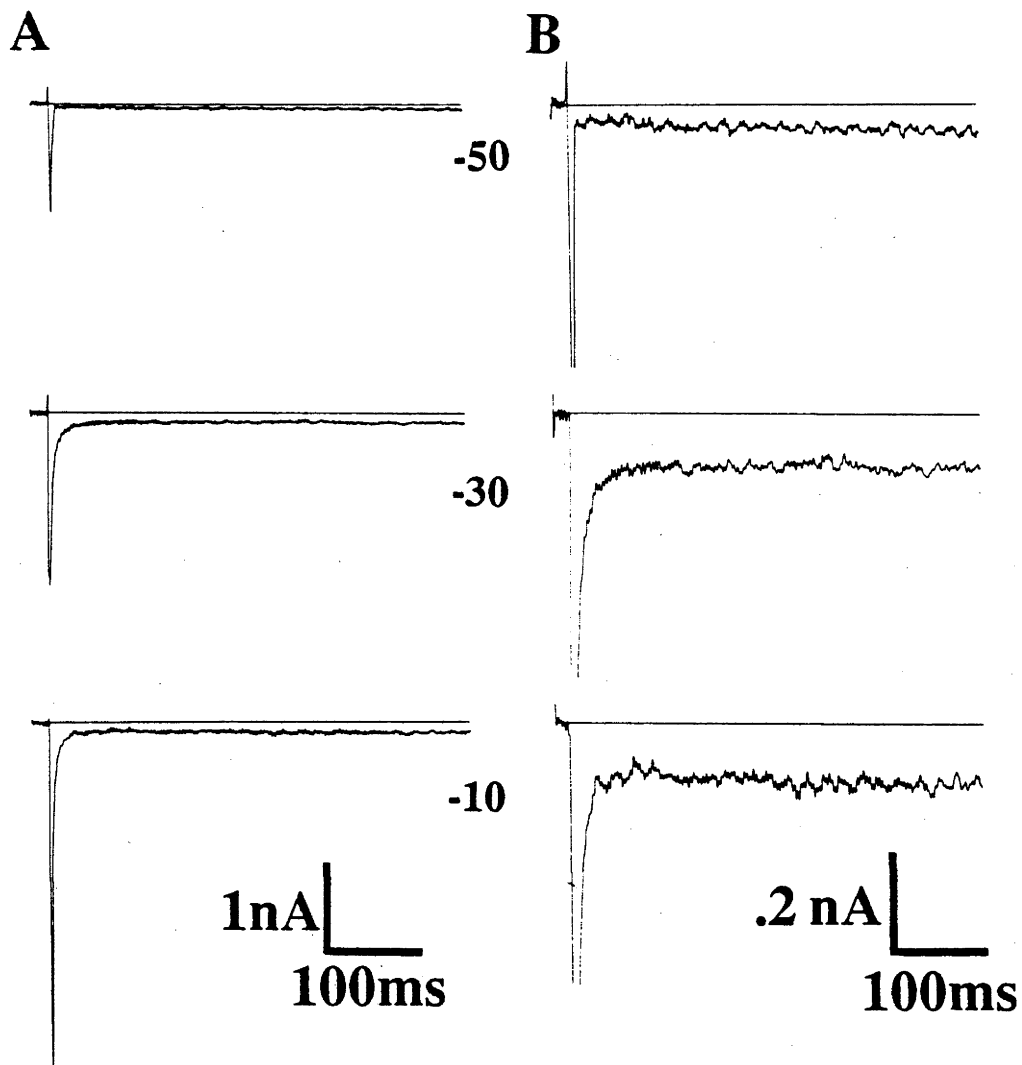
Assuming that the channels underlying the persistent current are ohmic, the conductance of the persistent channels can be obtained by using the equation:

$$g = I/(V - E_{Na}) \dots\dots\dots 5-1$$

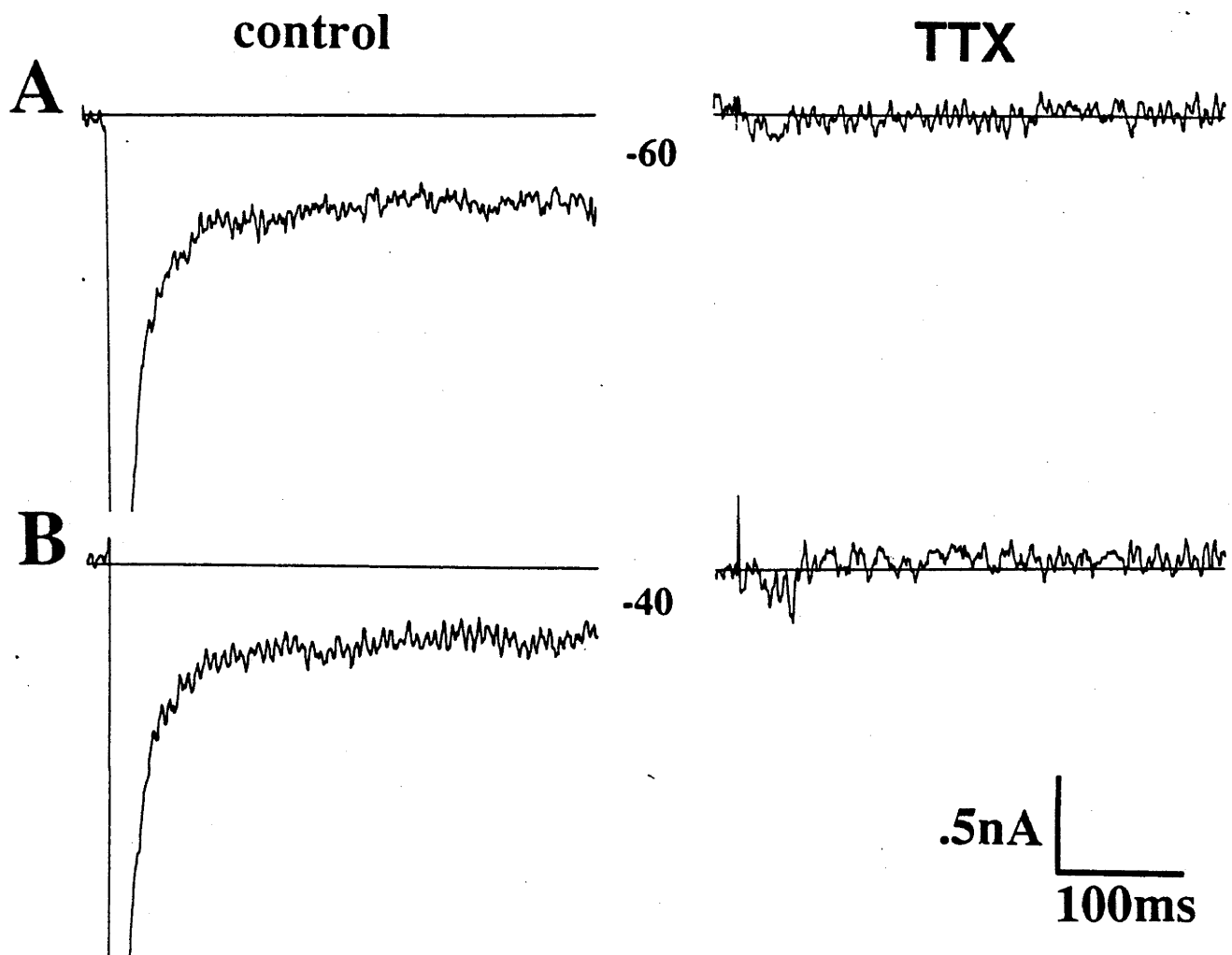
Where  $g$  is the conductance at membrane potential  $V$ , and  $E_{Na}$  is the sodium equilibrium potential. The conductance - voltage relation for this cell is shown in Figure 5.4B. The solid line through the data points is drawn according to the Boltzmann relation:

$$g = g_{max} / (1 + e^{(V'-V)/k}) \dots\dots\dots 5-2$$

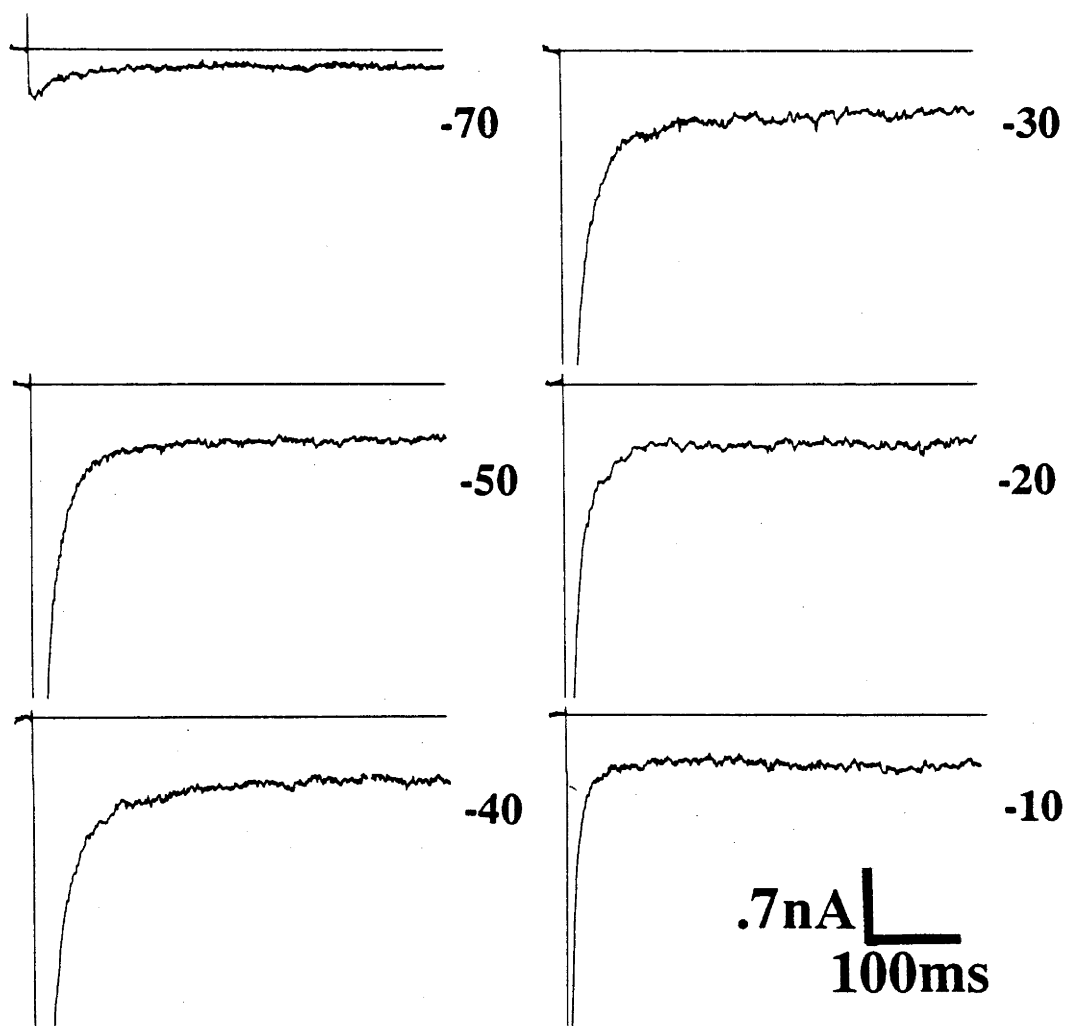
Where  $g_{max}$  is the maximum conductance,  $V'$  the voltage at which the conductance is half maximal and  $k$  is a slope factor. In the cell shown the values for  $g_{max}$ ,  $V'$  and  $k$  were 1.3 nS, -50 mV and 8 mV respectively. The average values for  $g_{max}$ ,  $V'$  and  $k$  obtained from 7 cells were  $2.7 \pm 0.9$  nS,  $-56.0 \pm 3.0$  mV and  $7.3 \pm 0.8$  mV respectively.



**Figure 5.1.** Persistent inward currents in a dissociated hippocampal CA1 neurones. Calcium currents have been blocked with 1mM cadmium in the external solution. **A.** Inward currents generated in response to depolarizing voltage steps to  $-50$ ,  $-30$  and  $-10$  mV from a holding potential of  $-100$  mV. These currents are shown on at higher gain in **B.** Vertical calibration 1 nA (A) and 0.2 nA (B). Horizontal calibration, 100 ms.

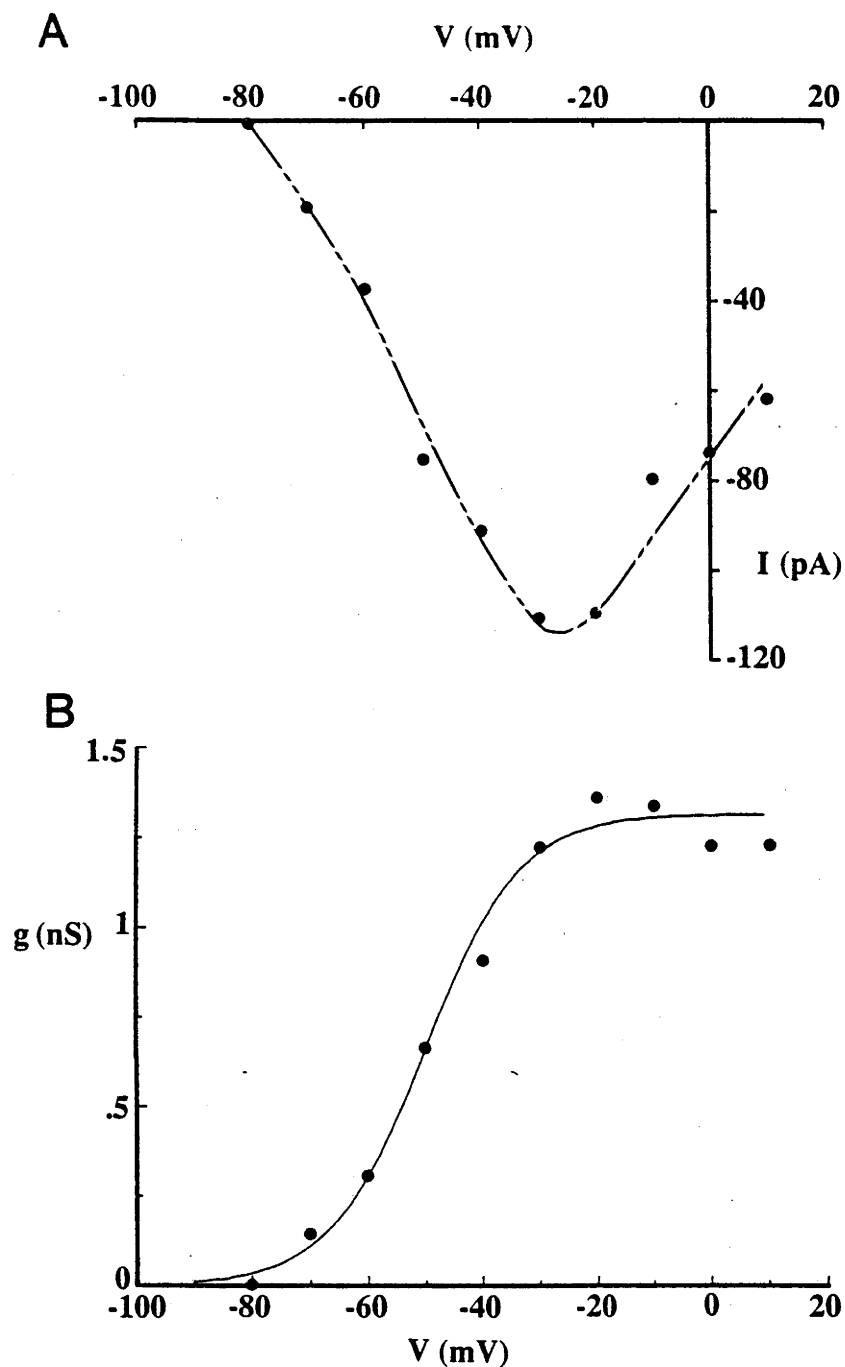


**Figure 5.2.** TTX blocks the persistent inward current. Inward currents generated in response to depolarizing voltage steps to  $-60\text{mV}$  (A) and  $-40\text{mV}$  (B). Traces in left panel were generated in control Ringer and traces in the right panel immediately after adding  $1\text{ }\mu\text{M}$  TTX to the external solution. Vertical calibration  $0.5\text{nA}$ . Horizontal calibration,  $100\text{ms}$ .



**Figure 5.3.** Persistent inward current recorded over a range of potentials. Traces show inward currents generated in response to depolarizing voltage steps to  $-70$ ,  $-50$ ,  $-40$ ,  $-30$ ,  $-20$  and  $-10\text{mV}$  from a holding potential of  $-100\text{mV}$ . Vertical calibration  $0.7\text{nA}$ . Horizontal calibration,  $2\text{ms}$ .





**Figure 5.4.** Properties of persistent inward current. **A.** Steady state current-voltage (I-V) relation for the currents generated in response to depolarizing voltage steps from a holding potential of  $-100\text{mV}$ . The extrapolated reversal potential was  $+50\text{mV}$ . **B.** Conductance ( $g$ ) calculated from the I-V relation shown in A using equation 5-1. The straight line is the line of best fit to equation 5-2 and gives a  $V'$  of  $-50\text{mV}$  and a  $k$  of  $8\text{mV}$ .

## DISCUSSION

The results presented in this chapter describe some of the properties of a TTX sensitive, noninactivating inward current in somata of hippocampal CA1 neurones. This current is activated at voltages more negative than required to activate the sodium current responsible for the action potential (see last chapter). It probably underlies the TTX sensitive component of the anomalous rectification that has been observed in these neurones in hippocampal slices (Hotson et al., 1979; Benardo et al., 1982; Connors and Prince, 1982).

### Properties of the Persistent Sodium Current.

The fact that the persistent inward current is blocked by micromolar concentrations of TTX, not affected by 1mM cadmium, and the extrapolated reversal potential is close to the sodium equilibrium potential strongly suggests that the current is carried by TTX-sensitive sodium channels. Furthermore, the persistent current described here is very similar to the noninactivating sodium current recorded from CA1 pyramidal neurones in hippocampal slices (French and Gage, 1985). The noninactivating inward current in dissociated cells is probably the same current. Therefore, this current will henceforth be called the persistent or "threshold" sodium current.

Average peak amplitudes for the persistent current measured in slices was  $0.39 \pm 0.04$  nA at -40mV (French and Gage, unpublished observations) and  $0.24 \pm 0.06$  nA ( $n=9$ ) for the cells reported here. This difference in average peak current suggests that a large part of the persistent sodium current may be localized to the dendrites of these cells. Peak conductance varied from 0.6 to 6.9nS with an average of  $2.7 \pm 0.9$  nS ( $n=7$ ). In six cells in which the area of the cells was estimated we calculate the maximal conductance due to the persistent sodium current in these cells to be  $0.17 \pm 0.06$  mS.cm<sup>-2</sup>.

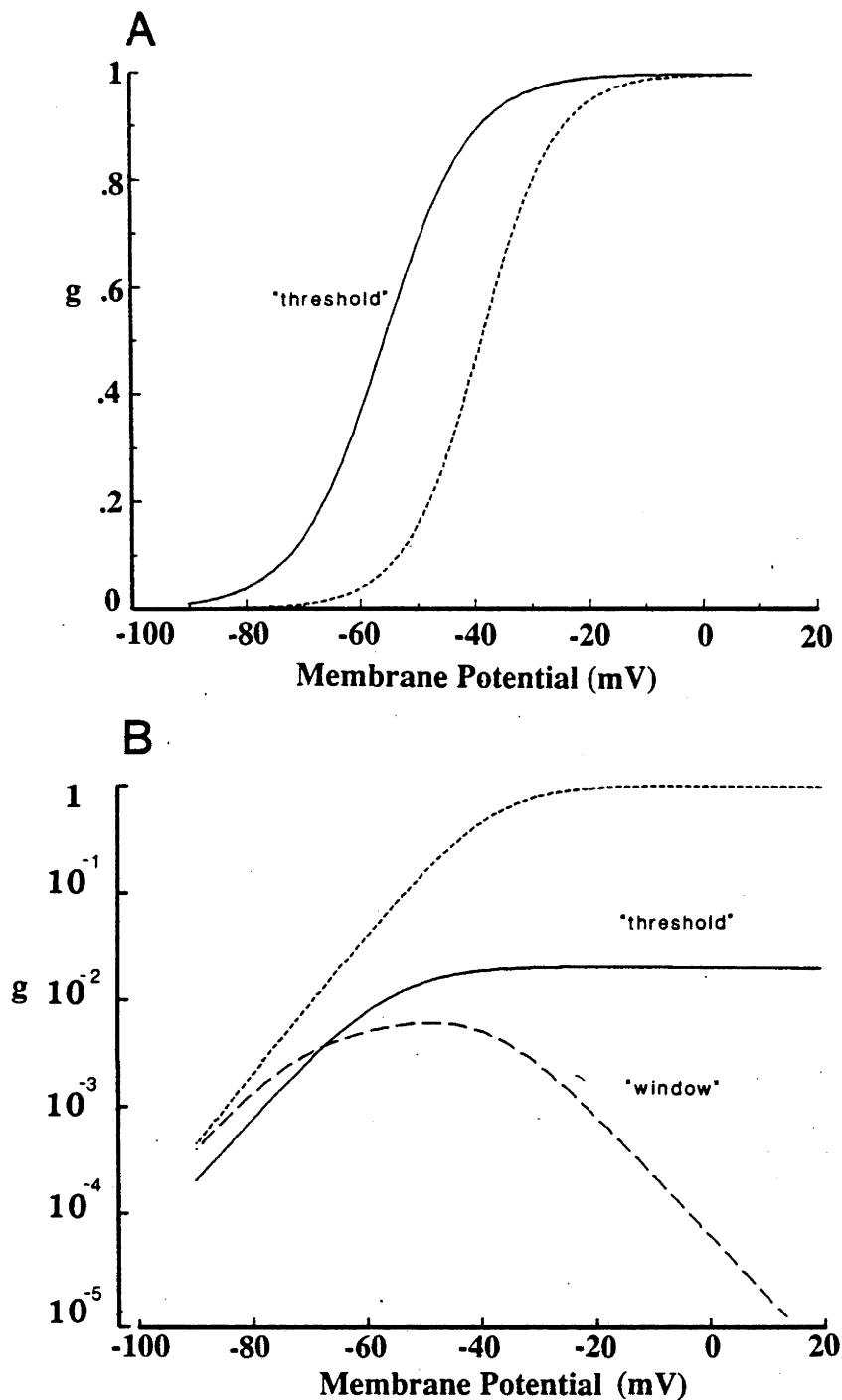
The normalized steady state conductance-voltage relations for the two types of sodium currents are plotted in Figure 5.5A. It can be seen that the activation curve of the persistent currents is shifted about 20mV in the hyperpolarized direction as compared to the action potential sodium channels. The threshold for the persistent current is about -80mV and half the channels

are open by  $-56\text{mV}$ . By comparison, the sodium channels underlying the action potential (Fig. 5.5A dotted line) have a threshold around  $-55\text{mV}$  and a half maximal conductance is obtained at  $-38\text{mV}$  (Chapter 4). The average conductance-voltage relation of the persistent sodium current (solid line), normalized to the peak conductance of the action potential sodium channels (dotted line), is plotted on the lower half of Fig 5.5. It can be seen that the conductance due to the persistent sodium channels is about 2% of the average peak conductance of the fast sodium current in these cells (see also Chapter 4).

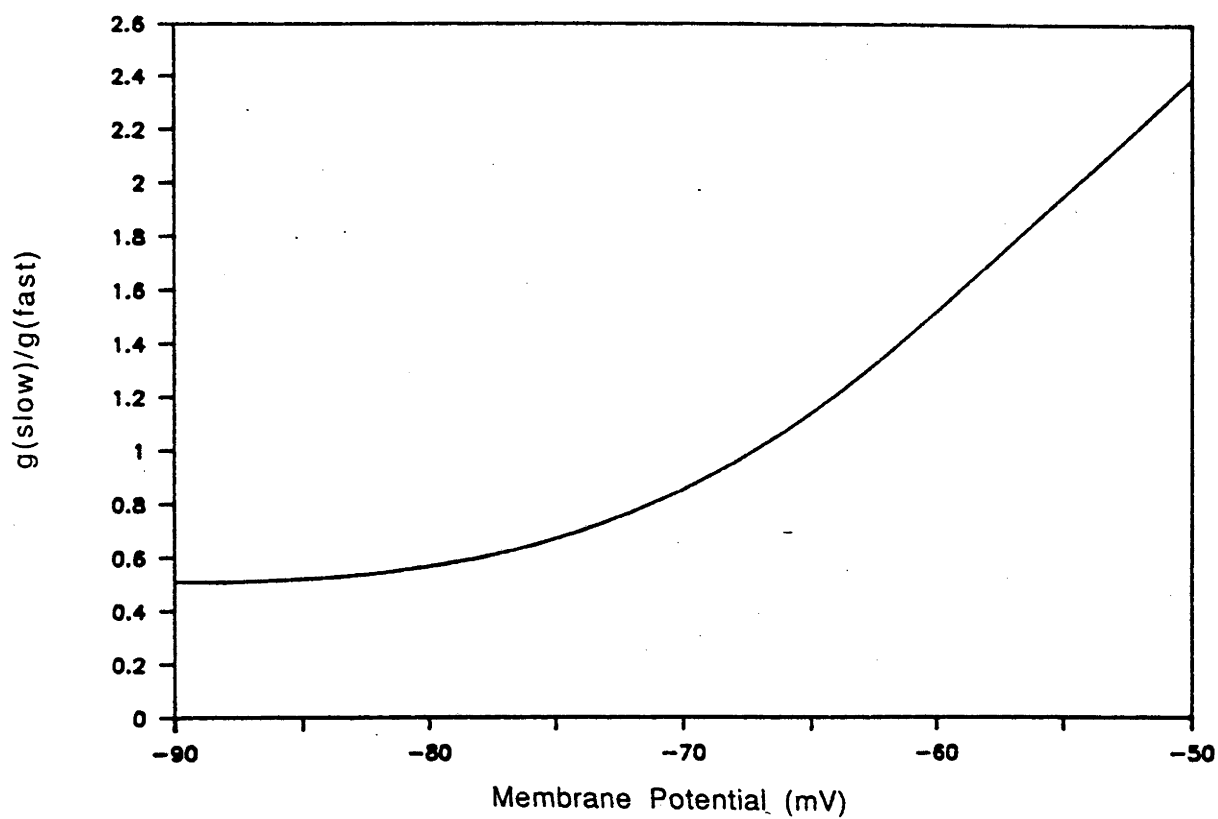
In order to compare the relative contribution made by each of the sodium currents to the total membrane current flux, the ratio of the two conductances (persistent / fast) in the steady state is plotted over a range of potentials in Fig. 5.6. It is clear that over the subthreshold range  $-70$  to  $-50\text{mV}$ , the persistent sodium conductance is the dominant inward current. Thus small sustained depolarizations of the neurone to the region subthreshold for action potential generation, as may happen with a barrage of asynchronous inputs (Granit, Kernell and Shortess, 1963) would selectively activate the persistent channels. Although the total conductance contributed by the persistent channels is small, they will provide enough current to depolarize the cell to threshold. We estimate that if a hippocampal neurone in situ (input resistance of  $50\text{M}\Omega$ ) is depolarized from a resting potential of  $-70\text{mV}$  to  $-55\text{mV}$ , the current contributed by the persistent channels will drive the cell a further  $20\text{mV}$  to  $-35\text{mV}$ . Thus the persistent current will play a significant role in controlling repetitive activity caused by small depolarizations. For example, it may underlie the persistent prepotentials seen in hippocampal pyramidal cells (Lanthorn, Størm and Anderson, 1984; see also Llinas and Sugimori, 1980a; Llinas and Yarom, 1981a). Given the role that this current would play in controlling cell firing and the finding that the purified sodium channel can be phosphoralated by endogenous kinases (Costa, Casnellie and Catterall, 1982), this current would be a likely candidate for modulation by neurotransmitters and/or second messengers.

### Comparison with Other Preparations.

A noninactivating TTX sensitive current has also been observed in cat neocortical neurons (Ståfstrøm, Schwindt and Crill, 1982, 1985) and similar currents have been inferred from current clamp data in cerebellar, thalamic and deep cerebellar neurones (Llinas and Sugimori, 1981a,b;



**Figure 5.5.** Comparison of persistent sodium current with the transient sodium current. Upper figure shows the average normalised conductance-voltage relations for the transient sodium current (dotted line) and the persistent sodium current (solid line).  $V'$  values for the transient and persistent currents were  $-54\text{mV}$  and  $-38\text{mV}$  respectively. The  $k$  values for the same curves are  $6.6\text{mV}$  and  $7.3\text{mV}$  respectively. Lower figure shows the same two curves normalised to the average peak conductance of the transient sodium current plotted on a semilogarithmic scale. The predicted window current (dashed line; see text) is also shown for comparison. Peak amplitude of the persistent current is about 2% that of the transient current.



**Figure 5.6.** Ratio of persistent sodium conductance to the transient sodium conductance in the steady state. The persistent component is the dominant component in the region immediately negative to threshold.

Jahnsen and Llinas, 1984a,b; Jahnsen, 1986a,b). A persistent, TTX sensitive sodium current has also been found in squid axon, which flows through channels distinct from the fast sodium channels, and which has a remarkably similar conductance-voltage relation to the current reported here (Gilly and Armstrong, 1984).

### **What Channels Underlie the Persistent Inward Current?**

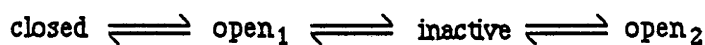
There are several possible explanations for the persistent currents reported here: (a) the persistent current is not due a sodium current; (b) the persistent current is a "window current"; (c) the persistent current is due to a second open state of the fast sodium channels; (d) the persistent currents represent a separate population of channels or sodium channels which can operate in more than one "mode". We will look at each of these hypotheses separately.

(a) The persistent current could be flowing through channels completely separate from sodium channels. The persistent current reported here is blocked by micromolar concentrations of TTX, furthermore the current in hippocampal slices has been shown to be sensitive to external sodium (French and Gage, unpublished observations). This current is seen even when calcium currents have been blocked with cadmium in the external solution. This evidence strongly suggests that the persistent currents are flowing through sodium channels.

(b) In cardiac tissue a persistent non-inactivating sodium current has been interpreted to be a "window" current, that is, the overlapping of the steady state activation and inactivation curves of the fast inward current give rise, according to the Hodgkin and Huxley formulation, to a small, sustained inward current (Attwell et al., 1979; Colatsky, 1982). We do not beleive that the current reported here is a window current for the following reasons. Firstly, when the kinetic parameters of peak activation and steady state inactivation of the fast sodium current are examined in dissociated hippocampal neurones (Chapter 4) there is minimal overlap of the two curves (see Figure 4.10). The window conductance predicted from the kinetics of the fast sodium current is plotted against potential along with the fast and persistent sodium current in Fig 5.5B (note logarithmic ordinate). The peak amplitudes of each current have been normalised to the peak amplitude of the fast sodium current. It is clear that the measured persistent sodium current is larger than the window current for all potential above  $-65\text{mV}$ , in addition, the window current

becomes smaller for potentials more positive than -40mV whereas the persistent conductance reaches a plateau level. It can be seen from Fig 5.4B that the experimentally measured persistent sodium conductance remains as a plateau at least until +10mV. Secondly, if the recent single channel data on the interpretation of the activation and inactivation of the fast sodium current is taken into account (Aldrich, Corey and Stevens, 1983; Horn and Vandenberg, 1987), the observation that once a sodium channel is opened by a depolarization, the inactivated state is essentially totally absorbing, such window currents may in fact be artefacts of the Hodgkin-Huxley equations.

(c) Sodium channels may have two open states. In squid axons, Chandler and Meeves (1970a,b) first proposed a second open state for sodium channels to account for their observation of steady sodium currents in axons perfused with NaF. These steady currents have subsequently been observed in nonperfused axons as well (Shoukimas and French, 1980). Two open states have also been included in models of the sodium channel in squid axons (Armstrong and Bezanilla, 1977) and frog node of Ranvier (Sigworth, 1981). Furthermore, a second open state has been directly observed in mouse neuroblastoma cells (Nagy, Kiss and Hof, 1983). All these models can be represented by the following scheme:



Where  $\text{open}_1$  represents the fast open state and underlies the action potential and  $\text{open}_2$  represents the second open state responsible for the persistent currents. This model requires the channel to pass through the first open state before it goes into the second (persistent) open state. Our observation that the voltage sensitivity of the persistent currents is quite different to the voltage sensitivity of the fast channels cannot be accounted for by this model.

(d) The persistent sodium currents may represent a second "mode" of channel activity as has been suggested for the operation of calcium channels (Hess, Lansman and Tsien, 1984). This second "mode" might represent a second fixed population of sodium channel with different activation and inactivation kinetics. Alternatively sodium channels might interconvert between the different modes. Based on single channel data, this model of channel activity has been invoked

by Patlak and Ortiz(1985,1986) to explain persistent currents through single sodium channel in rat heart and frog sartorius muscles.

For the persistent currents reported in this paper we favour the idea of a separate fixed population of sodium channels. The channels underlying these currents have activation and inactivation kinetics quite distinct from the fast channels. It has been found there are at least two and maybe three different sodium channel specific mRNA that can be isolated from rat brain (Noda et al., 1986a). On one of these messenger RNA's has been shown to code for the fast sodium channel (Stumer et al., 1987). It was shown in the last chapter that there appear to be two types of transient sodium currents in dissociated hippocampal cells. It is possible that each of the mRNA's may code for one of these three types of sodium channel.



## **Chapter 6**

### **A Voltage Dependent Potassium Current in Acutely Dissociated Guinea Pig Hippocampal CA1 Neurones.**

## INTRODUCTION

The delayed rectifier potassium channel responsible for repolarizing the membrane action potential has been well characterised in a number of preparations (for review see Hille, 1984). In the mammalian central nervous system, however, this conductance has been difficult to study because of the problems in separating this potassium current from other outward currents found in these neurones (Adams and Galvan, 1986; Barrett, Barrett and Crill, 1980; Halliwell and Adams, 1982; Segal and Barker, 1984; Chapter 2). The problem arises because as yet there are no known specific blockers for potassium currents, making it difficult to study the properties of any one current in isolation.

Previous studies of the delayed rectifier in mammalian neurons have applied one of two strategies to overcome this problem. Since many of the outward currents are calcium activated (Chapter 2; Galvan and Adams, 1986) one approach is to block the calcium activated currents with calcium channel blockers (Barrett, Barrett and Crill, 1980; Barker and Segal, 1984; Belluzzi, Sacchi and Wanke, 1984b). In these cases, although the delayed rectifier can be isolated, because of the technical problems in voltage clamping a large current with high resistance microelectrodes, it has only been possible to examine the current over a limited voltage range. Also, as shown below, calcium channel blockers appear to have direct effects on the delayed rectifier. The second approach is use cultured cells and use patch clamp techniques to examine the current at the single channel level (Gardner, 1986; Rogawski, 1986). At the single channel level, it is relatively simple to separate the delayed rectifier from other channels, however, as shown in this chapter some properties of ionic currents in cultured neurone may be different to neurones *in situ*. One therefore always has to compare data from a cultured system to cells *in vivo*.

The dissociated brain slice technique (Numann and Wong, 1984, Chapter 3) in conjunction with whole cell voltage clamp (Hamill et al., 1981) should allow easy separation of the delayed rectifier under conditions where a good voltage clamp can be achieved. In this chapter the properties of the delayed rectifier in acutely dissociated guinea pig hippocampal CA1 neurons have been examined. It will be shown that although there are some differences the current in

(Hodgkin and Huxley 1952c).

## METHODS

Details of preparation of cells and voltage clamp are the same as described previously (Chapter 3). The pipette solution consisted of (mM) KF 130, KCl 10, HEPES 10, EGTA 10, titrated to a pH of 7.2 with KOH. With the relatively high concentration of EGTA and the presence of fluoride, the free intracellular calcium concentration is expected to be less than  $2 \times 10^{-9}$  M (DeCoursey et al., 1984) thus minimising the contribution of calcium activated currents to the outward current (see discussion). Osmolality of intracellular solution was 290 - 300 mosmolar. The bathing solution consisted of (mM): NaCl, 125; KCl 5.0; NaHCO<sub>3</sub>, 25; MgSO<sub>4</sub>, 2; CaCl<sub>2</sub>, 2.5; NaHPO<sub>4</sub> 1.2; Glucose 11; pH 7.3 when bubbled with 95% O<sub>2</sub> / 5% CO<sub>2</sub>. When the potassium concentration was altered, KCl was substituted for NaCl in the required concentration. In experiments where calcium blockers were added, NaHCO<sub>3</sub> was replaced NaTRIS and the NaH<sub>2</sub>PO<sub>4</sub> was omitted. Calcium was replaced by either cobalt, magnesium or cadmium on an equimolar basis. Tetrodotoxin was routinely included at a concentration of 1  $\mu$ M to block inward currents.

Exponential functions were fitted to the currents using the Levenberg-Marquardt algorithm as described in Chapter 3. All experiments were done at room temperature 22 - 24 °C. All results are presented as  $\pm$  SEM.

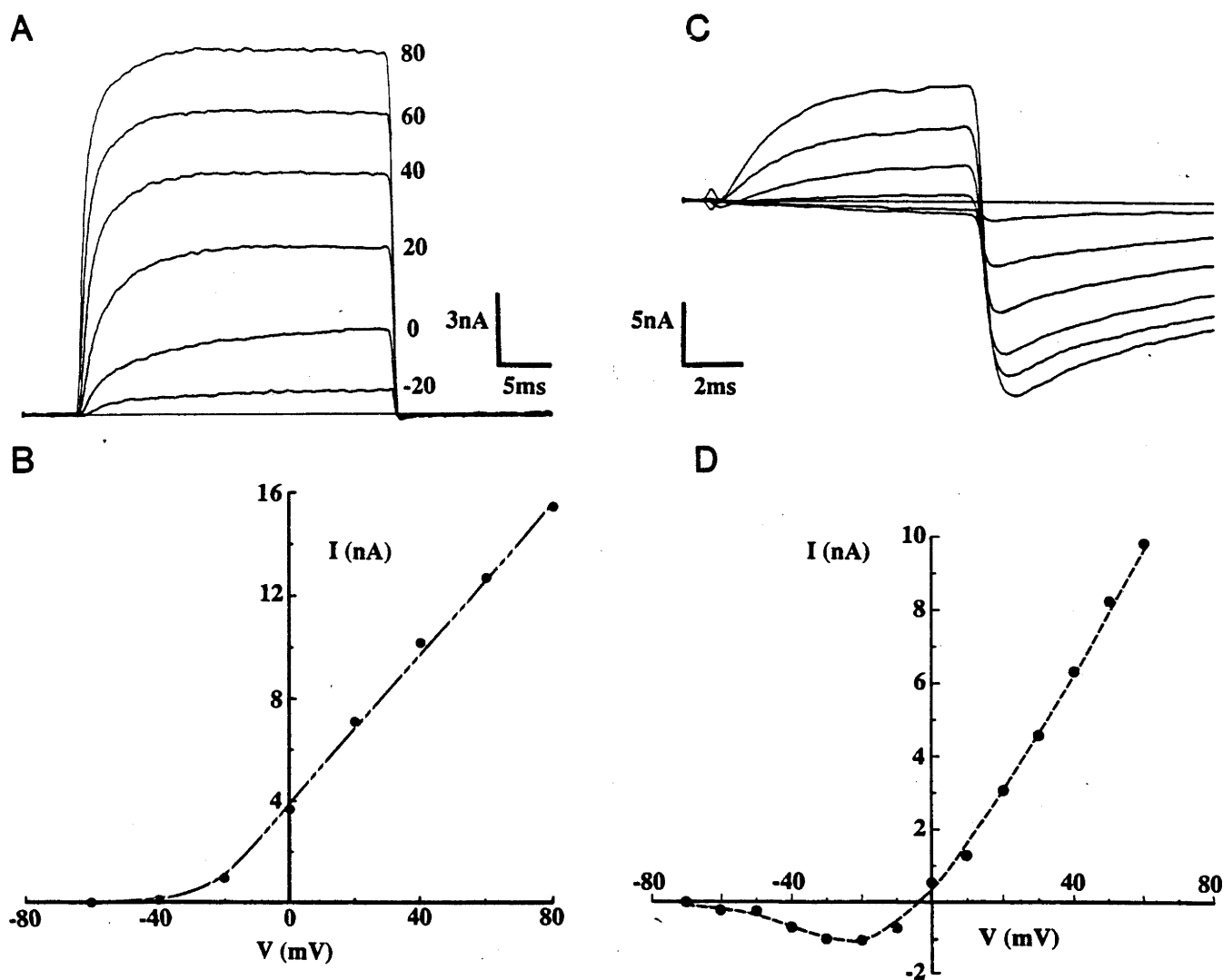
## RESULTS

### Outward Currents.

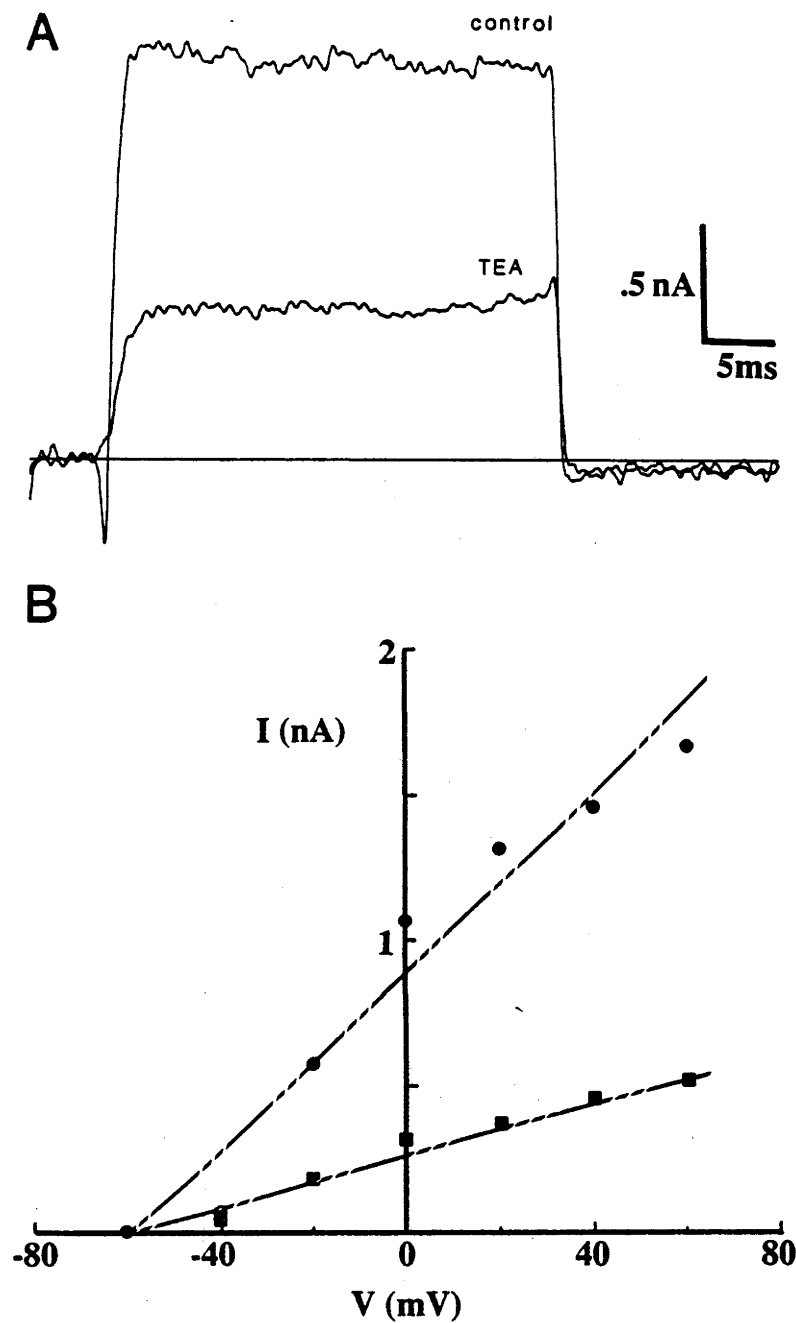
Figure 6.1 shows outward currents generated in an isolated hippocampal CA1 neurone in response to depolarizing voltage commands. The cell was voltage clamped at  $-80\text{mV}$  and given depolarizing voltage steps to potentials from  $-40$  to  $+80\text{mV}$  in  $20\text{mV}$  increments. The steady state current voltage relationship for this cell is shown in Fig. 6.1B. It can be seen that this outward current activates around  $-50\text{mV}$  and then increases steadily with increasingly depolarized voltage steps. These outward currents can be completely blocked by perfusing the cell with a solution containing  $\text{Cs}^+$  ions (Figure 4.1). When the external potassium concentration is raised to  $100\text{mM}$  the current is inward at potentials below  $-10\text{mV}$  (Fig 6.1C, different cell). This can be more clearly seen in the steady state current voltage relation as a region of negative slope conductance (Fig 6.1D). The reversal potential obtained in  $100\text{mM K}^+$  was  $-9.5\text{mV}$  which is close to the potassium equilibrium potential of  $-7\text{mV}$ . These observations suggest that the outward current is predominantly carried by potassium ions.

### Effects of 4-AP and TEA.

Many potassium currents are known to be blocked by external and internal application of TEA (Armstrong, 1966; Hille, 1967; Segal and Barker, 1984). Figure 6.2 illustrates the effect of externally applied TEA on outward currents in these cells. The potassium current in response to a depolarizing step to  $+40\text{mV}$  from a holding potential of  $-80\text{mV}$  is shown in Figure 6.2A. The potassium current immediately after application of  $30\text{mM TEA}$  is shown superimposed in the same figure. It is clear that addition of TEA to the external solution resulted in a marked reduction in the outward current. The current voltage relation for the potassium current before and after TEA is shown in Figure 6.2B. Similar results were obtained in three other cells. Outward currents were also markedly reduced with intracellular perfusion of  $30\text{mM TEA}$  (not illustrated). 4-AP at a concentration of  $0.5\text{ mM}$  had no detectable effect on the outward current.



**Figure 6.1.** Outward currents are potassium currents. **A.** Current traces generated in response to depolarizing voltage steps from  $-20\text{mV}$  to  $+80\text{mV}$  (holding potential  $-80\text{mV}$ ) in a cell bathed in  $5\text{mM K}^+$ . Vertical calibration  $3\text{ nA}$ . The peak current-voltage (I-V) relation for this cell is shown in **B**. Horizontal calibration,  $5\text{ ms}$ . **C.** Current traces generated in response to depolarizing voltage steps from  $-20$  to  $+60\text{mV}$  (holding potential  $-80\text{mV}$ ) in a different cell bathed in  $100\text{mM K}^+$ . The peak I-V relation for this cell is shown in **D**. The measured reversal potential was  $-4\text{ mV}$ . Vertical calibration  $5\text{ nA}$ . Horizontal calibration,  $2\text{ ms}$ .



**Figure 6.2.** External TEA blocks the outward current. **A.** Currents generated in response to a voltage step to +40 (holding potential  $-80 \text{ mV}$ ) before and after addition of  $30 \text{ mM}$  TEA. Vertical calibration  $0.5 \text{ nA}$ . Horizontal calibration,  $5 \text{ ms}$ . **B.** Peak  $I$ - $V$  relation measured in the same cell before (circles) and after (squares) addition of TEA. Broken lines were drawn by eye.

### Conductance-Voltage Relations.

Instantaneous current voltage relations were obtained using a two pulse procedure (Hodgkin and Huxley, 1952b). The cell was voltage clamped at  $-100\text{mV}$  and stepped to  $+40\text{mV}$  for  $10\text{ms}$  to activate the potassium conductance, the membrane potential was then repolarized to various potentials from  $+30$  to  $-130\text{mV}$  in  $10\text{mV}$  increments (Fig 6.3). Instantaneous currents were measured  $400\mu\text{s}$  after the voltage step and are plotted against the potential of the second step in Figure 6.4A. It is clear that the relations are linear over the voltage range where the potassium current is activated. At very negative potentials, the channel appears to rectify (cf. Binstock and Goldman, 1971). The straight line is the linear regression line of best fit to the data points between  $-80\text{mV}$  and  $+40\text{mV}$  and gives a reversal potential of  $-68\text{mV}$ . In some cells the tail currents were fitted with a single exponential and extrapolated back to the time of the step to obtain the instantaneous current. Figure 6.5 compares the instantaneous current voltage relations measured directly and by extrapolation in another cell. The circles are the values of the instantaneous current measured at  $400\mu\text{s}$  as described above. The triangles are the extrapolated peaks of the tail currents at each potential. It is clear that there is no significant difference between the two sets of data.

The peak conductance-voltage relation can be obtained by applying the formula

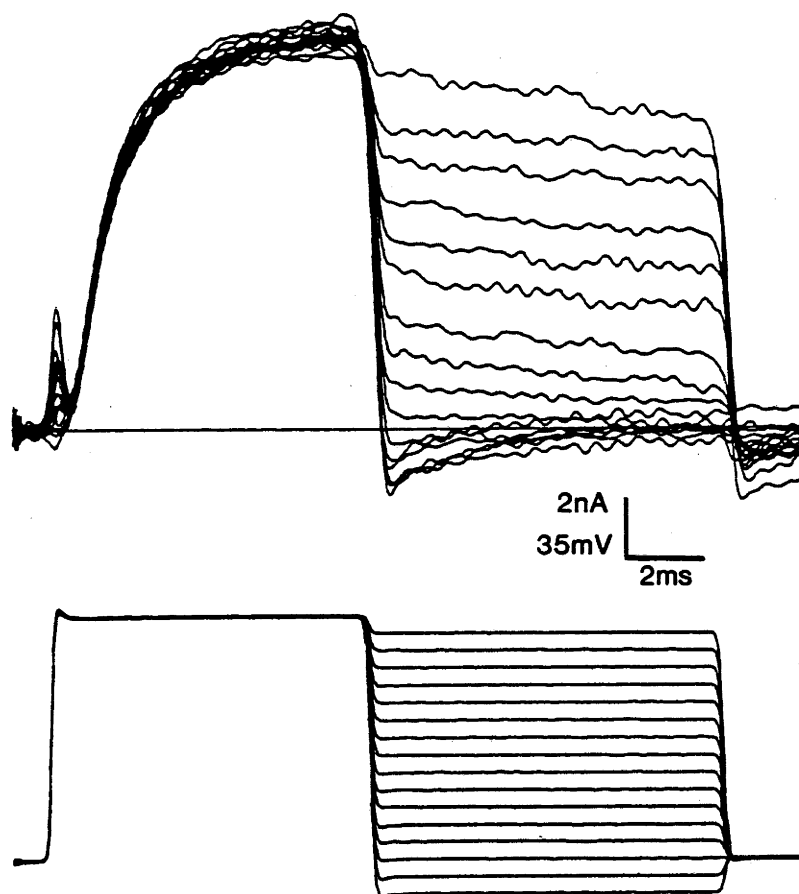
$$g = I/(V - E_0) \quad \dots\dots\dots 6-1$$

where  $g$  is the conductance,  $I$  the peak current,  $V$  the membrane potential and  $E_0$  the reversal potential (Hodgkin and Huxley 1952b). The conductance-voltage relation for the cell in Fig 6.3 is shown in Fig 6.4B. The solid line through the points is the line of best fit to a Boltzmann relation:

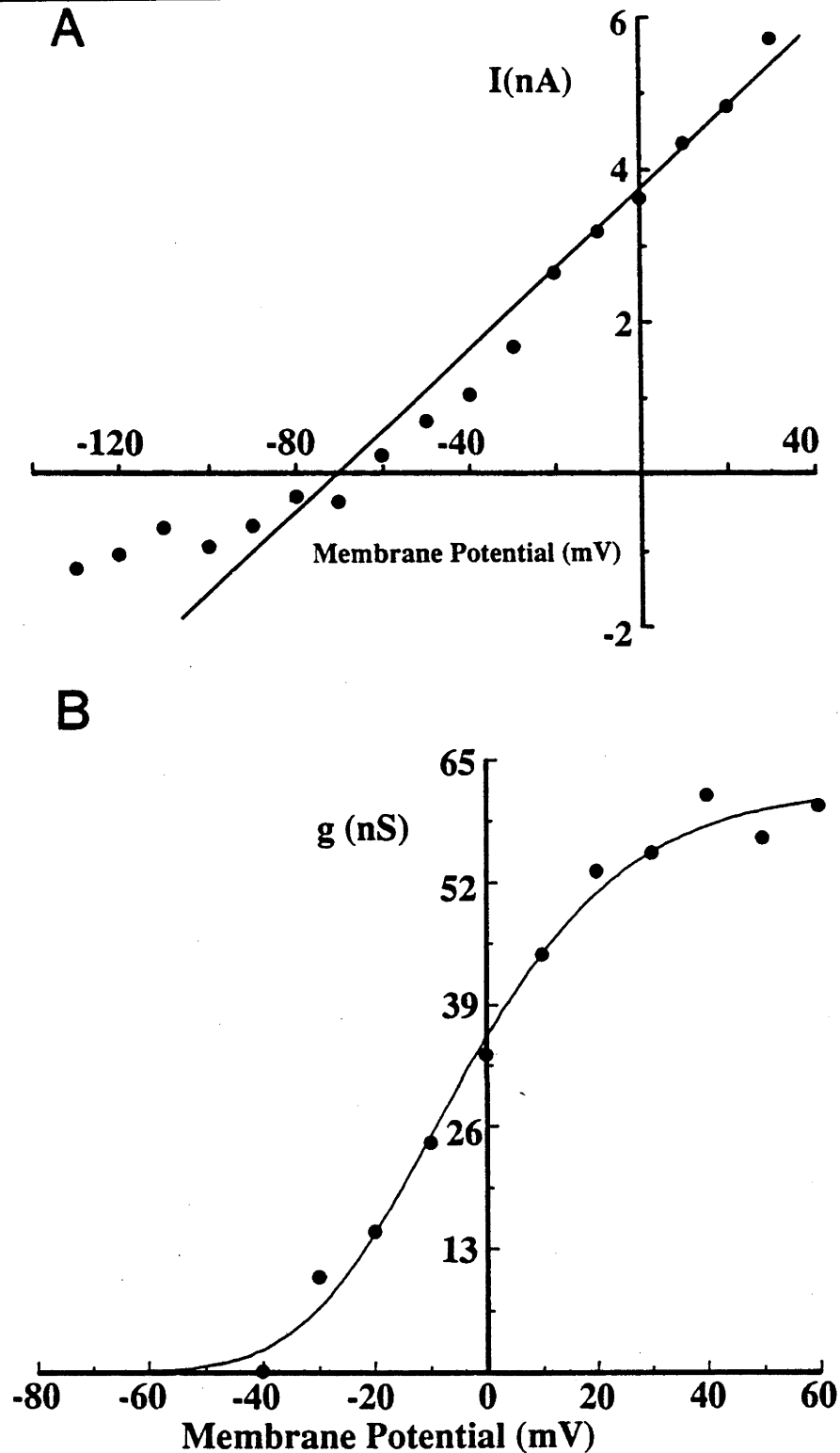
$$g_K = g_{K(\text{max})}/(1 + e^{(V' - V)/k}) \quad \dots\dots\dots 6-2$$

where  $g_K$  is the potassium conductance at voltage  $V$ ,  $g_{K(\text{max})}$  is the peak potassium conductance,

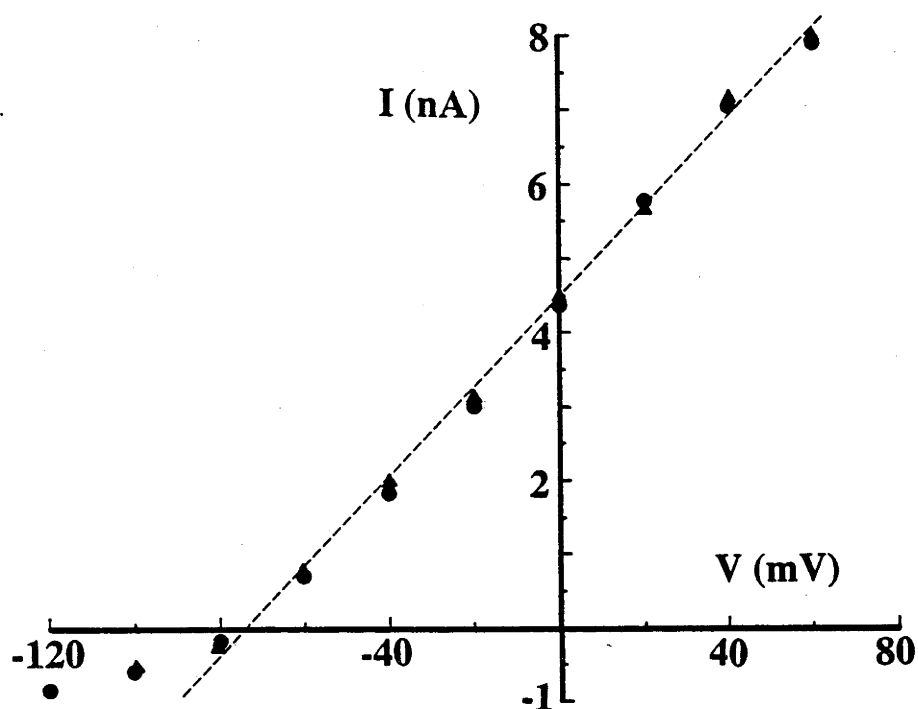




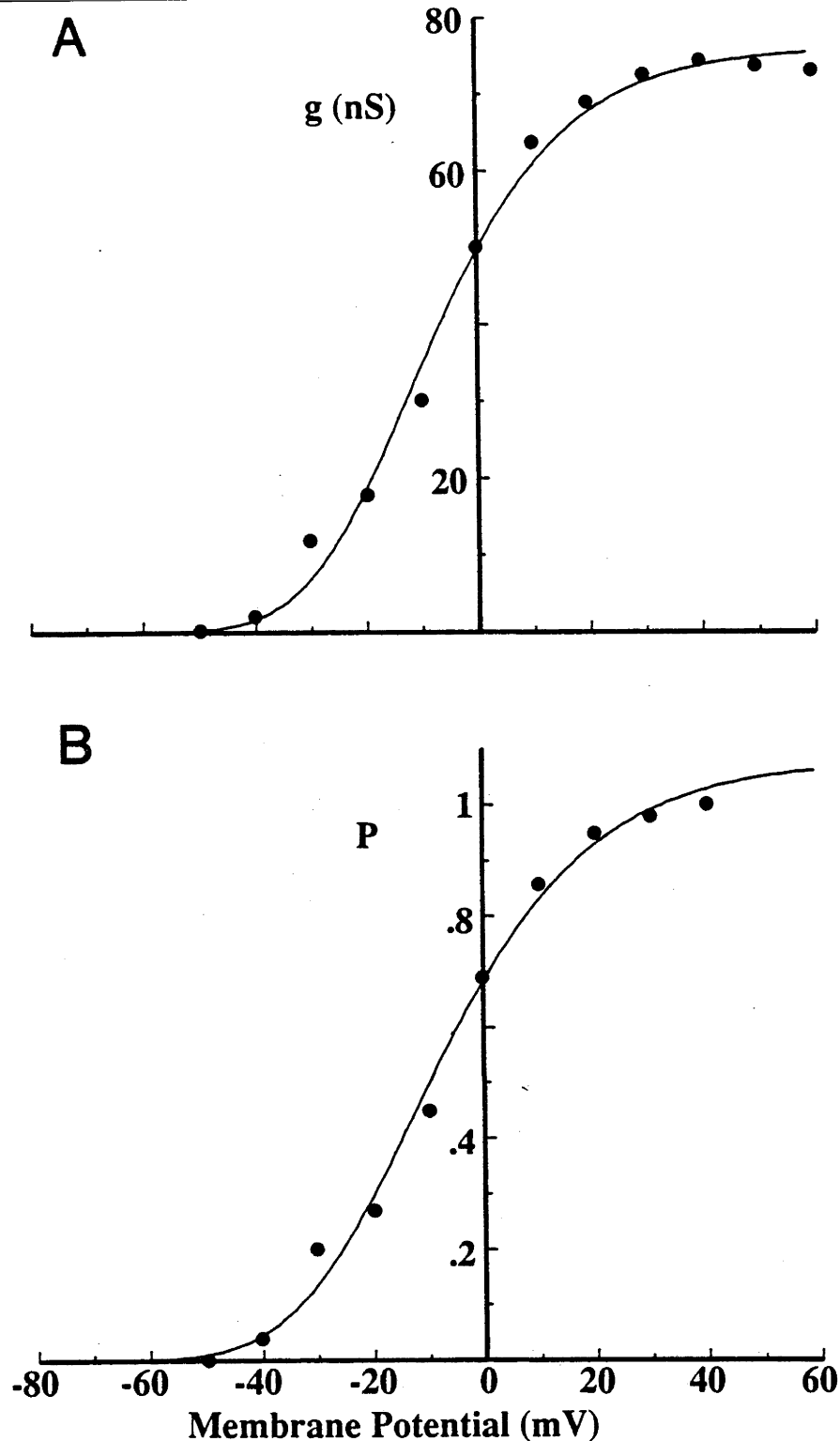
**Figure 6.3.** Instantaneous potassium currents. Upper panel shows instantaneous currents generated by depolarizing steps (lower panel) from a holding potential of  $-100\text{mV}$  (see text for details). Vertical calibration 2 nA for upper traces, and 35 mV for lower traces. Horizontal calibration, 2 ms.



**Figure 6.4.** Conductance-voltage relation. **A.** Instantaneous currents measured 400 $\mu\text{s}$  after the second voltage step. Solid line through the points is the linear regression line of best fit to the data points between  $-80$  and  $+40\text{mV}$  and gave a reversal potential of  $-68\text{mV}$ . **B.** Peak conductance ( $g$ ) calculated using equation 6-1 with a  $E_0$  of  $-68\text{mV}$ . Solid line is the linear regression of best fit to equation 6-2 and gave  $V'$  and  $k$  of  $-4.1\text{mV}$  and  $14.2\text{mV}$  respectively.



**Figure 6.5.** Comparison of instantaneous currents measured directly and obtained by extrapolation of the tail currents (see text). Circles represent instantaneous currents measured 400 $\mu$ s after the voltage step in one cell. Triangles are the instantaneous currents obtained by fitting the tail current with a single exponential and extrapolating it back to zero time. Straight line through the data points was drawn by eye.



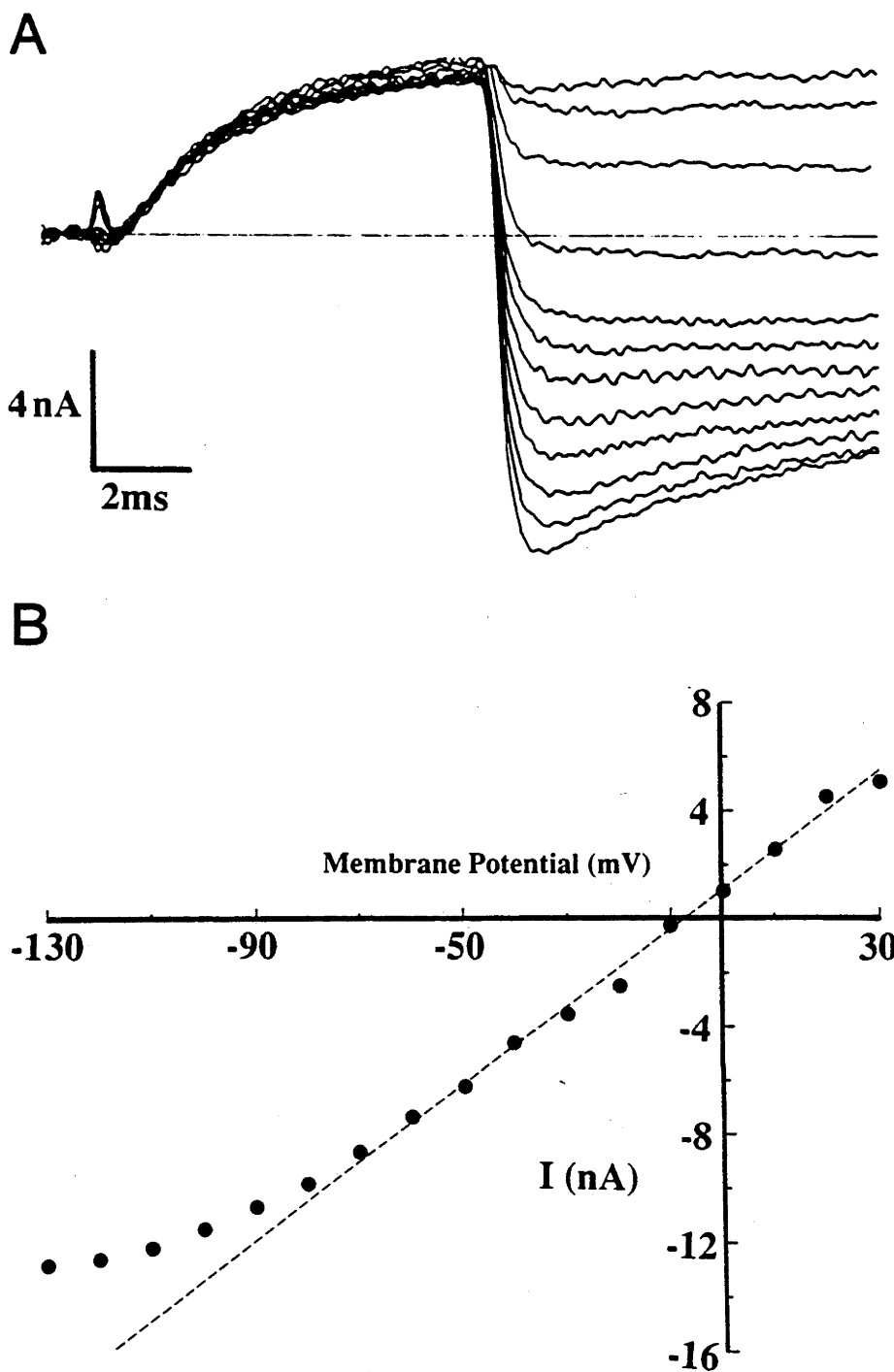
**Figure 6.6.** Conductance-voltage relation is an accurate reflection of the probability of channel opening. **A.** Conductance ( $g$ ) calculated for one cell according to equation 6-1. **B.** Probability of channel opening calculated by dividing the steady state current by the instantaneous current at that potential in the same cell as A. The holding potential was  $-100$  mV in each case. Solid lines through the data points are the lines of best fit to equation 6-2 (see text) and give values of  $V'$  and  $k$  of  $-7.41$  mV and  $10.8$  mV (A) and  $-8.3$  mV and  $11.8$  mV (B) respectively.

$V'$  the voltage at which  $g_K$  is half maximal and  $k$  a constant. In this cell the peak potassium conductance was 61 nS and the parameters  $V'$  and  $k$  were -4.1 mV and 14.2 mV respectively. The average values of  $V'$  and  $k$  from ten cells were  $-4.8 \pm 3.9$  mV and  $13.6 \pm 1.3$  mV respectively.

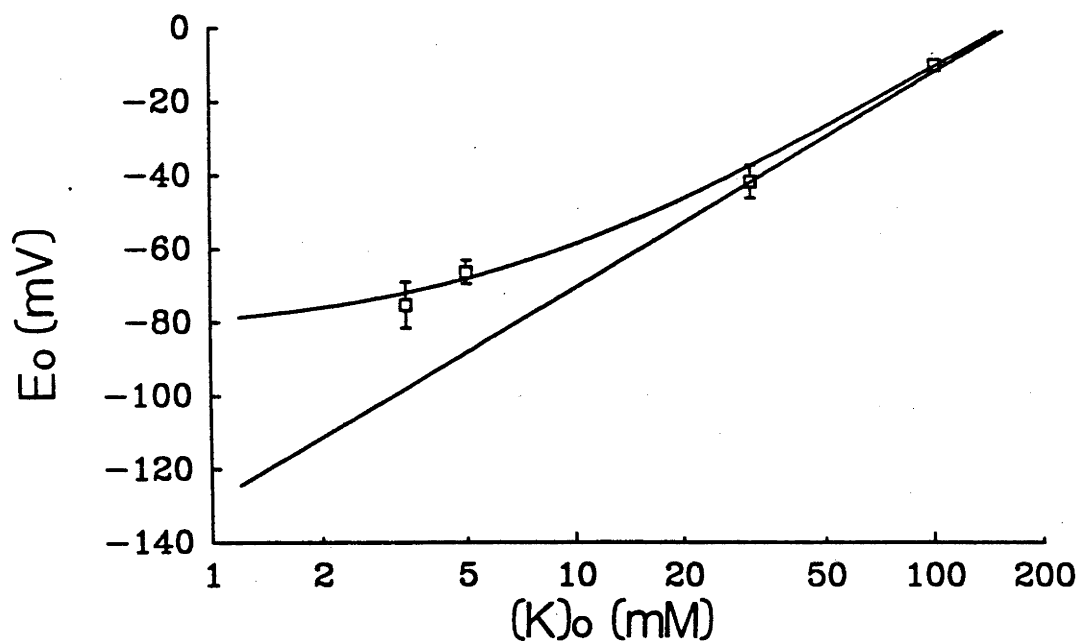
As shown above the instantaneous current-voltage relations for the potassium channels are not linear but rectify at negative potentials. One way to correct for this in the construction of conductance-voltage relations is to divide the steady state current voltage relation by the instantaneous current-voltage relation. This gives us a direct measure of the probability of the channel opening. An example of this is shown in Figure 6.6. In Fig 6.6A is plotted the conductance-voltage relation for a cell obtained as described above. The probability of opening of the underlying channels, obtained by dividing the steady state by the instantaneous current-voltage relation, is shown in Fig 6.6B. The lines through both sets of data have been drawn according to equation 6-2. The values of  $V'$  and  $k$  obtained for the conductance measurements were -7.41 mV and 10.8 mV respectively and the values for the probability of opening were -8.3 mV and 11.8 mV respectively. The conductance-voltage curves thus accurately describe the channel open probability.

### **Ion Selectivity.**

As expected for a potassium selective channel, the reversal potential shifted in a depolarizing direction as the external potassium concentration was raised. Instantaneous currents and the instantaneous current-voltage relation measured in a cell bathed in 100 mM potassium are shown in Fig. 6.7A and B respectively. The reversal potential calculated from the fitted line was -9 mV, close to the potassium equilibrium potential of -7 mV. It is clear that the reversal potential has shifted to a more depolarised potential as compared to a cell bathed in 5 mM potassium (Fig. 6.3). Reversal potentials measured at four different potassium concentrations are plotted against the external potassium concentration in Fig 6.8. The straight line is the prediction from the Nernst equation. The curved line was calculated using the Goldman-Hodgkin-Katz (GHK) equation (Goldman, 1943, Hodgkin and Katz, 1949):



**Figure 6.7.** Instantaneous I-V relation measured in high external potassium. **A.** Current traces generated as described in Fig. 6-3, in a cell bathed in 100mM  $K^+$ . The instantaneous I-V relation is shown in **B.** The straight line is the linear regression line of best fit to data points between  $-60$  and  $+30$ mV and gives a reversal potential of  $-9$ mV.



**Figure 6.8.** Ionic selectivity of channel underlying the potassium current. Each data point represents the average  $\pm 1$  SEM of reversal potentials measured in different cells for external potassium concentrations of 100mM (n=4), 50mM (n=2), 5mM (n=16) and 2mM (n=2). The straight line is the prediction from the Nernst equation. The curved line is drawn according to the GHK equation (equation 6-3 in text) with a  $P_{Na}/P_K$  of 0.04.

$$E_0 = \frac{RT}{F} \ln \left( \frac{[K]^o + p[Na]^o}{[K]^i + p[Na]^i} \right) \quad \dots\dots\dots 6-3$$

where  $p$  represents the permeability ratio  $P_{Na}/P_K$ . The permeability ratio ( $P_{Na}/P_K$ ) used to fit the data in Fig 6.8 was 0.04 (cf. Hodgkin and Horowicz, 1957).

As described above the instantaneous current-voltage relation rectifies at negative membrane potentials. The accuracy of the permeability ratio obtained by fitting the reversal potential data can now be checked by using the GHK equation for the ionic current to fit the instantaneous current-voltage relations. In Fig 6.9A the current-voltage relation shown in 6.3 is replotted. In Fig 6.9B the same data has been fit with the GHK equation (Hodgkin and Katz, 1949):

$$I = \frac{F^2 V}{RT} \frac{w - y e^{-VF/RT}}{1 - e^{-VF/RT}} \quad \dots\dots\dots 6-4$$

where

$$w = [K]^o + \frac{P_{Na}}{P_K} [Na]^o$$

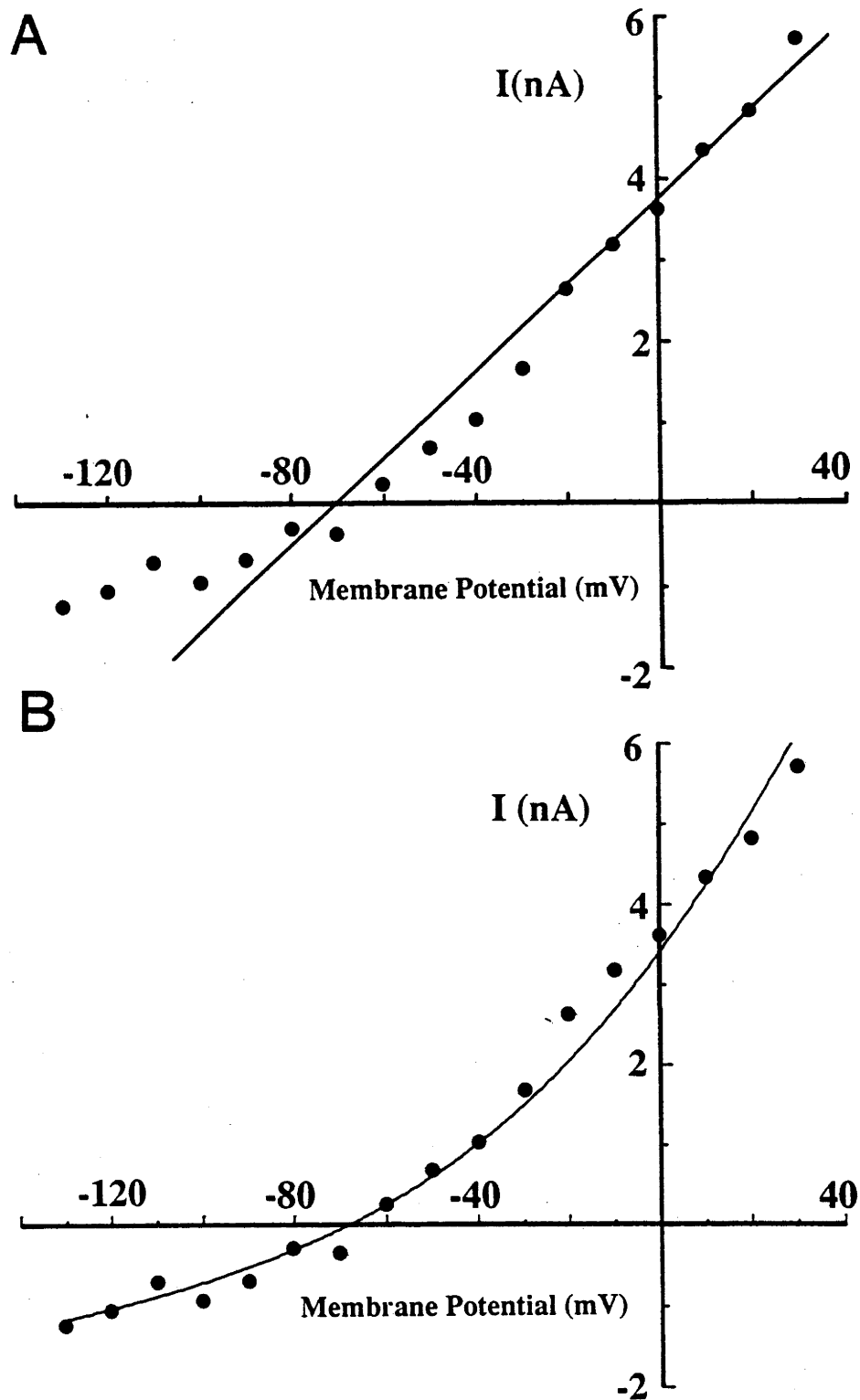
$$y = [K]^i + \frac{P_{Na}}{P_K} [Na]^i$$

where  $P_{Na}$  and  $P_K$  are the permeabilities to sodium and potassium respectively,  $F$  is the faraday,  $R$  the universal gas constant and  $V$  the voltage. It is clear that the GHK equation fits the data well with  $P_{Na}/P_K$  equal to 0.04 and the reversal potential obtained in this way is not significantly different to the simple straight line extrapolation.

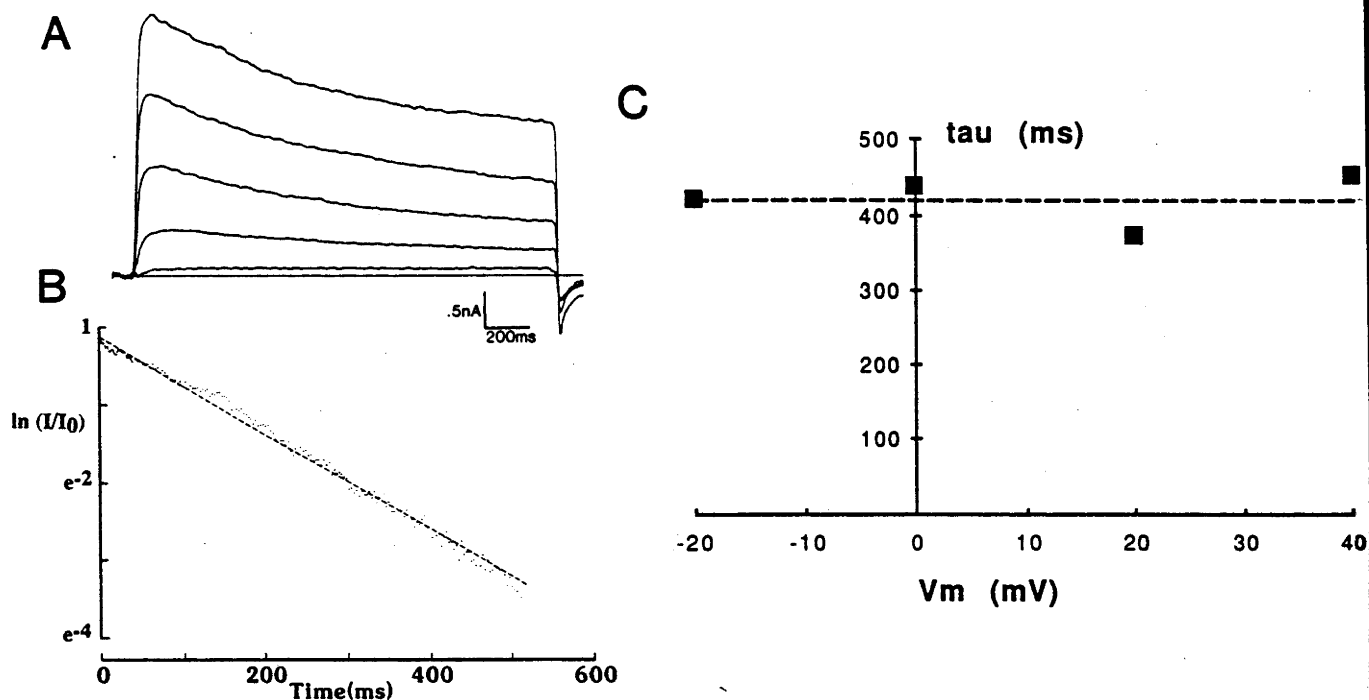
### Inactivation.

In response to prolonged depolarizing voltage steps the potassium currents showed a slow inactivation. This is illustrated in Figure 6.10A where potassium currents activated by 1 second depolarizations are shown. The inactivation was never complete but progressed to a plateau level and was well described by a single exponential process as shown in Figure 6.10B. The time constant of inactivation obtained by fitting a single exponential to the decay phase of the current





**Figure 6.9.** Goldman-Hodgkin-Katz formulation is a good description of the instantaneous I-V relation. **A.** The instantaneous I-V from Fig. 6-4 has been replotted. **B.** Same data points as in A, the curved line has been drawn according to the GHK equation (equation 6-4 in text) with  $P_{Na}/P_K$  equal to 0.04. The reversal potential in both cases was  $-68\text{mV}$ .



**Figure 6.10.** Inactivation kinetics of potassium current. **A.** Currents generated in response to 1 second depolarizing voltage commands from  $-40\text{mV}$  to  $+40\text{mV}$  in  $20\text{mV}$  increments from a holding potential of  $-100\text{mV}$ . Vertical calibration  $1\text{ nA}$ . Horizontal calibration,  $200\text{ms}$ . **B.** Data points show the decay of the current at  $+40\text{mV}$  plotted on a semilogarithmic scale. Broken line is the line of best fit to an single exponential and gave a decay time constant of  $450\text{ ms}$ . Decay time constants over a range of potentials are plotted in **C.** The straight line has been drawn by eye.

was about 450ms and independent of voltage as illustrated in Figure 6.10C. The inactivation seen above could be mimicked by accumulation of potassium ions at the external surface of the membrane (eg. see Frankenhauser and Hodgkin, 1956). We think that this is unlikely here because as discussed in Chapter 3, these cells do not appear to have any membrane infoldings and the cells are bathed in a Ringer which is continually being stirred by the stream of O<sub>2</sub>/CO<sub>2</sub>.

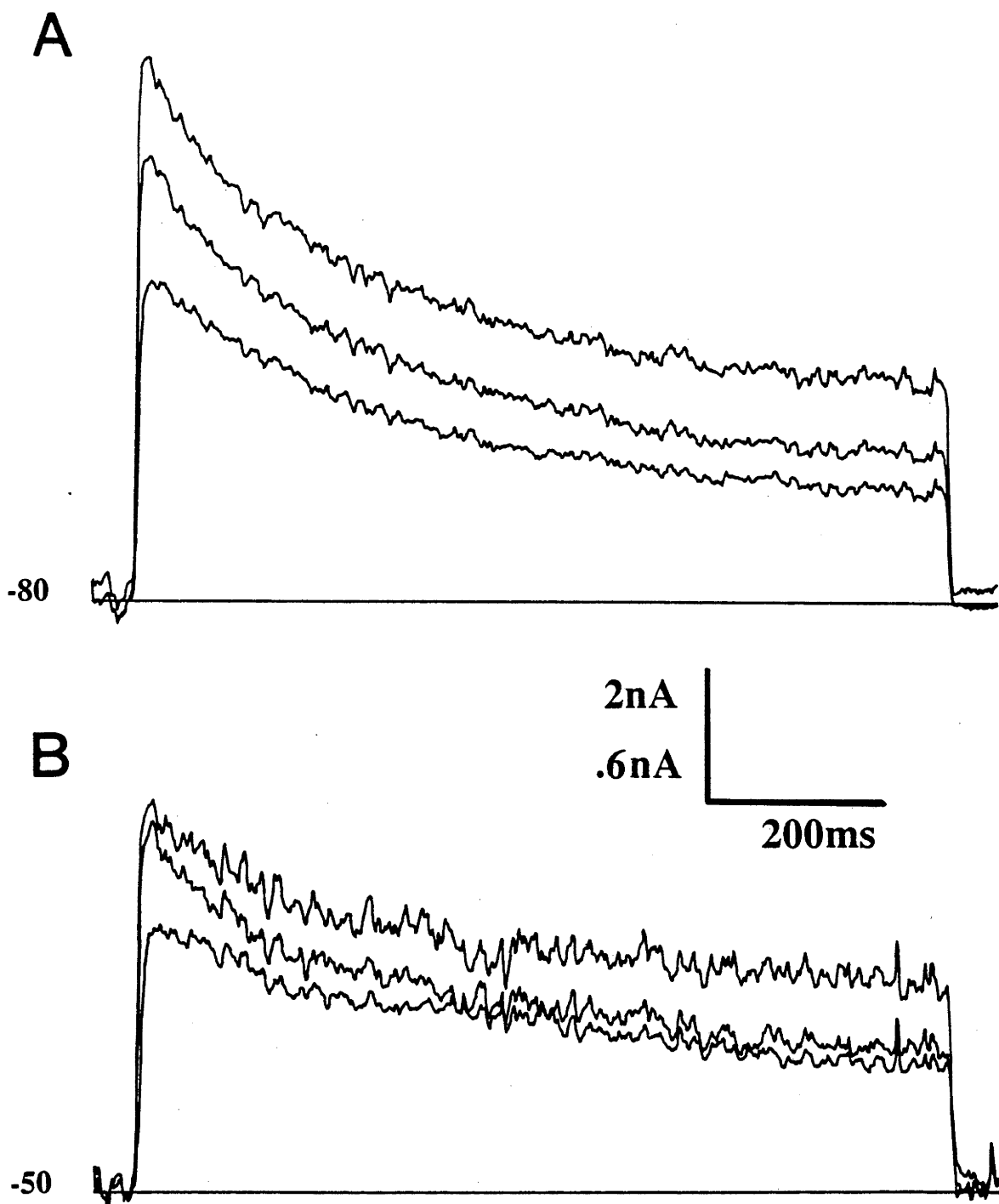
A fast activating and fully inactivating potassium current, the A current, I<sub>A</sub>, has been characterized in CA3 (Gustaffson et al., 1984) and CA1(Chapter 2) neurones in hippocampal slices. This current is sensitive to 4-AP and is completely inactivated at a holding potential of -50mV (Gustaffson et al., 1984; Barker and Segal, 1984). 1mM 4-AP did not affect the currents reported here (not shown). Voltage clamping the cells at -50mV did not block the decay of the outward current. Currents recorded by holding the cell at -100mV are shown in Fig 6.11A, when the holding potential is moved to -50mV Fig (6.11B) it can be seen that the outward currents are smaller because of the steady state inactivation (see below), the decay of the current is however unchanged. Thus the inactivating current is not the A- current.

### Steady State Inactivation.

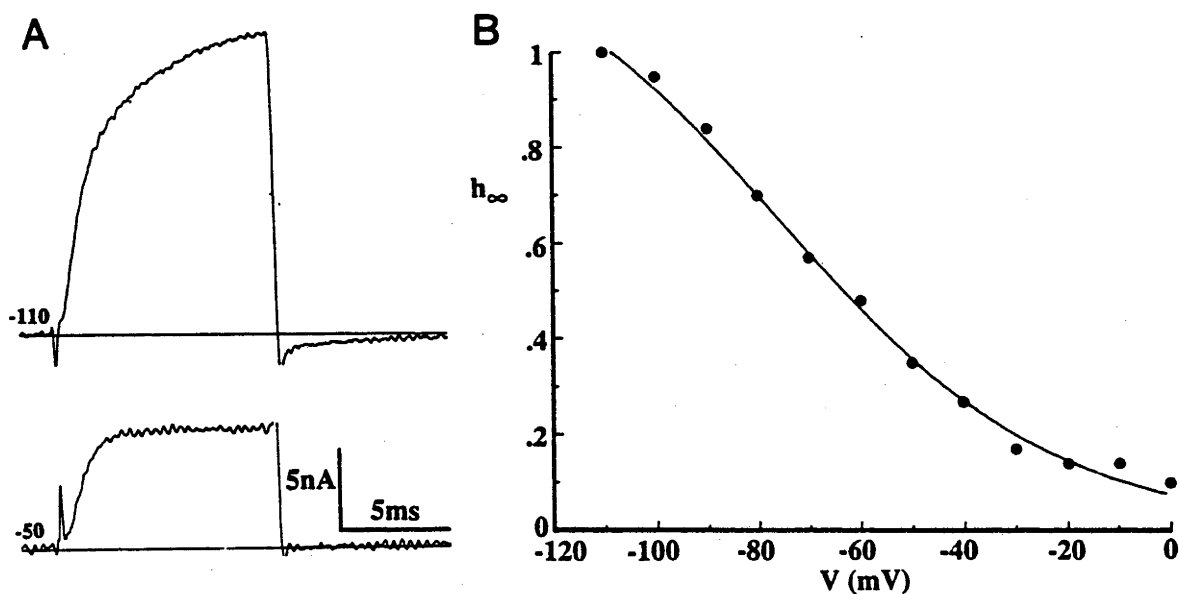
Steady state inactivation was studied by holding the cell for two seconds at different membrane potentials and then activating the potassium current with a 10ms step to +40mV. Potassium currents recorded in one cell held at -110 and -50mV are shown in Figure 6.12A. The steady state inactivation parameter  $h_{\infty}$  was then calculated by dividing the peak current at each potential by the peak current at -110mV. Results from one cell are shown in Figure 6.12B. The solid line through the points is drawn according to the relation (Hodgkin and Huxley, 1952c)

$$h_{\infty} = 1/(1 + e^{(V - V')/k}) \quad \dots\dots\dots 6-5$$

where V is the membrane potential, V' the potential at which  $h_{\infty}$  is 0.5 and k a constant. For the cell shown in Figure 6.12 the parameters V' and k were -65.9 mV and 26.9 mV respectively. Average values measured from four cells were  $-67.7 \pm 11.6$ mV and  $26.6 \pm 6.6$  mV respectively.



**Figure 6.11.** Outward current is not an A-current. A. Currents generated in response to depolarizing voltage steps to 0, +20 and +30mV from a holding potential of -80mV. B. Currents generated in the same cell in response to depolarizing voltage steps to the same potentials from a holding potential of -50mV. Vertical calibration 2 nA for A and 0.6 nA for B. Horizontal calibration, 200 ms.



**Figure 6.12.** Steady-state inactivation of potassium currents. **A.** Currents generated in response to depolarizing voltage steps to +40 mV from holding potentials of -110 and -50 mV. Vertical calibration 5 nA. Horizontal calibration, 5 ms. **B.** Data points represent the ratio ( $h_{\infty}$ ) of the peak current generated by a voltage step to +40 mV from holding potential of  $V$  to the current generated with a holding potential of -110 mV. The solid line is the line of best fit to equation 6-5 and gives a  $V'$  of -65.9 mV and a  $k$  of 26.9 mV.

### Activation Kinetics.

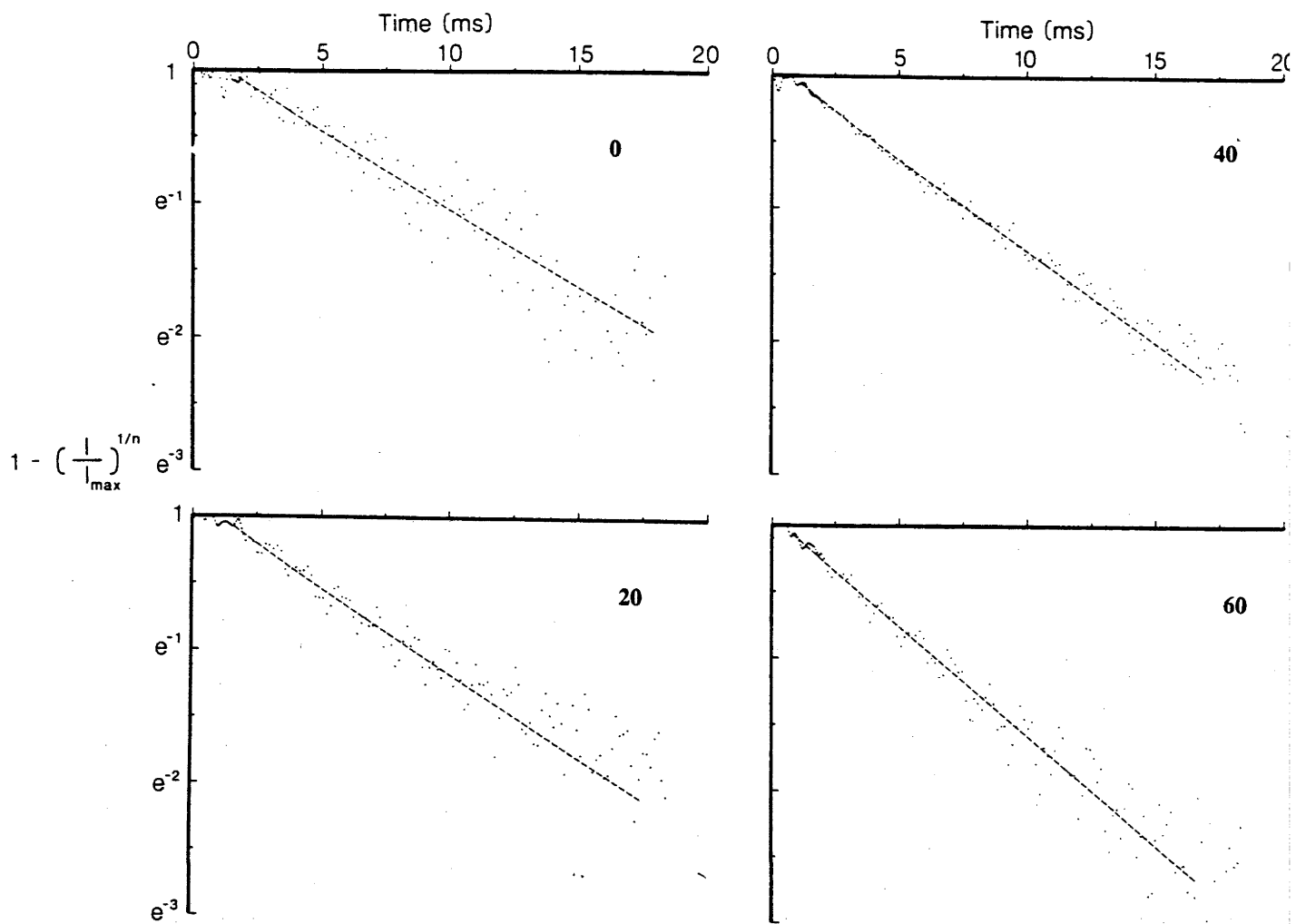
In order to compare our results with other studies of the delayed rectifier K<sup>+</sup> channel, the activation of the potassium current was fitted with a Hodgkin - Huxley type of formulation (Hodgkin and Huxley 1952d). Potassium currents were fitted according to the equation

$$I_K = I_{\max} \cdot (1 - e^{-t/\tau})^n \quad \dots\dots\dots 6-6$$

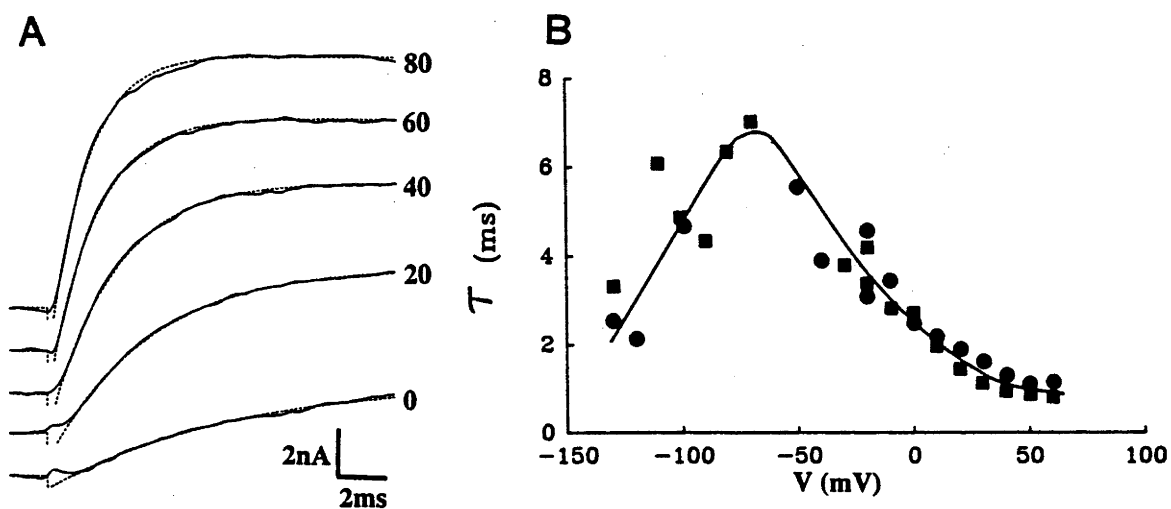
The value of the exponent  $n$  was empirically determined by plotting  $(1 - (I_K/I_{\max})^{1/n})$  against time on a semilogarithmic plot for various values of  $n$ . In ten different neurones the value of  $n$  which gave the best straight line was 1.0 (Fig 6.12). The value of  $\tau$  was estimated by fitting a single exponential to the rising phase of the current. Figure 6.13A shows potassium currents in one cell fitted according to equation 6-6 ( $n=1$ ). Tail currents obtained on repolarization to different potentials could also be fit with a single exponential (not shown). Values of  $\tau$  obtained from the activation and inactivation from two cells (circles and squares) are shown in Fig. 6.14B. The line through the points has been drawn by eye.

### Effects of Calcium Channel Blockers.

In other mammalian preparations where the delayed rectifier has been studied, calcium channel blockers have been used to block the calcium and calcium activated currents (Barker and Segal, 1984; Galvan and Sedlmeir, 1984; Belluzzi, Sacchi and Wanke, 1984b). In human T lymphocytes, calcium channel blockers have been shown to block the delayed rectifier (DeCoursey et al., 1985). Dissociated neurones under the recording conditions used here have a very small or absent calcium current (Chapter 4) and the calcium activated currents are absent (see below and discussion). We therefore examined the effects of cobalt and cadmium on the potassium current in these cells. Because of the problem of rundown, potassium currents were first examined in a Ringer solution in which the calcium had been replaced with Co<sup>2+</sup>, Ringer containing normal calcium (2.5mM) and no cobalt was then washed in and the potassium currents again measured. Results from one such experiment are shown in Fig 6.15A. The steady state

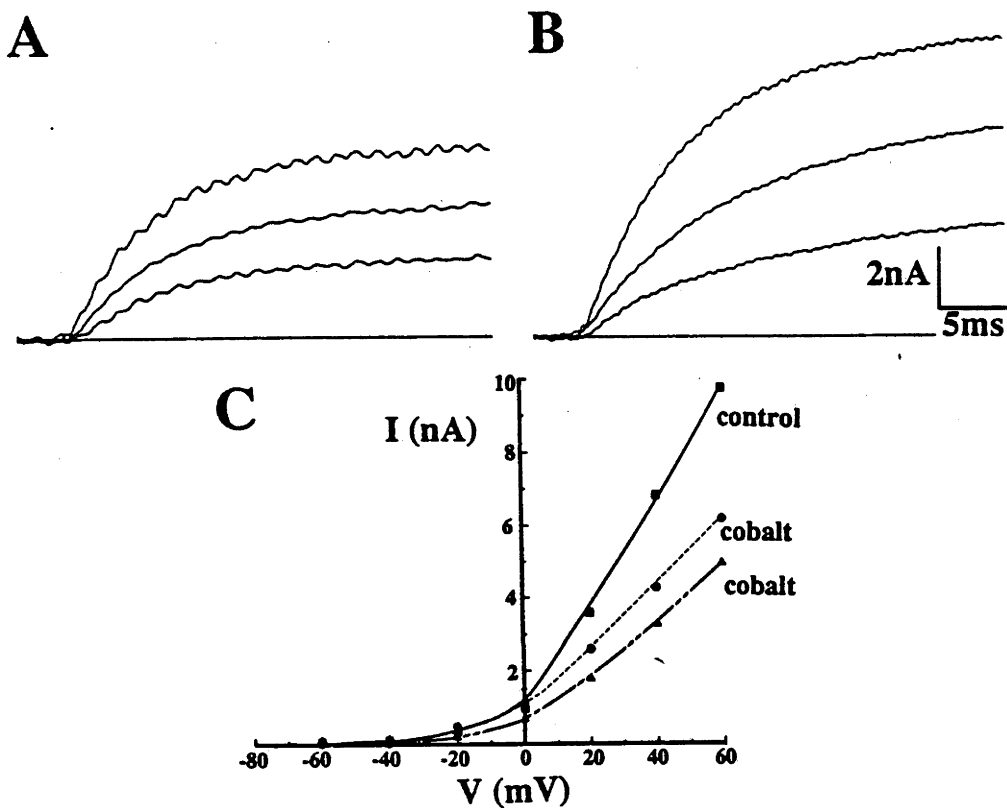


**Figure 6.13.** Potassium current activation. Currents generated in response to depolarizing voltage steps to 0, +20, +40 and +60mV (holding potential -80mV) are plotted against time according to the equation  $(1 - (I_K/I_{\max})^{1/n})$ . The straight lines through the data points have been drawn by eye.



**Figure 6.14.** Activation kinetics. **A.** Currents generated in response to depolarizing voltage steps from a holding potential of -80mV to potentials from 0mV to +60mV. The broken lines are the lines of best fit to equation 6-6 ( $n=1$ , see text). Vertical calibration 2nA. Horizontal calibration, 2ms. **B.** Activation time constants and time constant of decay of tail currents measured in two cells (circles and squares). The solid line has been drawn by eye.



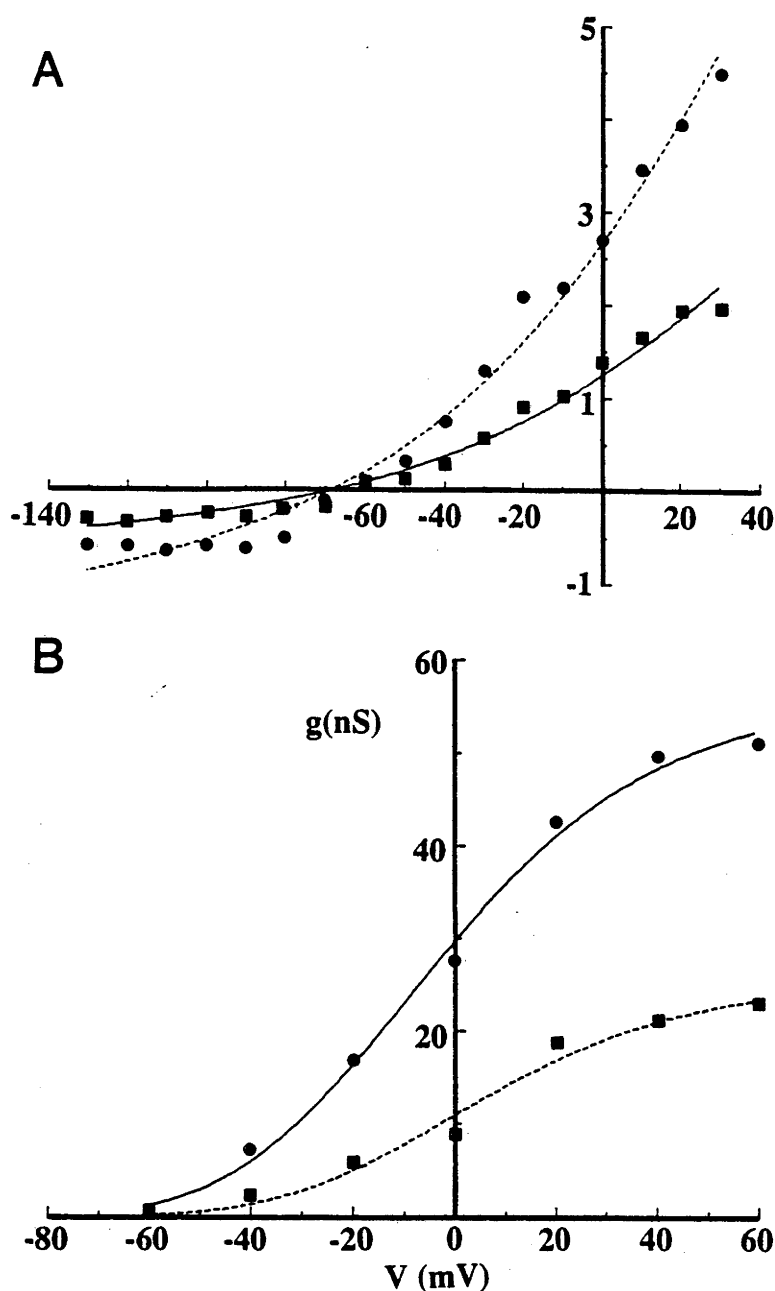


**Figure 6.15.** Effect of cobalt on outward currents. **A.** Current traces generated by depolarizing voltage steps to +20, +40 and +60 mV (holding potential -80 mV) in a cell bathed in cobalt Ringer (0 Ca<sup>2+</sup>, 2.5 mM Co<sup>2+</sup>). **B.** Traces generated in the same cell as (A) immediately after changing the external solution to a calcium Ringer (2.5 mM Ca<sup>2+</sup>). Vertical calibration 2 nA. Horizontal calibration, 5 ms. **C.** Peak I-V relations measured in the same cell first in cobalt Ringer (circles) then in calcium Ringer (squares) and then after changing back to a cobalt Ringer (triangles). Lines through the data points have been drawn by eye.

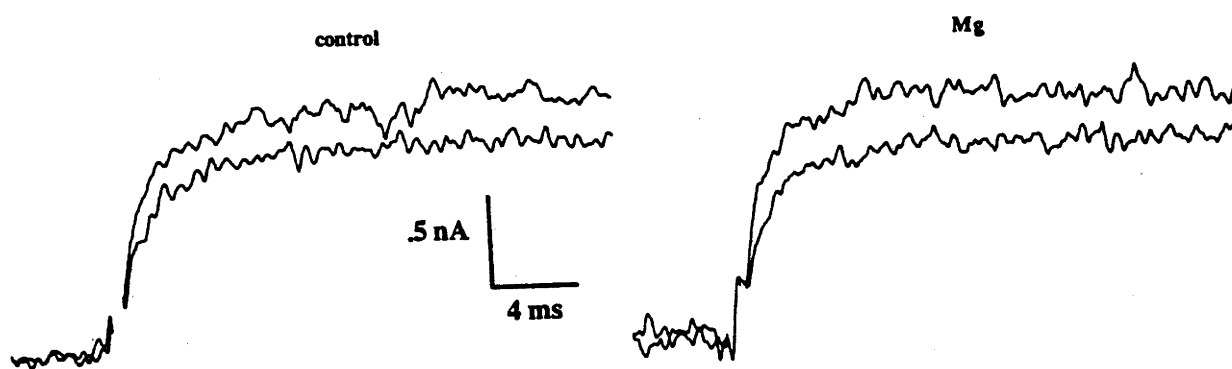
current-voltage relations are shown in Fig 6.15B. It is clear that cobalt reduced the potassium currents over the whole voltage range. This effect of cobalt is reversible as can be seen in Fig 6.15B where the data on returning back to a  $\text{Co}^{2+}$  Ringer is also plotted. To ensure that this effect was not due to a change in surface charge (Frankenhauser and Hodgkin, 1957) instantaneous current-voltage relations were obtained (Fig 6.16A, different cell) and conductance voltage curves constructed (Fig 6.16B). The measured reversal potentials before and after cobalt were  $-69\text{mV}$  and  $-70\text{mV}$  respectively and half maximal conductance was obtained at  $3.0\text{mV}$  and  $4.6\text{mV}$  respectively. Thus it is clear that the effect of cobalt cannot be explained by a change in surface charge. The activation and inactivation kinetics were also not significantly different in the presence of cobalt (data not shown).

One explanation of this effect of  $\text{Co}^{2+}$  may be that we are seeing a blockade of a residual calcium activated potassium current (eg. Meech and Standen, 1975). To test this hypothesis experiments were done in which the calcium in the external solution was replaced by magnesium. Out of five cells in which this was tested,  $\text{Mg}^{2+}$  Ringer caused an increase in the peak potassium current in two cells, had no effect in a further two cells and decreased the peak current in the fifth cell. The data from one cell in which  $\text{Mg}^{2+}$  had no effect is shown in Fig 6.17. Thus it seems that cobalt has a blocking effect on the delayed rectifier.

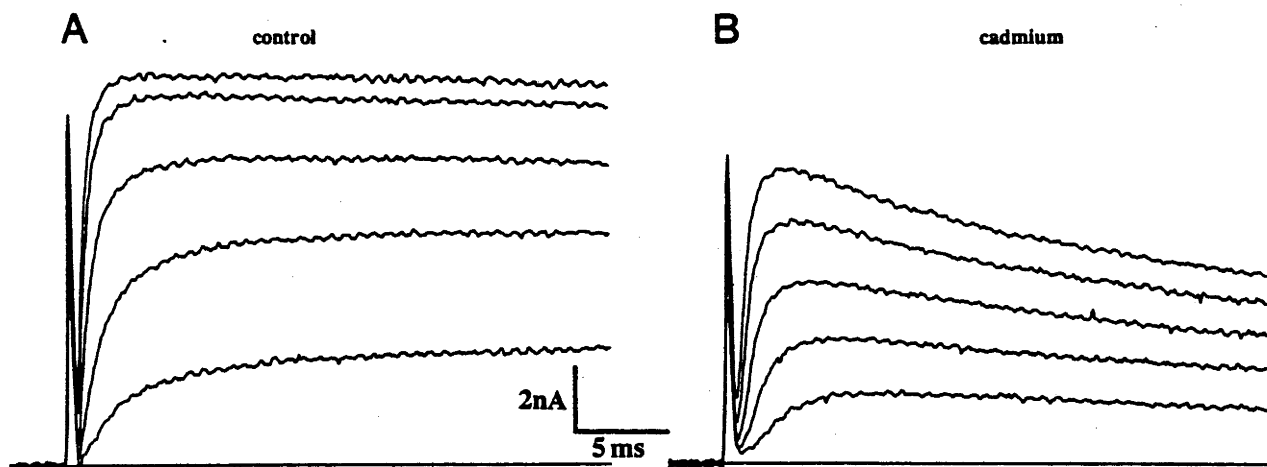
The effect of replacing all the calcium with cadmium was examined in two cells. In both cells there was a reduction in the peak amplitude of the outward current and there was an increase in the rate of inactivation (Fig 6.18). Lower concentrations of cadmium ( $100 - 500\mu\text{M}$ ) had no effect on the potassium current.



**Figure 6.16.** Cobalt effect is not due to surface charge. **A.** Instantaneous I-V relations measured in a cell bathed in calcium Ringer (circles) and immediately after changing over to a cobalt Ringer (squares). The lines through the data points have been drawn according to equation 6-4 (see text) with  $P_{Na}/P_K$  equal to 0.033. Reversal potentials obtained were  $-68\text{mV}$  (calcium Ringer) and  $-70\text{mV}$  (cobalt Ringer). **B.** Conductance ( $g$ ) calculated according to equation 6-1 in the same cell in calcium Ringer (circles) and in cobalt Ringer (squares). The lines through the data points are the linear regression lines of best fit to equation 6-2 and give values of  $V'$  and  $k$  of  $3.0\text{mV}$  and  $20.4\text{mV}$  (calcium ringer) and  $4.6\text{mV}$  and  $17.1\text{mV}$  (cobalt ringer) respectively.



**Figure 6.17.** Effect of magnesium Ringer on outward currents. Current traces in left panel were generated in response to a depolarizing voltage steps to +20 and +40mV from a holding potential of -80mV. Traces in right panel were recorded in the same cell after replacing the external ringer with a magnesium Ringer (0  $\text{Ca}^{2+}$ , 4.5mM  $\text{Mg}^{2+}$ ). Vertical calibration 0.5nA. Horizontal calibration, 4ms.



**Figure 6.18.** Effect of cadmium ringer. **A.** Current traces generated in response to depolarizing voltage steps to potentials between  $-40$  and  $+40\text{mV}$  from a holding potential of  $-80\text{mV}$ . **B.** Current traces in the same cell immediately after replacing the external solution with cadmium Ringer ( $0\text{ Ca}^{2+}$ ,  $2.5\text{ Cd}^{2+}$ ).

## DISCUSSION

The results presented in this chapter show that the voltage activated potassium current, the delayed rectifier ( $I_K$ ), in hippocampal CA1 neurones is very similar to same current studied in other cells. The current is activated with a delay at potentials more positive than  $-50\text{mV}$ , reaches a peak conductance around  $+40\text{mV}$  and can be fitted with a Hodgkin-Huxley type formulation. With prolonged depolarizations, the current inactivates with a time constant of about  $450\text{ms}$ .

### Comparison with Other Preparations.

Estimating the area of the cell from the input capacitance, average peak conductance was  $7.7 \pm 2.0 \text{ mS.cm}^{-2}$  ( $n=9$ ). This value is similar to the peak conductance of the delayed rectifier in rat sympathetic neurones (Belluzzi, Sacchi and Wanke, 1985b), cultured neuroblastoma cells (Moolenaar and Spector, 1978) and snail neurons (Reuter and Stevens, 1980), human T-lymphocytes and cultured hippocampal neurons (Rogawski, 1986) but is less than the peak conductance found in squid axon (Hodgkin and Huxley, 1952c). Assuming a single channel conductance of  $18\text{pS}$  (Conti and Neher, 1980, Rogawski, 1986) we calculate that there are about 4 delayed rectifier potassium channels per square micron on the soma of CA1 pyramidal neurones. This value compares well with the  $1\text{-}2 \text{ channels}/\mu^2$  reported from single channel from cultured hippocampal neurones (Rogawski, 1986) but much less than the density of the channels in the frog node of Ranvier (Begneish and Stevens, 1975). Single channel properties and densities of delayed rectifier channels in different preparations are compared in Table 6.1.

The currents reported here activate with an exponential time course as reported for the delayed rectifier in rat sympathetic neurons (Belluzzi, Sacchi and Wanke, 1985b). By comparison, a fourth power ( $n^4$ ) or higher formulation has been necessary to describe the delayed rectifier in squid axon (Hodgkin and Huxley, 1952d; Gilly and Armstrong, 1982), skeletal muscle (Adrian, Chandler and Hodgkin, 1970a; Stanfield, 1975; Pappone, 1980; Beam and Donaldson, 1983) and human T-lymphocytes (Cahalan et al., 1985) and  $n^2$  kinetics in cultured mouse neuroblastoma cells (Moolenaar and Spector, 1982).

**TABLE 6.1 Comparison of delayed rectifier channels.**

| Preparation                       | $\gamma$<br>(pS) | T<br>(°C) | Channel Density<br>(channels/ $\mu\text{m}^2$ ) |
|-----------------------------------|------------------|-----------|-------------------------------------------------|
| Squid Axon <sup>1</sup>           | 12               | 9         | 65                                              |
| Squid Axon <sup>2</sup>           | 18               | 5.5       | 72                                              |
| Snail Neuron <sup>3</sup>         | 2.4              | 12-16     | 7.2                                             |
| Frog Node <sup>4</sup>            | 2.7-4.6          | 15-18     | 760-1100                                        |
| Frog Node <sup>5</sup>            | 13,60            | 15        | 240                                             |
| Rabbit Node <sup>6</sup>          | -                | 14        | 0                                               |
| T-lymphocyte <sup>7</sup>         | 9,16             | 23        | 3                                               |
| Sympathetic Neuron <sup>8</sup>   | 18               | 37        | 9                                               |
| Hippocampus cultured <sup>9</sup> | 18               | 23        | 1-2                                             |
| Hippocampus CA1 <sup>10</sup>     | 18               | 23        | 4                                               |

References:<sup>1</sup> Conti et al.(1975), <sup>2</sup> Conti and Neher (1980),  
<sup>3</sup> Reuter and Stevens (1980),<sup>4</sup> Begenish and Stevens (1975),  
<sup>5</sup> Neumke et al.(1980),<sup>6</sup> Chiu et al. (1979), <sup>7</sup> Cahalan et al.(1985),  
<sup>8</sup> Belluzzi et al.(1985), <sup>9</sup>Rogawski (1986), <sup>10</sup>This study.  
<sup>2,7,9</sup> were single channel data. <sup>1,3,4</sup> were fluctuation analysis.  
<sup>8,10</sup> calculated from voltage clamp assuming 18pS single channel conductance.

Peak conductance was found to increase  $e$ -fold/13mV. This is similar to the voltage sensitivity reported for cultured hippocampal neurons (Rogawski, 1986) and rat sympathetic neurons (Belluzzi, Sacchi and Wanke, 1986b). By comparison the potassium conductance in squid axon (Hodgkin and Huxley, 1952b), skeletal muscle (Pappone, 1980; Beam and Donaldson, 1983), human (Chalan et al, 1985) and mouse T-lymphocytes (McKinnon and Ceredig, 1986) are much more steeply voltage dependent, increasing  $e$ -fold for about every 5mV.

The activation time constants of the currents described here are about two orders of magnitude faster than the kinetics of the delayed rectifier reported from cultured hippocampal neurons (Segal and Barker, 1984, Rogawski, 1986) at the same temperature. It is not clear why the currents reported here are so much faster than seen in cultured hippocampal cells. It may be that the internal perfusion alters the kinetics of the delayed rectifier. For example, in the squid giant axon (Adams and Oxford, 1983) it has been shown that internal perfusion with fluoride solutions slows the activation kinetics of the delayed rectifier. The activation time constants of the delayed rectifier in rat sympathetic neurons (Belluzzi, Sacchi and Wanke, 1985b) and cat motoneurons (Barrett, Barrett and Crill, 1980) are similar to those reported here. In addition, data obtained on the delayed rectifier from CA1 neurones in hippocampal slices in our laboratory (French and Gage, unpublished observations) suggests that the delayed rectifier in these cells has kinetics similar to the data presented here. Developmental studies on the delayed rectifier in cultured amphibian spinal neurones have demonstrated that the kinetics of this current change with maturation (Barish, 1986). It is thus possible that the difference in kinetics between the results presented here and the results obtained from cultured neurones are due to differences in cell maturity.

The ionic selectivity of the channel of potassium over sodium ( $P_{Na}/P_K$ ) is about 0.04, similar to that seen in squid axon (Binstock and Goldman, 1971; Bezanilla and Armstrong, 1972), snail neurons (Reuter and Stevens, 1980) and skeletal muscle (Gay and Stanfield, 1978). Thus, although there are many similarities between the properties of the delayed rectifier in hippocampal CA1 cells and other preparations there are also some differences. It is interesting to



Beluzzi, Sachhi and Wanke, 1985a). Calcium sensitive currents are not activated under the present recording conditions (see below). This could explain the absence of the A-current. Other possibilities are that perhaps like the calcium current (Kostyuk, 1984), the A-current is labile and therefore washed out during the dissociation and recording procedure, or that the A-current is present only on dendrites and so removed during the dissociation procedure. It is interesting that in a patch clamp study of potassium currents in the soma of cultured hippocampal cells Rogawski (1986) has reported that no single channel events corresponding to the A-current were found, despite the fact that A-currents can be recorded from the same cells using a two microelectrode voltage clamp (Segal and Barker, 1984). It is possible that the A-currents which can be recorded in the soma via the microelectrode are actually present in the dendrites of the cell thus not seen in patches of the soma.

We did not specifically look for the presence of the M-current (Halliwell and Adams, 1982), however its activation kinetics are slow and therefore unlikely to affect our analysis. The recently described voltage activated chloride current (Madison, Makenka and Nicoll, 1986) was also not seen. Reasons for this were discussed in Chapter 4.

### **Calcium Activated Currents.**

Hippocampal pyramidal neurones have also been shown to have several calcium activated potassium currents which activate over the same voltage range as the the delayed rectifier (Chapter 2; Brown and Griffith, 1982b; Lancaster and Adams, 1986; Lancaster and Nicoll, 1987). In dissociated cells, under the present recording conditions however there is no evidence to suggest the presence of such currents.

It can be seen from Figure 6.1 that the steady state current continued to increase until +80mV. It is clear that there is no region of negative slope resistance in the steady state current voltage relation as expected if there was a calcium activated component to the current (Meech and Standen, 1975; Galvan and Sedlmeir, 1984; Marty and Neher, 1985; Ritchie, 1987). In one cell in which the potassium current was followed to +100mV; again no N-shaped region was seen in the current-voltage relation. There are several reasons which can explain the absence of the calcium activated potassium currents in these cells. Firstly, the calcium current in these cells is

very small or in many cases absent (Chapters 3 & 4). Secondly, it has been shown by other investigators that the calcium activated potassium current can be suppressed by including strong calcium buffers in the pipette solution (Marty and Neher, 1985; Ritchie, 1987). The presence of a high concentration of fluoride as well as EGTA in the perfusing solution means that the intracellular  $\text{Ca}^{2+}$  concentration in our cells is strongly buffered to  $< 2\text{nM}$  (DeCoursey et al., 1984). Calcium activated potassium channels from muscle and sympathetic neurones have been found to require a free calcium concentration of at least  $100\text{nM}$  before they are activated (Barrett, Magelby and Pallota, 1982, Gardner, 1986). Thus the contribution of the calcium activated potassium current to the outward current recorded here would be very small.

### **Effects of Intracellular Perfusion.**

Intracellular perfusion of cells has been found to alter the voltage dependence and kinetics of ionic currents (Fernandez, Fox and Krasne, 1984; Horn and Vandenberg, 1986) and solutions high in fluoride have been shown to alter the kinetics of activation (Chandler and Meeves, 1970a; Adams and Oxford, 1983) and inactivation (Cahalan et al., 1985) of the delayed rectifier. We did not systematically look for shifts in the voltage dependence of the delayed rectifier in this study. Many cells were however recorded from for a period of up to 30 minutes. We found that, over this time, there was a gradual rundown of the peak current, however, no significant shifts in voltage dependence were observed.

### **Effects of Calcium Channel Blockers.**

It was surprising that the inorganic calcium channel blockers cobalt and cadmium (Hagiwara and Byerly, 1981) were found to block the delayed rectifier. Cobalt, manganese and nickel have been reported to depress a noninactivating voltage activated potassium conductance in cat motoneurons (Schwindt and Crill, 1981), in addition, these cations have been found to suppress the delayed rectifier in human T lymphocytes (Matteson and Deutsch, 1984; Decoursey et al., 1985). Cadmium and some of the heavier divalent cations (eg.  $\text{La}^{3+}$ ) were also found to speed up the decay kinetics of the delayed rectifier in human T lymphocytes (Decoursey et al., 1985). Experiments on squid axons also suggest that there may be an external divalent cation

binding site on the delayed rectifier which can alter the kinetics of the channel (Stanfield, 1975; Eaton and Brodwick, 1980; Gilly and Armstrong, 1982). Extracellular barium, for example has been shown to block the delayed rectifier (Armstrong and Taylor, 1980; Armstrong, Swenson and Taylor, 1982). Furthermore, Armstrong and Lopez-Barneo (1987) have recently suggested that the binding  $\text{Ca}^{2+}$  ions may be essential in the controlling the kinetics and selectivity of the delayed rectifier. It is possible that in hippocampal neurons this binding site could also alter the conductance or opening probability of the delayed rectifier channel. Another possible explanation is that, as suggested for delayed rectifiers in other preparations, the population of channels underlying the current is not homogeneous (eg. Dubois, 1981; Conti, Hille and Nonner, 1984, Gardner, 1986) and the calcium channel blockers are affecting one of the subpopulations. This result suggests that in any study in which the delayed rectifier is being examined in the presence of calcium channel blockers some caution must be exercised in interpreting the results obtained.

## **Chapter 7**

### **Discussion.**

It is now more than 30 years since Hodgkin and Huxley described the mechanisms underlying action potential generation and propagation in nerve membranes. When people began to look at neuronal cell bodies the discovery of the voltage gated calcium current and the various potassium current soon made it obvious that neuronal cells are much more complex than the squid axon studied by Hodgkin and Huxley. In recent years, the number of ion channels found in central neurones has rapidly escalated so that there are now thought to be several inward voltage dependent currents and many outward currents present in these cells. The work by Connor and Stevens (1971c) and Connor, Walter and McKown (1977) clearly demonstrate these different conductances are required by neurones in order to generate the complex spatiotemporal trains of membrane potential seen in these cells.

A few of these ionic conductances have been well characterised in some preparations, however, only qualitative descriptions exist for the majority of the known currents. In this thesis, the properties of several voltage gated ion channels present in the cell bodies of hippocampal CA1 pyramidal neurones have been examined. The ultimate goal of the type of work described here is the development of a complete mathematical description of central neurones based on the combination of the cells' passive properties and the known ionic conductances (ala the Hodgkin-Huxley axon). With an understanding of the electrical behavior of single neurones we will be in a position to explore several interesting questions: We could begin to try and predict the properties of networks of neurones with the hope of trying to understand the function of some areas of the nervous system. Secondly, we could look at the effects of pharmacological agents on specific neurones and assemblies of neurones. This would have important implications for evaluation and design of new neuroactive agents.

In order to produce a complete model of the active properties of a central neurone several pieces of information are needed: Firstly, we need to know the exact anatomy and passive membrane properties of the cells of interest. Secondly, we need to know the density and distribution of ionic channels present on the surface of these neurones. Thirdly, we need to understand the mechanisms whereby the function of these channels may be modified, and finally, we need to know the composition of the microenvironment of the neurones. The discussion of all these aspects of neuronal function are beyond the scope of this thesis. In this chapter, I will

TABLE 7.1 Voltage Activated Currents in Hippocampal Neurones.

|         | Current    | Ion | Activation | Inactivation | Blocker                                   |
|---------|------------|-----|------------|--------------|-------------------------------------------|
| Inward  | $I_{Na,t}$ | Na  | very fast  | fast         | TTX                                       |
|         | $I_{Na,s}$ | Na  | fast       | none         | TTX                                       |
|         | $I_{Ca,s}$ | Ca  | fast       | very slow    | Cd,DHP*                                   |
|         | $I_{Ca,t}$ | Ca  | fast       | fast         | Cd                                        |
|         | $I_Q$      | K   | slow       | none         | Cs                                        |
|         | $I_{Cl}$   | Cl  | slow       | none         | phorbol ester,?PKC†                       |
| Outward | $I_K$      | K   | fast       | slow         | Cs,TEA                                    |
|         | $I_A$      | K   | fast       | fast         | Cs,4-AP                                   |
|         | $I_C$      | K   | fast       | ?none        | Cs,TEA                                    |
|         | $I_M$      | K   | slow       | none         | Cs,TEA, carbachol                         |
|         | $I_{AHP}$  | K   | slow       | ?none        | Cs,carbachol,apamin<br>noradrenaline,cAMP |

\*DHP = Dihydropyridines. †PKC = Protein Kinase C

discuss the known distribution of voltage gated ion channels in vertebrate central neurones with a particular emphasis on hippocampal pyramidal neurones. For those channels for which no quantitative data exist, I will examine some strategies one could use to obtain this data.

### **Distribution and Density of Ion Channels.**

Neurones can be divided up into four regions based on their morphology: The dendrite, the soma, the axon hillock or initial segment and the axon (eg. Eccles, 1964). In vertebrate central neurones, at the present time, thirteen different voltage activated conductances have been described. There are two different sodium currents, three different calcium currents, six different potassium currents and two chloride currents. Voltage activated currents for which there is good evidence in hippocampal neurones are listed in Table 7.1. What is the distribution of these conductances over the surface of the neurone?

There are basically three approaches one can take in trying to map the distribution of ion channels over the surface of neurones. The first and traditional method is to look for the presence of the channels using electrophysiological techniques. The second approach is to label the channels with a high affinity probe and then look for the distribution of the probe. The last method that is available is to use ion sensitive dyes to look for changes in the concentration of various ionic species and to correlate this with ion channel distribution. All three of these methods can give us some idea of the channel density as well.

### **Electrophysiological Techniques.**

Because of the difficulties in voltage clamping vertebrate neurones, most of the studies discussed in this section have relied on voltage recordings. Early intracellular studies on spinal motoneurones revealed that these cells showed regional variability in electrical excitability (Coombs, Curtis and Eccles, 1957). These workers, and others (eg. Fatt, 1957a,b) found that when recording with a microelectrode in or near the soma, an antidromically evoked response showed an inflection in the rising phase of the action potential. If the soma is hyperpolarized, then the inflection becomes exaggerated, and eventually the large spike fails leaving a smaller regenerative response. This smaller spike has been called the IS (for initial segment) or A spike and Eccles and colleagues postulated that it arose in the region of the initial segment due to its

lower threshold for action potential generation. The larger response was said to be due to activation of the soma and proximal dendrites. Distal dendrites were suggested to not support sodium action potentials (Eccles, 1957, 1964; Dodge and Cooley, 1973). If we assume that the membrane properties of the neurone are isotropic, then these observations suggest that the density of sodium channels must be higher at the axon hillock. Although very indirect and qualitative, these early observations on the distribution of sodium channels over neurones have stood the test of time and motoneurones are still believed to lack sodium channels in their dendrites (Crill, 1983). A similar situation has been suggested to hold in the cerebellum (Llinas and Sugimori, 1981a,b), the deep cerebellar nuclei (Jahnsen 1986, a,b), thalamic neurones (Mulle, Madariaga and Desch enes, 1986) and substantia nigra neurones (Llinas, Greenfield and Jahnsen, 1984).

In hippocampal neurones on the other hand, sodium dependent spikes in the dendritic trees have been suggested by several different groups (Spencer and Kandel, 1961; Jeffries, 1979; Benardo, Masuwaka and Prince, 1982; Anderson, St orm and Wheal, 1987). Sodium spikes have also been directly recorded from isolated dendrites (Benardo, Masuwaka and Prince, 1982). At the present time there is no explanation as to why hippocampal neurones appear to have sodium dependent action potential in their dendrites whereas neurones from many other regions of the brain only seem to have them on the soma. Results reported in this thesis (Chapters 3,4,5) support the view that the fast sodium conductance is found on the soma of hippocampal pyramidal neurones, however, as the dendrites are removed during acute dissociation nothing can be added to the information on dendrites. We calculate that the density of fast sodium channels on the soma of hippocampal neurones is between 5 and 10 channels per  $\mu\text{m}^2$ . Assuming that all the channels are concentrated in the initial segment, and that the initial segment : soma ratio is similar to that in motoneurones, 0.08 (calculated from data in Conradi and Ronnevi, 1977) this means that the sodium channel density at the initial segment is between 65 and 130 channels/ $\mu^2$ .

Based on current clamp data, Llinas and Sugimori (1980a) were the first to suggest the presence of a noninactivating sodium current in cerebellar purkinje cells. Recording directly from the soma and dendrites they suggested that this conductance was distributed mainly over the soma and proximal dendrites (Llinas and Sugimori, 1980b). Evidence for a persistent sodium current has also been obtained in thalamic neurones (Janhsen and Llinas, 1984a), cortical neurones



(Connors, Gutnik and Prince, 1982; Stafstrøm, Schwindt, Flatman and Crill, 1984) and deep cerebellar (Jahnsen, 1986a) neurones. In some of these cases these authors have suggested that the persistent sodium current is also located mainly in the soma (Jahnsen, 1986b). Under voltage clamp, noninactivating sodium currents have been recorded at the soma in cortical (Stafstrøm, Schwindt and Crill, 1982; Stafstrøm et al., 1985) and hippocampal pyramidal (French and Gage, 1985) neurones. In Chapter 5, a noninactivating sodium current was demonstrated in isolated soma from hippocampal pyramidal cells. Thus, this conductance is clearly present in the soma and possibly the proximal dendrites of hippocampal CA1 neurones at a density of about 1 channel per  $10\mu^2$ . As there was a significant discrepancy between peak currents recorded in slices and dissociated cells a dendritic component cannot, however, be ruled out.

The distribution of calcium conductances has been examined in cerebellar purkinje cells (Llinas and Sugimori, 1980a,b), inferior olivary neurones (Llinas and Yarom, 1981a,b), thalamic neurones (Jahnsen and Llinas, 1984a,b; Mulle, Madariga and Deschênes, 1986), deep cerebellar neurones (Jahnsen, 1986a,b), nigral neurones (Llinas, Greenfield and Jahnsen, 1984) and hippocampal pyramidal cells (Benardo, Masuwaka and Prince, 1982). In all cases, high threshold calcium spikes (presumably mediated by the 'L' channel) have been suggested to be present in the distal dendritic trees. For all neurones it has been argued that these conductances are preferentially located in the dendrites. In hippocampal neurones, TTX resistant spikes have been reported in the isolated soma and isolated dendrites (Benardo, Masuwaka and Prince, 1982). The 'isolated somata' in the above study still had the proximal portion of the apical dendritic tree and the whole of the basilar dendritic tree attached. Thus it is possible that these TTX resistant spikes are generated in these distant regions. Persistent calcium currents have been recorded at the soma in hippocampal slices (Brown and Griffith, 1983b; Chapter 2) and acutely isolated neurones (Kay, Miles and Wong, 1986; Chapter 4). As discussed in Chapters 2 and 4, however, although slow inward calcium currents can be recorded at the isolated soma of these cells, it is probable that most of the current is located in the dendritic tree.

The low threshold calcium spike (activation of the 'T' channel) has also been seen in inferior olivary neurones (Llinas and Yarom, 1981b), thalamic neurones (Jahnsen and Llinas, 1984a,b; Mulle, Madariga and Deschênes, 1986) and dorsal horn neurones (Muraş and Randić,

1983). In all cases, it has been suggested to be located in the soma and proximal dendrites. The one exception is the report by Mulle et al. (1986) which suggests that in thalamic pacemaker neurones this conductance is located in the dendrites. In voltage clamp studies, a 'T' type calcium current has been reported in hippocampal CA1 cells in the slice (Halliwell, 1983; Chapter 2) and in the cell soma of cultured hippocampal neurones (Yarri, Hamon and Lux, 1987). In dissociated hippocampal neurones we found no evidence of a transient calcium current. Thus it appears that at least a large fraction of the high threshold calcium conductance may be located in the distal dendritic tree. The low threshold calcium current on the other hand seems to be located at or near the soma.

Potassium currents are more difficult to examine because current clamp techniques can only be used to examine the distribution of conductances which can generate active responses. Based on extracellular recording of fields, Llinas and Yarom (1981b) and Jahnsen (1986b) have argued that the calcium activated potassium conductance is located on the dendrites. In voltage clamp studies, calcium activated potassium conductances ( $I_C$  and  $I_{AHP}$ ) have been recorded at the soma of hippocampal pyramidal cells (Brown and Griffith, 1983b; Lancaster and Adams, 1986; Chapter 2). In this thesis calcium activated conductances were not examined. The dissociated cell technique should allow us to get more quantitative data on the properties and location of these currents at the cell soma.

The voltage activated potassium current ( $I_K$ ) has been usually assumed to follow the distribution of the fast sodium current (eg. Llinas, Greenfield and Jahnsen, 1984; Jahnsen, 1986b). A voltage activated potassium current with the properties of  $I_K$  has been recorded at the soma in spinal motoneurones (Barrett, Barrett and Crill, 1980).  $I_K$  can also be recorded in dissociated hippocampal neurones (Chapter 6), where the density of potassium channels was calculated to be about 4 channels per  $\mu m^2$ .

The A-current can be recorded at the soma of sympathetic neurones (Belluzzi, Sacchi and Wanke, 1984a; Galvan and Sedlmeir, 1985), hippocampal neurones both in slices (Gustafsson et al, 1982; Zbciz and Weight, 1985; Chapter 2) and in culture (Segal, Barker and Rogawski and Barker, 1984; Segal and Barker, 1984) and in cerebellar granule neurones in short term culture (Cull-Candy, Marshall and Ogden, 1987). There was no evidence for  $I_A$  is dissociated

hippocampal CA1 neurones and possible reasons for this were discussed in Chapter 6. Thus it is likely that this current is present in the soma of cortical neurones, however a dendritic  $I_A$  cannot as yet be excluded.

There are as yet no data on the possible distribution or density of the other potassium currents or chloride currents. This is largely because in most preparations it is only possible to record at the soma of the cell. With currents with slow kinetics, we expect most of the cell to be space clamped thus the current we see at the soma could have been generated anywhere on the surface of the neurone. The voltage activated chloride current has been suggested by Madison, Malenka and Nicoll (1986) to be located on the dendrites of hippocampal CA1 neurones. We found no evidence for this current in dissociated hippocampal neurones which have their dendritic trees missing. Thus the evidence seems to point to a dendritic location for this current. This assertion needs to be examined further using other techniques.

With the introduction of patch clamp techniques (Neher and Sakmann, 1983) and tissue culture methods (Nelson and Lieberman, 1981) and acute dissociation (Chapter 3) it may now be possible to map the distribution of many of the channels which have not as yet been examined. With the patch electrode it should be possible to directly examine the membrane of cultured neurones in all the areas of interest. Although one must be careful in extrapolating the results from cultured cells to neurones *in vivo* (see below), some of these uncertainties could be resolved using acutely dissociated neurones. As described in Chapters 4,5 and 6 we have for the first time some idea of the channel densities present in the somatic membrane of hippocampal pyramidal neurones. The application of patch clamp techniques to cultured and dissociated neurones promises some interesting results in the near future.

### **High Affinity Probes.**

As discussed above electrophysiological techniques which rely on recordings obtained with a single electrode in the soma are of limited value in obtaining information about the distribution or the density of ion channels over the neuronal, and particularly the dendritic membrane. An alternative approach is to take a molecule which can bind with high affinity to the channel of interest, label it with a probe, usually a radioactive group (eg.  $^{125}\text{I}$ ) or a fluorescent dye (eg. fluorescein 5-isothiocyanate), and then examine the distribution of the probe using biochemical

and histological assays. This technique was first used to map the distribution of acetylcholine receptors in skeletal muscle using  $^3\text{H}$ -labelled  $\alpha$  - bungarotoxin (Barnard, Wieckowski and Chiu, 1971). Subsequently, radiolabelled neurotoxins have been used to calculate the density and distribution of sodium channels in peripheral nerve and muscle (Ritchie and Rogart, 1977; Anglides, 1986). Catterall and Morrow (1978) examined [ $^3\text{H}$ ]saxitoxin binding on neuroblastoma (N18) cells and concluded that there were approximately 78 sodium channels per  $\mu\text{m}^2$  of membrane. More recently, Catterall (1981) has used  $^{125}\text{I}$ -scorpion toxin to localize sodium channels in cultured neuroblastoma cells and spinal neurones using light microscopic autoradiography. In this study it was found that in cultured spinal neurones, sodium channels were localized in high density in initial segments of individual neurites of many neurones. Although sodium channels were found all over the neurone, only one or two neurites had a high density of sodium channels. Catterall calculated that the density of sodium channels was approximately seven times higher in the initial segment than on the cell body. Except for the wide distribution of the sodium channel these results are entirely consistent with the electrophysiological observations of Eccles and colleagues as described above. As shown in Chapter 5, TTX also blocks a slow noninactivating sodium channel. Presumably, saxitoxin will bind to these channels as well. This might explain the wide distribution of the sodium channel seen by Catterall. Using data obtained from the above studies and combining this with data obtained from myelinated nerves (Ritchie and Rogart, 1977), Catterall (1984) has suggested the following distribution of sodium channels in a 'typical' neurone. Channel densities are given in channels/ $\mu\text{m}^2$ .

|                   |               |
|-------------------|---------------|
| Cell Body         | 50 to 75      |
| Axon hillock      | 350 to 500    |
| Unmyelinated axon | 110           |
| Node of Ranvier   | 2000 to 12000 |
| Internode         | <25           |
| Nerve terminal    | 20 to 75.     |

For comparison, the sodium channel density in dissociated hippocampal cells ranged between 5 and 10 channels per  $\mu\text{m}^2$ . This is an order of magnitude smaller than the cell body figures of Catterall. Even assuming that all the channels are concentrated at the initial segment the upper channels density is  $130/\mu^2$ . The reason for this discrepancy are not obvious. In motoneurons (Eccles, Libet and Young, 1958) and squid giant fibre lobe (Brismar and Gilly, 1987) it has been shown that the distribution of sodium channels dramatically changes following axotomy. Thus the channel distribution in cultured neurones may be more representative of axotomized neurones. Another possibility is that the channels in hippocampal cells are localized to the soma and we have overestimated the area of distribution due to the dendritic stumps still attached to the neurone.

For calcium channels, it has been shown that the dihydropyridines (DHP) bind with high affinity to one class of calcium channel (Miller, 1984, 1987). These drugs have been used for photoaffinity labelling and purification of calcium channels from nerve and muscle (eg. Flockerzi et al., 1986; Takahashi and Catterall, 1987), and have been used to localize calcium channels in T-tubules in muscle (eg. Fosset et al., 1983). In the central nervous system, three types of calcium channels have been found (see Chapter 1). Based on electrophysiological and biochemical evidence, Miller and colleagues have argued that DHP's only block one type of channel - the 'L' calcium channel. Furthermore, they have suggested that these channels are preferentially localized in the cell soma whereas the 'N' type channel is found on the nerve terminal and mediates transmitter release (Nowycky, Fox and Tsien, 1985; Kongsamut and Miller, 1986; Perney et al., 1986; Miller, 1987). The dihydropyridines do not bind to the 'T' type of calcium channels thus their distribution has not been explored. These data are at odds with the distribution of the high threshold calcium spike measured with electrophysiological techniques (see above), however, several reservations must be kept in mind. Firstly, it must be remembered that the evidence leading to the above conclusions is circumstantial and as yet there are no reports which have actually mapped the distribution of dihydropyridine binding sites on neuronal membranes. Secondly, most of the biochemical experiments have been done on cultured neurones and, as discussed above, these may have properties different to neurones *in vivo* (see below). Lastly, the DHP's do not bind as strongly to neuronal tissue as they do to muscle thus nonspecific effects must be rigourously excluded.

High affinity probes for the various potassium channels are just starting to be found. Apamin, a 18-amino acid peptide component of bee venom has been found to block a small conductance calcium activated potassium channel in several preparations (Burgess, Claret and Jenkinson, 1981; Romey and Lazdunski, 1984; Blatz and Magleby, 1986) and has been shown to block the conductance underlying the long lasting afterhyperpolarization seen in many cells (Romey and Lazdunski, 1984; Pennefather et al., 1985; Lancaster and Adams, 1986). A  $^{125}\text{I}$ -iododerivative has been prepared (Hugues, Duval et al., 1982) and used to affinity label and study the properties of the apamin binding site (Hugues, Schmid et al., 1982; Schmid-Antomarchi et al., 1984). In a preliminary study on cultured rat neurones Seagar et al. (1984) have reported there is a homogenous distribution of apamin receptors over the cell body of these cells and that their density is about 5% of the sodium channel density. There is as yet no definitive proof that the apamin binding site is the small calcium activated channel, however, this toxin is undoubtedly going to prove useful in future studies of this channel. Recently, a toxin (Charybdotoxin, CTX), has been isolated from the venom of scorpions (Miller et al., 1985) and found to selectively block the large calcium activated potassium channel in skeletal muscle. CTX has also been shown to slow the repolarization of the action potential in hippocampal pyramidal neurones (Størm, 1987; Lancaster and Nicoll, 1987), and has been suggested to block  $I_C$  in these cells. It remains to be seen whether this toxin will be useful in the purification and mapping of  $I_C$ .

For the rest of the potassium channels, no specific blockers have yet been found. Dendrotoxin (DTX), a venom prepared from the Green Mamba snake, has been suggested to selectively block the delayed rectifier in frog nerve (Weller et al., 1985) and guinea pig dorsal root ganglion neurones (Penner et al., 1986). Halliwell et al., (1986) have also reported an effect of DTX on the A-current in hippocampal pyramidal cells. DTX did not affect the delayed rectifier in rat T-lymphocytes (D. McKinnon, personal communication). It remains to be seen whether DTX will prove to be a useful tool for further characterization of the delayed rectifier.

Thus we can see that provided there is a high affinity molecule available to bind to the channels, this method can be useful in getting some idea of the channel distribution.

Unfortunately, except for the sodium channel, such tools have not been available for many of the

other channels. The didydropyridines and apamin may turn out to be useful in mapping the distribution of some calcium channels and the  $I_{AHP}$  channel, but this remains to be seen. For the rest of the channels, unless we find selective blockers it is going to be difficult to find their distribution. One hope is that once the protein structures of these channels are determined, perhaps by using molecular biological techniques (eg. see Jan et al., 1983), specific antibodies can be generated against the channels, and labelled antibodies can be used to find their distribution.

There are several reservations one must consider with this technique. In mapping channels using affinity probes one is forced to work with isolated cells - usually in culture. This is because as the cells are packed with high density in situ it is impossible to examine one cell in isolation. Neurones in the central nervous system are intimately associated with many glia, and are bathed in a 'soup' consisting of hormones and modulators which alter channel function. Cells grown in culture lack this. There is already some evidence that lack of glial cells alters the channel distribution of neural cells (Bostok and Raminsky, 1983). One solution to this problem may be to compare data obtained from cultured cells with acutely dissociated neurones. It is clear, however, that any result obtained in cultured cells will have to be verified with a noncultured preparation. The second problem with cultured cells is that one has no idea what type of cell one is dealing with. Any region of the brain is full of different types of cells. For example, in the hippocampus we have CA1, CA3 cells and interneurones which are known to have quite different electrophysiological properties and presumably a different complement of ion channels. It may be possible to get around this problem with the use of specific markers and sort the cells prior to culture with fluorescence (Gibb and Walmsley, 1987) or flowmicrofluorometry (St. John et al., 1986). This technique, however, remains to be proven with neurones. An alternate solution might be to use thin slices of brain tissue where the individual neurones can be visualised using interference optics (eg. Llinas and Sugimori, 1980a). If single neurones were to be highlighted using for example lucifer yellow (applied via a microelectrode), it may be possible to map the channel distribution in single, intact neurones from adult brain tissue.

## Optical Methods.

One of the biggest problems in studying ion channels in neurones is that the dendrites, which constitute the largest part of the neurone, are not easily accessible for electrophysiological recording. Also, as discussed above, binding studies with probe molecules are not really feasible with whole tissue. One way around some of these problems may be to use techniques which do not rely on easy access to individual neurones. Optical probes are now available which can measure membrane potential and calcium concentrations. These dyes change their optical properties (either changes in fluorescence or transmission) in response to changes in membrane potential or calcium concentration. Monitoring these dyes with optical recording techniques might allow us to examine the properties of single neurones without having to get too close to them.

Intracellular calcium concentration is very tightly regulated to a low concentration. Any influx of calcium from the extracellular fluid should therefore produce increases in calcium concentration. Any molecule which can indicate a change in calcium concentration will thus be a valuable indicator of calcium fluxes. Towards this end Tsien and colleagues have developed a series of photoactive calcium chelators which can act in the millisecond range (Grynkiewicz, Pognie and Tsien, 1985). Esterified derivatives of these compounds can cross the cell membrane and once inside the cell, the ester groups are cleaved by cytoplasmic esterases leaving the dye trapped in the cell. The dye now changes its fluorescence with the calcium concentration (for review see Tsien, Pozzan and Rink, 1984). This technique has been used in many cell types to examine the distribution and control of intracellular calcium (Tsien, Pozzan and Rink, 1984) and is now being applied to cultured neurones to look at the properties of voltage activated calcium channels (Perney, Dinderstein and Miller, 1984; Miller, 1987). Although still in its infancy, calcium activated fluorescent dyes should be able to answer many questions about the properties of calcium channels in small processes such as dendrites.

The second class of dyes that are being developed are those that change their properties in response to changes in membrane potential. Voltage sensitive dyes can either be applied extracellularly (eg. see Grinvald, 1984) or injected intracellularly via microelectrodes (Grinvald et al., 1987). These dyes have a time resolution on the order of microseconds and using



photodiode arrays to detect the optical changes a spatial resolution of up to  $50\mu\text{m}$  can be obtained (Grinvald, 1984). These dyes can therefore be used to study potential changes in many parts of the neurone simultaneously. In principle, it should be possible to carry out experiments which examine changes in action potential shape and thus infer membrane channel properties (eg Llinas and Sugimori, 1980a,b). In preliminary studies in hippocampal slices, it seems that this is possible (Grinvald and Segal, 1984). The problems with these dyes are firstly that it is very difficult to quantitate the potential changes and secondly, the signal to noise ratio obtained is very poor. Optical recording is still in its infancy and improvements in these techniques and in the types of dyes available should allow us to address some interesting questions about neurones in the future.

### **Conclusion.**

In conclusion, it is clear from the above discussion that although much is known about the function and distribution of voltage gated ion channels in vertebrate neurones, we are still a long way from constructing a realistic and useful model to describe the active properties of these cells. Techniques are now at hand to fill in many of the gaps in our understanding of these cells. In order to achieve this goal we will have to combine different electrophysiological, biochemical and molecular biological techniques.

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