VOLTAGE GATED POTASSIUM CHANNELS
OF CULTURED RAT CENTRAL NEURONS

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Whole cell and single channel patch clamp recording were used to study the properties of two types of voltage gated potassium channels in rat neurons. Neurons were dissociated from either the locus coeruleus or hippocampus of neonatal rats and grown in primary cell culture for 6 to 17 days before recording. Cultured neurons from both these brain areas were found to express whole cell A-currents and delayed rectifier currents, similar to those seen in other neurons. The conductance of single A-current channels in locus coeruleus neurons was 14.8pS, although this declined at positive membrane potentials. This reduction in unitary amplitude was shown to be due to voltage dependent block of the channel by intracellular magnesium and sodium ions. The \( K_v \) values of the blocking reactions at 0mV membrane potential were 15.7mM for magnesium and 76.0mM for sodium, the \( K_v \) of each block decreasing with increasing membrane depolarisation. The conductance of single delayed rectifier channels in hippocampal neurons was 18.4pS. Gating of this channel could be explained by a model in which the channel had one open state, four closed states and three inactivated states. This scheme was not consistent with a Hodgkin-Huxley model of voltage dependent gating, but instead favoured a model whereby gating occurred in a cooperative manner. Gating could be altered by changing the permeant ion from potassium to the less permeable rubidium; this had the effect of specifically slowing all channel closing rate constants. The rubidium permeability of this channel was 75% of the potassium permeability. External tetraethylammonium ions blocked delayed rectifier channels at two independent sites, each block having different kinetic properties. The \( K_v \) of the kinetically faster block was 53.4\( \mu \)M, whilst that of the slower block was estimated to be between 400 and 800\( \mu \)M.
FOREWORD

The techniques of whole cell and single channel patch clamp recording were used to study some of the properties of A-current and delayed rectifier potassium channels in neurons dissociated from the locus coeruleus and hippocampal CA1 areas of neonatal rat brains, grown in primary cell culture. All of the experiments were carried out in the Department of Physiology at the University of Leicester between October, 1989 and September, 1992. Those experiments carried out on locus coeruleus neurons were carried out in collaboration with Dr. Ian Forsythe.

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CHAPTER ONE: INTRODUCTION

Ionic Basis of Neuronal Excitability

The ability of nerve cells to pass electrical signals depends on ionic concentration gradients across the neuronal membrane and the relative permeability of the membrane to these ions. At rest, the membrane has a relatively large permeability to potassium ions and only a slight sodium permeability. Metabolic processes constantly pump sodium out of the cell, so that the extracellular sodium concentration greatly exceeds the intracellular concentration. Potassium concentration is higher inside the cell than outside, as the concentration gradient tending to drive potassium ions out of the cell is matched by the potential gradient tending to drive them in. Thus the inside of the cell is negatively charged with respect to the outside. The membrane potential (in mV) at which there is no net flux of potassium ions is the potassium equilibrium potential ($E_K$), as given by the Nernst equation for potassium ions:

$$E_K = \frac{RT}{zF} \ln \frac{[K^+]_o}{[K^+]_i},$$

(1 - 1)

where $R$ is the gas constant (units J K$^{-1}$ mol$^{-1}$), $T$ is the temperature (in Kelvins), $F$ is Faraday's constant (the total charge carried by one mole of monovalent ions; units C mol$^{-1}$) and $z$ is the valency of potassium ions (+1). $RT/F$ is termed the thermodynamic potential, and is the potential needed to balance an e-fold ionic concentration ratio across the membrane; at 20°C, this has the value of approximately 25 mV. $[K^+]_o$ and $[K^+]_i$ are the concentrations of potassium ions outside and inside the cell respectively.

During an action potential, the membrane transiently becomes more permeable to sodium. Sodium ions therefore flow into the cell, down both concentration and potential gradients, causing the membrane potential to rapidly become more depolarized, as it moves towards the sodium equilibrium potential. Increases in membrane potassium permeability cause the membrane potential to move towards the potassium equilibrium potential, causing a hyperpolarisation from resting levels.

It is now known that the ionic currents which underlie electrical excitability flow through ion channels, membrane-spanning proteins which form aqueous pores across the
membrane. These pores are usually highly selective for one particular ion type, allowing that ion to pass through the membrane at a very high rate (see below) whilst excluding most other ions. In different channel types, pore opening and closing is controlled by changes in membrane potential (see below), neurotransmitters (for example the nicotinic acetylcholine receptor) or intracellular factors (for example calcium-activated potassium channels). Because ionic currents are carried passively through pores and not by some active process, ions will always flow down their electrochemical gradient through open channels.

**Techniques for Studying Ionic Currents**

The study of the electrical currents which flow in nerves began in earnest following the development of the voltage clamp technique by Cole (1949) and Hodgkin, Huxley and Katz (1949). Voltage clamp allows the currents flowing across a cell membrane to be measured whilst controlling the membrane potential. Using this technique, Hodgkin and Huxley (1952a-d) gave the first description of the action potential mechanism, in the squid giant axon. They identified two ionic currents activated by membrane depolarization, one carried by sodium ions and the other by potassium. They showed that when the membrane was depolarized, the inward sodium current was activated rapidly but transiently, leading to the depolarizing phase of the action potential; the outward potassium current was activated only after a delay, and acted to repolarize the membrane and so keep the action potential short. Hodgkin and Huxley termed this the “delayed rectifier” potassium current.

In the early 1970s, a new experimental technique, that of fluctuation analysis, was applied to the study of acetylcholine-activated current at the frog neuromuscular junction (see Stevens, 1975). It was shown that the addition of acetylcholine to voltage-clamped muscle fibres not only caused a steady inward current, but also induced marked fluctuations around the mean current (Anderson & Stevens, 1973). Fourier analysis of these fluctuations yields power spectra (the square of the fluctuation amplitude in each frequency interval analysed), which can give information on how long individual channels stay open and on the conductance of single channels. Anderson & Stevens (1973) estimated the conductance of these channels at the frog neuromuscular junction to be around 30pS. A conductance of 12pS was found when fluctuation analysis was first used to study delayed rectifier potassium channels in squid giant axon (Conti, DeFelice & Wanke, 1975). This was the first direct evidence that the ion channels which carry ionic currents are indeed pores, as
the ionic fluxes suggested by these conductance measurements were too large for any carrier mechanism. For example, a current of 1pA corresponds to a net flux of approximately $6 \times 10^4$ monovalent ions per second, which is much faster than the fastest known carrier or enzyme reactions (Hille, 1984).

Hodgkin and Huxley (1952d) suggested that the voltage-dependent activation of the sodium and delayed rectifier currents in the squid giant axon were due to the movement of charged “particles” in the membrane due to the electric field (see below). If the actual ionic currents across a membrane are blocked, by replacing permeant ions with impermeant ones and by applying channel blocking drugs, the tiny “gating current” due to movement of such particles may be recorded using appropriate voltage clamp techniques (Armstrong & Bezanilla, 1973, 1974). The existence of the gating current has since been exploited extensively in the study of voltage-dependent channels.

The existence of ion channels as discrete pores was finally and unequivocally proven following the development of the patch clamp technique (Neher & Sakmann, 1976; Hamill, Marty, Neher, Sakmann & Sigworth, 1981; see chapter 2), which allowed the current through individual channels to be measured directly in a small patch of voltage clamped membrane.

**VOLTAGE ACTIVATED POTASSIUM CURRENTS IN MAMMALIAN CENTRAL NEURONS**

**Role of Potassium Channels in the Central Nervous System**

A large number of different potassium channels exist in electrically excitable cells, showing a far greater diversity than that of sodium, calcium or chloride channels (Hille, 1984; Rudy, 1988). Many different classes of potassium channels have now been identified in the mammalian central nervous system (CNS; Halliwell, 1989; see below). All potassium channels have a basically inhibitory role, as their activation tends to draw the membrane potential towards the potassium equilibrium potential and so further away from the threshold for action potential firing. Potassium channels therefore play an important role in regulating the excitability and firing patterns of central neurons. This excitability may also be greatly influenced by neurotransmitters and second messengers which modulate potassium channel function in the brain.
Properties

The delayed rectifier was the first potassium current to be demonstrated and to have its kinetic properties described (Hodgkin & Huxley, 1952a-d). On depolarization, this current increased with an S-shaped time course, and on repolarization it decayed with a single exponential. Hodgkin and Huxley (1952d) suggested that the current was controlled by four similar membrane-bound "particles", and that for potassium ions to flow each particle must occupy a specified position in the membrane. If the proportion of particles in the correct position is given by \( n \), the form of the conductance change could be described by:

\[
\tilde{g}_K = \tilde{g}_K n^4
\]

(1 - 2)

where \( \tilde{g}_K \) is the maximum possible conductance. Each of the four particles is assumed to carry a charge, so that their distribution in the membrane is sensitive to the membrane potential. The rate at which each particle moves from the incorrect position for conduction to the correct position is \( \alpha_n \), and the rate for moving from the correct to the incorrect position is \( \beta_n \). Because of the charge on each particle, the rate \( \alpha_n \) is increased by depolarization, and \( \beta_n \) is decreased.

The resting value of \( n \), \( n_0 \), is described by:

\[
n_0 = \frac{\alpha_{n_0}}{\alpha_{n_0} + \beta_{n_0}}
\]

(1 - 3)

If the membrane potential is now suddenly changed, \( \alpha_n \) and \( \beta_n \) will be changed immediately, and \( n \) will change at a rate:

\[
\frac{dn}{dt} = \alpha_n (1 - n) - \beta_n n
\]

(1 - 4)

The solution of this differential equation with respect to time is, if \( n = n_0 \) when \( t = 0 \):

\[
n = n_\infty - (n_\infty - n_0) e^{-t/\tau_n}
\]

(1 - 5)

where
As \( n \) increases exponentially on depolarization (according to equation 1-5), \( n^4 \) will rise with an S-shaped time course, leading to the observed delayed increase in \( g_K \). On repolarization, both \( n \) and \( n^4 \) decrease exponentially towards zero. Hodgkin and Huxley derived a similar set of expressions to describe the sodium current in the squid giant axon (Hodgkin & Huxley, 1952d). Together, their expressions for just these two ionic currents were sufficient to predict the major features of electrical excitability in the squid giant axon, such as action potential threshold, shape and duration, and conduction velocity.

Although the electrical responses of the squid giant axon were described quite adequately by just two ionic currents, when the voltage clamp technique was applied to other excitable cells it was soon discovered that many had membrane currents not seen in squid axons. For example, crab muscle fibres were shown to exhibit calcium spikes (Fatt & Ginsborg, 1958) and an inwardly rectifying potassium current was discovered in frog skeletal muscle (Katz, 1949).

A second class of voltage gated potassium current, activated alongside the delayed rectifier, was first described in neurons of the mollusc *Onchidium verruculatum* (Hagiwara, Kusano & Saito, 1961) and subsequently in supramedullary neurons of the puffer fish (Nakajima & Kusano, 1966; Nakajima, 1966). This current differed from the delayed rectifier in that it was activated more rapidly following depolarization, but subsequently inactivated, giving the current a transient nature. A similar current was observed in neurons of the mollusc *Anisodoris*, where it was named the A-current (or \( I_A \)) (Connor & Stevens, 1971a). The general voltage-dependent properties of the A-current were first described by Connor & Stevens (1971a) in *Anisodoris* neurons, and by Neher (1971) in neurons from the snail *Helix pomatia*. In both cases the A-current was activated more rapidly than other outward currents following depolarization, and it subsequently inactivated with an exponential time-course. The A-current showed a high level of steady-state inactivation, such that when the membrane was voltage clamped close to the resting potential and then
depolarized, only delayed outward currents were seen. Steady-state inactivation was removed by hyperpolarizing the membrane prior to depolarization, with the degree of removal of inactivation depending on both the magnitude and the duration of the hyperpolarizing step.

Delayed rectifier and A-currents have since been shown to coexist in many different cells, including mammalian peripheral (Galvan & Sedlmeir, 1984; Belluzzi, Sacchi & Wanke, 1985a,b) and central neurons (see below), as well as astrocytes (Bevan & Raff, 1985; Tse, Fraser, Duffy & MacVicar, 1992). Both current types can be identified in many animal phyla, suggesting a diversification of voltage gated potassium channels occurred early in evolution (Hille, 1984).

Several different mammalian central neurons appear to have a very similar A-current, which reaches a peak amplitude within 10ms of membrane depolarization, and then inactivates exponentially with a time constant of 20-40ms. Such rapidly inactivating A-currents have been reported in hippocampal (Segal, Rogawski & Barker, 1984; Numann, Wadman & Wong, 1987; Ficker & Heinemann, 1992; Wu & Barish, 1992), dorsal raphe (Aghajanian, 1985), neostriatal (Surmeier, Bargas & Kitai, 1988), cortical (Zona, Pirrone, Avoli & Dichter, 1988; Spain, Schwindt & Crill, 1991), cerebellar (Cull-Candy, Marshall & Ogden, 1989), thalamic (Huguenard, Coulter & Prince, 1991) and hypothalamic neurons (Müller, Misgeld & Swandulla, 1992), and also in neurohypophyseal nerve terminals (Thor, Wang & Lemos, 1991). The activation threshold of these currents is between -60 and -70mV, and half the maximum current is activated at between -20 and -35mV. As in molluscan neurons, these A-currents are inactivated at relatively depolarized membrane potentials; half inactivation occurs at holding potentials between -50 and -85mV.

However, rapidly activating potassium currents which inactivate more slowly (over hundreds of milliseconds) have been described in hippocampal (Gustafsson, Galvan, Grafe & Wigström, 1982; Zbicz & Weight, 1985; Storm, 1988; Ficker & Heinemann, 1992; Wu & Barish, 1992), locus coeruleus (Williams, North, Shefner, Nishi & Egan, 1984), hypothalamic (Greene, Haas & Reiner, 1990) and thalamic neurons (McCormick, 1991). In some cases, such currents have been described as A-currents, whilst in others (eg. Storm, 1988; Ficker & Heinemann, 1992; Wu & Barish, 1992), slowly inactivating currents co-exist with fast A-currents.

Voltage activated potassium currents which inactivate only over several seconds
have been described in hippocampal (Segal & Barker, 1984; Numann et al., 1987; Sah, Gibb & Gage, 1988), cortical (Zona et al., 1988; Spain et al., 1991), cerebellar (Cull-Candy et al., 1989), thalamic (Huguenard & Prince, 1991) and hypothalamic neurons (Müller et al., 1992). These currents have a higher activation threshold than the A-current in the same cell (between -30 and -50mV). Unlike delayed rectifier currents in other cells, these currents show steady-state inactivation at depolarized membrane potentials, which is removed by hyperpolarization.

Although voltage gated potassium currents in mammalian central neurons can be broadly separated into these categories, there is a wide spread of properties, particularly in the rate of inactivation, between different neurons. A potentially very large number of different voltage dependent potassium channels are encoded by the rat genome (see below), and different potassium channel genes are differentially expressed in different areas of the rat brain (Beckh & Pongs, 1990; Drewe, Verma, Frech & Joho, 1992; Rettig, Wunder, Stocker, Lichtinghagen, Mastiaux, Beckh, Kues, Pedarzani, Schröter, Ruppersberg, Veh & Pongs, 1992; Sheng, Tsaur, Jan & Jan, 1992; Tsaur, Sheng, Lowenstein, Jan & Jan, 1992). Different brain areas also show different densities of binding sites for certain high-affinity potassium channel probes (Awan & Dolly, 1991) and for antibodies to specific potassium channel gene products (Trimmer, 1991; Sheng et al., 1992). It is therefore possible that different central neurons contain a wide range of potassium channels with very slightly different properties which exactly suit their needs in terms of firing patterns.

Pharmacology

The first blocker of a potassium channel to be discovered was the tetraethylammonium ion (TEA), which was shown to block the delayed rectifier current of squid giant axons when injected into the axon, whilst having no effect when applied externally (Tasaki & Hagiwara, 1957). TEA and other quaternary ammonium ions have since been shown to block delayed rectifier channels in most preparations, usually acting from either side of the membrane (Stanfield, 1983). However, TEA is not a specific blocker of the delayed rectifier current; it is also known to block inward rectifier and some calcium-activated potassium channels (Stanfield, 1983), ATP-sensitive potassium channels (Spruce, Standen & Stanfield, 1987a; Davies, Spruce, Standen & Stanfield, 1989), and also neuronal chloride channels (Sanchez & Blatz, 1992).
The delayed rectifier current in mammalian central neurons has been found to be sensitive to external TEA at concentrations of 10-30mM (Segal & Barker, 1984; Numann et al., 1987; Sah et al., 1988; Zona et al., 1988; Cull-Candy et al., 1989; McLarnon, 1989). However, 10mM TEA has been reported to have no effect when applied to the intracellular face of rat hypothalamic neuronal membranes (McLarnon, 1989).

Early experiments showed that the A-current was less sensitive to block by external TEA than the delayed rectifier (Connor & Stevens, 1971a; Neher & Lux, 1972). The first selective blocker of the A-current to be described was 4-aminopyridine (4-AP), which blocked the A-current in molluscan (Tritonia) neurons at millimolar concentrations, without affecting either delayed rectifier or calcium-activated potassium currents (Thompson, 1977). 4-AP has since been shown to block A-currents in a number of preparations, including mammalian central neurons (Segal et al., 1984; Storm, 1988; Surmeier et al., 1988; Cull-Candy et al., 1989; Huguenard et al., 1991; Thorn et al., 1991; Wu & Barish, 1992) at concentrations of 1-3mM. Lower concentrations of 4-AP (30-50μM) are required to block the slowly inactivating current of rat hippocampal neurons (Storm, 1988; Ficker & Heinemann, 1992). A similar current in rat nodose ganglion cells is also sensitive to very low concentrations of 4-AP (Stansfeld, Marsh, Haliwell & Brown, 1986). The increase in evoked neurotransmitter release (Thesleff, 1980; Buckle & Haas, 1982) and central convulsant activity (Galvan, Grafe & ten Bruggencate, 1982) caused by low concentrations of 4-AP have often been suggested to be due to block of presynaptic and postsynaptic A-current channels. However, as pointed out by Stansfeld et al. (1986), the concentration required to block the A-current is far greater than the convulsive threshold, and block of a more slowly inactivating current may contribute to these effects.

The delayed rectifier current in squid giant axons (Yeh, Oxford, Wu & Narahashi, 1976) and murine B lymphocytes (Choquet & Korn, 1992) is also blocked by 4-AP. However, the delayed rectifier in mammalian central neurons is reported to be insensitive to 4-AP block (Segal & Barker, 1984; Sah et al., 1988; Zona et al., 1988; McLarnon, 1989; Huguenard & Prince, 1991). Tetrahydroaminoacridine (THA), which has a similar structure to 4-AP, is a more potent blocker of the A-current in cultured rat hippocampal neurons (Rogawski, 1987), but at higher concentrations also blocks sodium and delayed rectifier channels.

Transmitter release at the neuromuscular junction is also facilitated by dendrotoxin
(DTX), a peptide component of the venom of the eastern green mamba (*Dendroapsis angusticeps*; Harvey & Karlsson, 1980). DTX has also been shown to block the A-current in rat central neurons at nanomolar concentrations (Halliwell, Othman, Pelchen-Matthews & Dolly, 1986). In rat hippocampal neurons (Wu & Barish, 1992) and also in rat peripheral neurons (Stansfeld *et al.*, 1986; Stansfeld & Feltz, 1988), slowly inactivating potassium currents are more sensitive to DTX than A-currents. Toxin I, an homologous peptide to DTX from the black mamba (*Dendroapsis polylepis*), has similar actions but at even lower concentrations (Harvey & Anderson, 1985). Mast cell degranulating peptide (MCP), a protein from bee venom, blocks the slowly inactivating potassium current in rat peripheral neurons without affecting the A-current (Stansfeld, Marsh, Parcej, Dolly & Brown, 1987).

Different peptides isolated from scorpion toxins have been reported as being selective blockers of A-current, delayed rectifier or calcium activated potassium channels in rat brain synaptosomes (Blaustein, Rogowski, Schneider & Krueger, 1991).

A number of other drugs have been reported to block neuronal voltage gated potassium channels. The sulphonylurea glibenclamide, which is usually described as a selective blocker of ATP-sensitive potassium channels, also blocks the slowly-inactivating potassium current in rat hippocampal neurons (Crepel, Kmjevic & Ben-Ari, 1992) and a delayed rectifier-like current in SH-SY5Y human neuroblastoma cells (Reeve, Vaughan & Peers, 1992). Quinidine blocks both the A-current and delayed rectifier in rat hippocampal neurons (Oyama, Harata & Akaike, 1992). The class III antidysrhythmic drug clofilium, the therapeutic effects of which are due at least in part to block of cardiac potassium channels, also blocks the delayed rectifier current of NG 108-15 mouse neuroblastoma x rat glioma hybrid cells (Reeve & Peers, 1992).

**Neurotransmitter Modulation**

In rat hippocampal neurons, noradrenaline acting on β adrenoceptors (Sah, French & Gage, 1985) and acetylcholine acting on muscarinic receptors (Nakajima, Nakajima, Leonard & Yamaguchi, 1986) have been shown to decrease the amplitude of the A-current, as have noradrenaline acting on α₁ adrenoceptors in rat dorsal raphe neurons (Aghajanian, 1985) and 5-HT in rat cerebellar Purkinje cells (Wang, Strahlendorf & Strahlendorf, 1992).

All of these neurotransmitters may therefore increase neuronal excitability via their effects on the A-current. Cholecystokinin causes a depolarizing shift in both the activation and inactivation of the A-current in rat hippocampal neurons (Saint & Bucket, 1991), which
may underlie the excitatory effects of cholecystokinin on the hippocampus (Dodd & Kelly, 1981). The amplitude of the A-current in rat hippocampal neurons is increased by GABA acting on GABA_{A} receptors, due to a depolarizing shift in the voltage dependence of inactivation (Saint, Thomas & Gage, 1990), an effect which would lead to a decrease in the excitability of these cells.

Acetylcholine acting on muscarinic receptors causes a hyperpolarizing shift in both activation and inactivation of the A-current in rat neostriatal neurons (Akins, Surmeier & Kitai, 1990). This leads to a differential effect of acetylcholine on these cells, depending on the state of the neuron; when it is relatively hyperpolarized, acetylcholine reduces excitability by shifting the activation range of the A-current to membrane potentials which are subthreshold for action potential firing. However, when the neuron is more depolarised, acetylcholine makes it more excitable by shifting the inactivation range of the A-current, increasing the level of steady-state inactivation. Thus it was shown that at different membrane potentials, application of acetylcholine could increase or reduce the amplitude of the A-current (Akins et al., 1990). This study emphasises the importance of the role played by the A-current in controlling the excitability of this central neuron.

Neurotransmitter receptors which are not in themselves ion channels (in the way that, for example, the nicotinic acetylcholine receptor is) may affect channels via the actions of GTP-binding proteins (G-proteins; Brown & Birnbaumer, 1990; Brown, 1990). These may affect ion channels directly or via enzymes involved in second messenger production, such as adenylate cyclase, cyclic GMP phosphodiesterase, phospholipase C or phospholipase A\textsubscript{2}. The link between receptor activation and functional modification of A-current channels has not been demonstrated for any of the above examples. However, protein kinase C, which is the target of several second messengers, has been shown to reduce the amplitude of the A-current in Xenopus oocytes injected with chick brain total RNA (Lotan, Dascal, Naor & Boton, 1990) or mRNA transcribed from an identified Drosophila potassium channel gene (Moran, Dascal & Lotan, 1991).

Direct modulation of the delayed rectifier current in mammalian central neurons has not been demonstrated. However, the delayed rectifier current expressed by a cloned mouse brain potassium channel gene in Xenopus oocytes is suppressed by 5-HT, when coexpressed with the gene for a cloned mouse brain 5-HT\textsubscript{1c} receptor (Hoger, Walter, Vance, Yu, Lester & Davidson, 1991). The effect on the delayed rectifier current was
suggested to be due to the activation of a calcium-calmodulin activated phosphatase, and recovery of the current from suppression could be due to a protein kinase.

However, modulation of the delayed rectifier current does occur in other tissues. The delayed rectifier current of guinea pig myocytes is enhanced by the peptide endothelin, this modulation occurring via activation of phospholipase C, leading to protein kinase C activation and release of calcium from intracellular stores, both of which appear to modulate channel activity (Habuchi, Tanaka, Furukawa, Tsujimura, Takahashi & Yoshimura, 1992). The delayed rectifier channel in the squid giant axon is modulated by phosphorylation of its intracellular face, which results in a shift of all voltage dependent properties towards more positive potentials (Perozo, Bezanilla & Dipolo, 1989; Perozo, Jong & Bezanilla, 1991), probably due to electrostatic interactions between the channel voltage sensor and the negatively charged phosphate groups (Perozo & Bezanilla, 1990).

**Single Channel Studies**

Delayed rectifiers were the first potassium channels to be studied at single-channel level, in internally perfused squid giant axons (Conti & Neher, 1980). These channels were estimated to have a conductance of 9-11pS. In the cut-open squid giant axon, delayed rectifier channels with a conductance of 20pS have been recorded (Llano, Webb & Bezanilla, 1988). Similar channels have been observed from lobster axon membranes (when incorporated into lipid bilayers), where the conductance was 28pS (Coronado, Latorre & Mautner, 1984), in frog skeletal muscle, where it was 15pS (Standen, Stanfield & Ward, 1985), in bovine chromaffin cells (18pS; Marty & Neher, 1985), embryonic chick heart (15pS; Clapham & Logothetis, 1988), and in canine and porcine trachealis cells (13pS; Boyle, Tomasic & Kotlikoff, 1992). Single channels underlying a delayed rectifier-like current in rat phaeochromocytoma (PC-12) cells have a conductance of 7pS (Hoshi & Aldrich, 1988a). T lymphocytes have two delayed rectifier-type channels, with conductances of 9 and 16pS in human (Cahalan, Chandy, DeCoursey & Gupta, 1985) and 12 and 21pS in mouse (DeCoursey, Chandy, Gupta & Cahalan, 1987). Two delayed rectifier channels in *Drosophila* neurons have conductances of 10-16pS and 20-40pS respectively (Sole & Aldrich, 1988). Delayed rectifier channels with greater conductance (over 50pS) have been reported in rabbit smooth muscle (Benham & Bolton, 1983), embryonic chick heart (Clapham & DeFelice, 1984) and chick ciliary ganglion cells (Gardner, 1986). Single delayed rectifier channels have also been recorded in rat...
hypothalamic neurons, where the conductance was 48pS (McLarnon, 1989), and in rat cerebellar Purkinje cells, where it was 28pS (Gähwiler & Llano, 1989).

Single A-current channels have been recorded in snail (*Helix aspersa*) neurons, where the conductance was 14pS (Taylor, 1987), in molluscan neurons, where it was 9pS (Premack, Thompson & Coombs-Hahn, 1989) and in chick dorsal root ganglion neurons, where it was between 18 and 32pS (Florio, Westbrook, Vasko, Bauer & Kenyon, 1990). In *Drosophila*, two different A-current channels have been identified; a neuronal type with a conductance of 6-8pS (Solc, Zagotta & Aldrich, 1987; Solc & Aldrich, 1988), and a muscle type with a conductance of 12-16pS (Solc et al., 1987; Zagotta & Aldrich, 1990a). A-current channels in mammalian peripheral neurons have a conductance of approximately 20pS (Cooper & Shrier, 1985, 1989; Kasai, Kameyama, Yamaguchi & Fukuda, 1986; McFarlane & Cooper, 1991), similar to that found in PC-12 cells (18pS; Hoshi & Aldrich, 1988a) and rat olfactory receptor neurons (17 and 26pS; Lynch & Barry, 1991). Single channels underlying the more slowly inactivating potassium current in rat dorsal root ganglion cells have a conductance of 5-10pS (Stansfeld & Feltz, 1988).

All single channel conductances quoted were measured at physiological potassium concentrations, where the intracellular potassium concentration greatly exceeds the extracellular concentration. This is a necessary requirement for the comparison of conductances between different preparations, as the conductance of ion channels is increased by increasing the concentration of permeant ions on either side of the membrane.

Roles

The role originally demonstrated for the delayed rectifier current was to repolarise the membrane after an action potential (Hodgkin & Huxley, 1952a-d), and such a role is still usually ascribed to similar currents in other cells (Hille, 1984), although other potassium channels have been shown to be involved in action potential repolarization in central neurons (see below). Connor & Stevens (1971b) demonstrated that the role of the A-current in *Anisodoris* neurons is in the control of action potential frequency. They showed that the steady-state inactivation of the A-current was only removed during the period of hyperpolarization immediately following an action potential (the afterhyperpolarization). Therefore, when the membrane was next depolarized, the A-current would be activated and would act against the depolarization, slowing the approach of the membrane potential to threshold and so reducing action potential
frequency. A similar role was demonstrated in crab axons, where the repetitive firing behaviour of different classes of axons is determined by the presence or absence of the A-current (Connor, 1975).

In the mammalian central nervous system, the roles of voltage dependent potassium channels have been studied under conditions where their influence is reduced by neurotransmitters or blockers. This has often involved current clamp recording, in which constant current is injected into the cell and changes in membrane potential are measured. As direct neurotransmitter modulation of the delayed rectifier in the mammalian CNS has not been demonstrated, and no selective blockers are known, its role is not well understood. External TEA does prolong the action potential in hippocampal neurons (Segal & Barker, 1984; Storm, 1987), implying that it is blocking a current involved in action potential repolarization. However, this effect may be due to blockade of a fast calcium dependent potassium current which has been suggested to perform the function of action potential repolarization in mammalian central neurons (Zbicz & Weight, 1985; Storm, 1987).

Several different roles have been put forward for the A-current in the mammalian CNS. In rat dorsal raphe neurons, which act as pacemaker cells, inhibition of the A-current by noradrenaline shortens the normally long-lasting plateau between action potentials and so accelerates pacemaker activity (Aghajanian, 1985). In rat hippocampal neurons, where the A-current is inhibited by muscarinic agonists, both acetylcholine and 4-AP induce the firing of trains of action potentials when the membrane is current-clamped close to threshold (Nakajima et al., 1986). Action potential frequency is also increased by application of 4-AP in rat neostriatal neurons (Bargas, Galarraga & Aceves, 1989).

4-AP also reduces the long firing latency of rat neostriatal neurons on depolarisation (Bargas et al., 1989), suggesting that this latent period is due to activation of the A-current during the approach to threshold (Bargas et al., 1989). Inhibition of the A-current by 5-HT or 4-AP in rat cerebellar Purkinje cells similarly reduces firing latency (Wang et al., 1992). The latency to the first action potential is also reduced by 4-AP in rat peripheral neurons (Galvan & Sedlmeier, 1984).

As well as reducing the latency to action potential firing in response to a constant stimulus, block of the A-current can also reduce the firing threshold in some neurons. 4-AP causes action potential firing in current-clamped guinea-pig (Gustafsson et al., 1982) and rat (Segal et al., 1984) hippocampal neurons in response to depolarizing currents which
are normally sub-threshold. DTX similarly reduces firing threshold in rat hippocampal neurons (Halliwell et al., 1986).

In some neurons, the A-current may also have a role in the repolarization of the action potential. 4-AP increases spike duration in rat hippocampal (Storm, 1987) and amygdala neurons (Gean & Shinnick-Gallagher, 1989), and also in rat neurohypophyseal nerve terminals (Thorn et al., 1991). The A-current has also been implicated in action potential repolarization in a rat peripheral neuron (Belluzzi et al., 1985a). 4-AP also blocks the accommodation of action potential firing seen during long depolarizations of rat amygdala neurons (Gean & Shinnick-Gallagher, 1989).

Obviously, in neurons which contain both a fast A-current and a more slowly inactivating potassium current, the effects of 4-AP and DTX could be due to the block of either or both currents. By studying the effects of concentrations of 4-AP lower than that required to block the A-current, Storm (1988) showed that the slowly inactivating potassium current is responsible for the long delay to the first action potential when current-clamped hippocampal neurons are injected with a depolarizing current. The slow recovery from inactivation of this current also enables these neurons to integrate separate subthreshold depolarizing inputs over time (Storm, 1988).

OTHER POTASSIUM CHANNELS IN MAMMALIAN CENTRAL NEURONS

Calcium Activated Potassium Channels

A potassium current activated by an increase in cytosolic calcium concentration was first described in Aplysia neurons (Meech, 1972), and calcium-activated potassium currents have since been identified in almost every excitable cell examined (Hille, 1984). Calcium-activated potassium channels have been separated into two main types (see Rudy, 1988): a small conductance channel which is voltage insensitive and is blocked by apamin (a small peptide from bee venom) but not by TEA; and a large conductance channel which is both calcium and voltage sensitive, and is blocked by low concentrations of TEA and charybdotoxin (a component of scorpion venom).

Two different calcium-activated potassium channels have been identified in hippocampal neurons, with conductances of around 200pS and 19pS (Franciolini, 1988; Lancaster, Nicoll & Perkel, 1991). The large conductance channel is voltage-dependent and TEA-sensitive, and underlies the rapidly activating and inactivating calcium-activated
potassium current in these cells (Zbicz & Weight, 1985; Numann et al., 1987). This current is responsible for the fast afterhyperpolarization (around 10ms) that follows single action potentials in hippocampal neurons, and is also involved in spike repolarization (Storm, 1987). Four different calcium-activated potassium channels, each with similar properties to the large-conductance channel of hippocampal neurons, have been isolated from rat brain membrane vesicles (Reinhart, Chung & Levitan, 1989). The smaller conductance channel identified in hippocampal neurons is voltage- and TEA-insensitive and is responsible for the long afterhyperpolarization (several seconds) which causes accommodation of spike firing in response to a long depolarizing stimulus (Madison & Nicoll, 1984; Lancaster & Adams, 1986). This slow calcium-activated potassium current is modulated by the neurotransmitters acetylcholine, noradrenaline, histamine, 5-HT, dopamine and adenosine in hippocampal neurons (Halliwell, 1989).

A third type of calcium-activated potassium channel may underlie afterhyperpolarizations of medium duration (around 200ms) in hippocampal neurons (Storm, 1989). This current may be separated from that responsible for the slow afterhyperpolarization described above as it is both voltage- and TEA-sensitive (Brown & Griffith, 1983; Numann et al., 1987).

**Inward Rectifier Potassium Channels**

The phenomenon of inward (or anomalous) rectification was first described by Katz (1949), who noted that skeletal muscle fibres passed inward but not outward current when bathed in high potassium concentration solutions. Inward rectifier potassium channels are activated by hyperpolarization and allow inward potassium current to flow, but outward currents are prevented by blockade of the channel by intracellular magnesium ions (Matsuda, Saigusa & Irisawa, 1987; Vandenberg, 1987). Inwardly rectifying potassium currents have been identified in neurons of the olfactory cortex (Constanti & Galvan, 1983), raphe nucleus (Williams, Colmers & Pan, 1988) and globus pallidus, where it is reduced by substance P (Stanfield, Nakajima & Yamaguchi, 1985).

Inward rectification in mammalian central neurons may also be due to another hyperpolarization-activated current which is permeable to both potassium and sodium ions, which is known as $I_q$ (Halliwell & Adams, 1982; Mayer & Westbrook, 1983; Spain, Schwindt & Crill, 1987). A similar current is found in the heart, where it contributes to pacemaking (DiFrancesco, 1981; DiFrancesco, Ferroni, Mazzanti & Tromba, 1986).
M-Current Channels

The M-current is a slowly activating, non-inactivating voltage dependent potassium current, which was first discovered in frog sympathetic neurons (Brown & Adams, 1980). In these cells the M-current is turned off by muscarinic receptor agonists, a property which gave the current its name. In hippocampal neurons, the M-current is inhibited by muscarinic agonists (Halliwell & Adams, 1982) and 5-HT (Colino & Halliwell, 1987) and is activated by somatostatin (Moore, Madamba, Joels & Siggins, 1988). Muscarinic inhibition of the M-current in hippocampal neurons is thought to involve the phosphatidyl inositol pathway (Dutar & Nicoll, 1988), whilst augmentation by somatostatin may involve the phospholipase A2-arachidonic acid pathway (Schweitzer, Madamba & Siggins, 1990).

ATP-Sensitive Potassium Channels

Potassium channels which are inhibited by intracellular ATP have been studied in cardiac muscle, skeletal muscle and pancreatic β cells (Ashcroft, 1988). Single ATP-sensitive potassium channels have been identified in cultured rat cortical neurons (Ashford, Sturgess, Trout, Gardner & Hales, 1988) and in neurons from the ventromedial hypothalamic nucleus in rat brain slices (Ashford, Boden & Treherne, 1990). These channels have a greater conductance (about 50pS) and lower sensitivity to ATP (half blocked by 2-3mM) than those studied in other cell types (Ashford et al., 1988, 1990; cf. Ashcroft, 1988). Potassium channels with a conductance of approximately 26pS are activated by the potassium channel opener cromakalim or by energy-depleting conditions in cultured rat hippocampal neurons, and these channels are blocked by the ATP-sensitive potassium channel blocker glyburide (Politi & Rogawski, 1991). There is also electrophysiological and pharmacological evidence that ATP-sensitive potassium channels are present on some central presynaptic terminals (Häusser, de Weille & Lazdunski, 1991).

Agonist-Activated Potassium Channels

Central neurons may be hyperpolarized by neurotransmitters which act by opening potassium channels, for example GABA, adenosine, 5-HT, noradrenaline, acetylcholine, dopamine, opiates and somatostatin (Nicoll, 1988; Halliwell, 1989; Brown, 1990). In many cases, responses elicited by co-application of two different agonists are non-additive, indicating that they activate the same channels. Application of the G-protein $G_\alpha$ to the
intracellular face of hippocampal neuronal membranes activates four different, presumably agonist-sensitive, potassium channels (VanDongen, Codina, Olate, Mattera, Joho, Bimbaumer & Brown, 1988). The potassium conductance activated by neurotransmitters shows many of the characteristics of an inward rectifier current: rectification dependent on both voltage and the potassium equilibrium potential, blockade by barium and caesium ions, and impermeability to rubidium. In *Aplysia* neurons, 5-HT may activate inward rectifier channels by removing the voltage sensitivity of gating (Benson & Levitan, 1983), and it is possible that some neurotransmitters may also act in such a way in mammalian central neurons. Neurotransmitters may also activate neuronal M-current channels (see above).

**PROPERTIES OF ION CHANNEL PORES**

**Constant Field Theory and Independence**

The most commonly used model to describe the ionic permeability of membranes is the constant field theory developed by Goldman (1943) and Hodgkin & Katz (1949). Assuming that ions partition directly into a homogenous membrane from the bulk solution on either side, that ions can cross the membrane in either direction independently of each other, and that the electric field falls linearly across the membrane, then the net ionic flux inside the membrane will be determined solely by the concentration gradient and the electric field, and is given by the Goldman-Hodgkin-Katz (GHK) current equation:

$$I_S = \frac{P_S z_S^2 F E^2 [S]_i - [S]_e e^{-2(\sigma E F RT)}}{RT}$$

(1 - 8)

$I_S$ is the current carried by a permeant ion $S$ with a permeability $P_S$, a valency $z_S$ and internal and external concentrations $[S]_i$ and $[S]_e$ respectively. Hodgkin & Katz (1949) defined $P_S$ as:

$$P_S = \frac{D_S^* \beta_S^*}{l}$$

(1 - 9)

where $D_S^*$ is the diffusion coefficient for $S$ within the membrane (units cm$^2$/s), $\beta_S^*$ is the water-membrane partition coefficient for $S$ (dimensionless), and $l$ is the thickness of the membrane (in cm).
The GHK current equation predicts non-linear or “rectifying” current-voltage relationships when the permeant ion concentrations on either side of the membrane are unequal, such that the conductance is larger when current flows from the more concentrated side. Under these conditions, more ions are brought into the membrane, and so the effective membrane resistance is decreased; when current flows from the more dilute side, fewer ions enter the membrane, and so the effective membrane resistance is increased.

Deviation from Independence

The constant field theory assumes that the probability of an ion crossing the membrane is independent of the presence of other ions. If such independence is the case, then changing the permeant ion concentrations from \([S]_i\) and \([S]_o\) to \([S']_i\) and \([S']_o\) should change the current from \(I_s\) to \(I'_s\) by the ratio:

\[
\frac{I'_s}{I_s} = \frac{[S']_i - [S']_o e^{-(\alpha D F / RT)}}{[S]_i - [S]_o e^{-(\alpha D F / RT)}}
\]  (1 – 10)

This is the independence relation of Hodgkin & Huxley (1952a). However, when permeant ion concentrations are raised high enough, this relationship breaks down and ionic currents begin to saturate. Saturation is presumed to arise when the binding of permeant ions to sites in the channel becomes rate limiting. Other deviations from independence include the competition which takes place between different permeant ions for binding sites in the channel, and the competitive block of these sites by impermeant ions. These findings suggest that ions do not diffuse freely through the membrane, as in constant field theory, but that they must bind to certain sites in the pore, and that these binding sites can hold only one ion at a time.

Ion permeation through channels is now usually considered in terms of Eyring rate theory (Glasstone, Laidler & Eyring, 1941), which describes any rate process as a series of jumps over energy barriers. Thus, the pore is represented by a sequence of energy peaks and wells (which equate to ion binding sites) across the membrane (Eyring, Lumry & Woodbury, 1949). These energy profiles correspond to the Gibbs free energy of an ion at different positions across the membrane. The Gibbs free energy of an ion within the membrane is the sum of a fixed “chemical” component and an electrostatic component, which is due to the membrane electric field and (where the channel can hold more than
one ion at a time; see below) electrostatic repulsion between different ions. If the number
of energy barriers in the pore is large and each has the same height, the pore becomes more
uniform, and the flux equations derived by Eyring et al (1949) to describe ionic movements
across a neuronal membrane converge to the GHK equation (Woodbury, 1971).

When the concentration of permeant ions is low, the binding sites within the pore
are rarely occupied, and so the rules of independence are obeyed. However, at high
permeant ion concentrations, the binding sites are occupied most of the time, and so current
through the pore will begin to saturate.

Single-ion and Multi-ion Pores

Since the GHK current equation assumes independence of ionic movements, it can
be split into two expressions describing the ionic flux across the membrane in either
direction:

\[ I_s = P_s z_s^2 \frac{EF^2 [S]}{RT (1 - e^{-\Delta G_{1}E/FRT})} \] (1-11)

\[ I_s = P_s z_s^2 \frac{EF^2 [S]}{RT (1 - e^{\Delta G_{o}E/FRT})} \] (1-12)

\[ I_s \] and \[ I_s \] represent the efflux and influx of \( S \) across the membrane. The ratio of these two
unidirectional fluxes is the same as the Ussing flux ratio for free-diffusion systems (Ussing,
1949):

\[ \frac{I_s}{I_s} = \frac{[S]_i}{[S]_o} e^{\left(\Delta G_{i}E/FRT\right)} \] (1-13)

This ratio still holds for an ion channel pore containing several ion binding sites at all
permeant ion concentrations, provided that the pore can contain only one ion at a time (a
so called "single-ion pore", eg. Hille, 1975).

In cuttlefish axons, it has been found that potassium ion fluxes (carried primarily
through delayed rectifier channels) do not obey the Ussing flux ratio, unless the right hand
side of the equation is raised to a power \( n' \) (Hodgkin & Keynes, 1955):
The value of \( n' \) in cuttlefish axons was around 2.5. Values of \( n' \) greater than one suggest that the diffusing particle consists of more than one ion, and is evidence that the channel holds more than one ion at a time, the ions moving simultaneously in single file.

Other lines of evidence suggest that some channels have such “multi-ion single-file pores”. The evidence for some types of potassium channels has been summarised by Hille & Schwarz (1978). Briefly, the apparent effective valency of a blocking reaction by a monovalent cation (the valency of the blocking ion multiplied by the fraction of the total membrane electric field through which it moves) may be greater than one in a multi-ion pore, due to repulsion between ions within the pore. Also, the degree of block may show a greater than linear dependence on the blocker concentration. Thirdly, adding potassium ions to the opposite side of the membrane to which a blocker has been applied may relieve the block and even allow more current to flow from the side with the blocker, probably due to potassium ions entering the pore and repelling the blocking ion. A fourth line of evidence is the so-called “anomalous mole-fraction” effect, first observed in anion-selective channels from crayfish (Takeuchi & Takeuchi, 1971) and fish muscle fibres (Hagiwara & Takahashi, 1974a), and also seen in inward rectifier channels of starfish eggs (Hagiwara & Takahashi, 1974b; Hagiwara, Miyazaki, Krasne & Ciani, 1977). In these channels, conductance is higher and reversal potential more positive when all external potassium is replaced by thallous (Tl⁺) ions, indicating that thallous ions are more permeant than potassium. However, when thallous and potassium solutions are mixed together, conductance is less and the reversal potential more negative than when either ion is added alone. When either of these values goes through a minimum or maximum as a function of the ratio of permeant ion concentrations, the channel concerned shows anomalous mole-fraction dependence.

All of the above effects may be explained by assuming that the channel pore is able to hold more than one ion at a time, passing through in single file. It may also be necessary to assume a high probability that more than one ion is in the pore at any time, that ions in the pore repel one another, and that energy barriers within the pore are lower than those which determine exit from the pore (see Hille & Schwarz, 1978).
Selective Permeability

Much of the selectivity of ion channels can be accounted for in terms of the size of the pore and the radii of dehydrated ions. Thus, channels are permeable only to ions below a certain size, presumably due to a narrow region within the pore; this fact may be used to give an estimate of the dimensions of the narrowest part of the pore (Hille, 1984). However, pore size alone cannot explain the sequence of selectivity among permeant ions. For example, sodium channels are highly selective for sodium over (slightly larger) potassium ions, whereas potassium channels are highly selective for potassium over sodium. Permeability ratios are usually calculated by measuring the change in reversal potential when all ions of one type on one side of the membrane are replaced by another type (see Hille, 1984; chapter 7).

In terms of free energy profiles within pores, selectivity is described by the difference between the Gibbs’ free energy lost in dehydrating the ion and that gained by interaction with polar sites within the pore. For group I cations, only eleven selectivity sequences occur as a function of the Gibbs’ free energy differences; these are the so-called “Eisenman sequences” (Eisenman, 1962):

\[
\begin{align*}
I & : \text{Cs}^+ > \text{Rb}^+ > \text{K}^+ > \text{Na}^+ > \text{Li}^+ \\
II & : \text{Rb}^+ > \text{Cs}^+ > \text{K}^+ > \text{Na}^+ > \text{Li}^+ \\
III & : \text{Rb}^+ > \text{K}^+ > \text{Cs}^+ > \text{Na}^+ > \text{Li}^+ \\
IV & : \text{K}^+ > \text{Rb}^+ > \text{Cs}^+ > \text{Na}^+ > \text{Li}^+ \\
V & : \text{K}^+ > \text{Rb}^+ > \text{Na}^+ > \text{Cs}^+ > \text{Li}^+ \\
VI & : \text{K}^+ > \text{Na}^+ > \text{Rb}^+ > \text{Cs}^+ > \text{Li}^+ \\
VII & : \text{Na}^+ > \text{K}^+ > \text{Rb}^+ > \text{Cs}^+ > \text{Li}^+ \\
VIII & : \text{Na}^+ > \text{K}^+ > \text{Rb}^+ > \text{Li}^+ > \text{Cs}^+ \\
IX & : \text{Na}^+ > \text{K}^+ > \text{Li}^+ > \text{Rb}^+ > \text{Cs}^+ \\
X & : \text{Na}^+ > \text{Li}^+ > \text{K}^+ > \text{Rb}^+ > \text{Cs}^+ \\
XI & : \text{Li}^+ > \text{Na}^+ > \text{K}^+ > \text{Rb}^+ > \text{Cs}^+
\end{align*}
\]

Sequence IV corresponds most closely with the observed permeability sequences of most potassium channels, and sequence X with sodium channels. Sequence I applies to the smallest increase in Gibbs’ free energy associated with the interaction of the ion with the pore (where the binding site is a “weak site”), which is one likely to be the anion of a
strong acid), and sequence XI to the largest increase (for interaction with a “strong site”, likely to be the anion of a weak acid). For this reason, sodium channels are relatively easily blocked by protons compared with potassium channels. For a weak site, the Gibbs’ free energy associated with the electrostatic interaction with a cation is small in relation to that of dehydration, so ions which are more easily dehydrated will interact most strongly with the site. For a strong site, the Gibbs’ free energy of interaction is large in relation to that of dehydration, so the smallest ion will approach closest to a strong site and interact most strongly with it.

It appears that the selectivity filters of potassium and sodium channels act by excluding impermeant ions rather than preferentially binding permeant ones within the pore; it has been demonstrated that preferential binding of an ion does not necessarily make the pore selective for that ion (Bezanilla & Armstrong, 1972; Armstrong, 1975). Instead, it is the height of the largest energy barrier within the pore for different ions which determines selectivity; for potassium-selective channels, this barrier would have to correspond to interaction of the permeating ion with a relatively weak site in the pore, such that the Gibbs’ free energy gained by the ion during the interaction was enough to stabilise potassium, but not sodium ions during a dehydration step. The physical nature of this site is not known, although it has been proposed that permeant ions may interact with oxygen atoms in the pore wall, for example in protein carboxy groups, in both potassium (Armstrong, 1975) and sodium channels (Hille, 1975).

GATING OF SINGLE ION CHANNELS

Kinetic Models of Ion Channel Gating

Although the Hodgkin-Huxley model for the activation of the delayed rectifier current (Hodgkin & Huxley, 1952d) did not postulate the existence of channels, it can be interpreted as a model for the gating of a single delayed rectifier channel, with four hypothetical particles controlling channel opening and closing. It is assumed that the channel can exist in one of a number of discrete open or closed states (Armstrong, 1969):
C₁ to C₄ represent closed states of the channel, and O the open state. Multiples of α and β represent transition rates between these discrete kinetic states. The different closed states have different numbers of particles in the correct position for channel opening; from none in C₁ to three in C₄. At rest, as α is small and β is large, most channels will be in state C₁. On depolarization, α becomes large and β small, so the channels will tend to move towards the open state, but opening will occur after a delay, leading to S-shaped macroscopic current activation.

**Open and Closed Time Distributions**

Since the duration of individual ion channel openings and the time between openings can now be measured using patch-clamp recording, kinetic models such as the one above have been put forward for a number of different channel types, both voltage- and chemically-activated, and attempts have been made to calculate the transition rates between various states. The transitions of a single ion channel between discrete states may be treated as a Markov process (Colquhoun & Hawkes, 1977), meaning that the lifetime of each state is a memoryless random variable. “Memoryless” here means that state durations and the probability of a transition taking place are not affected by the past history of the channel, only by its present state. The probability density function (p.d.f.) of the lifetime of any state will have an exponential distribution, with a mean lifetime of the reciprocal of the sum of all the transition rates that lead away from that state (Colquhoun & Hawkes, 1977, 1983). For a channel with the kinetic scheme shown above, it would therefore be possible to measure the durations of a large number of openings, and fit an exponential function to the open time distribution histogram; the time constant of this exponential will then be the mean open time, which will correspond to the reciprocal of the transition rate.
Obviously, patch clamp data can only give information in the form of open and closed times, so where multiple closed (or open) states are proposed, the durations of each state cannot be determined directly. In this case, the closed (or open) time distribution histogram will be fitted by the sum of several exponentials, with the number of exponentials needed being the number of closed (or open) states (Colquhoun & Hawkes, 1981, 1982). This of course assumes that all the exponential components of the time distribution histogram have been resolved; since this cannot be proven to be the case, the number of exponential functions actually gives the minimum number of states.

For voltage-activated channels, the time between the start of a voltage step and the first opening of the channel (known as the first latency) can also give information about the number of closed states (Fukushima, 1981; Patlak & Horn, 1982; Aldrich & Yellen, 1983). For a linear kinetic scheme such as that shown above, the channel is assumed to reside in the closed state $C_1$ at rest, and must therefore pass through all the other closed states before the channel can open when the membrane is depolarized. The time taken to pass through all these states will be the first latency, and its probability density function will reflect the number of closed states which must be passed through during activation (see chapter 2).

**Bursting Behaviour of Channels**

In many cases where single ion channels have been studied, including delayed rectifier and A-current channels, several channel openings have been seen to occur in rapid succession, followed by a longer gap before another burst begins (Sakmann, Patlak & Neher, 1980; Conti & Neher, 1980; Coronado et al., 1984; Standen et al., 1985; Hoshi & Aldrich, 1988b; Zagotta & Aldrich, 1990a; Solc & Aldrich, 1990). Such behaviour may be interpreted as rapid, repeated transitions between the open state and the first (short-lived) closed state ($C_4$ in the above scheme); the burst is terminated when the channel moves to $C_3$, a longer-lived closed state. However, at depolarised potentials where $\alpha$ is much greater than $\beta$, the Hodgkin-Huxley model predicts that $C_4$ will be the longest-lived closed state. As pointed out by Conti & Neher (1980), the Hodgkin-Huxley model is insufficient to explain the gating of delayed rectifier channels at the single channel level.

The presence of bursting behaviour is evidence for the existence of more than one closed state, and studying only the closed times which occur within a burst may give some
information on one of the closed states in isolation (Colquhoun & Hawkes, 1981). The number of openings per burst, the burst length, the total open time per burst, total closed time per burst, and closed times within and between bursts may all be useful in studying the mechanisms of ion channel opening and closing (Colquhoun & Hawkes, 1982). For example, the number of exponential components found in the distributions of the open times, of the number of openings per burst and of the total open time per burst should all be equal to the number of open states.

In some cases, a number of bursts of openings may be followed by a very long closed time before several more bursts occur, indicating that the bursts occur in clusters (Sakmann et al., 1980). By direct analogy to the occurrence of bursts, clustering is evidence for the existence of at least three distinct closed states, and the analysis of clusters may be carried out using the same principles as the analysis of bursts (Colquhoun & Hawkes, 1982).

**Testing Hypothetical Kinetic Schemes**

Any postulated kinetic scheme for ion channel opening and closing which can be described as a Markov process may be tested using methods derived by Colquhoun & Hawkes (1977, 1981, 1982, 1983, 1987), and it should also be possible to estimate rate constants for some of the transition rates. If the number of kinetically distinguishable states in which the system can exist is k, then we can define a k x k matrix, P(t), whose elements are the probabilities of a transition between two states in time t. It can then be shown (Colquhoun & Hawkes, 1977, equation 9) that:

\[
dP(t)/dt = P(t)Q
\]

since \( P(0) = I \), the unit matrix, this may be simplified to:

\[
P(t) = e^{Qt}
\]

where \( Q \) is a matrix containing elements \( q_{ij} \) which are the rate constants for transitions between state i and state j. The diagonal elements of the matrix are constructed such that each row of \( Q \) sums to zero, so that \( q_{ii} \) is minus the sum of the rate constants for all transitions leaving state i. Using Armstrong's (1969) model of delayed rectifier channel gating as an example (see above), the \( Q \) matrix is:
It is convenient to subdivide $Q$ up into smaller submatrices containing one or more states. For example, in the analysis of bursts, the $k$ kinetic states may be divided into three subsets: open states, denoted $A$ ($k_A$ in number), short-lived closed states (i.e., closed states within a burst) denoted $B$ ($k_B$ in number) and long-lived closed states (closed states between bursts) denoted $C$ ($k_C$ in number). For example, in the above scheme, $A$ would contain the single open state $O$ ($k_A=1$), $B$ would contain the rightmost closed state $Q_4$ ($k_B=1$), and $C$ would contain the closed states $Q_1, Q_2$ and $C_3$ ($k_C=3$). The submatrices of $Q$ are therefore:

$$Q = \begin{pmatrix} Q_A & Q_{AB} & Q_{AC} \\ Q_{BA} & Q_B & Q_{BC} \\ Q_{CA} & Q_{CB} & Q_C \end{pmatrix}$$  \hspace{1cm} (1-19)$$

Both $Q_{AC}$ and $Q_{CA}$ contain only zero elements, as direct transitions between open and long-lived closed states are not possible in this scheme.
Numerical Evaluation of Results

Numerical evaluation of the p.d.f.s for any submatrix requires the spectral expansion of that submatrix (see Colquhoun & Hawkes, 1977, equations 13-17):

$$Q_{AA} = \sum_{m=1}^{k} A_m \rho_m$$ (1 - 21)

where $\rho_m$ are the eigenvalues of $Q_{AA}$, and the matrices $A_m$ can be calculated from the eigenvectors of $Q_{AA}$. Eigenvalues and eigenvectors can be calculated by computer subroutines. More useful are the eigenvalues of $-Q_{AA}$, denoted $\lambda_m (\lambda_m = -\rho_m)$, which are positive rate constants. Once the eigenvalues and eigenvectors are known, they can be substituted into the matrix given by (Colquhoun & Hawkes, 1982, equation 1.30):

$$P_{AA}(t) = \sum_{m=1}^{k} A_m e^{-\lambda_m t}$$ (1 - 22)

From this, all p.d.f.s can be expressed in the form (Colquhoun & Hawkes, 1982, equation 1.31):

$$f(t) = bP_{AA}(t)c$$ (1 - 23)

where $b$ is a single vector derived from all the factors which pre-multiply $P_{AA}(t)$, and $c$ is a post-multiplier vector. This p.d.f. can be expressed in scalar form as the weighted sum of $k_A$ exponentials (Colquhoun & Hawkes, 1982, equation 1.32):

$$f(t) = \sum_{m=1}^{k_A} w_m e^{-\lambda_m t}$$ (1 - 24)

The scalar coefficients are given by (Colquhoun & Hawkes, 1982, equation 1.33):

$$w_m = bA_m c$$ (1 - 25)

$$= \sum_{i \in A} \sum_{j \in A} b_i a_{ijm} c_j$$ (1 - 26)

where $b_i$, $c_j$ and $a_{ijm}$ are elements of $b$, $c$ and $A_m$ respectively.
Open Time Distribution

In the above kinetic example (from Armstrong, 1969), there is only one open state, so the submatrix $Q_{AA}$ contains only one term:

$$Q_{AA} = -4\beta$$

(1-27)

In general, the distribution of all open times is given by (Colquhoun & Hawkes, 1983, equation 86):

$$f(t) = \Phi P_{AA}(t) (-Q_{AA}) u_A$$

(1-28)

$$= \Phi e^{Q_{AA}t} (-Q_{AA}) u_A$$

(1-29)

with the mean open time (Colquhoun & Hawkes, 1983, equation 87):

$$m = \Phi (-Q_{AA}^{-1}) u_A$$

(1-30)

The initial vector, $\Phi$, specifies the relative probabilities of an opening starting in each of the open states, and the final vector, $u_A$, has unit elements. Both $\Phi$ and $u_A$ can be omitted where there is only a single open state. Therefore, substituting (1-27) into (1-29) and (1-30) gives the result that the open time is described by a single exponential with a mean of $1/4\beta$.

Bursts of Openings

The subdivision of the $Q$ matrix described above allows evaluation of the transition rates within subsets of states. The probability density which describes the transition between a state $i$ in one subset (say $A$) and a state $j$ in a different subset ($B$) is defined as $g_{ik}(t)$, and the $k_A \times k_B$ matrix of such quantities is denoted $G_{AB}(t)$. This matrix is given by (Colquhoun & Hawkes, 1982, equation 1.20):

$$G_{AB}(t) = P_{AA}(t) Q_{AB}$$

(1-31)

Multiplication of the Laplace transformations of expressions such as (1-31) can give the distributions of events such as the burst length.

$G_{AB}$, the matrix of transition probabilities between states $A$ and states $B$ regardless of when this transition occurs, is given by (Colquhoun & Hawkes, 1983, equation 92):
\[ G_{AB} = -Q^{-1}_{AA}Q_{AB} \]  \hspace{1cm} (1-32)

which can be found directly from the relevant submatrices of \( Q \). From (1-32), we can derive a general expression describing the probability that a burst contains \( r \) openings, which is relevant to any mechanism (Colquhoun & Hawkes, 1983, equation 93):

\[ P(r) = \Phi_b(G_{AB}G_{BA}^{-1})(I - G_{AB}G_{BA})u_b \]  \hspace{1cm} (1 - 33)

with the mean number of openings per burst (Colquhoun & Hawkes, 1983, equation 94):

\[ m_r = \Phi_b(I - G_{AB}G_{BA})^{-1}u_b \]  \hspace{1cm} (1 - 34)

The initial vector \( \Phi_b \) gives the relative probabilities of a burst starting in each of the open states (where multiple open states exist) and the vector \( u_b \) has all elements unity.

**STRUCTURE OF VOLTAGE GATED POTASSIUM CHANNELS**

Isolation of Potassium Channel Genes

Recent advances in molecular biology have allowed the primary amino acid sequence of ion channel proteins to be elucidated (Claudio, 1986). mRNA is extracted from a tissue thought to contain the channel of interest, and is copied into complementary DNA (cDNA) using a reverse transcriptase. The cDNA is not the same as the genomic DNA as it contains no introns, but it will code for the same protein. Once the cDNA clone of interest has been identified from all the clones in this "cDNA library", its nucleotides can be sequenced, and with knowledge of the genetic code, this can be used to determine the primary amino acid sequence of the protein it encodes.

The first potassium channel genes for which cDNA clones were isolated were from the fruitfly *Drosophila melanogaster*. The *Shaker* mutant of *Drosophila* causes the alteration or loss of the A-current (Salkoff & Wyman, 1981), and the gene responsible for this mutation was isolated (Papazian, Schwarz, Tempel, Jan & Jan, 1987; Kamb, Iverson & Tanouye, 1987; Baumann, Krah-Jentgens, Müller, Müller-Holtkamp, Seidel, Kecskemethy, Casal, Ferrus & Pongs, 1987) and used to identify clones prepared from a *Drosophila* head cDNA library (Tempel, Papazian, Schwarz, Jan & Jan, 1987; Kamb, Tseng-Crank & Tanouye, 1988; Pongs, Kecskemethy, Müller, Krah-Jentgens, Baumann, Kiltz, Canal, Llamazares & Ferrus, 1988). Several of these cDNA clones coded for proteins
highly homologous to a single domain of the voltage dependent sodium (Noda, Shimizu, Tanabe, Takai, Kayano, Ikeda, Takahashi, Nakayama, Kanaoka, Minamino, Kangawa, Matsuo, Raftery, Hirose, Inayama, Hayashida, Miyata & Numa, 1984) and calcium channel α subunits (Tanabe, Takeshima, Mikami, Flockerzi, Takahashi, Kangawa, Kojima, Matsuo, Hirose & Numa, 1987), both of which are made up of four such repeated domains. Each Shaker cDNA product, and each domain of the sodium and calcium channels, contains six hydrophobic segments capable of forming α-helices which could span the membrane, known as S1 to S6. In each case, the S4 segment contains a number of positively charged amino acids, suggesting that this region could form the voltage sensor in all of these channels, moving in response to a change in membrane potential to lead to channel opening (Noda et al., 1984).

It was subsequently shown that a number of mature Shaker mRNAs, to which these cDNA clones are complementary, are made from a single gene by alternative splicing of a primary RNA transcript (Schwarz, Tempel, Papazian, Jan & Jan, 1988). Each Shaker protein contains a common central portion, to which variant amino and carboxy terminal ends are added. At present, five different amino termini and two carboxy termini are known, and any combination of these termini around the common core region is thought to be possible (Stocker, Stühmer, Wittka, Wang, Müller, Ferrus & Pongs, 1990), so that theoretically the single Shaker gene could code for up to ten different proteins.

The Shaker cDNA clones were proven to code for potassium channels by expression in Xenopus oocytes. In this procedure, the cDNA is subcloned into a plasmid expression vector and mRNA is produced by in vitro transcription. This mRNA is then injected into the oocytes, where it is translated into the appropriate protein, which is inserted into the membrane. This leads to the expression of transient potassium currents in the oocytes when examined under voltage clamp (Timpe, Schwarz, Tempel, Papazian, Jan & Jan, 1988; Iverson, Tanouye, Lester, Davidson & Rudy, 1988).

Shaker cDNA probes have been used to isolate three other potassium channel genes from Drosophila, known as Shab, Shal and Shaw (Butler, Wei, Baker & Salkoff, 1989). Injection of Shab, Shal or Shaw mRNA into Xenopus oocytes also leads to the expression of voltage activated potassium currents (Wei, Covarrubias, Butler, Baker, Pak & Salkoff, 1990).

Drosophila potassium channel cDNA probes have now been used to isolate
mammalian potassium channel genes. Those genes so far isolated may be divided into four classes, depending on their homology to different \textit{Drosophila} potassium channel genes; the \textit{Shaker}-like genes in rat (St{"u}hmer, Ruppersberg, Schr{"o}ter, Sakmann, Stocker, Giese, Perschke, Baumann & Pongs, 1989; Christie, Adelman, Douglass & North, 1989; McKinnon, 1989), in mouse (Tempel, Jan & Jan, 1988; Chandy, Williams, Spencer, Aguilar, Ghanshani, Tempel & Gutman, 1990) and in human (Kamb, Weir, Rudy, Varmus & Kenyon, 1989; Grupe, Schr{"o}ter, Ruppersberg, Stocker, Drewes, Beckh & Pongs, 1990); the \textit{Shab}-like genes in rat (Frech, VanDongen, Schuster, Brown & Joho, 1989; Hwang, Glatt, Bredt, Yellen & Snyder, 1992) and mouse (Pak, Covarrubias, Ratcliffe & Salkoff, 1991); the \textit{Shaw}-like genes in rat (McCormack, Vega-Saenz de Miera & Rudy, 1990; Schr{"o}ter, Ruppersberg, Wunder, Rettig, Stocker & Pongs, 1991; Rettig \textit{et al.}, 1992; Vega-Saenz de Miera, Moreno, Fruhling, Kentros & Rudy, 1992;) and in NG 108-15 mouse neuroblastoma x rat glioma hybrid cells (Yokoyama, Imoto, Kawamura, Higashida, Iwabe, Miyata & Numa, 1989); and the \textit{Shal}-like genes in rat (Baldwin, Tsaur, Lopez, Jan & Jan, 1991) and mouse (Pak, Baker, Covarrubias, Butler, Ratcliffe & Salkoff, 1991). In many of these cases, the proteins encoded by these genes have been expressed in \textit{Xenopus} oocytes or other expression systems to yield a number of functionally diverse potassium currents. In contrast to the situation in \textit{Drosophila}, gene duplication rather than alternative splicing appears to underlie most of the potassium channel diversity in mammals; so far only \textit{Shaw}-like mammalian potassium channels have been demonstrated to be formed by alternative splicing (Luneau, Williams, Marshall, Levitan, Oliva, Smith, Antanavage, Folander, Stein, Swanson, Kaczmarek & Buhrow, 1991; Rettig \textit{et al.}, 1992).

Because each potassium channel gene, both in \textit{Drosophila} and mammals, encodes a protein homologous to one of the four domains of a sodium or calcium channel, it has often been suggested that functional potassium channels may be made up of four independent subunits (Tempel \textit{et al.}, 1987; Agnew, 1988). Indeed, by injecting two different potassium channel mRNAs into the same oocyte, it has been shown that heteromultimeric \textit{Shaker} (Isacoff, Jan & Jan, 1990) and mammalian (Christie, North, Osborne, Douglass & Adelman, 1990; Ruppersberg, Schr{"o}ter, Sakmann, Stocker, Sewing & Pongs, 1990) potassium channels may be formed, greatly increasing the potential number of different potassium channels which may exist in \textit{Drosophila} and mammals \textit{in vivo}. Even structurally similar \textit{Drosophila} and rat potassium channel subunits can join together.
to form functional heteromultimers in oocytes (Isacoff et al., 1990). However, in *Drosophila* only subunits from the same subfamily (*Shaker, Shab, Shal or Shaw*) can form heteromultimers together (Covarrubias, Wei & Salkoff, 1991), and heteromultimer formation may be similarly restrained in mammals (Rettig et al., 1992). Even formation of heteromultimers from two different *Drosophila Shaker* subunits can only take place if they have closely related structures in the region which forms the six membrane-spanning domains (McCormack, Lin, Iverson & Rudy, 1990), suggesting that this region controls heteromultimer formation. The suggestion that potassium channels are tetramers of such subunits has been substantiated by studying the interaction of charybdotoxin with wild-type and toxin-insensitive mutant *Shaker* channel subunits coexpressed in oocytes (MacKinnon, 1991)

A completely unrelated gene encoding a voltage dependent potassium channel has been identified in rat (Takumi, Ohkubo & Nakanishi, 1988), human (Murai, Kakizuka, Takumi, Ohkubo & Nakanishi, 1989) and mouse (Honore, Attali, Romey, Heurteaux, Ricard, Lesage, Lazdunski & Barhanin, 1991). This gene encodes a much smaller protein (129 or 130 amino acids, compared with between 500 and 900 amino acids for the cloned rat brain potassium channels described above) with only one putative transmembrane domain. When expressed in *Xenopus* oocytes, this protein forms a potassium channel which activates very slowly on depolarization, with activation being incomplete even after several seconds (Takumi et al., 1988). The activity of this channel in oocytes is enhanced by elevation of intracellular calcium concentration and inhibited by activation of protein kinase C (Honore et al., 1991; Busch, Kavanaugh, Varnum, Adelman & North, 1992). Expression of the gene encoding this channel has not been demonstrated in nervous tissue.

Several cDNAs encoding calcium-activated potassium channels have recently been isolated from the *Drosophila Slo* locus (Atkinson, Robertson & Ganetzky, 1991; Adelman, Shen, Kavanaugh, Warren, Wu, Lagrutta, Bond & North, 1992). These cDNAs encode proteins of approximately 1200 amino acids with little primary sequence homology to the cloned voltage gated potassium channels, although they do have six putative transmembrane domains. The most highly conserved region between cloned voltage- and calcium-activated potassium channels is in the region between the S5 and S6 transmembrane domains, which is thought to be important in pore formation (see below).
Structure-Function Relationships in Potassium Channels

The elucidation of the primary structure of a number of both *Drosophila* and mammalian potassium channels, and the availability of various expression systems for electrophysiological recording, has allowed some investigation of the role of different regions of the proteins in channel function. For example, it was noted that different *Shaker* channels with variant amino or carboxy terminal ends differ in their rates of inactivation, and it was implied that these intracellular domains are involved in the processes of fast and slow inactivation respectively (Timpe, Schwarz *et al.*, 1988; Timpe, Jan & Jan, 1988; Iverson *et al.*, 1988; Iverson & Rudy, 1990). Mutations replacing any of the first nineteen amino acids at the amino terminus of one *Shaker* channel causes a significant slowing or removal of inactivation, whilst mutations affecting the next sixty amino acids cause either a speeding up (deletions) or a slowing down (insertions) of inactivation (Hoshi, Zagotta & Aldrich, 1990). Inactivation could be restored to a noninactivating mutant *Shaker* channel by application of a synthetic peptide corresponding to the first twenty amino acids of the normal amino terminus to the cytoplasmic face of the membrane (Zagotta, Hoshi & Aldrich, 1990). It was suggested by Hoshi *et al.* (1990) that inactivation of *Shaker* potassium channels could occur by a similar mechanism to the “ball and chain” theory proposed for the inactivation of sodium channels (Armstrong & Bezanilla, 1977). The nineteen amino acids at the extreme end of the amino terminus would form the “ball” which interacts with a receptor near the cytoplasmic face of the channel pore to block conduction. This “ball” would be tethered to the membrane-spanning regions of the channel by the sixty or more amino acid “chain”, additions to or deletions from which would also affect the rate of inactivation. This twenty amino acid *Shaker* “ball” peptide also blocks calcium-activated potassium channels when applied to the cytoplasmic face of membrane patches (Foster, Chung, Zagotta, Aldrich & Levitan, 1992; Toro, Stefani & Latorre, 1992), suggesting that the binding site for this “ball” peptide is highly conserved between different classes of potassium channel.

Inactivation of wild-type *Shaker* channels is slowed by internal application of TEA (Choi, Aldrich & Yellen, 1991), suggesting that TEA competes with the inactivation “ball” for the binding site on the intracellular face of the channel. However, the binding sites for TEA and the inactivation “ball” may be different, but close enough to prevent both binding at once, since a mutation of a *Shaker* channel which affects internal TEA block
does not alter fast inactivation (Yellen, Jurman, Abramson & MacKinnon, 1991; see below). Mutation of conserved amino acid residues in the cytoplasmic region between the S4 and S5 transmembrane domains of a Shaker channel affects the stability of the inactivated state (Isacoff, Jan & Jan, 1991), suggesting that this part of the channel may form the receptor for the inactivation "ball".

Even in Shaker channels where the amino terminal has been removed, a slower inactivation process still takes place (Hoshi, Zagotta & Aldrich, 1991). This slow inactivation does take place when the amino terminal is still present, and it is also slowed when the amino terminal is removed (Hoshi et al., 1991). This suggests that the fast and slow inactivation processes are independent of each other, but that they are partially coupled, such that slow inactivation takes place more easily from the fast inactivated state than from any open or closed states.

The rates of entry into and recovery from the slow inactivated state vary greatly between Shaker channels with different carboxy termini (Iverson & Rudy, 1990; Wittka, Stocker, Boheim & Pongs, 1991; Hoshi et al., 1991). However, large deletions in the carboxy end have no effect on slow inactivation, suggesting that it does not occur by a "ball and chain" mechanism (Hoshi et al., 1991). Instead, it appears to be controlled by a single amino acid residue in the S6 transmembrane domain, which also differs between different Shaker channels with different carboxy termini (Hoshi et al., 1991; Wittka et al., 1991). Slow inactivation is also pharmacologically distinct from fast inactivation; it is insensitive to internal TEA, but is slowed by external TEA, in a manner suggesting that blocked channels cannot enter the slowly inactivated state (Choi et al., 1991).

Those amino acids which form the presumed extracellular link between the S5 and S6 transmembrane regions have been implicated in channel pore formation. Many mutations in this region of cloned Shaker or rat brain potassium channels affect external binding of the channel blockers charybdotoxin (MacKinnon & Miller, 1989; Goldstein & Miller, 1992), TEA (MacKinnon & Yellen, 1990; Kavanaugh, Varnum, Osborne, Christie, Busch, Adelman & North, 1991; see chapter 8) and DTX (Hurst, Busch, Kavanaugh, Osborne, North & Adelman, 1991; Stocker, Pongs, Hoth, Heinemann, Stühmer, Schröter & Ruppersberg, 1991), channel conductance (MacKinnon & Yellen, 1990) and ionic selectivity (Yool & Schwarz, 1991; Kirsch, Drewe, Taglialatela, Joho, DeBiasi, Hartmann & Brown, 1992). Mutation of another amino acid in the S5-S6 region affects internal TEA
block, suggesting that this part of the protein must cross the membrane, possibly not spanning the entire membrane thickness, to form at least parts of the internal and external mouths of the pore (Yellen et al., 1991). It has also been shown that chimeric channels made up of parts of two different rat potassium channel proteins adopt the single channel conductance and external and internal TEA block characteristics of the channel from which the S5-S6 region of the protein is taken (Hartmann, Kirsch, Drewe, Taglialetela, Joho & Brown, 1991). Point mutations in the same region of a sodium channel can confer calcium channel-like selectivity properties (Heinemann, Terlau, Stühmer, Imoto & Numa, 1992).

The widely-held belief that the positively charged S4 sequence common to voltage dependent sodium, calcium and potassium channels forms the voltage sensor in these channels (Noda et al., 1984; Tempel et al., 1987) and is responsible for the gating current detected during channel activation (Armstrong & Bezanilla, 1973, 1974; Bezanilla, White & Taylor, 1982) has been supported by site-directed mutagenesis experiments in both Shaker (Papazian, Timpe, Jan & Jan, 1991; Lopez, Jan & Jan, 1991) and rat cloned potassium channels (Liman, Hess, Weaver & Koren, 1991; Logothetis, Movahedi, Satler, Lindpaintner & Nadal-Ginard, 1992). Mutations which replace positively charged amino acids in the S4 region specifically alter voltage-dependent activation and decrease the gating charge (Papazian et al., 1991; Liman et al., 1991; Logothetis et al., 1992). However, mutations which affect uncharged residues within S4 have just as great an effect on voltage-dependent activation (Lopez et al., 1991), and it was suggested that these residues may interact non-electrostatically with other parts of the channel to affect the stability of open and closed states.

It has been suggested that the opening of the channel following translocation of the S4 region may involve the so-called "leucine zipper" motif (McCormack, Campanelli, Ramaswami, Mathew, Tanouye, Iverson & Rudy, 1989; McCormack, Tanouye, Iverson, Lin, Ramaswami, McCormack, Campanelli, Mathew & Rudy, 1991). This motif consists of four or five leucine residues repeated every seventh amino acid, and it is fully conserved in all potassium channels cloned (except Shaw, which has limited amino acid substitutions). It is also found in some DNA binding proteins, where it mediates protein dimerization (Kouzarides & Ziff, 1988), and has also been implicated in other protein-protein interactions (McCormack et al., 1989). The leucine zipper motif is located immediately adjacent to the S4 region in voltage-dependent sodium, calcium and potassium channels.
The $Sh^5$ mutation in *Drosophila*, which causes a single amino acid substitution in the leucine zipper region of a *Shaker* channel (Gautam & Tanouye, 1990), leads to the expression of A-type channels in muscle with differences in the voltage dependence of activation and inactivation consistent with the alteration of a structure within the channel closely associated with the movement of the gating charge during activation (Zagotta & Aldrich, 1990b). Mutation of leucine residues in this region of a *Shaker* channel (McCormack *et al.*, 1991) and a rat sodium channel (Auld, Goldin, Krafte, Catterall, Lester, Davidson & Dunn, 1990) cause similar large changes in the voltage dependence of activation and inactivation. It was suggested that voltage-dependent translocation of the S4 region may be transduced into channel opening via subunit interactions mediated by the leucine zipper (McCormack *et al.*, 1991).

**INTRODUCTION TO WORK CARRIED OUT**

The aims of the work described were to characterise two types of voltage gated potassium channels in mammalian central neurons: A-current channels and delayed rectifier channels. A convenient preparation on which to carry out this study was primary cultures of neonatal rat central neurons, which ensured a reliable source of cells with generally good sealing properties. Neurons were prepared from the locus coeruleus, a small brainstem nucleus thought to be involved in attention, orientation, anxiety, stress, learning and memory, brain plasticity and sleep-waking activities (Barnes & Pompeiano, 1991), and from the hippocampus, which is known to play a pivotal role in memory (Eichenbaum, Otto & Cohen, 1992). Because the hippocampus is such a large brain structure, it was possible to prepare cells specifically from one area, the CA1 region; in this way, it was hoped to obtain a more homogeneous population of cells.

The electrophysiological properties of both locus coeruleus and hippocampal CA1 cells were first examined using conventional whole cell patch recording. As is described in chapter 4, this showed that both cell types had an A-current and a delayed rectifier current, although there were some differences in the properties of these currents between cell types. However, during single channel recordings from these two cell types, usually only A-current channels were found in locus coeruleus neurons, whereas only delayed rectifier channels were routinely found in CA1 neurons. Although the reason for this remains unclear, it made it convenient to study the unitary properties of these different channels in different cells. The properties of single A-current channels in locus coeruleus
neurons are described in chapter 5, and those of single delayed rectifier channels in CA1 neurons are described in chapter 6.

After the description of the unitary properties of these two channel types had been carried out, further experiments examined the properties of single delayed rectifier channels in CA1 neurons under different conditions. Specifically, the effects external potassium and rubidium as the permeant ions are described in chapter 7, and the block of these channels by unusually low concentrations of external tetraethylammonium ions is described in chapter 8.
CHAPTER TWO: ELECTROPHYSIOLOGICAL METHODS

THE PATCH CLAMP TECHNIQUE

All of the current recordings described were made using the patch clamp technique (Neher & Sakmann, 1976; Neher, Sakmann & Steinbach, 1978; Hamill et al., 1981), either in the single-channel or whole-cell recording modes. Single channel patch clamp recording involves electrically isolating a small patch of membrane from the extracellular solution. This is done by pressing the tip of a fire polished glass pipette against the cell membrane, and applying light suction to the inside of the pipette; this leads to the formation of a seal between pipette and membrane such that the electrical resistance of the seal is in the order of tens of gigohms ($10^{10}$-$10^{11}$ Ω). A high seal resistance minimises the background noise, which is essential in resolving the tiny current through a single channel. The variance of the current noise through a resistor ($\sigma^2$; units $A^2$) is related to resistance ($R$) by:

$$\sigma^2 = 4kTB/R$$

(2 - 1)

where $k$ is Boltzmann’s constant, $T$ is the temperature in Kelvins, and $B$ is the bandwidth of the current recording (which is equal to the low pass filter frequency). Therefore, for a seal resistance of $10G\Omega$ at $20^\circ$C, the standard deviation of the current noise ($\sigma$) at 2kHz will be 0.06pA, whereas for a seal resistance of $100M\Omega$ (which was typical in the earliest patch clamp experiments) it will be 0.57pA. A high seal resistance also ensures good electrical isolation of the patch, by allowing less of the current flowing through the patch to be lost across the seal resistance. However, this error can be corrected when the seal resistance is low, for example as it is in “loose-patch” recording (Almers, Stanfield & Stühmer, 1983; Stühmer, Roberts & Almers, 1983).

Configurations of Patch Clamp Recording

The high resistance seal obtained between pipette and membrane in patch clamp recording is mechanically as well as electrically stable, so that single channel events may be recorded from a patch of membrane which is still attached to the cell (“on-cell” recording), or from an excised, cell-free patch.

During on-cell recording, the pipette interior is in contact with the extracellular face of the membrane; to reproduce physiological ionic gradients across the patch, the pipette
solution should therefore be an “extracellular” solution, similar in ionic concentrations to
the bathing solution (the composition of solutions used in recording is set out below, in
table 1). The permeability of ion channels under non-physiological conditions may be
examined by changing the concentration or nature of permeant ions in the pipette solution.

In on-cell recording, the potential across the patch will be the sum of the pipette
potential and the resting membrane potential of the cell. For example, for a cell with a
resting potential of -60mV, if the pipette potential is set to +20mV, the conventional patch
potential (inside with respect to outside) will be -80mV. If the pipette potential is -20mV,
the patch potential will be -40mV. Since the resting potential is not usually known
(although it can be measured using a patch clamp amplifier by breaking the patch, and
measuring the potential at which no current flows), patch potentials for on-cell recording
are usually quoted with respect to the resting potential. Mean resting potentials measured
in this way were -61.4±0.4mV (n=16) in locus coeruleus neurons and -62.1±0.7mV (n=20)
in hippocampal CA1 neurons.

If the pipette is withdrawn from the cell, an “inside-out” membrane patch is formed;
the extracellular face of the membrane is still in contact with the pipette solution, and the
intracellular face is in contact with the bathing solution. Therefore, to maintain
physiological ionic gradients, the pipette should again contain an “extracellular” solution,
and the bathing solution should be changed to an “intracellular” solution. This is usually
done by moving the tip of the pipette into a localised stream of artificial intracellular
solution, rather than changing the solution in the whole bath. An excised membrane patch
has no resting potential, so the patch potential is determined solely by the pipette potential.
Thus, for an inside-out patch, a pipette potential of +60mV gives a conventional patch
potential of -60mV.

From recording in the on-cell configuration, it is possible to rupture the patch of
membrane by applying extra suction, allowing direct access from the pipette to the
cytoplasm. This method is used to record whole cell currents with the patch clamp
technique (see below). If the pipette is withdrawn from the cell following patch disruption,
the membrane should reseal to give an excised, “outside-out” patch, such that single
channel events can be recorded with the pipette interior in contact with the intracellular
face of the membrane. For this method of recording, the pipette should contain an
“intracellular” solution; the extracellular face of the membrane should remain in the normal bathing solution. As there is no patch resting potential, the patch potential will be equal to the pipette potential.

**Whole Cell Recording**

As mentioned above, following disruption of a patch, whole cell currents may be recorded using the patch clamp technique. The equivalent circuit to this recording mode is shown in figure 2-1. Current in the pipette, \( i_p \), is the sum of two components, one due to the membrane resistance \( (i_m) \) and one due to membrane capacitance \( (i_c) \). \( i_p \) flows through a resistance \( R_s \) which is in series with the membrane. This series resistance, which exists between the pipette interior and the cell interior, is due in part to the resistance of the pipette, but is mainly due to the resistance of the broken patch. Series resistance has three detrimental effects: it leads to voltage errors between the pipette and the cell, it slows the charging of the membrane capacitance, and it contributes the major source of noise in whole cell recording.

The first of these effects is due to the voltage drop across \( R_s \) when current flows through it. This voltage drop causes a difference between \( V_p \), the pipette potential, and \( V_c \), the membrane potential:

\[
V_p = V_c + i_p R_s \quad (2-2)
\]

Thus, when \( i_p \) is large, a significant voltage error will occur, leading to an ineffectual voltage clamp of the cell.

Whenever \( V_C \) is changed, for example during a step voltage command, current will flow to charge the membrane capacitance \( C_m \). The total charge moved, \( Q \), during a step change in \( V_C \) of \( \Delta V \), is:

\[
Q = C_m \Delta V \quad (2-3)
\]

The capacitive current carrying this charge will decay after a step voltage change with a time constant \( \tau \), given by:

\[
\tau = R_s C_m \quad (2-4)
\]
Figure 2-1. The equivalent circuit to whole cell recording with the patch clamp technique. Current in the pipette, $i_p$, is the sum of the currents due to the membrane resistance ($i_m$) and capacitance ($i_c$). $i_p$ flows through an additional series resistance $R_s$, causing a difference between $V_p$ and $V_c$ equivalent to $i_p R_s$. 
Thus, a large value of $R_s$ increases $\tau$, slowing the decay of capacitive currents. $\tau$ will also influence the response of the cell to applied voltages and to membrane currents. This leads to effective low pass filtering of the recorded current with a bandwidth ($f$) of:

$$f = \frac{1}{2\pi \tau} \quad (2-5)$$

Marty & Neher (1983) have shown that the predominant source of background noise in whole cell recording is the combination of series resistance and membrane capacitance. For recording bandwidths above about $1\text{kHz}$, the variance of the current noise ($\sigma^2$) is related to these two parameters by:

$$\sigma^2 \propto R_s C_m^2 \quad (2-6)$$

To minimise these three effects of series resistance, low resistance pipettes are used for whole cell recording. The magnitudes of capacitive current transients and background noise are also minimised by using small cells, which have a lower membrane capacitance. The voltage error introduced by the series resistance may also be compensated electronically by the patch clamp amplifier (see below).

The size of the capacitive current transients seen following a step change in voltage may be used to estimate $C_m$ and $R_s$. The area of the transient, which may be found by integrating the current with respect to time, gives the charge $Q$, and the time constant of the exponential transient decay is equal to $\tau$; since $AV$, the size of the voltage step, is known, this allows $C_m$ and $R_s$ to be calculated using equations 2-3 and 2-4.

**Description of the Patch Clamp Amplifier**

The patch clamp amplifier acts as a current-to-voltage converter with a high resistance feedback resistor to convert the tiny membrane currents recorded by the pipette into voltage signals for recording and analysis. It also allows the pipette potential to be controlled, giving an effective voltage clamp of a patch of membrane or a whole cell. The patch amplifier used was an Axopatch 1B (Axon instruments) for recording from locus coeruleus neurons, and an EPC-7 (List-electronic) for recording from hippocampal neurons. The basic circuit for current recording in the EPC-7 is shown in figure 2-2.

The potential of the pipette is equal to $V_{\text{REF}}$, due to the actions of the feedback amplifier. Before obtaining a seal, $V_{\text{REF}}$ is set to zero using electrode potential offset in the "Search" mode of the EPC-7. This mode uses an integrator to provide negative feedback.
Figure 2-2. Circuit diagram of the EPC-7 current-to-voltage converter. The pipette potential is set to $V_{\text{REF}}$, which is set to zero by the electrode offset potential (in "Search" mode), and determined by the holding potential and the stimulus input in voltage clamp mode. Current in the pipette, $i_{\text{pip}}$, is converted to a voltage signal at the current monitor equal to $i_{\text{pip}}R_p$, as described in the text.
between the current monitor and the voltage signal. This has the effect of keeping the pipette current at zero whilst $V_{\text{REF}}$ is adjusted to zero voltage. After a seal has been achieved, the EPC-7 is switched to "voltage clamp" mode, where $V_{\text{REF}}$ is determined as the sum of the electrode holding potential, which is set by the EPC-7, and a stimulus input from an external source, through which step changes in electrode potential may be applied.

The feedback amplifier also measures the pipette current and converts it into a voltage signal. The output of the feedback amplifier will be equal to its non-inverting input minus its inverting input, ie

$$V_{\text{OUT}} = V_{\text{REF}} - i_p R_F$$  \hspace{1cm} (2-7)

where $V_{\text{OUT}}$ is the output, $i_p$ the pipette current and $R_F$ the resistance of the feedback resistor. A second amplifier is needed to subtract $V_{\text{REF}}$. Its output, the voltage at the current monitor ($V_{\text{CMP}}$), will be:

$$V_{\text{CM}} = V_{\text{REF}} - V_{\text{OUT}}$$

$$= i_p R_F$$  \hspace{1cm} (2-8)

The feedback resistance, $R_F$, will affect the recorded current noise in the same way that the seal resistance does. Therefore, a high value of $R_F$ is desirable to minimise this source of noise. However, since $V_{\text{CM}}$ cannot exceed the values of the EPC-7 power supply ($\pm 12V$), a high value of $R_F$ will restrict the amplitude of pipette currents which can be measured. Therefore, the EPC-7 has two different feedback resistors, which can be switched according to the type of experiment being carried out. For single channel recording, a 50GΩ feedback resistor is used. This has a very low noise level, but the maximum current amplitude is $\pm 240pA$. For whole cell recording, the feedback resistance is switched to 500MΩ, giving a current limit of $\pm 24nA$. This resistor introduces more background noise, but this is less critical in whole cell recording, where overall background current noise is dominated by the series resistance (see above).

During whole cell recording, the EPC-7 can also be used to provide series resistance compensation (see above). A fraction of the current monitor signal is scaled and added to $V_{\text{REF}}$, which changes the pipette potential to compensate for the potential drop across the series resistance. The EPC-7 also has a facility for the cancellation of transient capacitive currents. Separate mechanisms exist for the cancellation of the fast transient
due to the pipette capacitance, and the slow transient due to the cell membrane capacitance. Both act by injecting an equal and opposite current into the pipette, which sums with the capacitive current to zero.

THE RECORDING SET-UP

All recordings were made from cultured rat central neurons grown on 13mm glass cover slips (see chapter 3). Whole or fragments of cover slips were placed in a 35mm Petri dish in a Peltier-driven environmental chamber (Medical Systems), which was placed on the stage of a Zeiss inverted microscope. This arrangement was placed on an Ealing vibration isolation table, which was kept inflated to a pressure of approximately 4 bar (400kPa; 581b/in²) by an attached nitrogen cylinder, and was inside a well-earthed Faraday cage. The pipette holder was attached directly to the patch clamp headstage, which was attached to a motorised manipulator (Märzhauser) bolted onto the microscope stage. The pipette holder contained a chlorided silver wire, which made the electrical contact between the amplifier headstage and the pipette solution. This was made by briefly dipping a thin silver wire into molten silver chloride. When a pipette was in the bath solution, the electrical circuit was completed by a bath earth electrode which connected to an earthing point on the probe. This bath earth consisted of a silver-silver chloride pellet in a thin glass tube filled with artificial cerebrospinal fluid (ACSF; see below), which was in direct contact with the bath solution.

Currents recorded by the patch clamp amplifier were subsequently low-pass filtered as required using an eight-pole Bessel filter (Frequency Devices). Filtered currents were digitised at the required rate using a Cambridge Electronic Design (CED) 1401 interface, and written onto the hard disk of a Dell System 210 computer for subsequent analysis using the CED “patch” software. This software was also used to generate the voltage protocols which, via the 1401 interface, were the stimulus input to the amplifier. Occasionally, current signals were also recorded onto video tape using a digital audio processor (Sony PCM-701ES) connected to a VHS video cassette recorder (JVC), for subsequent analysis using a Dell System 325 or 450DE computer running “Tracan” and “Evtan” software (Davies, 1992; see below) or a Digital Equipment Company (DEC) PDP 11-73 computer running “Noise” software (see below). Currents recorded onto video tape were not passed through the eight-pole Bessel filter during recording, but were subsequently filtered at an appropriate cut-off frequency using this or an eight-pole

43
Butterworth filter (Frequency Devices; used only in the case of noise analysis experiments, see chapter 8) before being digitised onto the hard disk of the Dell (using an axon TL-1 DMA interface) or the PDP 11-73 (using a CED 502 interface). During recording, the current was also observed using a Tektronix storage oscilloscope, which was triggered to respond to voltage pulses by using a digital output of the 1401. When current was recorded onto video tape, it was also necessary to record this trigger pulse, in order to appropriately trigger the digitisation of the current records. The recording set-up is summarized diagrammatically in figure 2-3.

Traditionally, outward fluxes of cations across the membrane are assigned to be positive currents, and inward fluxes negative currents. Therefore, in the on-cell and inside-out patch configurations, where the pipette interior is in contact with the extracellular face of the membrane, current flowing into the pipette will be positive (outward with respect to the membrane), whereas in the whole-cell and outside-out patch configurations, where the pipette interior is in contact with the intracellular face of the membrane, current flowing into the pipette will be negative (inward with respect to the membrane). For this reason, currents recorded in the on-cell or inside-out patch configurations must be inverted before digitisation, so that currents recorded in all patch configurations have the same polarity. This inversion was achieved using a simple inverting amplifier, built in the laboratory using components obtained from RS Components. This amplifier also incorporated a leak subtraction facility, which could be used to compensate some of the leak current during voltage pulses. This leak current is due to the seal resistance and is therefore linearly proportional to pipette potential. When leak subtraction was employed, a variable proportion of the pipette potential could be subtracted from the current output of the patch clamp amplifier before digitisation.

Fabrication of Pipettes

Pipettes were made from hard borosilicate capillaries (Clark Electromedical). For pipettes to be used in whole cell recording, thin walled glass was used, whilst for single channel recording, pipettes were made from thick walled glass. Pipettes made from thin walled glass have a lower resistance, which is important for minimizing series resistance in whole cell recording. However, stray pipette capacitance to earth, most of which arises between the pipette solution and the bath solution, is greater when the solutions are separated by a thinner layer of electrode glass. Pipette capacitance contributes to
Figure 2-3 Diagrammatic representation of the current recording set-up. Voltage pulses are applied to the stimulus input of the patch clamp amplifier from a digital-to-analog converting (DAC) output of the 1401 interface. This is summed with the holding potential set by the amplifier to give the patch potential, which is the voltage output of the amplifier. This output is recorded by one analog-to-digital converting (ADC) input of the 1401, and also displayed on the storage oscilloscope. To coordinate triggering of the oscilloscope to the start of voltage pulses, a digital trigger pulse supplied from the 1401 is applied to the oscilloscope timebase. This trigger pulse is also recorded by the digital audio processor (PCM) and video recorder (VCR), for reasons set out in the text. Current in the pipette is recorded by the amplifier and converted into a scaled voltage signal, as described in the text; this is the signal at the current output of the amplifier. This output is recorded unfiltered by the PCM, and is also, after low-pass filtering by an 8-pole Bessel filter, displayed on the oscilloscope and recorded by an ADC input of the 1401.
To Computer

1401

ADC Inputs
Current Voltage

DAC Output

Digital Trigger Pulse

VCR

PCM Trigger Pulse
Input

Current Input

Bessel Filter
Input Output

Oscilloscope

Channel 1

Channel 2

Time Base

Pipette

Patch Clamp Amplifier

Current Monitor Current Output

Voltage Output Stimulus Input
high-frequency noise and also leads to capacitive currents when the pipette potential is changed, which may saturate the current-to-voltage converting amplifier. For these reasons, thick walled glass is preferable for single channel recording, and pipette capacitance may be further reduced by coating with Sylgard resin (see below).

Pipettes were pulled using a two-stage upright puller, where gravity alone provides the pulling force; either a Kopf Model 700D or a Narishige PP-83 puller was used. Immediately after pulling, pipette tip size was often tested by measuring the “bubble number”, a gauge of the pressure required to blow bubbles through the tip of the pipette into methanol; this pressure should be greater for pipettes with smaller tips. Thick walled pipettes for single channel recording were always subsequently coated with Sylgard (Dow Corning); thin walled pipettes were not coated, as in whole cell recording, pipette capacitance is small in relation to the membrane capacitance of the cell. Sylgard is an inert, hydrophobic silicone elastomer resin which reduces pipette capacitance in two ways. First, by effectively thickening the pipette wall it reduces the capacitive coupling between pipette and bath solutions. Secondly, as it is more hydrophobic than glass, it will prevent bath solution from creeping up the outside of the pipette, and it therefore reduces the area of contact between the pipette and the bath solution. For this reason, the Sylgard coating should continue above the level of the bath solution, which should also be kept as low as possible. Coating should also continue as close as possible to the pipette tip. Sylgard resin and curing agent were mixed together and stored in a freezer; under these conditions, the mixture remained runny and usable for many weeks. It was applied to pipettes from a 1ml syringe with a broken-off hypodermic needle, whilst observing under a dissecting microscope, and was quickly cured by placing the pipette within a heated electrode puller coil. On heating, the Sylgard formed a hard, inert coating on the pipette wall.

In order to obtain high-resistance seals, the tips of pipettes were fire polished before use, using a Narishige MF-83 polisher. The pipette tip is briefly brought close to a heated platinum wire, coated with glass to prevent platinum sputtering onto the pipette tip. A constant pressure airstream is directed at the platinum wire, which produces a steep local temperature gradient. Fire polishing smooths the tip of the pipette, and burns off any contaminants left near the tip by coating.

The electrode glass used had a fused internal filament which greatly facilitated pipette filling. Pipette solution was added to the back of the pipette through a drawn-out Pasteur
pipette; because of the filament, the tip of the pipette tended to fill first. Any remaining bubbles after filling could usually be removed by gently tapping the pipette with a finger. Only a small amount of solution was put into each pipette, to reduce capacitive coupling to the bath solution. It is also important not to overfill pipettes, because if solution gets into the pipette holder, noise can result from the thermal motion of ions in films of aqueous solution.

**Solutions**

The composition of solutions used for recording is set out in table 2-1 below:

<table>
<thead>
<tr>
<th>Table 2-1. Composition of Solutions Used For Recording</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Concentration (mM)</strong></td>
</tr>
<tr>
<td>-------------------------</td>
</tr>
<tr>
<td><strong>Bath Solutions</strong></td>
</tr>
<tr>
<td>ACSF</td>
</tr>
<tr>
<td>AICS</td>
</tr>
<tr>
<td><strong>Single Channel Pipette Solutions</strong></td>
</tr>
<tr>
<td>Low K+ Patch</td>
</tr>
<tr>
<td>High K+ Patch</td>
</tr>
<tr>
<td>Rb+ Patch</td>
</tr>
<tr>
<td><strong>Whole Cell Pipette Solutions</strong></td>
</tr>
<tr>
<td>K+ Gluconate WCS</td>
</tr>
<tr>
<td>K+ Aspartate WCS</td>
</tr>
</tbody>
</table>

Abbreviations: ACSF, artificial cerebrospinal fluid; WCS, whole cell recording solution; AICS, artificial intracellular solution; KGlucose, potassium gluconate; KAspartate, potassium aspartate; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid; EGTA, ethyleneglycol-bis-(β-aminoethyl ether) N,N,N',N'-tetraacetic acid; Glucose, glucose.

The osmolarity of all solutions was adjusted to approximately 320 mOsm, as measured by a Wescor 5100B vapour pressure osmometer, by addition of sucrose. The pH of all solutions was adjusted to 7.3 with NaOH, KOH or RbOH, depending on the major cation present in the solution, and measured with a Solex model 3100 pH meter. All extracellular solutions (ACSF, low K+ patch, high K+ patch, Rb+ patch) also contained 1µM tetrodotoxin (TTX; Sigma) to block voltage dependent sodium channels. All solutions were sterilized by passing through a 0.22µm filter under vacuum pressure; clean solutions are an essential requirement for the consistent formation of seals. Solutions were stored in a refrigerator at 4°C. TTX was stored frozen as a 1mM stock in ACSF, and added
For single channel recording, the pipette solution used was low K⁺ patch, high K⁺ patch or Rb⁺ patch. For whole cell recording, it was K⁺ gluconate WCS for locus coeruleus neurons, and K⁺ aspartate WCS for hippocampal neurons. 2mM disodium adenosine-5'-triphosphate (Na₂ATP; Sigma) was added to whole cell pipette solutions prior to use, and the pH was readjusted to 7.3 with KOH. For inside-out patch recording, the intracellular face of membrane patches was perfused with AICS, to which MgCl₂ or NaCl were added as required. Tetraethylammonium chloride (TEA; Sigma) was added to the pipette solution for some single channel experiments. During some whole cell experiments, ACSF containing either 4-aminopyridine (4-AP; Sigma) or TEA was applied by pressure ejection using a General Valve Picospritzer II from an unpolished patch pipette placed near the cell soma. Stock solutions of TEA (1M in ACSF, and also 1M and 10mM in Low K⁺ Patch Solution), 4-AP (100mM in ACSF) and Na₂ATP (10mM in the appropriate WCS) were stored frozen and added to solutions as required on the day they were to be used.

During recordings, the recording chamber was continually perfused with ACSF using a gravity-controlled perfusion system. AICS was applied to inside-out patches using a peristaltic pump (Gilson Minipuls 3). The chamber was constantly being aspirated (to keep the bath level low and to remove debris floating on top of the solution) using a bent hypodermic needle connected to another peristaltic pump (LKB Varioperpex).

Formation of Seals

Several procedures must be strictly adhered to in order to form high resistance seals routinely. As mentioned above, all solutions must be clean and pipette tips must be fire polished. Cell membranes must also be clean and free of debris; this is usually the case for cultured cells, provided that they are washed to remove serum. Under these conditions, seals with resistances in excess of 10GΩ could usually be obtained by the following method.

After filling the pipette (as described above), it was placed in the electrode holder so that the chlorided silver wire just dipped into the pipette solution, and the electrode holder was placed in the amplifier headstage. Whilst inserting the electrode holder in the probe, it is advisable to touch a grounded metal surface to avoid damaging the electronic circuitry of the probe with static electricity. Before the pipette was moved into the bath,
positive pressure was applied to it. This is primarily to keep the tip free of debris as it moves through the air-water interface, although it is helpful to keep the positive pressure on until the seal begins to form. The pipette was moved close to the cell using the manipulator. When it was very close, the pipette potential was set to zero using the search mode of the patch clamp amplifier, and a 1mV, 10ms test pulse was applied to the pipette at a frequency of 2.5Hz. This allowed the development of a seal to be monitored on the oscilloscope, as an increase in resistance causes a decrease in the current elicited by this pulse. The pipette was advanced very slowly towards the cell soma; the moment when a seal is about to form was judged both by monitoring the pipette current on the oscilloscope and by watching for deformations of the cell membrane as the electrode pressed against it. The positive pressure on the electrode was then released; sometimes, a seal formed immediately this was done, whilst on other occasions gentle suction was required. When a seal formed, the amplifier was switched into voltage clamp mode so that recording could begin.

To make a whole cell recording, the pipette potential was next set to the expected resting membrane potential (about -60mV here) and extra suction was then applied to the pipette. When the membrane patch became disrupted, large current transients due to the whole cell membrane capacitance appeared in response to the test pulse. These transients were cancelled by setting appropriate levels of cell membrane capacitance and series conductance (reciprocal of series resistance) compensation with the amplifier. First the value of series conductance compensation was adjusted to bring the initial amplitude of the transient to zero, and then the cell membrane capacitance compensation was adjusted to minimise overall transient area. In theory, once these controls have been correctly set for transient cancellation, they are also correctly set for series resistance compensation. Therefore, the series resistance compensation was next increased to approximately 90%. The value of series resistance compensation applied indicates the amount by which the membrane capacitance time constant and the voltage error due to series resistance are reduced.
**DATA ANALYSIS**

As stated above, current data was analysed using either a Dell System 210 computer running CED "Patch" software, a Dell System 325 or 450DE computer running "Tracan" and "Evtan" software (Davies, 1992; see below), or a DEC PDP 11-73 computer running "Noise" software (see below). Single channel data was in the form of a series of records obtained by applying the same voltage step to the pipette at regular intervals. For all single channel analyses, the first step was to digitally remove the remaining capacitive and leakage currents associated with stepping of the pipette potential. This was done by averaging records or portions of records which contained no channel openings, and then subtracting this average from all current records. Current records “cleaned” in this way were written to a new file, on which all subsequent analysis was carried out.

A different method had to be used to subtract capacitive and leakage currents from whole cell current records. Voltage steps which were too small to activate any membrane currents were applied to the cell, and the current responses to these steps, which consisted only of capacitive and leakage currents, were subtracted (after appropriate scaling) from those records in which membrane currents were activated. This method assumes that the amplitude of capacitive and leakage currents are linearly proportional to the size of the voltage step.

A potential difference occurs at the interface between two dissimilar salt solutions, due to the diffusion of ions with different mobilities in solution. Such liquid junction potentials may occur at the tip of microelectrodes, and can cause voltage errors in whole cell recording if not compensated for. In practice, liquid junction potentials were measured and added to voltages measured during whole cell experiments. Liquid junction potentials were measured by filling both an electrode and the bath with whole cell recording solution, and zeroing the pipette potential using the amplifier; under these conditions there is presumably no potential difference across the tip of the electrode. The bath solution was then replaced by the normal extracellular solution (ACSF; see table 2-1 above); the pipette potential measured under these conditions is therefore equal to the liquid junction potential. The mean liquid junction potential measured in this way was -8.5mV for K⁺ Gluconate WCS and -5.6mV for K⁺ Aspartate WCS (see table 2-1); appropriate corrections were made to whole cell data in chapter 4.
**Measurement of Single Channel Current Amplitude**

The amplitude of single channel currents was measured by plotting the distribution of the digitised current values as an “amplitude histogram”. This gives a peak corresponding to the channel closed level (zero current), and peaks corresponding to each open level. Gaussian distributions were fitted to each peak using a least-squares fitting routine of the CED software to give the mean and standard deviation of each current level, and of the noise (zero current level).

If the current records contain many closings which are too brief to reach full amplitude, this may smear the open level amplitude histogram so that Gaussian curves do not give a good fit (as well as shifting it towards the closed level). This fact may be used to study single channel events which are too rapid to be completely resolved (Yellen, 1984; see below), but usually the problem was avoided by selecting parts of the current record which contained long openings or closings. Also, parts of the current record where more than one channel was open were omitted, so that the amplitude histogram had only two peaks, corresponding to the closed and single open current levels.

**Ensemble Average Currents**

If a large number of single channel records due to the same voltage pulse are averaged, then the result should be equivalent to a macroscopic recording from a number of similar channels. This method was routinely used to check the identity of single channel currents, by their correspondence to currents seen during whole cell recording.

Such ensemble average currents can also be used to calculate channel open probability at any time during the voltage step, provided that the number of channels in the patch is known. Open probability, $P_o$, is given by:

$$P_o = \frac{i_m}{n.i} \quad (2-9)$$

where $i_m$ is the mean current per trace (measured from the ensemble average), $i$ is the unitary current amplitude (calculated from the amplitude histogram as described above), and $n$ is the number of channels in the patch.
Ensemble Ramp Currents

Unitary current-voltage relationships were also occasionally studied using ramp changes in membrane potential. A depolarising step was applied to activate the channels, and the membrane potential was then returned to the holding potential linearly over time. Currents elicited by such pulses had capacitive and leakage currents removed as described for unitary currents during step pulses. Ensemble averages were then formed, selecting only those parts of each current trace where a single channel was open. The ensemble currents thus formed therefore represented the current-voltage relationship of a single open channel.

Analysis of Open and Closed Times

The theoretical distribution of single channel open and closed times has been discussed in chapter 1. In practice, analysis of open and closed times was carried out only where the patch contained just one channel, to avoid any ambiguity concerning the origin of measured closed durations. Patches were judged to contain one channel only when no double open level currents were observed under conditions where the open probability was high. Open and closed times were measured by setting a cursor at an appropriate level between zero current and full unitary amplitude. An opening or closing was then deemed to have occurred each time the current record crossed this threshold. Traditionally, 50% of the unitary amplitude has been used as a threshold; however, in some cases channel substate behaviour made this impractical, and other threshold levels were used (see chapter 5). An idealised record was formed these transitions and checked against the current records by eye.

When a large number of open and closed times had been measured, histograms were formed to show the distribution of the times. These were fitted by one or the sum of several exponential distributions, using a least-squares fitting routine of the CED software. First latency histograms (see introduction) were also formed and fitted as described below; first latencies were not included in the overall closed time distribution. Similarly, open or closed durations which were truncated by the end of the voltage pulse were excluded.

A more detailed kinetic analysis of delayed rectifier channels in hippocampal CA1 neurons was carried out using “Tracan” and “Evtan” software, developed in Leicester using the AxoBASIC library by Dr. N.W. Davies (Davies, 1992), as described in chapter
The distribution of open times, closed times, durations of bursts of openings and durations of clusters of bursts were studied using logarithmic binning of the data (McManus, Blatz & Magleby, 1987), as described by Davies, Standen & Stanfield (1992), following imposition of a minimum resolution of 200µs (Colquhoun, 1987). The distributions are displayed with a logarithmic abcissa, and the ordinate is the square root of the number of events in a bin (see Sigworth & Sine, 1987). Displaying the data in this way allows different exponential components with very different time constants and areas to be identified in a single histogram. Histograms formed in this way were fitted with between one and five exponential components by the method of maximum likelihood, as described by Davies et al. (1992).

Correction for Missed Events

Many short closings were too brief to be detected, particularly after imposition of a minimum resolution (see above). Therefore, each apparent opening will probably be a burst of openings separated by closings too brief to be detected. The measured mean open time will therefore be an overestimate of the true mean open time. Mean open times measured using the "Tracan" software were therefore corrected by multiplying them by the proportion of closed events which were detected, found by integrating the fitted closed time distribution between the minimum resolution and infinity, as described by Davies et al. (1992). Mean open times measured using CED software were not routinely corrected for missed closures; nor was a minimum resolution usually applied. Mean closed times were never corrected for missed brief open events, which were much rarer than brief closed events.

Analysis of Bursts of Openings

As discussed in chapter 1, where channel openings occur in bursts, certain kinetic information can be gained by studying the distribution of parameters such as the burst length and the number of openings per burst. For the purposes of kinetic analysis, a burst is defined as a group of openings which are separated by closings shorter than some specified critical time t_c. Similarly, where bursts occur in clusters, a cluster is defined as a group of bursts separated by closings shorter than a critical time. In practice, critical times for burst analysis were chosen to make the proportion of short closings (closings within bursts) which were misclassified as long equal to the proportion of long closings.
(closings between bursts) misclassified as short (Colquhoun & Sakmann, 1985). This was done automatically for any number of components in the closed time distribution and for any number of closed components counted as closings within bursts within the “Evtan” software, by solving for \( t_c \) the equation:

\[
\frac{\sum_{j=1}^{n} a_j \exp(-t_c/\tau_j)}{\sum_{j=1}^{n} a_j \exp(-t_c/\tau_j)} = \frac{\sum_{i=n+1}^{k} a_i \left\{ \exp(-t_c/\tau_i) - \exp(-t_c/\tau_i) \right\}}{\sum_{i=n+1}^{k} a_i \exp(-t_c/\tau_i)}
\]

where \( k \) is the total number of components in the closed time distribution, \( n \) is the total number of closed components within a burst, \( a_i \) and \( a_j \) are the areas of closed time components \( i \) and \( j \), with time constants \( \tau_i \) and \( \tau_j \) respectively, and \( t_m \) is the imposed minimum resolution (see above).

**First Latency Distribution**

For a voltage-gated channel with a linear scheme of open and closed states (such as that proposed by Armstrong (1969); see equation 1-15, chapter 1), it is usually assumed that at rest (i.e., at negative membrane potentials), the channel resides in the closed state furthest away from the open state. On depolarisation, the channel must therefore pass through all the closed states on its way to opening. The distribution of the time taken for the first opening to occur following a depolarising step should therefore be made up of the sum of a number of exponential components equal to the number of closed states. This time is commonly referred to as the first latency.

The first latency distribution of sodium channels has previously been described by a model in which two closed states are connected in a linear way to the open state:

\[
\begin{align*}
& k_1 & k_2 \\
C_1 & \rightarrow & C_2 & \rightarrow & O \\
k_{-1} & & k_{-2}
\end{align*}
\]

It has been shown that under these conditions, the probability density function (p.d.f.) of the first latency is described by (Fukushima, 1981; Patlak & Horn, 1982):
\[ f(t) = \frac{R_1R_2}{R_1 - R_2} [\exp(-R_2t) - \exp(-R_1t)] \]  

(2 - 12)

where \( R_1 \) and \( R_2 \) are given by:

\[ R_1, R_2 = \frac{k_1 + k_2 + k_{-1} \pm \sqrt{(k_1 + k_2 + k_{-1})^2 - 4k_1k_2}}{2} \]  

(2 - 13)

These equations are the same as those given by Colquhoun & Hawkes (1981; equations 2.9 to 2.10 and 2.12) for the conditional p.d.f. of burst length in a linear three-state scheme, where the burst is constrained to start and end at the two most extreme states. \( R_1 \) and \( R_2 \) are in fact the minus eigenvalues of the \( Q_{bb} \) submatrix containing the transition rate constants between the different states leading up to the open state (see chapter 1, equations 1-18 to 1-20):

\[
Q = \begin{pmatrix}
Q_{AA} & Q_{AB} \\
Q_{BA} & Q_{BB}
\end{pmatrix}
\]  

(2 - 14)

\[
Q = \begin{pmatrix}
O & C_2 & C_1 \\
O & \begin{pmatrix}
-k_{-2} & | & k_{-2} & 0 \\
\vdots & | & \vdots & \vdots \\
C_2 & k_2 & | & -(k_2 + k_{-1}) & k_{-1} \\
C_1 & 0 & | & k_1 & -k_1
\end{pmatrix}
\end{pmatrix}
\]  

(2 - 15)

If there are three closed states connected in a linear way to the open state:

\[
C_1 \rightarrow C_2 \rightarrow C_3 \rightarrow O
\]  

(2 - 16)

The relevant \( Q \) matrix is now:
The first latency p.d.f. under these conditions will be given by an analogous expression to that given by Colquhoun & Hawkes (1981) for the conditional p.d.f. of burst length in a linear kinetic scheme where the burst is constrained to begin and end in the two most extreme states (Colquhoun & Hawkes, 1981, equation 3.25):

\[ f(t) = k_1 k_2 k_3 (e_1 + e_2 + e_3) \quad (2-18) \]

where \( e_1, e_2 \) and \( e_3 \) are related to the minus eigenvalues of the \( Q_{bb} \) submatrix of equation 2-16 which concern transitions between closed states by (Colquhoun & Hawkes, 1981, equation 3.20):

\[
e_1 = \frac{\exp(-\lambda_1 t)}{(\lambda_2 - \lambda_1)(\lambda_3 - \lambda_1)}
\]

\[
e_2 = \frac{\exp(-\lambda_2 t)}{(\lambda_1 - \lambda_2)(\lambda_3 - \lambda_2)} \quad (2-19)
\]

\[
e_3 = \frac{\exp(-\lambda_3 t)}{(\lambda_1 - \lambda_3)(\lambda_2 - \lambda_3)}
\]

where \( \lambda_1, \lambda_2 \) and \( \lambda_3 \) are the minus eigenvalues of equation 2-17, given by the roots of the cubic equation:

\[ \lambda^3 - b\lambda^2 + c\lambda - d = 0 \quad (2-20) \]

where
First latency distributions of this form are solved for the delayed rectifier channel of hippocampal CA1 neurons in chapter 6.

**Analysis of Fast Channel Block**

Many ion channel blockers interact with the channel with kinetics which are too fast to allow the individual fluctuations between open and blocked states to be resolved using patch clamp recording. The filtered current record under such conditions will therefore show a decrease in unitary current amplitude and an increase in open state noise. In such cases, the kinetics of the blocking reaction may be studied using amplitude distribution analysis (Yellen, 1984) or by analysis of the excess current variance seen in the presence of the blocker (Ogden & Colquhoun, 1985). Both methods were used to study the interaction of TEA with single delayed rectifier channels in hippocampal CA1 neurons (see chapter 8).

**Amplitude Distribution Analysis**

As mentioned above, repeated brief interruptions in the current record can smear the amplitude histogram. If the majority of the interruptions are due to fast channel block, this smearing can be used to calculate the channel blocking and unblocking rates. For a simple blocking reaction:

\[
\begin{align*}
\beta[\text{TEA}] \\
\text{Open} & \rightarrow & \text{Blocked} \\
& \leftarrow & \alpha
\end{align*}
\]

(2 – 24)
If the current record is filtered with a first-order filter of time constant $\tau$, then the amplitude histogram produced will show an open level current which is fit not by a Gaussian distribution, but by a $\beta$-distribution with the probability density function:

$$f(y) = \frac{y^{a-1}(1-y)^{b-1}}{B(a,b)} \quad (2-25)$$

where $a=\alpha \tau$, $b=\beta[TEA] \tau$ and

$$B(a,b) = \int_0^1 y^{a-1}(1-y)^{b-1}dy \quad (2-26)$$

If the current record is filtered with an 8-pole Bessel filter (as it was here) with a -3dB cut-off frequency of $f$, then the time constant of the equivalent first-order filter is given by (Yellen, 1984):

$$\tau = 0.228/f \quad (2-27)$$

$\beta$-distributions were fitted to the open level current amplitude histograms formed from current records in the presence of TEA using "Tracan" software. A Gaussian distribution was first fitted to the closed level amplitude histogram, which was then convolved with a $\beta$-function of the form given in equation 2-25. A least squares method was used to find the values of $\alpha$ and $\beta$ which gave the best fit of this $\beta$-function to the data. When $\alpha$ is greater than $\beta$, the $\beta$-distribution is skewed towards the open (full current in the absence of blocker) amplitude, and when $\beta$ is greater than $\alpha$ the distribution is skewed towards the blocked (zero current) amplitude.

**Analysis of Excess Current Variance**

If it is assumed that the excess open single channel noise introduced by a blocker is caused by brief, complete blockages of the channel, then the excess current variance seen in the presence of blocker ($\sigma^2$), from the binomial distribution, should be:

$$\sigma^2 = m_i(i-m_i) \quad (2-28)$$

where $i$ is the unitary current in the absence of blocker, and $m_i$ is the mean single channel current (whilst the channel is open or blocked) when blocker is introduced.

The observed current variance at different frequencies in the absence or presence of
TEA was measured using the PDP 11-73 computer program “Noise”, written by Professor D. Colquhoun, Dr. M. Tessier-Lavigne and Dr. A. Cachelin. Current variance was measured from current record subsamples 1/16 s long, and power spectra of the variance per unit frequency were formed from the average of between 20 and 243 subsamples (the mean number of subsamples used to form the power spectra was 79). For each experiment, separate power spectra were formed from parts of the current record where a single channel was open (or blocked), and where no openings occurred. Subtraction of this baseline spectrum from the open level spectrum gave a net spectrum of the noise associated with an open channel. This net spectrum was then fit with the sum of two Lorentzian curves, each of the form:

\[ S(f) = \frac{S(0)}{1 + (f/f_0)^2} \]  

(2 - 29)

where \( S(f) \) is the current variance per unit frequency range (units \( \text{A}^2/\text{Hz} \)) at each frequency \( f \) (Hz), \( S(0) \) is the maximum value of \( S \) (taken as \( f \) approaches zero), and \( f_0 \) is the frequency at which \( S(f) \) falls to half its maximum value. \( S(0) \) and \( f_0 \) were adjusted to give the best fit of the Lorentzian curves to the net spectrum by eye. The total current variance over all frequencies (\( \sigma^2 \)) associated with each Lorentzian is found by integrating \( S(f) \) with respect to frequency, which gives:

\[ \sigma^2 = \frac{S(0)\pi f_0}{2} \]  

(2 - 30)

The total observed variance is the sum of that associated with each Lorentzian. The excess variance associated with channel block was calculated as the difference between the total variance in the presence and absence of blocker under the same conditions.

In practice, not all of the predicted excess variance will be recorded, owing to the effects of low-pass filtering the current. Currents for excess variance analysis were filtered using an 8-pole Butterworth filter. Ogden & Colquhoun (1985) have shown that, for such a filter, the ratio of predicted to observed excess variance, \( R \), is given by:

\[ R = 1.561(f_e/f_f) \]  

(2 - 31)
where $f_f$ is the filter cut-off frequency and $f_c$ is the cut-off frequency associated with the channel blocking reaction, which is given (using the blocking reaction scheme shown in equation 2-24 above) by:

$$f_c = \frac{\alpha + \beta[\text{TEA}]}{2\pi} \quad (2 - 32)$$

The observed excess variance associated with TEA block was calculated according to equation 2-30, and compared with the predicted excess variance, calculated from equation 2-28, to give the ratio $R$, which was then used to calculate $f_c$ from equation 2-31. Ogden & Colquhoun (1985) have shown this method of calculating $f_c$ is accurate to within 5% if $R$ is greater than 2.5, which was always the case here, except at the lowest TEA concentrations studied (see chapter 8). $f_c$ was then used to calculate the sum of the blocking and unblocking rates, since from equation 2-32:

$$\alpha + \beta[\text{TEA}] = 2\pi f_c \quad (2 - 33)$$

To separate the blocking and unblocking rates, it was first necessary to calculate $K_d$, the affinity of TEA for the channel, which is equal to $\alpha/\beta$. This was done by measuring the reduction in mean current amplitude by TEA (see chapter 8). Substituting $\beta = \alpha/K_d$ into equation 2-33 gives:

$$\alpha = \frac{2\pi f_c}{(1 + [\text{TEA}]/K_d)} \quad (2 - 34)$$
CHAPTER THREE: CELL CULTURE METHODS

Introduction

Cells grown in culture have been widely used for patch clamp recording, as they are readily accessible to the patch pipette and have clean membrane surfaces, important for the formation of seals. For these reasons, low density primary cell cultures of rat hippocampal CA1 and locus coeruleus neurons were prepared for electrophysiological recording. The methods used for culturing neurons were similar to those previously described for hippocampal (Banker & Cowan, 1977; Forsythe & Westbrook, 1988) and locus coeruleus neurons (Masuko, Nakajima, Nakajima & Yamaguchi, 1986). Neurons were plated onto a confluent glial cell layer previously prepared from rat hippocampi. Glial cells may provide conditioning factors which help in the survival and development of neurons in culture, and they also form an adhesive surface to which neurons become firmly attached. Although most tissue culture methods in the past have used cells from embryonic tissue, which survive better in culture, both neurons and glial cells here were prepared from neonatal Lister Hooded rats (aged 1 to 5 days). This provided more mature cells, and also minimised the use of adult animals. All cultured cells were prepared and grown under sterile conditions and maintained in an incubator at 35°C and in a 5% CO₂ atmosphere.

Preparation of the Growth Surface

Glial cells were grown on 13mm glass cover slips coated with collagen and poly-L-lysine. The cover slips were put into 16mm multi-well dishes, and briefly immersed in 0.5ml of collagen solution (see appendix). The collagen was then removed, and the cover slips left to dry at least overnight, although they could be stored in a dry container for several weeks. On the day of use, 0.5ml of poly-L-lysine solution (see appendix) was added to each well, and removed after one hour. The wells were then rinsed three times with “MilliQ” sterile water.
Glial Cell Culture

Glial cells were prepared from neonatal Lister Hooded rat hippocampi as follows. Two rat pups were killed by decapitation, and the heads were immediately placed in ice cold D1 solution (see appendix). For dissection, the heads were placed into 60mm Petri dishes containing 5ml of D1 solution, and observed under a dissecting microscope. The skin was peeled back to reveal the top of the skull, which was cut in both the transverse and sagittal planes, dividing it into four parts. These four sections could then be peeled apart to reveal the brain. The whole brain was then carefully lifted out, and the cranial nerves cut. The cerebral hemispheres were separated by running a pair of forceps along the interhemispheric fissure, and they were then cut away from the rest of the brain. The meninges were then carefully stripped from both the medial and lateral surfaces of each cerebral hemisphere using forceps. Although these meninges are extremely thin, they may be visualised by the blood vessels which pass through the subarachnoid space. At this stage, it is possible to see the hippocampus as a fairly large, crescent-shaped body in the medial surface of each cerebral hemisphere. Fine forceps may then be placed laterally to the hippocampus, between it and the rest of the cerebral hemisphere, and used to unfold and separate the hippocampus. The hippocampus is finally removed by cutting in the region of the entorhinal cortex.

Once the whole hippocampus had been removed, it was placed in a fresh 60mm Petri dish containing 5ml of D1 solution. All four hippocampi (from two rats) were placed in the same Petri dish, chopped up with fine scissors, and 5ml of 0.25% trypsin solution (see appendix) was added. This Petri dish was then placed in the incubator for 25 minutes at 35°C, to allow the trypsin to begin to digest the tissue.

After removal from the incubator, the tissue was placed in 10ml of a 10% foetal calf serum based medium (FCS; see appendix) for 5 minutes. This was in order to inactivate the trypsin, as foetal calf serum contains endogenous trypsin inhibitors. The tissue was subsequently transferred to a 10ml conical tube containing 2ml of FCS, and was triturated gently through a fire-polished Pasteur pipette, in order to dissociate the tissue into single cells. After approximately 20 triturations, the remaining tissue was left for three minutes to settle, and then the supernatant (containing dissociated cells) was removed to another 10ml conical tube. A further 1ml of FCS was added to the tissue pellet, and the trituration procedure was repeated, but using a finer bore Pasteur pipette. Again, the supernatant was
removed after three minutes, and added to that already obtained. This procedure was then repeated with a further 1ml of FCS and an even finer bore Pasteur pipette, so that eventually 4ml of cell suspension was obtained. Three triturations was usually enough to completely dissociate the tissue. A small volume of the cell suspension was taken and placed on a haemocytometer slide for examination under a microscope. By counting the number of cells on the slide, the concentration of cells in the cell suspension could be calculated, so that the suspension could be diluted with an appropriate volume of FCS to give the required final concentration of approximately 125000 cells per ml. 0.5ml of this final suspension was placed into each 16mm well, so that approximately 62500 cells were plated onto each cover slip.

After one day in culture, the medium was removed and replaced with fresh FCS; this “full feeding” was subsequently repeated three times per week. This treatment destroyed the neurons, so that only glial cells survived. The cells were examined daily under a microscope, and when the glial cell layer was judged to be confluent (usually after approximately 10 days), further cell division was stopped by adding a solution of the antimitotic agent 5-fluoro-2'-deoxyuridine (FdUr, see appendix; added at 7μl per ml of medium), and the feeding medium was changed to 10% heat inactivated horse serum (10HS; see appendix).

**Hippocampal CA1 Neuronal Culture**

After the glial cell cultures had become confluent, neurons prepared from either the CA1 region of the hippocampus or from the locus coeruleus of neonatal Lister Hooded rats were plated onto them. For CA1 neuronal cultures, the hippocampus was removed as described for the preparation of hippocampal glial cells (see above), and the CA1 region was then isolated by carefully splitting along the hippocampal fissure using forceps. Usually the CA1 regions of two or four hippocampi were taken and subjected to trypsin treatment as described above. The tissue was subsequently triturated as for glial cell preparation, except that all trituration was done in a serum-free medium (see appendix), rather than FCS. For this reason, directly following incubation in trypsin the tissue was left for five minutes in serum free medium containing 1mg/ml trypsin inhibitor (Sigma T6522) prior to trituration. The dissociated cells were counted on a haemocytometer slide and diluted using 5% heat inactivated horse serum (5HS; see appendix) to a final concentration of 50000 cells per ml. 0.5ml of this final cell suspension was added to each
well without removing the medium already present. The following day, half the medium in each well was removed, and 0.5ml of 5HS containing FdUr solution (7μl/ml) was added. Once per week thereafter, half the medium in each well was removed and 0.5ml of fresh 5HS was added. Neuronal cultures were always given a “half-feed” in this way in order to maintain any glial conditioning factors which may be present. CA1 neurons prepared in this way were used for electrophysiological recording after 6-17 days in culture.

**Locus Coeruleus Neuronal Culture**

The locus coeruleus is located in the pontine tegmentum, at the ventrolateral border of the central grey rostrally, and the lateral border of the central grey caudally, and is the largest noradrenergic nucleus in the rat brain. Neurons from this region of neonatal Lister Hooded rat brains were also plated onto confluent hippocampal glial cell feeder layers. Because of the small size of the locus coeruleus (about 1650 cells), sixteen nuclei (from eight rats) were usually taken. The whole brain was removed as for the preparation of glial cells (see above). The meninges were stripped from the ventral surface of the brain stem, and a section about 1mm thick was then taken by cutting first just anterior to the pyramidal decussation, and then anterior to the pons, this cut passing between the cerebellum and the superior colliculus. The sections obtained were pinned out, caudal side uppermost, on a silicone rubber coated dish filled with D1 solution. The locus coeruleus was then removed from the outer wall of the fourth ventricle in each slice using a tissue punch (diameter 0.5-0.75mm). The sixteen nuclei were placed in a 60mm Petri dish containing 5ml of D1 solution, to which 5ml of 0.25% trypsin solution was added, as for hippocampal neurons. However, the locus coeruleus was more difficult to dissociate than the hippocampus, so the tissue was left in trypsin solution for 45 minutes at 35°C. As with hippocampal neurons, the trypsin was subsequently neutralized with serum free medium containing 1mg/ml trypsin inhibitor (Sigma T6522) and trituration was carried out in serum free medium. Because of the toughness of the locus coeruleus, five separate triturations were necessary to completely dissociate the tissue. The cell suspension was diluted to a final volume of 6ml with 5HS, and 0.5ml was then added to each well, without removing the medium already present. As with CA1 neurons, locus coeruleus neurons were half-fed after 24 hours with 5HS containing 7μl/ml FdUr solution, and thereafter half-fed once per week with 5HS. Locus coeruleus neurons were used for electrophysiological recording after 6 to 16 days in culture.
Immunohistochemical Localization of Cultured Neurons

Cultured neurons were stained histochemically using primary antibodies raised against neuron specific enolase (NSE) and dopamine β hydroxylase (DBH), obtained from Applied Protein Products. NSE is a commonly used neuronal marker, and is expected to be present in neurons but not glial cells (Marangos, Polak & Pearse, 1982). DBH is the enzyme which synthesizes noradrenaline from dopamine, and should therefore be present only in noradrenergic neurons. These primary antibodies are recognised by a secondary, or “bridging” antibody. The secondary antibody used is biotinylated, so it binds with high affinity to an avidin-horseradish peroxidase complex, which is added as a third layer reagent. This complex may then be visualised by addition of a chromogenic substrate, diaminobenzidine (DAB), which reacts with the peroxidase in the presence of hydrogen peroxide to give a coloured precipitate.

Cover slips to be used for immunohistochemical staining were removed from culture and placed in another 16mm well containing cold minimum essential medium (MEM; see appendix). The MEM was subsequently removed and replaced with ice cold methanol, to fix the cells. After 10 minutes, the methanol was removed and the preparation was washed three times with phosphate buffered saline (PBS; see appendix). The PBS was then replaced by approximately 100μl (just enough to cover the cover slip) of PBS containing 0.3% hydrogen peroxide and 0.1% of the detergent Triton-X. Hydrogen peroxide was added to block endogenous peroxidases, and Triton-X to permeabilize the cells. After 30 minutes, the cover slips were again washed three times with PBS, and left in approximately 200μl of PBS containing 5% swine serum and 0.1% Triton-X for 20 minutes. Swine serum was included to block non-specific staining. The solution is then removed, but the cover slips were not washed at this stage, as it is important not to wash away the swine serum. The primary antibody (rabbit anti-NSE or rabbit anti-DBH) was then added in PBS with 0.1% Triton-X. It is important that enough antibody solution is added to completely cover the cover slip, to ensure even staining. Approximately 300μl was added to each cover slip. The cover slips were then incubated in the antibody solution for 24 hours at 4°C.

After incubation, the cover slips were washed three times in cold PBS before the secondary antibody (biotinylated swine anti-rabbit IgG in PBS) was added, approximately 100μl to each cover slip. The secondary antibody was left on for one hour at room
temperature, and the cover slips were then washed three times in PBS. 100μl of the third layer reagent (horseradish peroxidase avidin D in PBS) was then added to each cover slip and left at room temperature for 20 minutes. The cover slips were again washed three times in PBS before PBS containing 0.6mg/ml DAB and 0.3% hydrogen peroxide was added. The cells were then examined under a microscope, and a brown stain was seen to develop within a few minutes. When the stain was fully developed, the reaction was stopped by rinsing in tap water. The cover slips were then dehydrated by passing through ethanol baths of increasing concentration and mounted on microscope slides for subsequent examination and photography. Some examples of neurons stained in this way are shown in figures 3-1 to 3-3.
Figure 3-1. Single cultured hippocampal CA1 (above) and locus coeruleus neurons (below), stained immunohistochemically for the presence of NSE, as described in the text. In each case, it can be seen that the neurons are stained far more heavily than the glial cell substrate. These cells were grown in culture for 2-3 weeks before staining.
Figure 3-2. Single cultured hippocampal CA1 (above) and locus coeruleus neurons (below), stained immunohistochemically for the presence of DBH, as described in the text. Higher levels of DBH are detectable in locus coeruleus neurons, suggesting that these neurons continue to synthesise the neurotransmitter noradrenaline in culture. As in figure 3-1, these cells were grown in culture for 2-3 weeks.
Figure 3-3  Lower-power photograph of cultured hippocampal CA1 (above) and locus coeruleus neurons (below), stained immunohistochemically for the presence of NSE as in figure 3-1.
CHAPTER FOUR: WHOLE CELL RECORDING OF VOLTAGE GATED POTASSIUM CURRENTS FROM LOCUS COERULEUS AND HIPPOCAMPAL CA1 NEURONS

INTRODUCTION

Many different classes of potassium channels coexist in individual mammalian central neurons (Halliwell, 1989; see chapter 1), where they may play different roles in regulating excitability and patterns of action potential firing. Several different potassium currents have been identified in many central neurons using microelectrode techniques, and various strategies have been used to separate those currents and so study their properties in isolation. Depolarisation-activated potassium currents which do not require intracellular calcium for activation were originally described as A-currents or delayed rectifiers, on electrophysiological (time course of activation and inactivation, dependence on the resting potential) and pharmacological (sensitivity to 4-aminopyridine and tetraethylammonium) grounds (eg. Segal & Barker, 1984; Numann et al., 1987). However, as more studies have been carried out, it has become increasingly apparent that voltage gated potassium channels in mammalian central neurons show a spread of properties which invalidate such clear-cut distinctions (eg. Storm, 1988; Picker & Heinemann, 1992; Wu & Barish, 1992; see chapter 1).

At the same time, the identification of a large number of genes for voltage gated potassium channels which are expressed in both rat and mouse central nervous systems (see chapter 1) has indicated the potential diversity of potassium channels which may exist in central neurons in vivo. Different potassium channel genes are expressed differentially throughout the rat brain (Beckh & Pongs, 1990; Drewe et al., 1992; Rettig et al., 1992; Sheng et al., 1992; Tsaur et al., 1992), which may be one mechanism by which different patterns of electrical activity are conferred on different neuronal cells.

Another way in which functionally diverse potassium channels may be formed in different parts of the brain is by formation of heterooligomers (Christie et al., 1990; Ruppersberg et al., 1990; see chapter 1). Voltage gated potassium channels are tetramers (Mackinnon, 1991; see chapter 1), and so subunits encoded by different genes may aggregate to form channels whose properties are different from those of homotetrameric
channels. This may explain the difficulty in matching the properties of native channels with those of one particular cloned channel; since we do not know which subunits go together to form channels in vivo, or how such aggregation is controlled, we cannot tell how the expression of certain potassium channel genes in certain neurons will affect their electrophysiological properties.

**RESULTS**

Whole cell patch clamp recordings were made from both locus coeruleus neurons (after 6-16 days in culture) and hippocampal CA1 neurons (after 6-13 days in culture), as described in chapter 2. Current records were filtered using an 8-pole Bessel filter set at 1kHz (-3db filter rate) and digitised at 5kHz, except where noted. In some instances, 4-aminopyridine (4-AP) or tetraethylammonium (TEA) were applied from a puffer pipette placed close to the cell soma, as described in chapter 2.

**Locus Coeruleus Neurons**

Locus coeruleus neurons exhibit both transient and sustained voltage gated outward currents, as shown in figure 4-1. This figure shows the currents activated in the same neuron when it is depolarised from a holding potential of -40mV (upper current records) or -100mV (lower current records). On depolarisation from -40mV, the sustained current only is activated; as the upper currents in figure 4-1 show, this current is activated over several milliseconds after depolarisation, and is practically non-inactivating over the time course of the pulse (90ms). On depolarisation from -100mV, both transient and sustained currents are activated. Comparison of the two sets of current records in figure 4-1 shows that the transient current is activated more rapidly following a depolarising step, reaching a peak within a few milliseconds, and that it subsequently inactivates fully within tens of milliseconds. The steady-state inactivation seen on holding at a relatively depolarised potential is characteristic of the A-current (Connor & Stevens, 1971a; Segal et al., 1984). The amplitude of the sustained current, measured near the end of the voltage step, is approximately the same when the holding potential is -40 or -100mV, indicating that it is not subject to steady-state inactivation over this voltage range. This is also shown in the whole cell current-voltage relationship for this cell (figure 4-2). The transient current is activated at a more negative voltage than the sustained current, the activation thresholds being close to -60mV and -40mV respectively (see figure 4-2); this means that between
Figure 4-1 Whole cell currents activated in a locus coeruleus neuron on depolarisation from holding potentials of -40mV (upper current records) and -100mV (lower current records) to test potentials between -30 and +10mV. Leak and capacitive currents have been subtracted as described in the methods, which is the case for all whole cell current records shown (except for figures 8 and 14, where no leak subtraction has been done).
Figure 4-2 Whole cell current-voltage relationships for a locus coeruleus neuron, from the current traces shown in figure 1, showing the amplitude of the transient current activated from -100mV (●), and that of the sustained current activated from 100mv (■) and from -40mV (□); it can be seen that the amplitude of the sustained current is the same under these conditions. The amplitude of the sustained current was measured near the end of the depolarising step, where the transient current is assumed to have fully inactivated. The amplitude of the transient current activated by steps from -100mV was measured at the peak, and has had the amplitude of the sustained current at the same point during a step to the same potential from -40mV subtracted. The data have been corrected for a liquid junction potential of -8.5mV, as described in chapter 2.
these potentials, the transient current only is activated (see figure 4-1, lower current records). A few cells had hardly any sustained current, such that the transient current could be activated virtually in isolation (eg. figure 4-4).

The voltage dependence of activation for both transient and sustained currents, and the voltage dependence of inactivation of the transient current, were examined using different experimental protocols. Activation was studied by holding at -120mV to ensure complete removal of the inactivation of the transient current, and then stepping to different test potentials and measuring the amplitude of each current elicited (as described in the legend to figure 4-2). These currents were used to calculate relative conductances at the different test potentials, assuming a reversal potential of -80mV (see chapter 5) and a linear unitary current-voltage relationship. Inactivation of the transient current was studied by holding at -100mV and then giving a 500ms prepulse to different potentials before stepping to a constant test potential (+10mV), and examining the relationship between relative conductance during the test pulse (calculated as for activation) and prepulse potential. The results of these experiments are shown in figure 4-3. Both activation and inactivation curves for the transient current (figure 4-3A) have been fitted by a Boltzmann expression of the form:

$$G = \frac{G_{\text{MAX}}}{1 + \exp[(V - V')/k]} \quad (4-1)$$

where $G$ is conductance (relative to a maximum $G_{\text{MAX}}$), $V'$ is the potential at which the value of $G$ is one-half of $G_{\text{MAX}}$ (ie the potential at which the current is half maximally activated or inactivated), and $k$ is a slope factor (the voltage change for an e-fold increase in conductance). For activation, the fitted curve has a $V'$ of -23.7mV and a $k$ of -8.1mV; for inactivation, $V'$ is -91.8mV and $k$ is 10.4mV. However, this curve may not represent the true activation process of the channels underlying the transient current, which do not have a linear current-voltage relationship (see chapter 5). The activation curve for the sustained current (figure 4-3B) could not be fitted by a Boltzmann expression, since the current was probably not fully activated even at the most positive test potentials studied.

Transient and sustained currents could also be separated by their sensitivity to block by 4-AP, a selective blocker of transient voltage gated potassium channels in other cells (Thompson, 1977; Segal et al., 1984). In five cells, puffer application of 2.5mM 4-AP
Figure 4-3 Activation and inactivation curves for whole cell currents in locus coeruleus neurons. A.: activation (○; mean of data from 5 cells) and inactivation (●; mean of data from 3 cells) of the transient current. Both curves have been fitted by a Boltzmann expression (equation 1 of text); for activation $V'=-23.7\text{mV}$ and $k=-8.1\text{mV}$, and for inactivation $V'=-91.8\text{mV}$ and $k=10.4\text{mV}$. B.: activation of the sustained current, showing the mean of the data from 7 cells. In both A. and B., error bars represent ± one S.E.M.; where no error bar is shown, this is smaller than the size of the symbol. As in figure 2, a correction for a liquid junction potential of -8.5mV has been made.
reduced the amplitude of the transient current by 19.3±4.5% without affecting the sustained current. The effect of 4-AP on the transient current is shown in figure 4-4; the current-voltage relationship for this cell is shown in figure 4-5.

**Hippocampal CA1 Neurons**

CA1 neurons also exhibit both transient and sustained outward currents on depolarisation. Figure 4-6A shows the currents activated in one neuron during depolarising steps from a holding potential of -40mV (upper current records) and -80mV (lower current records). As with locus coeruleus neurons, both transient and sustained currents are activated on depolarisation from the more negative holding potential, and the sustained current alone is activated on depolarisation when the holding potential is more positive. However, in contrast to locus coeruleus neurons, the sustained current in CA1 neurons is also sensitive to the holding potential; its amplitude is approximately 40% less on depolarisation from a holding potential of -40mV than it is during steps to the same test potential when the holding potential is -80mV. This can also be seen from the current-voltage relationships for this cell, which are shown in figure 4-7. The activation thresholds for both currents are more positive than those for the corresponding currents in locus coeruleus neurons; close to -50mV for the transient current, and -20mV for the sustained current.

Comparison of the current records in figure 4-6A with those from figure 4-1 show that the time course of activation and inactivation of both transient and sustained currents is similar in CA1 and locus coeruleus neurons. The slow inactivation of the sustained current in CA1 neurons during prolonged depolarising pulses is shown in figure 4-6B.

Activation of the two currents was studied by holding at -80mV and stepping to different test potentials, as in the lower current traces in figure 4-6A. Inactivation of both currents was also studied. Because of the incomplete inactivation of the sustained current during a depolarising pulse, it was not possible to use a similar protocol to that used to study inactivation of the transient current in locus coeruleus neurons. Instead, CA1 neurons were held at 0mV, so that both currents were almost totally inactivated, and then hyperpolarised to different potentials for 500ms to remove inactivation, before stepping to a constant test potential of +40mV; an example of the test currents elicited by this protocol is shown in figure 4-8. Activation and inactivation curves were constructed from these current records in the same way as described for locus coeruleus neurons, and these
Figure 4-4 Effect of 4-AP on the transient current in a locus coeruleus neuron. The currents were elicited by steps to between -40 and 0mV from a holding potential of -100mV, before (top), during (middle) and after (bottom) application of 2.5mM 4-AP to the cell soma from a localised puffer pipette. In this cell 4-AP reduced the amplitude of the transient current by around 36%. This cell lacked much sustained current; in cells where the sustained current was more prominent, its amplitude was unaffected by this concentration of 4-AP.
Figure 4-5. Current-voltage relationships constructed from the data shown in figure 4, showing the effect of 4-AP on the transient current in a locus coeruleus neuron. Current values, measured as for figure 2, show control (●), 2.5mM 4-AP (■) and recovery (○). As in figure 2, a correction for a liquid junction potential of -8.5mV has been made.
Figure 4-6 Whole cell currents activated by depolarisation in a hippocampal CA1 neuron. A. shows the currents in one neuron activated by steps to between -20 and +40mV, from a holding potential of -40mV (upper current records) and -80mV (lower current records). B. shows the currents activated in another neuron by longer steps to between -20 and +60mV, from a holding potential of -80mV; the currents here were filtered at 100Hz and digitised at 500Hz.
Figure 4-7 Current-voltage relationships constructed from the data shown in figure 6A, for both transient and sustained currents in CA1 neurons. Curves represent the transient current activated from -80mV (●), and the sustained current activated from -80mV (■) and -40mV (□); at all test potentials, the amplitude of the sustained current activated from -40mV was approximately 40% less than that activated from -80mV. For this reason, the amplitude of the two currents could not be separated as easily as in locus coeruleus neurons. As with locus coeruleus neurons, the amplitude of the sustained current was measured near the end of the depolarising step. The amplitude of the transient current activated from -80mV was measured at the peak; the amplitude of the sustained current at this point was calculated by appropriately scaling up the current at the same point during a step to the same potential from -40mV, and was subtracted to give the amplitude of the transient current alone. This method assumes that although the amplitude of the sustained current is reduced by holding at a more positive potential, its timecourse is unchanged. The data have been corrected for a liquid junction potential of -5.6mV, as described in chapter 2.
Figure 4-8 An experiment to study the inactivation of both transient and sustained currents in CA1 neurons. The currents are activated by a step to +40mV following a 500ms prepulse to between -120 and 0mV; the holding potential is 0mV. When the prepulse is to a more negative potential, more of each current is activated during the test pulse.
are shown in figure 4-9. The inactivation curve for the transient current (figure 4-9A) has been fitted by a Boltzmann expression (equation 4-1), with a \( V' \) of -73.4mV and a \( k \) of 14.2mV; the activation curve could not be fitted by a Boltzmann expression. Both activation and inactivation curves for the sustained current (figure 4-9B) have been fitted by a similar Boltzmann expression; for activation, this fitted curve has a \( V' \) of 4.6mV and a \( k \) of -10.1mV, whilst for inactivation \( V' \) is -62.8mV and \( k \) is 17.4mV. However, the inactivation curve for the sustained current is inconsistent with the data obtained from experiments such as that shown in figure 4-6A, which show that the amplitude of the sustained current which can be activated from a holding potential of -40mV is approximately 40% less than that activated from -80mV; in contrast, figure 4-9B suggests that such a change in holding potential should cause around a 67% reduction in amplitude. This indicates that the protocol used to study inactivation does not reproduce steady-state conditions, a 500ms hyperpolarising prepulse probably not being long enough to fully remove the inactivation of the sustained current developed by holding at 0mV for prolonged periods.

Another difference between the sustained current in CA1 neurons and that in locus coeruleus neurons was its sensitivity to block by 4-AP. In three CA1 neurons, puffer application of 1mM 4-AP reduced the amplitude of the transient current by 10.1±0.8%, and also reduced the amplitude of the sustained current by 31.5±2.9%; an example of this is shown in figure 4-10, and in the current-voltage relationships of the same cell (figure 4-11). The sustained current in these cells was also sensitive to block by TEA, which selectively blocks sustained voltage gated potassium channels in other cells (Neher & Lux, 1972; Thompson, 1977; Segal & Barker, 1984). In five cells, 30mM TEA reduced the amplitude of the sustained current by 73.9±3.8% (see figures 4-12 and 4-13). This concentration had no effect on the transient current on depolarisation from a more negative potential.

The time course of recovery from inactivation of the transient current which occurs at hyperpolarised potentials was also studied in CA1 neurons. Cells were held at a negative potential and given a 200ms test pulse to -20mV, which activated some transient current with practically no contamination by sustained current, by virtue of the difference in activation thresholds. Following this pulse the membrane potential was returned to the holding potential for a variable period of time, before a second identical test pulse was
Figure 4-9 Activation and inactivation curves for whole cell currents in CA1 neurons. 
A.: activation (○; mean of data from 13 cells) and inactivation (●; mean of data from 8 cells) of the transient current. The inactivation curve has been fitted by a Boltzmann expression (equation 1 of text) with \( V' = -73.4 \text{mV} \) and \( k = 14.2 \text{mV} \). 
B.: activation (○; mean of data from 17 cells) and inactivation (●; mean of data from 4 cells) of the sustained current. Both curves in B. have been fitted with Boltzmann expressions; for activation, \( V' = 4.6 \text{mV} \) and \( k = -10.1 \text{mV} \), and for inactivation \( V' = -62.8 \text{mV} \) and \( k = 17.4 \text{mV} \). In both A. and B., error bars represent ± one S.E.M.; where no error bar is shown, this is smaller than the size of the symbol. As in figure 4-7, a correction for a liquid junction potential of \(-5.6 \text{mV}\) has been made.
Figure 4-10 Effect of 4-AP on whole cell currents in CA1 neurons. The currents were elicited by steps to between -20 and +60mV from a holding potential of -80mV, before (top), during (middle) and after (bottom) application of 1mM 4-AP to the cell soma from a localised puffer pipette. The notch near the beginning of each of the uppermost current records is an artefact of the leak subtraction procedure.
Figure 4-11 Current-voltage relationships from the data shown in figure 10, illustrating the effect of 4-AP on both transient and sustained currents in CA1 neurons. The curves are the controls for transient (●) and sustained currents (■), and these currents following application of 1mM 4-AP (○,□). As in figure 4-7, a correction for a liquid junction potential of -5.6mV has been made.
Figure 4-12. Effect of TEA on the sustained current in CA1 neurons. The current records were activated by steps to between 0 and +60mV from a holding potential of -40mV, before (top), during (middle) and after (bottom) puffer application of 30mM TEA. Similar concentrations of TEA had the same effect on the sustained current activated from a more negative holding potential, but had no effect on the transient current under these conditions (not shown).
Figure 4-13  Current-voltage relationships for the data shown in figure 12, illustrating the effect of TEA on the sustained current in CA1 neurons. The curves show control (●), 30mM TEA (■) and recovery (○). As in figure 4-7, a correction for a liquid junction potential of -5.6mV has been made.
given. An example of the currents elicited by such a protocol is shown in figure 4-14, where the holding potential is -70mV. It can be seen that as the interval between the pulses is increased, so more transient current is activated during the second pulse, more inactivation having been removed. Figure 4-15A shows how the length of the interval between the two pulses affects the amplitude of the current during the second pulse, measured as a fraction of the current activated by the first pulse, in this same cell. Recovery from inactivation under these conditions follows a double exponential time course, which can be seen more clearly in figure 4-15B. Here the same data as in figure 4-15A have been fitted by eye by two exponential components, with time constants of 42 and 760ms. In several similar experiments, the shorter time constant was found to be strongly dependent on the holding potential, as illustrated in table 4-1. The longer time constant had values of between 88 and 760ms, and also showed some voltage dependence. This double exponential time course of recovery from inactivation suggests that the channels underlying the transient current have at least two inactivated states. The voltage dependence of both exponential components further suggests that recovery from both inactivated states is more rapid at more negative potentials. The presence of two inactivated states might be expected if the inactivation process is assumed to be similar to that seen in cloned \textit{Shaker} channels, where the amino terminus of each subunit mediates fast inactivation, whilst a slower inactivation process involves the S6 transmembrane region (Hoshi \textit{et al.}, 1990, 1991; see chapter 1).

\begin{table}[h]
\centering
\caption{Mean Time Constant of the Fast Component of Transient Current Recovery from Inactivation}
\begin{tabular}{l l l}
\hline
Membrane Potential (mV) & $\tau$ (ms) \\
\hline
-70 & 37 (n=1) \\
-80 & 30±2 (n=6) \\
-90 & 25 (21,29) \\
-100 & 22±3 (n=5) \\
-120 & 10 (n=1) \\
\hline
\end{tabular}
\end{table}
Figure 4-14  Recovery from inactivation of the transient current in CA1 neurons. The currents shown were elicited by pairs of identical pulses to -20mV of 200ms duration from a holding potential of -70mV; the interval between the pulses here was 5, 20, 60, 120, 180 and 250ms. It can be seen that as this interval was increased, the amplitude of the transient current activated during the second pulse also increased.
Figure 4-15. Timecourse of recovery from inactivation of the transient current in CA1 neurons, from the current records shown in figure 14. Since the test pulses in figure 14 activate the transient current virtually in isolation, its amplitude was measured as the difference between the peak current activated and that at the end of the pulse. Current amplitudes measured in this way were compared during the first and second pulse, and the ratio of the two currents was plotted as a function of the interpulse interval in figure 15A. This current ratio is also plotted on a logarithmic axis in figure 15B (●), which highlights the double exponential timecourse of recovery from inactivation. One exponential has been fitted by eye to the data for the longest interpulse intervals, and this has been subtracted from the data for shorter intervals to isolate the shorter exponential component (○), which was also fitted by eye. The time constants of the two fitted exponential components are 760 and 42 ms.
DISCUSSION

Both locus coeruleus and CA1 neurons exhibit two voltage-gated potassium currents. In both neuronal types, one current is rapidly activating and inactivating, most closely resembling an A-current (Connor & Stevens, 1971a; Neher, 1971; see chapter 1), and the other activates more slowly and inactivates only very slowly, most closely resembling a delayed rectifier (Hodgkin & Huxley, 1952a-d; see chapter 1). However, the properties of both current types showed some differences between locus coeruleus and CA1 neurons.

A-Currents

The kinetic properties of the A-current were very similar in both neuronal types; very rapid activation, with the current reaching a peak within a few milliseconds, and then inactivating fully over a few tens of milliseconds. A-currents with activation and inactivation kinetics of this time scale have previously been described in many rat central neurons (e.g., Segal et al., 1984; Surmeier et al., 1988; Zona et al., 1988; Cull-Candy et al., 1989; Huguenard et al., 1991; Lynch & Barry, 1991; Thorn et al., 1991; Ficker & Heinemann, 1992; Müller et al., 1992), and have also been expressed from the rat brain potassium channel gene (using the nomenclature of Chandy, 1991) Kv3.4 (Schröter et al., 1991; Rettig et al., 1992). Rapidly activating potassium currents which inactivate slightly more slowly (time constant of inactivation around 100 ms) have been expressed from the other so-called “A-type channel” genes Kv1.4 (Stühmer et al., 1989) and Kv4.2 (Baldwin et al., 1991), and also from expression of a heterotetrameric channel made up of Kv1.1 and Kv1.4 gene products (Ruppersberg et al., 1990). A fast A-type current is also expressed from the Kv4.1 gene in mouse (Pak, Baker et al., 1991), although the rat equivalent of this gene has not been isolated.

Both activation and inactivation of the A-current occurred at more positive potentials in CA1 neurons than in locus coeruleus neurons. The half-inactivation potentials, from the Boltzmann fits to figures 4-3A and 4-9A, were -91.8 mV for locus coeruleus neurons and -73.4 mV for CA1 neurons. Although this value for locus coeruleus neurons is unusually negative, possibly implying that the A-current is more inactivated under resting conditions in the locus coeruleus than in most central neurons, values for both neuronal types are close to those previously reported in neurons from rat hippocampus (75 mV, Segal et al., 1984; -81 mV, Ficker & Heinemann, 1992; -81 mV), neostriatum (-81 mV,
Surmeier et al., 1988), cerebellum (-72mV, Cull-Candy et al., 1989), and thalamus (-75mV, Huguenard et al., 1991). Activation thresholds are close to -60 and -50mV in locus coeruleus and CA1 neurons respectively, and are more negative than the threshold for delayed rectifier activation in both neuronal types; both these findings are in agreement with those of previous studies on rat (Segal & Barker, 1984; Segal et al., 1984; Zona et al., 1988) and other mammalian central neurons (Numann et al., 1987; Spain et al., 1991). The activation of the A-current could be fitted by a Boltzmann expression only in locus coeruleus neurons, although even this does not represent the true activation properties of the underlying single channels (see chapter 5). However, the half-activation potential of this fitted curve (-23.7mV) is similar to that previously reported for the A-current in neurons from rat neostriatum (-33mV, Surmeier et al., 1988), thalamus (-35mV, Huguenard et al., 1991), hippocampus (-19mV, Ficker & Heinemann, 1992; -17mV), and hypothalamus (-18mV, Müller et al., 1992), which might suggest that the single channels underlying these currents show similar current rectification to that seen in locus coeruleus neurons (see chapter 5).

The pharmacological profile of the currents identified here showed the classic characteristics of the A-current (Thompson, 1977; Segal et al., 1984; see chapter 1): block by low millimolar concentrations of 4-AP and (in CA1 neurons at least) insensitivity to TEA. All the evidence therefore suggests that the A-currents in cultured locus coeruleus and CA1 neurons described here are functionally very similar to each other and to A-currents in many other parts of the rat brain.

The similarity of A-currents throughout the rat brain might lead one to expect a common genetic background. In fact, the currents described here show far less similarity to those carried by any cloned rat brain potassium channel than they do to each other or to A-currents in many other rat central neurons. As stated above, the only cloned rat brain potassium channel gene which expresses a current which inactivates as fast as the A-current in locus coeruleus or CA1 neurons is Kv3.4 (Schröter et al., 1991; Rettig et al., 1992). However, activation and inactivation of the current expressed by this gene take place at much more positive potentials than native A-currents; the activation threshold is close to -30mV and the current is half-activated at 14.1mV, and the half-inactivation potential is -29.8mV (Schröter et al., 1991). This current is also sensitive not only to low concentrations of 4-AP (half blocking concentration, IC_{50} 0.5mM) but also to TEA (IC_{50} 0.3mM; Schröter
et al., 1991). Large differences also exist between the unitary properties of this cloned channel and those of the A-current channel in locus coeruleus neurons (see chapter 5).

Other potassium channel genes which express currents which inactivate slightly more slowly than native A-currents have previously been described as “A-type” (Stühmer et al., 1989; Ruppersberg et al., 1990; Baldwin et al., 1991; see above). Of these, the gene Kv1.4 (Stühmer et al., 1989) expresses a potassium current whose properties most closely resemble those of native A-currents; potentials for half-activation and half-inactivation are -21.7 mV and -73.6 mV respectively, and the current is blocked by 4-AP but not TEA (Stühmer et al., 1989). The unitary properties of this channel are, however, quite dissimilar from those of the A-current channel in locus coeruleus neurons (see chapter 5).

It is possible that the difficulty in matching the properties of native neuronal A-currents to those carried by any cloned channel type reflects the formation of heterotetrameric channels in neurons which may coexpress several different potassium channel genes. Although many rat brain potassium channel genes have now been identified, the properties of the currents carried by only a few heterotetrameric channels have been reported (Christie et al., 1990; Ruppersberg et al., 1990). However, it is likely that the formation of heterotetramers is tightly controlled in some way. *Drosophila* potassium channel subunits can only form heterotetramers with other subunits with which they share a high degree of structural homology, particularly in the membrane-spanning regions (K. McCormack et al., 1990; Covarrubias et al., 1991). Similarly, rat Kv3 gene products can only form heterotetramers with other subunits from the same gene subfamily (Rettig et al., 1992), a restriction which may also apply to other subfamilies.

Alternatively, it is possible that native voltage gated potassium channels are formed by a combination of four subunits of the type encoded by the potassium channel genes described above with other, as yet unknown proteins. Cloned potassium channels are homologous to a single domain of sodium and calcium channel α subunits (see chapter 1), and these α subunits alone are capable of forming functional sodium or calcium channels (see Catterall, 1988; Stühmer, 1991) in the same way that cloned potassium channel subunits are. However, native sodium and calcium channels purified using high affinity probes also contain smaller subunits which modify channel activity. The kinetic and pharmacological properties of the A-current expressed in *Xenopus* oocytes by injection of total rat brain mRNA are also different from those seen when low molecular weight
mRNAs are excluded, suggesting one or more smaller protein subunits may also contribute to mature A-current channels (Rudy, Hoger, Lester & Davidson, 1988). A voltage gated potassium channel purified from rat brain using the probes dendrotoxin, mast cell degranulating peptide and β-bungarotoxin contained not only subunits of a similar size to those of cloned rat brain potassium channel subunits, but also smaller subunits of unknown function (Rehm & Tempel, 1991).

**Delayed Rectifier Currents**

Although the A-currents of these locus coeruleus and CA1 neurons appear quite similar, greater differences existed between the two delayed rectifier currents identified. The most striking difference was the steady-state inactivation of the current seen in CA1, but not locus coeruleus neurons. Although this property is classically considered as characteristic of transient or slowly inactivating currents (Connor & Stevens, 1971a; Storm, 1988), it is seen in delayed rectifier currents in neurons from rat (Segal & Barker, 1984) and guinea pig hippocampus (Numann et al., 1987; Sah et al., 1988), rat cerebellum (Cull-Candy et al., 1989), rat thalamus (Huguenard & Prince, 1991) and cat cortex (Spain et al., 1991), although not in the delayed rectifier current of rat neocortical neurons (Zona et al., 1988).

The delayed rectifier current in locus coeruleus neurons had a more negative activation threshold (-40mV) than that seen in CA1 neurons (-20mV). The more negative of these two values is the more typical for delayed rectifier currents in rat central neurons (eg. Segal & Barker, 1984; Zona et al., 1988; Cull-Candy et al., 1989). The half-activation and half-inactivation potentials calculated for the delayed rectifier current in CA1 neurons (4.6mV and -62.8mV respectively; see figure 4-9B) were also more positive than have previously been reported in mammalian central neurons (eg. Sah et al, 1988; Huguenard & Prince, 1991; Spain et al., 1991), although both the activation threshold and half-activation potential are comparable to those reported in rat hypothalamic neurons (Müller et al., 1992).

The two delayed rectifier currents described also showed greatly different sensitivities to 4-AP; in locus coeruleus neurons it was unaffected at a concentration of 2.5mM, whereas in CA1 neurons the delayed rectifier was slightly more sensitive to 4-AP block than was the A-current. Rat brain delayed rectifier currents are usually described as being insensitive to 4-AP (Segal & Barker, 1984; Zona et al, 1988; McLarnon, 1989;
Huguenard & Prince, 1991), although this is not the first case in which 4-AP block has been observed (e.g., Cull-Candy et al., 1989). Sensitivity to 4-AP is more usually associated with A-currents (see above) or more slowly-inactivating voltage gated potassium currents (slow A-currents or D-currents; Williams et al., 1984; Zbicz & Weight, 1985; Storm, 1988; Greene et al., 1990; McCormick, 1991; Ficker & Heinemann, 1992). The delayed rectifier was also blocked by 30mM TEA, however, which usually blocks delayed rectifiers but not more rapidly inactivating voltage gated potassium currents.

Thus, although both delayed rectifier currents described in these neurons are activated more slowly and at more positive potentials than are the A-currents in the same neurons, and both inactivate only very slowly (over many seconds), some of their properties are significantly different. The current in locus coeruleus neurons is more similar to the classical profile of a delayed rectifier: it shows no apparent steady-state inactivation, begins to activate around -40mV, and is insensitive to 4-AP at concentrations which block the A-current. On the other hand, the delayed rectifier current described in CA1 neurons exhibits several of the properties usually associated with slowly inactivating potassium currents identified in many mammalian central neurons (see above), such as steady-state inactivation removed by hyperpolarisation and block by low concentrations of 4-AP. However, this current is still more properly classified as a delayed rectifier; figure 4-6B shows that inactivation is still far from complete even after a depolarising pulse lasting 5 seconds. Also, slowly inactivating currents usually activate at a more negative potential than A-currents (Storm, 1988; Greene et al., 1990), whereas the delayed rectifier current in CA1 neurons, as already stated, activates at an unusually positive potential. It is unlikely that the sustained current in CA1 neurons represents a mixture of a delayed rectifier and one or more other, more rapidly inactivating potassium currents. Activation and inactivation curves, shown in figure 4-9B, are both very well fitted by Boltzmann expressions, which strongly suggests the data are from only one population of channels.

During a maintained depolarisation, only two decay phases can be identified in the current record (figure 4-6B); one represents the inactivation of the A-current, the other the slow inactivation of the delayed rectifier. Also, 1mM 4-AP reduces the sustained current apparently without altering its time course (figure 4-10), again suggesting a uniform population of channels.

A number of rat brain potassium channel genes have been isolated which express
delayed rectifier-like currents. Those which activate slowly and inactivate only over several seconds include Kv1.1 (Christie et al., 1989; Stühmer et al., 1989), Kv1.2 (Stühmer et al., 1989), Kv2.1 (Frech et al., 1989), Kv3.2 (T. McCormack et al., 1990), and recently-identified members of the Shab (Kv2) (Hwang et al., 1992) and Shaw (Kv3) subfamilies (Rettig et al., 1992). Another delayed rectifier-like current is carried by heterotetrameric channels formed from Kv1.1 and Kv1.3 subunits (Christie et al., 1990). As is the case with A-currents (see above), although the currents expressed by these genes show a range of different properties, for example in activation thresholds, half-activation potentials and sensitivities to TEA and 4-AP, none closely match the properties of native rat brain delayed rectifier currents, either in locus coeruleus or CA1 neurons, or in other neuronal types previously described. Again by analogy with neuronal A-currents (see above), this suggests that the formation of potassium channels of unknown subunit structure may underlie the different delayed rectifier currents identified in rat central neurons.

**Expression of Potassium Channel Genes in the Rat Brain**

Although at least twelve separate genes encoding voltage-gated potassium channel subunits are expressed in the rat brain (Chandy, 1991), and the number of possible combinations of these subunits into mature heterotetramers is unknown (but presumably very large), most rat central neurons studied show only two (e.g., the locus coeruleus and CA1 neurons studied here) or three (e.g., Storm, 1988) voltage-gated outward potassium currents. This implies the formation of potassium channels in individual neurons must be very tightly controlled, at the level of subunit aggregation or gene expression. How mature potassium channels are assembled from their constituent subunits is not known, although it is clear that unrestricted heterotetramer formation does not take place (see above). More is known about the expression patterns of certain potassium channel genes in the rat brain. Distinct spatial distributions of Kv1.1, Kv1.2, Kv1.3, Kv1.4, Kv1.6, Kv2.1, Kv3.1, Kv3.4 and Kv4.2 gene expression have been demonstrated in adult rat brain (Beckh & Pongs, 1990; Drewe et al., 1992; Rettig et al., 1992; Sheng et al., 1992; Tsaur et al., 1992). Of these, Kv1.1, Kv1.2, Kv1.3, Kv1.4 and Kv2.1 are the most highly expressed in the hippocampus; Kv2.1 appears to be most significantly expressed in the CA1 region. Expression of the Kv3.4 gene, which encodes a rapidly inactivating “A-type” channel (see above), was not detected in the hippocampus (Rettig et al., 1992). Because of the small
size of the locus coeruleus, it has not been possible to demonstrate which potassium channel genes are most highly expressed there, although high levels of Kv1.1, Kv1.2 and Kv3.4 gene expression have been shown in the medulla-pons region, which includes the locus coeruleus. Of course, since the neurons used here were isolated from neonatal rat brains, expression of potassium channel genes may be different from those in adult neurons; patterns of potassium channel gene expression show both developmental (Beckh & Pongs, 1990; Drewe et al., 1992) and activity-dependent changes (Tsaur et al., 1992).

The localisation of Kv1.4, Kv2.1 and Kv4.2 potassium channel gene products has been studied at a higher resolution using immunohistochemistry, with antibodies raised specifically against these protein subunits (Trimmer, 1991; Sheng et al., 1992). These studies have shown that both Kv2.1 and Kv4.2 proteins are localised in the somata and dendrites of specific rat central neurons, whereas the Kv1.4 protein is present in axons and possibly presynaptic nerve terminals.

Electrophysiological investigations of the properties of voltage gated outward potassium currents in rat central neurons, such as those described here for locus coeruleus and CA1 neurons, have shown that although individual neurons usually require only two or three kinetically distinct potassium currents to determine their electrical properties, neurons from different parts of the brain exhibit a large number of potassium currents with subtly different properties. At the same time, investigations into the molecular biology of the channel proteins which underlie these currents have shown that we should expect a very large number of distinct potassium currents in the rat brain. As yet, however, it has proven difficult to tell which potassium channel genes encode which mature neuronal channels, due to a current lack of understanding of the way in which mature channels are formed from channel gene products.
CHAPTER FIVE: UNITARY PROPERTIES OF A-CURRENT CHANNELS IN LOCUS COERULEUS NEURONS

INTRODUCTION

Although many of the properties of ion channels can be studied using whole cell current recording (see chapter 4), it is often more informative to study the properties of the single channels which underlie these macroscopic currents. Whereas whole cell currents may be carried by a number of different channel types, necessitating the use of different protocols to examine the properties of one population of channels in isolation, unitary currents may easily be recorded from one type of channel alone, allowing its properties to be studied under a wide range of experimental conditions. The use of single channel recording is therefore an important test of whether the currents identified by whole cell recording are carried by a single type of channel. This is particularly important in studying cells such as central neurons, which have extensive neurite systems. During whole cell recording from the soma, the cable properties of the neurites mean that the quality of the voltage clamp deteriorates as one moves away from the soma. During single channel recording, one can be more certain that the much smaller area of membrane being recorded from is effectively voltage clamped.

The unitary properties of A-current channels have previously been described in mammalian peripheral neurons (Cooper & Shrier, 1985, 1989; Kasai et al., 1986; McFarlane & Cooper, 1991), in rat phaeochromocytoma (PC-12) cells (Hoshi & Aldrich, 1988a) and in rat olfactory receptor neurons (Lynch & Barry, 1991). Similar channels have been described in snail neurons (Taylor, 1987), molluscan neurons (Premack et al., 1989) and chick peripheral neurons (Florio et al., 1990). Different A-current channels are found in Drosophila neurons and muscle cells (Solc et al., 1987; Solc & Aldrich, 1988). This difference is due at least in part to the presence of multiple potassium channel genes in the Drosophila genome (Solc et al., 1987; Tempel et al., 1987; Butler et al., 1989; see chapter 1). It may reasonably be assumed that the presence of multiple potassium channel genes in the rat genome (see chapters 1 and 4) will also lead to the expression of different A-current channels. The properties of homomultimeric (Stühmer et al., 1989; Baldwin et
al., 1991; Schröter et al., 1991; Rettig et al., 1992) and heteromultimeric (Ruppersberg et al., 1990) A-current like channels expressed from cloned rat potassium channel genes have already been described.

RESULTS

Except where otherwise noted, experiments were carried out on locus coeruleus neurons, grown in culture for between 6 and 16 days, using the on-cell or inside-out configurations of the patch clamp technique (see chapter 2). The patch pipette contained either low K⁺ patch solution or high K⁺ patch solution (see chapter 2). During inside-out patch recordings, the intracellular face of the patch was continuously perfused with AICS (see chapter 2), to which MgCl₂ or NaCl were occasionally added. All current records illustrated were low-pass filtered using a Bessel filter set at 2kHz (-3dB filter rate) and digitised at 10kHz.

Because of the presence of a prominent subconductance state with an amplitude around 50% of the fully open state (see below), it was deemed impractical to use a 50% threshold for measurement of channel open times (see chapter 2). Instead, the threshold was set at approximately 30% of the fully open level, such that visits to this substate were classified as openings, even though this may lead to an overestimate of the duration of short openings which just reach this threshold. Where other substates could be identified, these were also classified as openings. Open times measured in this way were not corrected for missed closures (see chapter 2), nor was a minimum resolution applied to the data. Exponential curves were fitted to the open time histograms using a least squares fitting routine of the CED software.

Identification of Single A-Current Channels

Single channel currents underlying the A-current in locus coeruleus neurons were identified by the similarity of their electrophysiological properties to those of the whole cell A-current in these cells (see chapter 4). Thus, these channels were activated rapidly by depolarising steps from a holding potential more negative than the cell resting potential, and subsequently inactivated during the step, such that the ensemble average current showed the same transient nature as the whole cell A-current. Such unitary currents were recorded from on-cell patches when the pipette contained 3mM K⁺ (low K⁺ patch solution; see figure 5-1) or 140mM K⁺ (high K⁺ patch solution; see figure 5-2). Unitary
Figure 5-1. Unitary A-current channels recorded from an on cell patch with 3mM K⁺ in the pipette. The patch was held 40mV more negative than the cell resting potential before depolarising steps of 140mV (in A.) or 80mV (in B.) were applied. In both A. and B., the ensemble average current, formed as the average of 100 consecutive pulses in each case, is shown beneath the unitary currents.
Figure 5-2. Unitary A-current channels recorded from an on cell patch with 140mM K\(^+\) in the pipette. The voltage protocol used to activate the channels is the same as that used in figure 1: the patch was held 40mV more negative than the resting potential and then stepped positive by 140mV (A.) or 80mV. As in figure 1, the ensemble average current shown was formed from 100 consecutive pulses. During the larger depolarising step, outward current flows through the channels, whereas during the smaller step currents are inward. Note that the large inward current at the end of the last current record in B. is a tail current, where the channel was open at the end of the pulse, and not a channel reopening on repolarisation.
current-voltage relationships under these two conditions were calculated from Gaussian curves fitted to amplitude histograms as described in chapter 2. The current-voltage relationships for the two patches shown in figures 5-1 and 5-2 are shown in figures 5-3A and 5-3B respectively. These figures show that the reversal potential is shifted to a more positive potential when the external potassium concentration is raised, as would be expected for a potassium selective channel. With 140mM K⁺ in the pipette, the mean reversal potential was 65.7±4.1mV positive to the cell resting potential (n=6). With 3mM external K⁺, the reversal potential was more negative than the threshold for channel activation, and so could not be measured directly in this way; by extrapolation of the current-voltage relationship shown in figure 5-3, it was estimated to be approximately 15mV negative to the resting potential. It can also be seen from figure 5-3 that the unitary current-voltage relationship is not linear, but rectifies at the most positive potentials. This rectification is most marked when the pipette contained 3mM K⁺, when the current-voltage relationship shows a region of negative slope conductance. Mean unitary conductances, measured between 20 and 40mV positive to the resting potential, were 14.8±0.4pS (n=11) with 3mM K⁺ in the pipette, and 39.0±1.5pS (n=7) with 140mM K⁺.

Voltage Dependence of Activation and Inactivation

The voltage dependence of activation and inactivation of unitary A-currents were studied using similar protocols to those used to study activation and inactivation of the whole cell currents (see chapter 4). Thus to study activation, patches were held at a negative potential to fully remove inactivation before stepping to different test potentials, and to study inactivation patches were held at different potentials before stepping to a constant positive test potential. The degree of channel activation under these conditions was measured as the channel open probability, calculated from ensemble average currents constructed from at least 100 consecutive steps as described in chapter 2. Activation and inactivation curves calculated in this way for one patch which contained a single A-current channel are shown in figure 5-4. Both curves have been fitted with a Boltzmann expression similar to equation 4-1 (see chapter 4):

\[
P_o = \frac{P_{\text{MAX}}}{1 + \exp([(V - V')/k]}
\]  

(5 - 1)
Figure 5-3  Unitary current-voltage relationships, formed from the two patches illustrated in figures 1 and 2, when the pipette contained either 3mM (A.) or 140mM K⁺ (in B.). Voltage is given relative to the resting potential. This shows that the reversal potential is shifted to a more positive value by increasing external K⁺ concentration, as expected for a potassium selective channel. The current-voltage relationship also shows rectification, such that currents at the most positive potentials are less than expected; this rectification is most noticeable when the pipette contained 3mM K⁺ (A.).
Figure 5-4. Activation and inactivation curves for a single A-current channel, measured using the voltage protocols described in the text. Voltage is given relative to the resting potential. Both curves have been fitted by a Boltzmann expression (equation 1); for activation (●) $V' = 85.7 \text{mV}$ more positive than the resting potential, and $k = -26.8 \text{mV}$; for inactivation (○) $V' = 33.4 \text{mV}$ more negative than the resting potential, and $k = 12.7 \text{mV}$. 
where $P_o$ is the open probability, $P_{\text{MAX}}$, the maximum open probability, is taken as one in this instance, $V'$ is the voltage at which $P_o=0.5$, and $k$ is a slope factor. For inactivation, the fitted curve has a $V'$ of -33.4mV with respect to the resting potential, and a $k$ of 12.7mV; these values are similar to those calculated from the whole cell inactivation curve (see figure 3-3A, chapter 3) if a resting potential of -60mV is assumed. For activation, the fitted Boltzmann expression has a $V'$ of 85.7mV positive to the resting potential, and a $k$ of -26.8mV; this value of $V'$ is around 50mV more positive than that estimated from the whole cell activation curve (see figure 3-3A, chapter 3), and the value of $k$ calculated here is over three times greater than that estimated from the whole cell activation curve. This is presumably because the method of calculating the whole cell activation curve assumes a linear current-voltage relationship, which is clearly not the case for this channel (see figure 5-3); calculating channel activation directly from channel open probability avoids this problem, and in this case gives a more accurate measure of the activation process. The similarity of the whole cell activation curve to that reported in other cell types (see chapter 4) and the rectification of unitary current-voltage relationships for other A-current channels (Cooper & Shrier, 1985) suggest that this may be a common problem in studying the voltage dependence of channel activation solely from whole cell experiments.

When open probability was reduced by holding at a relatively positive potential to inactivate the channels, those depolarising steps which failed to activate channels were not randomly distributed, but tended to occur in groups, as has previously been described for other voltage gated channels (eg. Horn, Vandenbergh & Lange, 1984; Standen et al., 1985). Runs of consecutive pulses in which openings occurred, or runs in which no openings occurred, were examined in the patch used to construct figure 5-4, which contained a single A-current channel. If a run is defined as two or more consecutive pulses in which openings occur, or in which openings fail to occur, then the expected number of runs in a series of $n$ pulses is $2np(1-p)$ if openings occur at random, where $p$ is the probability of at least one opening occurring during a pulse. If the number of runs observed is $R$, then one may evaluate (from Gibbons, 1971, equation 2.9):

$$Z = \frac{R - 2np(1-p)}{2n^{1/2}p(1-p)}$$

(5-2)
where \( Z \) is a standardised random variable with a mean of zero and a standard deviation of one. When \( Z \) has a large, negative value, steps which activate or fail to activate channel openings are significantly grouped.

When the patch used to construct figure 5-4 was held 60mV more negative than the resting potential, where inactivation is almost totally removed, and a large depolarising step was applied (to 120mV positive to the resting potential), the channel opened during 94 of 99 consecutive pulses. The number of observed runs was 10, compared with an expected number (from \( 2np(1-p) \)) of 9.5. \( Z \), calculated from equation 5-2, was 0.53, consistent with openings being randomly distributed under these conditions. When the holding potential was changed to 25mV more negative than the resting potential, increasing the steady-state inactivation, steps to the same test potential elicited openings during only 73 of 200 consecutive pulses, indicating that steady-state inactivation was indeed increased. The number of runs in this case was 49, against an expected number of 92.7. Under these conditions \( Z \) was -6.67, indicating that openings were distributed non-randomly. It was shown that this grouping was indeed directly associated with the increase in inactivation, rather than with the decrease in open probability, by returning the holding potential to 60mV negative to the resting potential and stepping to a less depolarised potential (20mV positive to the resting potential), so that the proportion of pulses in which the channel opened was roughly the same (the channel opened during 35 out of 100 pulses here). The observed number of runs was 47, compared with an expected number of 45.5; \( Z \) was 0.33, again consistent with openings being randomly distributed. Thus, grouping of pulses which caused openings or failed to cause openings was increased only under conditions in which channel inactivation was also increased. Such grouping has previously been associated with slow steps in channel inactivation (Horn et al., 1984; Standen et al., 1985), and provides evidence for the existence of more than one inactivated state of this channel. The time course of current recovery from inactivation similarly suggests that the A-current channel of CA1 neurons has at least two inactivated states (see chapter 4).

**Subconductance States of the Channel**

Although most channel openings under constant conditions were to the same current level (see figures 5-1 and 5-2), occasionally openings to smaller subconductance states were observed. The most obvious substate (though not necessarily the most commonly
occurring; see below) had an amplitude of 55±4% of the fully open state in five patches. Two other substates could also be identified; openings to substates could be most clearly seen in inside out patches, where unitary currents are larger than in on cell patches (see below). An example is shown in figure 5-5; here openings to three different subconductance states, as well as the fully open state, can clearly be seen. By carefully constructing amplitude histograms from parts of the record where the channel is closed, fully open or in one of the substates, the amplitudes of the three substates in this patch were estimated to be approximately 23%, 43% and 73% of the fully open state amplitude (see figure 5-6). The overall amplitude histogram for this patch, including full openings and openings to all three substates, could best be fitted by the sum of the four Gaussian curves used to fit each individual open state in figure 5-6 by assuming that 85% of all open time is spent in the fully open state, 9% in the largest amplitude substate, and 3% in each of the smaller substates (see figure 5-5b).

Although subconductance states could most easily be identified in inside out patches, patch excision did not actually appear to alter the probability of the channel opening to a substate. Figure 5-7 shows the presence of substates in an on cell patch, where the pipette contained high K+ patch solution. On stepping to 100mV positive to the resting potential, three substates with amplitudes of approximately 26%, 52% and 70% of the fully open state were identified; these substates accounted for around 2, 4 and 6% of total open time respectively (figure 5-7A). However, when a much smaller depolarising step was applied (to 20mV positive to the resting potential), occupancy of substates accounted for a much larger proportion of total open time (figure 5-7B). Here the substates were found to have amplitudes of approximately 28%, 58% and 78% of the fully open state; around 6, 18 and 14% of the total open time was spent in each of these substates.

Block by Internal Mg2+ and Na+

The rectification of the unitary current-voltage relationships shown in figure 5-3, and the negative slope conductance at very positive potentials with 3mM external K+, are very reminiscent of the voltage dependent block of many ion channels by small cations (eg. Bezanilla & Armstrong, 1972; Gay & Stanfield, 1977; Hille & Schwarz, 1978), and are consistent with the voltage dependent block of the channel by internal cations. For these reasons, the effects of intracellular Mg2+ and Na+ applied to the intracellular face of inside out patches containing A-current channels were investigated. For all inside out
Figure 5-5  Subconductance states of a single channel in an inside out patch. A. shows unitary currents elicited by voltage pulses from a holding potential of -120mV to +60mV. The records have been specifically selected to show the presence of substates; in many other records, no substates were obvious (for example, see figure 13B.). Three substates can be identified from these records; in this patch, they were shown to be approximately 23%, 43% and 73% of the fully open state amplitude (see figure 6). B. shows the amplitude histogram for this patch, including all openings. It has been fitted by the sum of five Gaussian curves, of amplitudes (± standard deviation) of 0.1±0.2pA, 0.7±0.3pA, 1.3±0.3pA, 2.2±0.3pA and 3.0±0.3pA. The fit assumes that the relative areas of the last four of these Gaussian curves, which represent the three substates and the fully open state, are 0.03, 0.03, 0.09 and 0.85 respectively.
Figure 5-6  Amplitude histograms formed to measure the relative amplitude of subconductance states and the fully open state in the patch shown in figure 5. Each amplitude histogram was formed by carefully selecting parts of the current record where the channel was either closed, fully open or in one specific substate. In each case the peak corresponding to the fully open state had an amplitude of 3.0pA; the amplitude of the substate peak was 0.7pA in A. (ie approximately 23% of the fully open amplitude), 1.3pA in B. (approximately 43% of the fully open amplitude) and 2.2pA in C. (approximately 73% of the fully open amplitude).
Figure 5-7 Subconductance states in an on cell patch, where the pipette contained 140mM K\(^+\). A. shows unitary currents recorded during depolarising steps of 140mV from a holding potential 40mV more negative than the resting potential. Although the presence of substates is not as obvious as during recordings from an inside out patch, three substates were identified in this patch. The amplitude histogram here has been fitted by the sum of five Gaussian curves, as in figure 5. The amplitudes of the four Gaussian curves corresponding to the four open levels were 0.35±0.18pA, 0.70±0.18pA, 0.95±0.18pA and 1.35±0.18pA (measured as in figure 6), and their relative areas were 0.02, 0.04, 0.06 and 0.88. B. shows inward unitary currents recorded when the size of the depolarising pulse was reduced to 60mV. The amplitude histogram has again been fitted by the sum of five Gaussian curves. Those corresponding to open states of the channel had amplitudes of 0.54±0.19pA, 1.13±0.19pA, 1.51±0.19pA and 1.94±0.19pA, and their relative areas were 0.06, 0.18, 0.14 and 0.62, indicating an increase in the proportion of total open time spent in each substate.
patch recording, the pipette contained low K⁺ patch solution and the intracellular face of the patch was perfused with AICS (see chapter 2); therefore, a physiological potassium concentration gradient across the membrane (3mM K⁺ outside, 140mM K⁺ inside) was maintained.

The unitary current-voltage relationships of A-current channels in both on cell and inside out patches were routinely examined from ensemble ramp currents, as described in chapter 2. Figure 5-8 shows the ensemble ramp currents in an on cell patch, and in the same patch after excision in response to a similar voltage ramp. The current recorded on cell shows similar rectification and negative slope conductance to the current-voltage relationship shown in figure 5-3A. However, the current recorded following patch excision is almost linear; it actually shows a small degree of outward rectification, as expected from the Goldman-Hodgkin-Katz current equation (equation 1-8, chapter 1) when the external potassium concentration is much smaller than the internal concentration. Such near-linear ensemble ramp currents were always seen following patch excision when the AICS perfusing the patch contained neither Mg²⁺ nor Na⁺. The mean slope conductance under these conditions was 17.8±0.4pS (n=17), slightly higher than that seen on cell (around 14.8pS; see above), presumably due to the relief of channel block on patch excision. The reversal potential of the ensemble ramp currents in inside out patches, estimated by extrapolation as for on cell currents, was -79.8±0.9mV (n=17), close to that estimated from on cell currents (see above) assuming a cell resting potential of around -60mV.

The current rectification seen in on cell patches could be restored in inside out patches by the addition of Mg²⁺ or Na⁺ to the intracellular perfusate. Figure 5-9 shows the effects of adding either Mg²⁺ (5mM or 10mM) or Na⁺ (10mM or 20mM) to the AICS. It is presumed that internal Mg²⁺ and Na⁺ both cause a rapid, "flickery" block of the channel, such that individual blocking events are too fast to be resolved at the filter rate used. This will cause a reduction in the amplitude of the filtered current, such that (see also chapter 8):

\[
m_i/i = \frac{1}{1 + ([B]/K_d)}
\]

(5 - 3)
Figure 5-8. Removal of unitary current rectification following patch excision. The current records are the ensemble currents from a single A-current channel formed using a ramp protocol, as described in chapter 2. Whilst on cell, the patch was held 40mV negative to the resting potential, and then a step depolarisation of 180mV was given to activate the channel; the membrane potential was then returned to the holding potential linearly over the next 80ms, ie at a rate of 2.25mV/ms. Following excision the patch perfused with AICS, and the same ramp protocol was applied whilst holding at -100mV. The ensemble current measured on cell shows characteristic rectification with a region of negative slope conductance; however, following patch excision, the ensemble current is almost linear.
Figure 5-9  Restoration of unitary current rectification in inside out patches by intracellular application of Mg\(^{2+}\) or Na\(^{+}\). The ensemble ramp currents shown here were recorded from two inside-out patches, using the voltage protocol described in the legend to figure 8. Current rectification, similar to that seen during on cell recording, could be induced by addition of Mg\(^{2+}\) (upper current records) or Na\(^{+}\) (lower current records) to the AICS perfusing the intracellular face of the patch. The unitary current records used to construct these ensemble averages were filtered at 500Hz.
where $m_i$ is the mean unitary current in the presence of the blocking ion, $i$ is the control current amplitude, $[B]$ is the concentration of the blocking ion ($\text{Mg}^{2+}$ or $\text{Na}^+$), and $K_d$ is the voltage dependent dissociation constant of the blocking reaction. $m_i$ and $i$ were measured directly from ensemble ramp currents such as those shown in figure 5-9, and the voltage dependence of $m_i/i$ for the different concentrations of $\text{Mg}^{2+}$ and $\text{Na}^+$ studied are shown in figures 5-10A and 11A respectively. In each case the data have been fitted by equation 5-3, with the voltage dependence of $K_d$ given by a Boltzmann expression similar to that originally used by Woodhull (1973) to describe the proton block of $\text{Na}^+$ currents in frog node of Ranvier:

$$K_d = K_d(0) \exp\left(\frac{-z'VF}{RT}\right)$$

(5-4)

where $K_d(0)$ is the dissociation constant at a membrane potential of $0\text{mV}$, and $z'$ is the apparent valency of the blocking ion, which is equal to the real valency $z$ multiplied by $\delta$, the fraction of the membrane electric field experienced by the blocking ion. $K_d(0)$ and $z'$ were calculated by fitting the voltage dependence of $K_d$ using equation 5-4, as is shown in figures 5-10B and 5-11B. Mean values of $K_d(0)$, measured as the intercept of the fitted straight line with the ordinate in graphs such as those shown in figures 5-10B and 5-11B, were $15.7 \pm 0.7\text{mM} \ (n=6)$ for $\text{Mg}^{2+}$ and $76.0 \pm 4.2\text{mM} \ (n=3)$ for $\text{Na}^+$. $z'$ was calculated from the slope of this line, since the change in membrane potential required to produce an e-fold change in $K_d$ is equal to:

$$\Delta V = \frac{RT}{z'F}$$

(5-5)

For $\text{Mg}^{2+}$, $K_d$ changed e-fold for a $48.9 \pm 3.2\text{mV} \ (n=6)$ change in membrane potential, indicating a value for $z'$ of 0.51; since $\text{Mg}^{2+}$ has a valency of two, this suggests that $\text{Mg}^{2+}$ ions block the channel at a site approximately 26% of the way through the transmembrane electric field ($\delta=0.26$). For $\text{Na}^+$, $K_d$ changed e-fold for a $32.0 \pm 0.6\text{mV} \ (n=3)$ change in membrane potential, indicating that $z'$ is equal to 0.78; since $\text{Na}^+$ is a monovalent ion, this is equal to the value of $\delta$, suggesting that $\text{Na}^+$ ions enter further into the channel pore to cause block than do $\text{Mg}^{2+}$ ions, experiencing around 78% of the total transmembrane electric field.

Although the effects of other internal cations on unitary A-currents were not tested,
Figure S-10 Voltage dependence of the block by intracellular Mg$^{2+}$. Unitary current amplitudes were measured from the ensemble ramp currents shown in figure 9, and used to calculate the fractional current in the presence of Mg$^{2+}$ ($m/i$) at different voltages. This is shown in A, for both 5mM (●) and 10mM Mg$^{2+}$ (●). Values of $m/i$ were used to calculate the $K_d$ of the blocking reaction from equation 3 of the text; the voltage dependence of $K_d$ is shown in B. It can be seen that the $K_d$ values calculated in this way from the block by 5mM (●) and 10mM Mg$^{2+}$ (●) are in close agreement. The fitted straight line in B. gives a $K_d$ at 0mV of 15.8mM, and a slope of 44mV per e-fold change in $K_d$. These values were used to construct the fit shown in A., using equation 4 of the text. Note that this assumes that a single Mg$^{2+}$ ion blocks the channel; fitting the data assuming that two Mg$^{2+}$ ions were required for block gave an inferior fit (not shown).
Figure 5-11 Voltage dependence of the block by intracellular Na⁺. All fitting was done in the same way as for Mg²⁺ block, as described in the legend to figure 10. Data points in both A. and B. correspond to the block by 10mM (○) and 20mM Na⁺ (●). The fit to the data in B. gives a $K_d$ at 0mV of 68.2mM and a slope of 32mV per e-fold change in $K_d$. As with the block by Mg²⁺, the fitting here assumes one Na⁺ ion blocks the channel; this gave a superior fit than that given by assuming that block is by two ions (not shown).
it is likely that the current rectification seen in on cell recording can be accounted for solely in terms of block by Mg²⁺ and Na⁺. To test this hypothesis, an ensemble ramp current was constructed from an A-current channel in the on cell configuration, following which the patch was excised and the intracellular face of the membrane perfused with AICS containing concentrations of Mg²⁺ and Na⁺ close to what would be expected in the cell cytoplasm under normal conditions (2mM and 10mM respectively). The ensemble ramp current constructed under these conditions, as illustrated in figure 5-12, shows a similar degree of rectification to that of the on cell current, indicating that these concentrations of Mg²⁺ and Na⁺ alone are capable of mimicking on cell conditions in terms of voltage dependent channel block.

The scheme used to fit the reduction in mean unitary amplitude caused by Mg²⁺ and Na⁺ block (equation 5-3) assumes a rapid blocking reaction of the kind (see also chapter 8):

\[
C \quad O \quad B
\]  

where C represents channels which are closed, O open channels, and B blocked channels. Inactivated states have been omitted here. If channels which are blocked by Mg²⁺ or Na⁺ cannot close, as in the scheme of equation 5-6, then each apparent opening of the channel will actually be a burst of openings interrupted by brief, unresolved blockings, such that there will be an apparent increase in mean open time in the presence of internal Mg²⁺ or Na⁺. If, however, a similar scheme is postulated in which blocked channels can close normally:

\[
\begin{align*}
C & \quad \rightarrow \quad O \\
\uparrow \downarrow & \quad \uparrow \downarrow \\
CB & \quad \rightarrow \quad OB
\end{align*}
\]  

(5 - 7)
Figure 5-12 Near-physiological intracellular concentrations of Mg\(^{2+}\) and Na\(^+\) can mimic the current rectification seen during on cell recording. The upper current record is an ensemble ramp constructed from an on cell patch, held 40mV negative to the resting potential before stepping positive by 180mV and then returning to the holding potential at 2.25mV/ms, as in figure 8. The lower current record is an ensemble ramp from the same patch following excision and perfusion of the intracellular face of the patch with AICS containing both Mg\(^{2+}\) (2mM) and Na\(^+\) (10mM), close to the concentrations expected within neurons. The inside out patch was held at -100mV and the same voltage ramp was repeated. It can be seen that the current rectification is roughly the same under these two conditions, suggesting that these two cations alone may cause the rectification seen in on cell patches.
On Cell

Inside Out

0.5 pA

10 ms
where OB represents an open, blocked state and CB all closed, blocked states, then mean open time will be unchanged in the presence of blockers, although the effect on mean unitary current will be the same as in the scheme of equation 5-6.

These two schemes were tested by measuring mean open time under similar conditions in both the on cell configuration, where channels should be blocked by both Mg\(^{2+}\) and Na\(^{+}\), and in inside out patches where the AICS contained no Mg\(^{2+}\) or Na\(^{+}\). Following patch excision, unitary currents were larger, which was also reflected in an increase in the peak amplitude of the ensemble average current (see figure 5-13). However, the peak open probability, calculated as described in chapter 2, was unchanged; it was 0.72 in figure 5-13A (on cell) and 0.68 in figure 5-13B (inside out patch). It can be seen, however, that inactivation of the channel becomes more rapid following patch excision, which occurred routinely during inside out patch recording. Mean channel open time under these conditions, measured as described above, was unchanged following patch excision, which is indicated by the open time histograms shown in figure 5-14. This suggests that blocked channels can close normally, favouring the scheme shown in figure 5-7. The reason for the speeding up of channel inactivation in inside out patches is unclear, but one possibility is that while blocked channels can close normally, they cannot enter an inactivated state (I):

\[
\begin{align*}
\text{C} & \rightarrow & \text{O} & \rightarrow & \text{I} \\
\uparrow & \downarrow & \uparrow & \downarrow \\
\text{CB} & \rightarrow & \text{OB}
\end{align*}
\]

(5 – 8)

This hypothesis is supported by the fact that adding Mg\(^{2+}\) to the AICS slows inactivation slightly in inside out patches, without affecting either peak open probability or mean open time. The effect on inactivation is shown in figure 5-15, where a single A-current channel is recorded from an inside out patch under the same conditions when the AICS contained no Mg\(^{2+}\) (figure 5-15A) or 5mM Mg\(^{2+}\) (figure 5-15B); in each case the AICS contained no Na\(^{+}\). It can be seen that the amplitude of both the single channel currents and the ensemble average current are reduced by intracellular Mg\(^{2+}\); however, peak open probability is 0.71
Figure 5-13 Effects of patch excision on unitary A-current channels recorded during step changes in membrane potential. A. shows the activation of a single A-current channel in an on cell patch, held 40mV more negative than the resting potential and then stepped positive by 100mV. Below the unitary currents is an ensemble average formed from 100 such records, showing the transient nature of the current under these conditions. B. shows the same channel following patch excision. The inside out patch was held at -100mV and depolarised by 100mV to activate the channel. Its intracellular face was perfused with AICS containing neither Mg$^{2+}$ nor Na$^+$. Under these conditions, the unitary current is larger, reflected in an increase in the amplitude of both the single channel currents and the ensemble average. However, the channel open probability is unchanged by patch excision, being 0.72 in A. and 0.68 in B. It can also be seen from the ensemble average currents that the rate of channel inactivation is increased significantly by patch excision.
Figure 5-14. Mean channel open time is not affected by patch excision. The open time histograms were constructed from the patch shown in figure 13, before (A.) and after (B.) patch excision. In each case, the fitted line is a single exponential function (suggesting that the channel has only one open state) of the form \( f(t) = A \exp(-t/\tau_{\text{open}}) \), where \( A \) gives the intercept on the ordinate and \( \tau_{\text{open}} \) is 18.2 ms in A. and 18.5 ms in B.
Figure 5-15  Effects of 5mM internal Mg$^{2+}$ on single A-currents in an inside out patch. In both A. and B., the patch was held at -120mV and depolarised by 160mV. In A., the AICS contained neither Mg$^{2+}$ nor Na$^+$; in B., it contained 5mM Mg$^{2+}$ and no Na$^+$. Mg$^{2+}$ reduces the amplitude of the single channel currents and the ensemble average current, but does not alter peak open probability, which is 0.71 in A. and 0.74 in B. It can also be seen from the ensemble average currents that the rate of channel inactivation is slowed slightly by internal Mg$^{2+}$. 
in figure 5-15A and 0.74 in figure 5-15B. Inactivation of the ensemble average current
does appear slightly slower in figure 5-15B than in 5-15A. Mean channel open time is
unchanged, as shown in figure 5-16. Thus, it appears that whilst channels which are
blocked by Mg\(^2+\) can close normally, they are prevented from entering the inactivated state.
This is analogous to the effects of external tetraethylammonium ions on the inactivation
of potassium channels in human T lymphocytes (Grissmer & Cahalan, 1989a) and of
cloned Drosophila Shaker channels (Choi et al., 1991). It is possible that the presence of
a positively charged blocking ion in the pore could repel the inactivation “ball”, or even
bind to the same receptor.

Unitary A-Current Channels in CA1 Neurons

Although delayed rectifiers were the most common channel type seen during single
channel recording from CA1 neurons (see chapters 1 and 6-8), A-current channels were
also occasionally identified. These channels had very similar properties to those described
above in locus coeruleus neurons. The mean unitary conductance during on-cell recordings
was 14.7±1.5pS (n=4) with 3mM K\(^+\) in the pipette, rising to 35.7±1.7pS (n=3) when the
pipette contained 140mM K\(^+\). An example of an A-current channel recorded on-cell from
a CA1 neuron is shown in figure 5-17. One obvious difference from the locus coeruleus
A-current channel is that there is much less current rectification here, with no region of
negative slope conductance over the voltage range studied.

DISCUSSION

Comparison With Other A-Current Channels

The unitary properties of A-current channels in locus coeruleus neurons described
here are similar to those previously reported in mammalian peripheral neurons (Cooper
& Shrier, 1985, 1989; Kasai et al., 1986; McFarlane & Cooper, 1991), rat PC-12 cells
(Hoshi & Aldrich, 1988a) and rat olfactory receptor neurons (Lynch & Barry, 1991). In
all these cases, unitary conductance was in the range 17-22pS under conditions of
physiological potassium concentrations, compared with 14.8pS (on cell) and 17.8pS
(inside out patches) in locus coeruleus neurons. Interestingly, this conductance is also
close to that reported for cloned rat brain Kv3.4 channels (14pS; Schröter et al., 1991),
which have rapid, A-current like kinetics (see chapter 4). These channels also show unitary
current rectification with a region of negative slope conductance at very positive potentials
Figure 5-16 Mean channel open time is not affected by internal Mg$^{2+}$. The open time histograms were constructed from the patch shown in figure 15, when the AICS contained no Mg$^{2+}$ (A.) or 5mM Mg$^{2+}$ (B.) As with figure 14, each histogram has been fitted by a single exponential function of the form $f(t) = A \exp(-t/\tau_{\text{open}})$, where $A$ gives the intercept on the ordinate and $\tau_{\text{open}}$ is 12.7ms in A. and 12.4ms in B.
Figure 5-17 An example of a single A-current channel in a hippocampal CA1 neuron. A. shows a single A-current channel recorded on cell from a CA1 neuron where the patch contained 3mM K+. The patch was held 40mV more negative than the resting potential, and stepped positive by 120mV to activate the channel. B. shows the ensemble average current under these conditions, illustrating the transient nature of the channels. C. shows the unitary current-voltage relationship for this patch. It can be seen that although there is some current rectification at positive potentials, it is not as great as that seen in locus coeruleus neurons (see figure 3A.), with no region of negative slope conductance. The voltage values on the abcissa are relative to the resting potential. The maximum slope conductance of this relationship is 13.9pS, close to that seen in locus coeruleus neurons.
(Schröter et al., 1991), which is mimicked by application of 2mM Mg$^{2+}$ to the intracellular face of inside out patches (Rettig et al., 1992). The conductance of other cloned, “A-type” channels from rat brain is much smaller (less than 5pS; Stühmer et al., 1989; Baldwin et al., 1991). The conductance of heteromultimeric “A-type” channels formed from cloned Kv1.1 and Kv1.4 subunits is also less than measured here (Ruppersberg et al., 1990).

However, Kv1.4 channels, which have been suggested to form functional A-current channels in rat central neurons (Pardo, Heinemann, Terlau, Ludewig, Lorra, Pongs & Stühmer, 1992), and also Kv3.4 channels and Kv1.1/Kv1.4 heteromultimeric channels, show other properties not seen in native A-current channels from locus coeruleus neurons. The rate of inactivation of each of these cloned channels is slowed by patch excision to the inside out configuration (Ruppersberg, Stocker, Pongs, Heinemann, Frank & Koenen, 1991), in contrast to the speeding up of inactivation seen in locus coeruleus neurons on patch excision (see figure 5-13). The slowing of inactivation in cloned channels was shown to be due to loss of intracellular factors which oxidise or reduce cysteine residues in the inactivation “ball” of the channel, and so regulate the rate of inactivation (Ruppersberg, Stocker et al., 1991). Both Kv1.1 and Kv3.4 channels have cysteine residues in the putative inactivation “ball”, although there are no cysteine residues in the inactivation “ball” of Shaker channels (Zagotta et al., 1990). Following substitution of these cysteine residues in Kv1.1 or Kv3.4 channels, the rate of inactivation was unaltered by patch excision (Ruppersberg, Stocker et al., 1990). Clearly, similar regulation does not take place in A-current channels of locus coeruleus neurons. Kv1.4, Kv3.4 and Kv1.1/1.4 channels also reopen during the recovery from inactivation which takes place at hyperpolarised potentials, causing a long-lasting afterhyperpolarisation (Ruppersberg, Frank, Pongs & Stocker, 1991); it was shown that these reopenings were caused by voltage-dependent release of the inactivation “ball” from the channel pore on repolarisation. If such reopenings occurred in A-current channels from locus coeruleus neurons, they would have been easily identifiable from unitary current recordings where the patch pipette contained 140mM K$^+$; reopenings were not observed under these conditions (for example, see figure 5-2). As a result, it is concluded that recovery from inactivation in these channels does not cause channel reopening, suggesting that the channels close before the inactivation “ball” is released.
Subconductance states are frequently observed in ion channels activated by neurotransmitters such as acetylcholine (Hamill & Sakmann, 1981; Trautmann, 1982; Sachs, 1983), inhibitory amino acids (Hamill, Bormann & Sakmann, 1983; Bormann, Hamill & Sakmann, 1987) and excitatory amino acids (Cull-Candy & Usowicz, 1987; Jahr & Stevens, 1987). At least for the amino acid transmitters, different distributions of openings to substates occur with different agonists, probably due to activation of different channel subtypes (Bormann et al., 1987; Cull-Candy & Usowicz, 1989).

Several other channels show subconductance states, and in many cases there is evidence that the channel pore is made up of a number of similar conducting pathways in parallel (Miller, 1982; Kazachenko & Geletyuk, 1984; Geletyuk & Kazachenko, 1985; Krouse, Schneider & Gage, 1986; Hunter & Giebisch, 1987). It was suggested that in such channels, gating of each conducting pathway or “co-channel” was independent of the other co-channels and of a “main gate”, which could occlude all co-channels simultaneously (Krouse et al., 1986; Hunter & Giebisch, 1987). Thus, occupancy of each open level is given by a binomial distribution, and transitions between the closed level and any open sublevel could occur by opening and closing of the main gate.

The inward rectifier channel of guinea pig heart cells does not show substates under normal conditions, but low concentrations of the blockers Mg$^{2+}$, Cs$^{+}$ and Rb$^{+}$ reveal the presence of three equally conductive co-channels by blocking each co-channel independently (Matsuda, 1988; Matsuda, Matsuura & Noma, 1989). Under these conditions, occupancy of each open level was given by a binomial distribution. The absence of identifiable substates under control conditions indicates that the three co-channels open and close in a co-ordinated manner, such that there must be a high degree of cooperation between co-channels. It is possible that an analogous situation occurs in A-current channels of locus coeruleus neurons. The presence of three subconductance levels, with amplitudes close to 25%, 50% and 75% of the fully open level, might suggest the presence of four similar ion conducting pathways within the pore; this is a particularly attractive proposal when one considers that voltage dependent potassium channels are tetramers of independent subunits (MacKinnon, 1991; see chapter 1). If part of each subunit lined the pore of the channel (Guy & Conti, 1990), it could be proposed that the gating of each subunit could be partially independent, and could contribute roughly equally
to overall channel gating. It would be necessary for gating of all four subunits to be very highly co-ordinated, since the channel does not appear to have to visit each sublevel in turn during transitions between the closed and fully open states. The ability of the channel to pass directly from the fully open state to any subconductance state, and back again, also suggests a very high level of cooperativity between subunits. This scheme would be equally applicable if subconductance states were assumed to occur when one, two or three subunit pores were occluded by their own inactivation "ball", with a degree of cooperativity between individual balls. Whatever process leads to the occurrence of substates, it is clearly a voltage sensitive one, since occupancy of substates accounts for a greater proportion of overall open time at more negative potentials. Possibly, the degree of co-ordination between substates is less at these potentials.

If the pore of the A-current channel is made up of more than one ion conductive pathway, it is unlikely that the individual pores are blocked independently by internal Mg$^{2+}$ ions, in the way that inward rectifier channels in heart are (Matsuda, 1988). Clearly, the rate of channel blocking is much faster in A-current channels than in inward rectifiers, being of the "flickery" type, such that individual blocker-induced visits to substates could not be resolved as they are in inward rectifier channels. However, since block of the A-current channel by Mg$^{2+}$ and Na$^+$ is best fit assuming a Hill coefficient of one in each case, suggesting that the pore is blocked by a single Mg$^{2+}$ or Na$^+$ ion, it is considered unlikely that each blocking reaction is affecting only one pore of a multi-pore channel.

**Block by Internal Mg$^{2+}$ and Na$^+$**

The rectification of the unitary A-current seen during on cell recording was shown to be due to voltage-dependent block of the channel by internal Mg$^{2+}$ and Na$^+$. Both these ions blocked the channel at physiological internal concentrations; for Mg$^{2+}$, the $K_d$ at 0mV membrane potential was 15.7mM, and for Na$^+$ it was 76.0mM. The voltage dependence of block was consistent with one Mg$^{2+}$ or Na$^+$ ion blocking the channel, the two ions apparently acting at different sites, Na$^+$ ions being able to enter further into the channel pore.

Internal application of physiological concentrations of Mg$^{2+}$ and Na$^+$ also cause a voltage-dependent block of inward rectifier channels (Matsuda et al., 1987; Vandenberg, 1987; Matsuda, 1988, 1992) and ATP-sensitive potassium channels (Horie, Irisawa & Noma, 1987; Quayle & Stanfield, 1989). Internal Na$^+$ has also been shown to block single
calcium activated potassium channels (Marty, 1983; Yellen, 1984) and whole cell delayed rectifier currents (Bergman, 1970; Bezanilla & Armstrong, 1972; French & Wells, 1977). Internal Mg\(^{2+}\) ions also block some cloned rat brain potassium channels (Rettig et al., 1992), causing similar unitary current rectification during on cell recording to that seen here.

In contrast to the results shown here for the A-current channel, it was shown that in ATP-sensitive potassium channels of guinea pig heart cells, one Mg\(^{2+}\) ion or two Na\(^{+}\) ions bind to the same site to block the channel (Horie et al., 1987). In other cases, it has been shown that a single Na\(^{+}\) ion can block ATP-sensitive (Quayle & Stanfield, 1989) and inward rectifier potassium channels (Matsuda, 1992).
CHAPTER SIX: UNITARY PROPERTIES OF DELAYED RECTIFIER CHANNELS IN HIPPOCAMPAL CA1 NEURONS

INTRODUCTION

Delayed rectifier channels were the first potassium channels to be studied using single channel recording, in squid giant axons (Conti & Neher, 1980). The unitary properties of delayed rectifier channels have now been described in rat hypothalamic (McLarnon, 1989) and cerebellar Purkinje neurons (Gähwiler & Llano, 1989), and also in rat phaeochromocytoma (PC-12) cells (Hoshi & Aldrich, 1988a). Similar channels have been found in rabbit smooth muscle (Benham & Bolton, 1983), embryonic chick heart (Clapham & DeFelice, 1984; Clapham & Logothetis, 1988), lobster axons (Coronado et al., 1984), human (Cahalan et al., 1985; Lee, Levy & Deutsch, 1992) and murine T lymphocytes (DeCoursey et al., 1987), bovine chromaffin cells (Marty & Neher, 1985), frog skeletal muscle (Standen et al., 1985), chick ciliary ganglion cells (Gardner, 1986), Drosophila neurons (Solic & Aldrich, 1988) and canine and porcine airway smooth muscle cells (Boyle et al., 1992).

As is the case with A-current channels (see chapter 5), several different genes which are expressed in the rat brain encode potassium channel subunits with delayed rectifier-like properties. Some of the unitary properties of delayed rectifier-like channels expressed from cloned rat brain potassium channel genes have been described (Stühmer, Stocker, Sakmann, Seeburg, Baumann, Grupe & Pongs, 1988; Stühmer et al., 1989; Grupe et al., 1990; Kirsch, Taglialatela & Brown, 1991; Taglialatela, Vandongen, Drew, Joho, Brown & Kirsch, 1991; Rettig et al., 1992).

The voltage dependent gating of the delayed rectifier current was originally described in squid axons by the Hodgkin-Huxley model (Hodgkin & Huxley, 1952d; see chapter 1). This model was interpreted in terms of single channels by Armstrong (1969). In this scheme, the channel has four closed (C) states and one open (O) state connected together in a linear fashion (see chapter 1):
Multiples of $\alpha$ and $\beta$, as described by the Hodgkin-Huxley model (see chapter 1), represent the transition rates between the discrete states.

Although this model can correctly predict, with appropriate values of $\alpha$ and $\beta$, the voltage dependence of the macroscopic delayed rectifier current and its time course following changes in membrane potential, it was found to be insufficient to explain gating at the single channel level (Conti & Neher, 1980; see chapter 1). The Hodgkin-Huxley model is also inconsistent with the results of studies on the gating current of delayed rectifier channels (e.g., White & Bezanilla, 1985; see discussion). Most kinetic schemes for delayed rectifier channel gating still assume a sequential arrangement of closed and open states (e.g., Conti & Neher, 1980; Coronado et al., 1984; Standen et al., 1985; White & Bezanilla, 1985), although it is no longer presumed that the transition rates between the states are multiples of single underlying rate constants such as $\alpha$ and $\beta$ of equation 6-1.

RESULTS

All experiments were carried out on CA1 neurons grown in culture for between 6 and 17 days, using the on-cell configuration of the patch clamp technique (see chapter 2). The patch pipette contained either low K* patch solution or high K* patch solution (see chapter 2). All current records illustrated were low-pass filtered using an 8-pole Bessel filter set at either 2kHz or 100Hz (-3dB filter rate) and digitised onto the hard disk of the Dell System 210 computer (via the CED 1401 interface) at either 10kHz or 500Hz for subsequent analysis using CED “Patch” software.

For analysis of open and closed times, current signals were also recorded unfiltered onto video tape (via the digital audio processor), as described in chapter 2. These currents were subsequently played back through an 8-pole Bessel filter set at 2kHz (-3dB rate) and digitised at 10kHz onto the hard disk of a Dell System 325 computer. Analysis of open and closed times was carried out on these records using a Dell System 425DE computer running “Tracan” and “Evtan” software, as described in chapter 2. All currents used for the analysis of open and closed times were recorded during step membrane depolarisations.
lasting 4 seconds, to allow very long events to be detected. Because each 4 second pulse contained so much kinetic information, only a few pulses were recorded in each data file (between 8 and 78; most files contained fewer than 25 pulses). For this reason, analysis of first latency, which requires a large number of pulses but where pulse length is less critical, was carried out using the CED “Patch” software on currents recorded during briefer pulses.

Identification of Single Delayed Rectifier Channels

Single delayed rectifier channels were identified by the similarity of their electrophysiological properties to those of the whole cell delayed rectifier current in CA1 neurons. Thus, these channels were activated during depolarising steps and often stayed open throughout a 200ms pulse, so that the ensemble average current showed little or no inactivation during such a pulse. As with the whole cell delayed rectifier current, activation of single delayed rectifier channels was sensitive to the holding potential, indicating that the channels were nevertheless subject to steady-state inactivation. This inactivation manifested itself in a gradual reduction in the number of active channels in a patch when the membrane was held more positive than the resting potential for prolonged periods. Channel activity could only partially be restored by changing the holding potential to a more negative potential. This suggests that holding at a relatively positive potential for long periods can cause the channel to enter an extremely long-lived inactivated state.

Examples of unitary delayed rectifier channels recorded from on cell patches are shown in figures 6-1 (where the pipette contained low K⁺ patch solution, 3mM K⁺) and 6-2 (where the pipette contained high K⁺ patch solution, 140mM K⁺). On stepping to a relatively positive potential, activation of the channel was usually very rapid, such that the rise of the ensemble average current was also very rapid (eg. figures 6-1A and 6-2A). When the test potential is less positive, activation is slower (eg. figures 6-1B and 6-2B). Substate behaviour in these cells was much less marked than that seen in A-current channels in locus coeruleus neurons (see chapter 5). Unitary current-voltage relationships were calculated from Gaussian curves fitted to amplitude histograms formed from single channel currents such as those shown in figures 6-1 and 6-2, as described in chapter 2. Figure 6-3 shows the mean current-voltage relationships when the pipette contained either 3mM K⁺ (A.) or 140mM K⁺ (B.). The reversal potential is shifted from around 10mV negative to the resting potential (estimated by extrapolation) in figure 6-3A to 76.2mV positive to the
Figure 6-1  Unitary delayed rectifier channels recorded from an on cell patch with 3mM K⁺ in the pipette. The patch was held 20mV more negative than the cell resting potential before depolarising steps of 120mV (in A.) or 60mV (in B.) were applied. In both A. and B., the ensemble average current, formed as the average of 100 consecutive pulses, is shown beneath the unitary currents. These current records were filtered at 2kHz and digitised at 10kHz.
Figure 6-2 Unitary delayed rectifier channels recorded from an on cell patch with 140mM K\(^+\) in the pipette. The voltage protocol used to activate the channels was the same as in figure 6-1: the patch was held 20mV more negative than the resting potential and depolarised by 120mV (A.) or 60mV (B.). As in figure 6-1, the ensemble average current is formed from 100 consecutive pulses in each case. Filter rate 2kHz, digitisation rate 10kHz.
Figure 6-3  Mean unitary current-voltage relationships for the delayed rectifier channel, formed from patches where the pipette contained 3mM K⁺ (A.) or 140mM K⁺ (B.). Points are the mean values from 8-16 patches in A., 6-11 patches in B. Error bars show ± one S.E.M.; where no error bars are shown, this is less than the size of the symbol. Voltage is given relative to the resting potential. The reversal potential is shifted to a more positive value by increasing K⁺ concentration, as expected for a potassium selective channel. As with A-current channels (see chapter 5), there is marked rectification of the current at the most positive potentials, particularly when the pipette contains 3mM K⁺ (A.).
resting potential in figure 6-3B, consistent with current being carried by potassium ions. As with unitary A-current channels in locus coeruleus neurons (see chapter 5), the unitary current-voltage relationship is not linear, but rectifies at the most positive potentials, particularly when the pipette contained 3mM K⁺. It is presumed that the cause of this rectification is similar to that described for A-current channels in chapter 5, namely voltage-dependent block of the channel by internal cations, although this was not examined in these channels. Channel conductance, measured as the slope of the linear parts of the unitary current-voltage relationships in figure 6-3, was 18.4pS with 3mM K⁺ in the pipette, and 36.4pS with 140mM K⁺.

The ensemble average currents shown in figures 6-1A and 6-2A show slight inactivation of delayed rectifier channels may take place during a depolarising step lasting 200ms. This inactivation was examined further by giving much longer steps (4 seconds), as is shown in figure 6-4. It can be seen from the unitary current records that apparent bursts of openings lasting hundreds of milliseconds can occur, as can very long-lived closures. On some occasions, the channel reopened after being closed for several hundred milliseconds; this is suggested to be due to channels returning from relatively short-lived inactivated states (see below). The ensemble average current shows that significant inactivation of the channel takes place over four seconds.

**Kinetic States of the Channel**

Open and closed times measured using a 50% threshold crossing criterion were binned logarithmically after imposition of a minimum resolution of 200μs, and fitted by a probability density function which contained one or the sum of several exponential components, as described in chapter 2. Figures 6-5 and 6-6 show the distribution of open and closed times respectively, at voltages 60, 80 and 100mV positive to the resting potential. Each open time histogram has been fitted by a single exponential in each case, suggesting a single open state of the channel. The mean open time shows little voltage dependence over this range, as illustrated in table 6.1 below. The closed time histograms shown in figure 6-6 have been fitted by the sum of five exponential components; fitting these histograms with only four exponentials gave a less good fit of the data at long closed times. It can be seen from the form of the fitted distribution that most short closed times are not detected when a minimum resolution of 200μs is applied. However, if the same closed time data were binned without imposing a minimum resolution, the resulting closed
Figure 6-4  Activation of a single delayed rectifier channel by prolonged membrane depolarisation. The patch was held 20mV more negative than the resting potential and depolarised by 120mV for 4 seconds, this pulse being applied once every 12 seconds. It can be seen that the channel is usually activated in apparent bursts lasting for hundreds of milliseconds, and that very long closures can also occur. In the top trace, the channel falls silent after less than one second, probably due to it entering a very long-lived inactivated state (see text). The ensemble average current shown below is the sum of 18 such records, and illustrates the slow inactivation of the current. Filter rate 100Hz, digitisation rate 500Hz.
**Figure 6-5** Open time histograms, formed from single delayed rectifier channels recorded during 4 second pulses using “Tracan” and “Evtan” software, as described in the text. A minimum resolution of 200μs has been applied (see chapter 2). Voltages are 60mV positive to the resting potential in A., 80mV positive to the resting potential in B., and 100mV positive to the resting potential in C. For each histogram, the ordinate has a square root scale and the abscissa a logarithmic scale (see chapter 2). Each has been fitted by a single exponential function, suggesting the presence of a single open state, with a time constant of 10.97ms in A., 18.05ms in B., and 11.71ms in C, using a maximum likelihood fitting routine (see chapter 2).
A. Number of Events

B. Number of Events

C. Number of Events

Time (ms)
Figure 6-6  Closed time histograms from the same patches as used for figure 6-5, formed as described in the legend to figure 6-5. Each has been fitted by the sum of five exponential distributions, with time constants (and relative areas) of 0.0891ms (0.8806), 0.693ms (0.0797), 5.50ms (0.0280), 37.1ms (0.0109) and 300ms (0.0006) in A., 0.0619ms (0.9037), 0.343ms (0.0628), 3.75ms (0.0158), 44.9ms (0.0147) and 446ms (0.0027) in B., and 0.0617ms (0.9024), 0.203ms (0.0891), 1.65ms (0.0054), 37.7ms (0.0024) and 484ms (0.0004) in C.
time histogram was still fitted by the sum of five exponentials with similar time constants and areas as those found with a minimum resolution of 200μs. This is demonstrated in figure 6-7, which shows the closed time histogram of figure 6-6B after (A.) and before (B.) imposition of a 200μs minimum resolution.

The mean parameters of the fitted exponential components to open and closed time histograms in 3-10 patches at different voltages are illustrated in table 6-1. Because the number of events recorded from different patches at the same voltage varied considerably (from 221 to 8693), the values shown in table 6-1 are weighted means calculated by weighting the values from each patch in proportion to the number of events included in the distribution for that patch, according to the equation:

\[
\bar{x}_w = \frac{\sum_{i=1}^{N} x_i n_i}{\sum_{i=1}^{N} n_i}
\]  

(6 - 1)

where \(\bar{x}_w\) is the weighted mean of each parameter, \(x_i\) is the mean from patch \(i\), \(n_i\) is the total number of events from that patch, and \(N\) is the total number of patches included in the weighted mean. The weighted mean values in table 6-1 are given ± one standard deviation, calculated as the square root of the weighted variance, which is given by:

\[
\sigma^2 = \frac{\sum_{i=1}^{N} (x_i - \bar{x}_w)^2 w_i}{N - 1}
\]  

(6 - 2)

where \(w_i\) is the weight of each patch, given by:

\[
w_i = \frac{n_i}{\sum_{i=1}^{N} n_i}
\]  

(6 - 3)
Figure 6-7 Closed time data from the same patch (that illustrated in figure 6-6B.), after (A.) and before (B.) imposition of a minimum resolution of 200μs. Each has been fitted by the sum of five exponential distributions. The time constants (and relative areas) of each exponential in A. are as described in the legend to figure 6-6; in B., they are 0.107ms (0.8817), 0.6919ms (0.0487), 4.89ms (0.0400), 54.5ms (0.0235) and 429ms (0.0058). Since many closed events less than 200μs are likely to have been missed during the analysis, the short closed times are likely to have overestimated time constants and underestimated areas in B.
Table 6-1. Voltage Dependence of Open and Closed Time Distributions

<table>
<thead>
<tr>
<th>Voltage (mV)</th>
<th>60mV</th>
<th>80mV</th>
<th>100mV</th>
<th>120mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>4</td>
<td>10</td>
<td>8</td>
<td>3</td>
</tr>
<tr>
<td>$n_o$</td>
<td>2837</td>
<td>15562</td>
<td>9852</td>
<td>12767</td>
</tr>
<tr>
<td>$\tau$</td>
<td>1.53±0.41</td>
<td>1.53±0.30</td>
<td>1.38±0.24</td>
<td>1.19±0.16</td>
</tr>
</tbody>
</table>

**Open Times**

<table>
<thead>
<tr>
<th>Voltage (mV)</th>
<th>60mV</th>
<th>80mV</th>
<th>100mV</th>
<th>120mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>$n_e$</td>
<td>2840</td>
<td>15571</td>
<td>9860</td>
<td>12770</td>
</tr>
<tr>
<td>$\tau$</td>
<td>0.0762±0.0084</td>
<td>0.0635±0.0044</td>
<td>0.0619±0.0029</td>
<td>0.0509±0.0017</td>
</tr>
<tr>
<td>$a_1$</td>
<td>0.889±0.012</td>
<td>0.886±0.016</td>
<td>0.847±0.024</td>
<td>0.812±0.019</td>
</tr>
<tr>
<td>$\tau_2$</td>
<td>0.508±0.108</td>
<td>0.311±0.058</td>
<td>0.224±0.048</td>
<td>0.141±0.004</td>
</tr>
<tr>
<td>$a_2$</td>
<td>0.079±0.008</td>
<td>0.095±0.015</td>
<td>0.137±0.024</td>
<td>0.176±0.019</td>
</tr>
<tr>
<td>$\tau_3$</td>
<td>5.17±0.75</td>
<td>2.57±0.47</td>
<td>1.86±0.44</td>
<td>0.723±0.068</td>
</tr>
<tr>
<td>$a_3$</td>
<td>0.023±0.004</td>
<td>0.012±0.002</td>
<td>0.011±0.002</td>
<td>0.008±0.002</td>
</tr>
<tr>
<td>$\tau_4$</td>
<td>38.5±3.0</td>
<td>36.8±6.8</td>
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<td>34.1±4.8</td>
</tr>
<tr>
<td>$a_4$</td>
<td>0.008±0.002</td>
<td>0.005±0.002</td>
<td>0.003±0.000</td>
<td>0.002±0.000</td>
</tr>
<tr>
<td>$\tau_5$</td>
<td>324±130</td>
<td>312±84</td>
<td>386±74</td>
<td>328±119</td>
</tr>
<tr>
<td>$a_5$</td>
<td>0.001±0.000</td>
<td>0.001±0.001</td>
<td>0.001±0.000</td>
<td>0.001±0.000</td>
</tr>
</tbody>
</table>

$N$ is the number of patches at each voltage included in the mean distributions; $n_o$ and $n_e$ are the total number of openings and closings from all $N$ patches; and for the closed times, $\tau$ and $a$ are the mean time constants and areas of the five exponential time constants fitted to the closed time distributions.

**Burst Kinetics**

Examination of the current traces in figures 6-1 and 6-2 show that openings of the delayed rectifier channel occur in bursts, as has previously been described in other cases (eg. Conti & Neher, 1980; see chapter 1). Bursts were defined as described in chapter 2, as a group of openings separated by closings shorter than a critical time $t_c$. $t_c$ was chosen for each patch according to equation 2-10 (see chapter 2). For bursts of openings, the shortest of the five components of the closed time histogram was defined as closings within bursts, and the other four closings between bursts (in equation 2-10, $k=5$ and $n=1$). Values of $t_c$ used to delimit bursts in different patches were between 0.25 and 0.37ms. Clusters of bursts were analysed in the same way by calculating $t_c$ from equation 2-10 where $k$ was 5 and $n$ was 2, so that the two shortest components of the closed time histogram were
defined as closings within clusters. Values of \( t \) used to delimit clusters of bursts in different patches were between 0.39 and 1.13 ms. The distributions of burst duration and cluster duration are shown in figures 6-8 and 6-9 respectively. Each has been fitted by the sum of two exponential components, which should be equal to the total number of states within the burst or cluster (Colquhoun & Hawkes, 1982). There may be a third component to the distribution of cluster length which has an area too small to be detected, as there are three states (one open and two closed) within clusters.

Mean durations of bursts of openings and of clusters of bursts are shown in table 6-2. As with the durations of open and closed times, the values given are weighted means (calculated according to equation 6-1) ± the square root of the weighted variance (calculated according to equation 6-2). Also shown are the mean number of openings per burst, which was measured directly and corrected for missed closed events in exactly the same way as mean open time. Again, the values are weighted means ± the square root of weighted variance; individual values were weighted according to the total number of openings in each patch.

### Table 6-2. Voltage Dependence of Burst Parameters

<table>
<thead>
<tr>
<th>Voltage</th>
<th>60mV</th>
<th>80mV</th>
<th>100mV</th>
<th>120mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>( N )</td>
<td>4</td>
<td>10</td>
<td>8</td>
<td>3</td>
</tr>
<tr>
<td>( n_b )</td>
<td>1023</td>
<td>5523</td>
<td>3554</td>
<td>4883</td>
</tr>
<tr>
<td>( \tau_b )</td>
<td>26.8±2.1</td>
<td>39.6±3.6</td>
<td>41.7±8.7</td>
<td>58.4±9.3</td>
</tr>
<tr>
<td>( o/b )</td>
<td>19.2±5.0</td>
<td>33.1±5.5</td>
<td>27.5±2.4</td>
<td>37.2±3.1</td>
</tr>
<tr>
<td>( n_e )</td>
<td>550</td>
<td>2658</td>
<td>1500</td>
<td>2416</td>
</tr>
<tr>
<td>( \tau_e )</td>
<td>49.5±6.6</td>
<td>82.1±5.2</td>
<td>99.1±15.5</td>
<td>117.3±31.4</td>
</tr>
</tbody>
</table>

\( N \) is the total number of patches at each voltage, \( n_b \) and \( n_e \) are the total number of bursts and clusters respectively from all \( N \) patches, \( \tau_b \) and \( \tau_e \) are the mean burst length and the mean cluster length respectively, and \( o/b \) is the mean number of openings per burst.
Figure 6-8 Burst length histograms for the same patches as used for figures 6-5 and 6-6, formed as described in the legend to figure 6-5. Each has been fitted by the sum of two exponential distributions (see text), with time constants (and relative areas) of 28.7ms (0.9172) and 0.0791ms (0.0827) in A., 47.9ms (0.9481) and 0.0869ms (0.0518) in B., and 60.2ms (0.9519) and 0.205ms (0.0480) in C. This gives overall mean burst durations of 26.3ms in A., 45.4ms in B. and 57.3ms in C. The critical times used to delimit the bursts (see chapter 2) were 0.37ms in A., 0.32ms in B. and 0.35ms in C.
Figure 6-9  Cluster length histograms for the same patches as used for figures 6-5 to 6-8, formed as described in the legend to figure 6-5. Each has been fitted by the sum of two exponential distributions, with time constants (and relative areas) of 57.6ms (0.9547) and 0.122ms (0.0452) in A, 77.6ms (0.9939) and 0.214ms (0.0060) in B, and 153ms (0.8129) and 0.0610ms (0.1870) in C. This gives overall mean cluster durations of 55.0ms in A, 77.1ms in B, and 124ms in C. The critical times used to delimit the clusters (see chapter 2) were 1.02ms in A, 0.76ms in B, and 0.64ms in C.
A. Number of Clusters vs. Time (ms)

B. Number of Clusters vs. Time (ms)

C. Number of Clusters vs. Time (ms)
A Simple Kinetic Model for the Delayed Rectifier Channel

The distribution of channel open and closed times shown in table 6-1 suggest that the channel has one conducting (open) state and at least five non-conducting (closed or inactivated) states. Of these five non-conducting states, it was assumed that the three shortest-lived were closed states (corresponding to the time constants \( \tau_1, \tau_2 \) and \( \tau_3 \) in table 6-1) and the two longer-lived were inactivated states (corresponding to \( \tau_4 \) and \( \tau_5 \) in table 6-1). There are two reasons behind this assumption. First, the durations of the three shortest components of the closed times (\( \tau_1, \tau_2 \) and \( \tau_3 \)) are clearly voltage-dependent, consistent with the lifetime of each of these states decreasing at more positive potentials, whereas the time constants \( \tau_4 \) and \( \tau_5 \) vary little with voltage (see table 6-1). For a channel with strongly voltage-dependent activation, such as the delayed rectifier channel, the rate constants for transitions from closed to open states would also be expected to be strongly voltage-dependent. Secondly, components with time constants similar to \( \tau_4 \) and \( \tau_5 \) of table 6-1 could not be identified in the first latency histograms for the channel at all potentials (see below); these histograms should show components with time constants similar to those of all closed (but not inactivated) states.

The simplest kinetic model which can account for the distributions of open and closed states is therefore a linear one with three closed states (\( C_1, C_2, C_3 \)), one open state (\( O \)) and two inactivated states (\( I_1, I_2 \)):

\[
\begin{array}{cccccc}
& k_1 & k_2 & k_3 & k_4 & k_5 \\
C_1 & \rightarrow & \rightarrow & \rightarrow & \rightarrow \rightarrow & \rightarrow \\
& k_{-1} & k_{-2} & k_{-3} & k_{-4} & k_{-5} \\
\end{array}
\]

The question of whether or not channels can inactivate directly from closed states was not addressed, although this clearly can occur in sodium channels (Horn, Patlak & Stevens, 1981; Aldrich, Corey & Stevens, 1983), and has been suggested for some voltage-dependent potassium channels (e.g. Solc & Aldrich, 1990; Zagotta & Aldrich, 1990a).

Values of the transition rates (the \( k_5 \) in scheme 6-4) which best fit the mean open and closed time distributions given in table 6-1 were estimated using a computer program written by Dr. N.W. Davies. The voltage dependence of all ten transition rates in scheme 6-4: $\text{Scheme 6-4}$
6-4 is shown in figure 6-10; those rates leading towards the open state \((k_1, k_2, k_3, k_4\) and \(k_5\)) in figure 6-10A, and those leading away from the open state \((k_1, k_2, k_3, k_4\) and \(k_5\)) in figure 6-10B. It can be seen from figure 6-10A that all the forward rates corresponding to transitions from closed states towards the open state \((k_1, k_2\) and \(k_3\)) are increased by depolarisation, which underlies the voltage-dependent activation of the channel. Of these rates \(k_i\), which corresponds to the first step in channel activation (presuming that the channel resides in state \(C_1\) under resting conditions) is both the slowest and the most voltage-dependent step during activation, whereas the last step in activation, which has the rate \(k_3\), is the fastest and least voltage-dependent. Reopening following visits to either \(I_1\) or \(I_2\) appears to be independent of voltage.

In contrast, all the backward rates corresponding to transitions away from the open state and towards state \(C_1\) \((k_1, k_2\) and \(k_3\)) show very little voltage dependence. It is not clear if the apparent voltage dependence of the transition rates leading towards the inactivated states \((k_4\) and \(k_5\)) represents a true voltage dependence of inactivation or is a result of the difficulty in calculating these rates accurately, due to the small number of visits to inactivated states during the recordings.

This scheme does not, however, account for all of the bursting properties of the channel. A burst is described as a series of transitions between states \(C_3\) and \(O\), terminated when the channel enters either \(C_2\) or \(I_1\). The ratio of the transition rates \(k_3\) and \(k_2\) is such that almost all bursts will actually be terminated by transitions to \(C_2\), even though during a burst much more time is spent in state \(O\) than in \(C_3\). In this case, the number of closings per burst should be approximately equal to the ratio \(k_3/k_2\), and the number of openings per burst \(1+(k_3/k_2)\). For the transition rates shown in figure 6-10, the number of openings per burst should therefore be equal to 12.3 at 60mV positive to the resting potential, 14.2 at 80mV positive to the resting potential, 12.2 at 100mV positive to the resting potential, and 12.8 at 120mV positive to the resting potential; this compares with mean measured values (see table 6-2) of 19.2, 33.1, 27.5 and 37.2 openings per burst at these potentials. The observed (but not predicted) voltage dependence of the number of openings per burst also underlies the increase in burst length at more positive potentials, as mean open time shows a slight decrease at these potentials (see table 6-1). Cluster length also increases at more positive potentials (see table 6-2). However, this would be predicted from the calculated transition rates. If a cluster is described as transitions between states \(C_2, C_3\) and \(O\), usually
Figure 6-10 Voltage dependence of the ten transition rates of equation 6-4, estimated as described in the text. A. shows the rates leading towards the open state: these are \( k_1 \), \( k_2 \), \( k_3 \), \( k_4 \), and \( k_5 \). The voltage dependence of each rate has been fitted by linear regression analysis, assuming that each rate changes exponentially with voltage (since the ordinate has a logarithmic scale). The slope of the fits suggest that the increase in membrane potential (depolarisation) required to cause an e-fold increase in rate is 80.8mV for \( k_1 \), 91.3mV for \( k_2 \), 413mV for \( k_3 \), 1450mV for \( k_4 \), and 4000mV for \( k_5 \). B. shows the rates leading away from the open state: \( k_1 \), \( k_2 \), \( k_3 \), \( k_4 \), and \( k_5 \). As in A., the slope of the straight line fits to the data suggest that the increase in membrane potential required to cause an e-fold increase in rate is 1540mV for \( k_1 \), 403mV for \( k_2 \), 529mV for \( k_3 \), and 221mV for \( k_5 \). The rate \( k_4 \) appears to decrease e-fold for a 121mV increase in membrane potential.
terminated by visits to $C_1$, then the number of bursts per cluster should be equal to $1 + (k_{ij}/k_e)(k_{ij} + k_{2j})$ (Colquhoun & Hawkes, 1982; Spruce, Standen & Stanfield, 1989), which (from the calculated transition rates) is increased from 4.2 at 60mV positive to the resting potential to 16.1 at 120mV positive to the resting potential. An increase in the number of bursts per cluster at more positive potentials will obviously cause an increase in cluster length, whether or not burst length is increased.

The computer program used for the fitting of transition rates also allowed calculation of the eigenvalues from the $Q$ matrix of these rates (see Colquhoun & Hawkes, 1977, and chapter 1). These eigenvalues were used to fit first latency histograms formed from patches recorded at different potentials with equation 2-18 of chapter 2, which assumes that there are three closed states leading to the open state, as there are in scheme 6-4. These first latency histograms are shown in figure 6-11. It can be seen that the distribution of first latencies is extremely voltage dependent, much more so than can be explained by scheme 6-4. The most likely explanation for this is that there are more than three closed states of the channel, for example:

$$\begin{array}{cccc}
C_0 & C_1 & C_2 & C_3 & O \\
k_{00} & k_{11} & k_{22} & k_{33} & k_{30} \\
\end{array}$$

(6-5)

The presence of even more closed states cannot be ruled out. In this scheme, if the transition rate $k_{ij}$ were slower and more voltage dependent than $k_i$ (which might be expected, since the other forward transition rates get faster and less voltage dependent as one moves closer to state O; see above), then this scheme would predict a longer, more voltage dependent first latency distribution than that predicted by scheme 6-4. If the ratio of the rates $k_i$ and $k_{00}$ were such that a channel in state $C_1$ were more likely to move to $C_2$ than to $C_0$ at depolarised potentials (which would be expected to be the case; from the transition rates shown in figure 6-10 at the least depolarised potential studied, 60mV positive to the resting potential, a channel in $C_2$ is more than three times more likely to move to $C_3$ than to $C_1$, and a channel in $C_3$ is more than eleven times more likely to move to $O$ than to $C_2$, both these ratios increasing at more positive potentials), then it may be expected that too few visits would be made to state $C_0$ to be identified in the closed time histogram.
Figure 6-11 First latency histograms formed for single delayed rectifier channels recorded at 60mV positive to the resting potential (A.), 80mV positive to the resting potential (B.), and 100mV positive to the resting potential (C.). Note the different abcissa in each case, which indicates that the distribution of first latencies is strongly voltage dependent. Each has been fitted with equation 2-18 of chapter 2, which assumes that there are three closed states leading up to channel opening on depolarisation, using eigenvalues calculated as described in the text. It can be seen that at negative voltages, a slower component of the first latency histogram is seen, suggesting the presence of a fourth closed state (see text).
Scheme 6-4 is also insufficient to explain the slow inactivation of the ensemble average current (see figure 6-4). From the transition rates shown in figure 6-10, the channel will eventually reach a steady state at which channel open probability will be between 0.605 (at 60mV positive to the resting potential) and 0.793 (at 120mV positive to the resting potential), whereas the open probability after four seconds in figure 6-4 is obviously less than this, and might be expected to fall to zero if the pulse were maintained for long enough. Also, as described above, channels could fall silent for very prolonged periods, which also suggests the presence of a far longer-lived inactivated state than either $I_1$ or $I_2$ of scheme 6-4. The simplest amendment to scheme 6-4 to take account of slow inactivation would be to include a third inactivated state, $I_3$, recovery from which was so slow as to make it effectively absorbing:

$$
\begin{align*}
O & \xrightarrow{k_4} I_1 & \xrightarrow{k_5} I_2 & \xrightarrow{k_6} I_3 \\
& \xleftarrow{k_4} I_1 & \xleftarrow{k_5} I_2 & \xleftarrow{k_5} I_3
\end{align*}
$$

(6-6)

**DISCUSSION**

Comparison with Other Delayed Rectifier Channels

The conductance of the delayed rectifier channel identified in CA1 neurons was around 18pS with a physiological potassium gradient (3mM K⁺ in the pipette), increasing to 36pS with a symmetrical potassium gradient (140mM K⁺ in the pipette). The value measured with a physiological potassium gradient is less than that previously reported in rat cerebellar Purkinje (28pS; Gähwiler & Llano, 1989) or hypothalamic neurons (48pS; McLarnon, 1989). However, delayed rectifier channels with a similar conductance have been identified in human T lymphocytes (16pS; Cahalan et al., 1985), bovine chromaffin cells (18pS; Marty & Neher, 1985), frog skeletal muscle (15pS; Standen et al., 1985), murine T lymphocytes (21pS; DeCoursey et al., 1987), embryonic chick heart (15pS; Clapham & Logothetis, 1988) and squid giant axon (20pS; Llano et al., 1988). A-current channels in a number of cells, including locus coeruleus neurons (see chapter 5), also have a similar conductance. Delayed rectifier-like channels expressed from cloned Kv1 subfamily gene products have a lower conductance than reported here (around 10pS; Sühmer et al., 1988, 1989; Grupe et al., 1990).
Kinetic Modelling of Voltage-Gated Potassium Channels

The simplest model consistent with all the kinetic features described for the delayed rectifier channel of CA1 neurons is of the form:

\[
\begin{array}{cccccccc}
C_0 & \rightarrow & C_1 & \rightarrow & C_2 & \rightarrow & C_3 & \rightarrow & O & \rightarrow & I_1 & \rightarrow & I_2 & \rightarrow & I_3 \\
& k_0 & & k_1 & & k_2 & & k_3 & & k_4 & & k_5 & & k_6 & \\
\end{array}
\] (6-7)

Those states and rates which are printed in bold are those for which there is direct evidence from the distributions of open and closed times; evidence for additional closed and inactivated states comes from the distribution of first latencies and from slow channel inactivation (see above). All the opening transition rates \((k_0, k_1, k_2, k_3)\) appear to be voltage dependent, with earlier steps in activation being both more voltage dependent and slower than later steps. This in contrast to the Hodgkin-Huxley model of delayed rectifier channel gating, in which earlier steps in activation must be faster than later steps, (Hodgkin & Huxley, 1952d; see chapter 1), suggesting that the steps in the gating of the delayed rectifier channel in CA1 neurons may be cooperative rather than independent (as they are in the Hodgkin-Huxley model). This model is consistent with previous studies of delayed rectifier channels in frog skeletal muscle (Spruce et al., 1989) and A-current channels in Drosophila neurons (Solc & Aldrich, 1990), in which the first transition in the gating process was described as being the most voltage dependent (although at least in the case of Drosophila neurons, not necessarily the slowest). It is possible, considering the tetrameric structure of voltage-gated potassium channels (see chapter 1), that each step in the activation of the channel represents the gating of one subunit, and that the gating of the four subunits is an allosterically cooperative process. Alternatively, it is possible that the gating of each subunit involves a series of voltage dependent steps, the first of which is the slowest and the most voltage dependent. Cooperativity of gating between the subunits of a voltage-gated potassium channel has also been demonstrated by studying the interaction of subunits with different gating properties in Xenopus oocytes (Tytgat & Hess, 1992). Studies of the gating currents of a number of different voltage-gated potassium channels have also suggested that the activation “gates” are not independent, that several voltage dependent steps lead up to opening, and that early steps in activation are more

Although the proposed simple model is sufficient to explain most of the kinetic properties of the delayed rectifier channel in CA1 neurons, it is unlikely that it is a unique solution. In particular, errors introduced by the presence of noise and by filtering, which cannot be accounted for using conventional techniques, may make discrimination between different kinetic models impossible. A more rigorous test has been introduced by Magleby & Weiss (1990a,b) to take account of these distorting effects. Their technique is based on the analysis of both real and simulated single channel records using exactly the same methods, which allows the effects of noise and filtering to be more accurately modelled. This method also uses two-dimensional distributions of real and simulated open and closed times to allow correlations between successive open and closed times to be more easily identified.
CHAPTER SEVEN: EFFECTS OF EXTERNAL RUBIDIUM ON DELAYED RECTIFIER CHANNEL GATING IN CA1 NEURONS

INTRODUCTION

Potassium channels are among the most selective channels known, and the pattern of ionic permeabilities between different classes of potassium channel are very similar (see Hille, 1984), suggesting some structural similarity between potassium channel pores. In most potassium channels studied the most permeant ion is thallous, followed by potassium, rubidium and ammonium. The permeability to sodium and lithium is too small to be measured in most cases, and caesium usually blocks potassium channels. This sequence of permeabilities is most similar to Eisenman sequence IV or V (see chapter 1).

It was originally assumed that the gating of ion channels was not affected by the concentration (Hodgkin & Huxley, 1952a) or nature (Chandler & Meves, 1965) of permeant ions. However, when the technique of noise analysis was introduced to study membrane currents, it was shown that the open lifetime of channels could be affected by the permeant ions. This effect was first noted in channels activated by acetylcholine in toad skeletal muscle (Van Helden, Hamill & Gage, 1977; Gage & Van Helden, 1979), where open time is increased when lithium rather than sodium carries the current, and decreased when caesium or potassium carry current. Similarly, openings of channels activated by acetylcholine in Aplysia neurons are prolonged when caesium, magnesium or calcium rather than sodium carry current (Ascher, Marty & Neild, 1978). These effects were first proposed to be due to permeant ions binding to sites within the pore, with the channel being unable to close whilst this binding site is occupied (Marchais & Marty, 1979). Permeant ions which bind more tightly in the pore will therefore hinder closing and increase open time, in the same way in which some open-channel blockers prevent channel closure (eg. Neher & Steinbach, 1978). However, in acetylcholine activated channels of frog muscle, channel conductance when current is carried by organic cations is limited by binding to a site within the pore, but binding to this site does not seem to influence open time; instead, it was suggested that permeant ions could affect open time by acting at modulatory sites on the internal or external face of the channel (Adams, Nonner, Dwyer & Hille, 1981). Permeant ion effects also occur in inward rectifier potassium...
channels, where gating is dependent on voltage and external potassium concentration, but is independent of internal potassium (Hagiwara & Yoshii, 1979; Leech & Stanfield, 1981) and is also profoundly altered by the permeant ion thallous (Stanfield, Ashcroft & Plant, 1981; Ashcroft & Stanfield, 1983); in chloride channels, where gating is dependent on voltage and internal, but not external chloride concentration (Chesnoy-Marchais, 1983); in calcium channels, where open time is different when calcium, barium, strontium or magnesium carry current (Nelson, French & Krueger, 1984; Chesnoy-Marchais, 1985); in sodium channels, where internal sodium and caesium ions alter inactivation gating (Oxford & Yeh, 1985); and in calcium activated potassium channels, where both permeant and blocking ions modulate gating by occupying a site near the outer mouth of the channel, primarily due to an increase in channel opening rate when this site is occupied (Miller, Latorre & Reisin, 1987; Neyton & Miller, 1988a,b; Neyton & Pelleschi, 1991).

The first example of permeant ion effects on delayed rectifier potassium channels was the finding that channel gating was dependent on the external potassium concentration (Dubois & Bergman, 1977). It was subsequently found that replacement of external potassium by the permeant ion rubidium had a greater effect on gating, prolonging tail currents through delayed rectifier channels on membrane repolarisation from a depolarised potential in *Xenopus* nerve fibres (Århem, 1980) and squid axons (Swenson & Armstrong, 1981), which was presumed to be due to a prolongation of channel open time when rubidium carried current. Similar effects of rubidium on delayed rectifier channel gating have since been described in rat (Beam & Donaldson, 1983) and frog skeletal muscle (Spruce et al., 1989), frog myelinated nerve (Cahalan & Pappone, 1983; Plant, 1986), human (Cahalan et al., 1985) and murine lymphocytes (Shapiro & DeCoursey, 1991b), rat brown fat cells (Lucero & Pappone, 1989), and toadfish pancreatic islet cells (Sala & Matteson, 1991). Rubidium also increases the open time of calcium activated potassium channels from guinea pig smooth muscle (Hu, Yamamoto & Kao, 1989) and rat skeletal muscle (Demo & Yellen, 1992). The effects of rubidium ions on delayed rectifier channel gating have been interpreted both in terms of less permeant rubidium ions binding more tightly than potassium inside the channel pore and so preventing channel closure (eg. Swenson & Armstrong, 1981; Beam & Donaldson, 1983; Sala & Matteson, 1991), and rubidium acting at an internal or external modulatory site on the channel (eg. Clay, 1988; Shapiro & DeCoursey, 1991b). However, other possible mechanisms have also been put
forward. Armstrong & Matteson (1986) have shown that external calcium antagonises the effects of rubidium on the closing of delayed rectifier channels in squid axons. They suggested that calcium ions normally occupy closed channels, and that rubidium ions could compete with calcium for its site of action within the channel. Channels occupied by rubidium rather than calcium would then close more slowly and reopen more readily. Supporting this hypothesis are the findings that these channels close more securely when they are occupied by barium ions (Armstrong, Swenson & Taylor, 1982), and that delayed rectifier channels in squid neurons (Armstrong & Lopez-Barneo, 1987) and cloned Shaker A-current channels (Armstrong & Miller, 1990) require external calcium ions for normal gating in the absence of external potassium ions. Similarly, slowly inactivating potassium channels in human T lymphocytes inactivate more rapidly when calcium or barium ions are bound in the channel, and this effect is antagonised by permeant potassium and rubidium ions (Grissmer & Cahalan, 1989b). Calcium activated potassium channels from rat skeletal muscle can also close whilst blocked by barium; closed channels containing barium reopen more readily (Miller et al., 1987).

RESULTS

All experiments were carried out on hippocampal CA1 neurons, grown in culture for between 6 and 17 days, using the on-cell patch clamp technique. The patch pipette contained either high K⁺ patch solution or Rb⁺ patch solution (see chapter 2), such that the extracellular face of the patch was exposed to either 140mM K⁺ or 140mM Rb⁺. The bath contained ACSF (see chapter 2). All current records illustrated were filtered using an 8-pole Bessel filter set at 2kHz (-3dB filter rate) and digitised at 10kHz. Measurement of open and closed times was carried out using a 50% threshold crossing criterion, as described in chapter 2. Open and closed times were not corrected for missed events; nor was a minimum resolution applied to the data (see chapter 2). All curve fitting was done using a least squares fitting routine of the CED software.

Rubidium Permeability

The relative permeability of the channel to Rb⁺ and to K⁺ was estimated by measuring the difference in reversal potential when either ion was present in the pipette. The reversal potential of unitary currents was measured from ensemble ramp currents, constructed as described in chapter 2. Ensemble ramp currents formed using the same voltage protocol.
when the pipette contained either K\(^+\) or Rb\(^+\) are shown in figure 7-1. It can be seen that Rb\(^+\) carries less inward current through the channel than K\(^+\), and that the current reverses at a more negative potential when the pipette contains Rb\(^+\) (assuming that there is no change in the resting potential), indicating that Rb\(^+\) is less permeant than K\(^+\). This protocol gave a reversal potential of 76.2±0.7mV positive to the cell resting potential (n=11) with K\(^+\) in the pipette, and 68.9±0.8mV (n=8) with Rb\(^+\). This shift in reversal potential was used to calculate the relative permeability of Rb\(^+\) and K\(^+\), using the equation (see Hille, 1984):

\[
\Delta E_{REV} = \frac{RT}{zF} \ln \frac{P_{Rb^+}[Rb^+]_o}{P_{K^+}[K^+]_o}
\]

(7 - 1)

where \(\Delta E_{REV}\) is the difference between the reversal potentials when the pipette contains Rb\(^+\) or K\(^+\), and \(P_{Rb^+}/P_{K^+}\) is the permeability of Rb\(^+\) relative to that of K\(^+\). The observed value of \(\Delta E_{REV}\) of -7.3mV gives a permeability ratio \((P_{Rb^+}/P_{K^+})\) of 0.75. This is very close to the value observed for delayed rectifier channels in snail neurons (0.74; Reuter & Stevens, 1980), human (0.77; Cahalan \textit{et al.}, 1985) and murine lymphocytes (0.76; Shapiro & DeCoursey, 1991a), frog node of Ranvier (0.76; Plant, 1986), and rat brown fat cells (0.81; Lucero & Pappone, 1989). It is also similar to values reported for other types of potassium channel, for example calcium activated potassium channels in rat skeletal muscle (0.67; Blatz & Magleby, 1984) and guinea pig smooth muscle (0.65; Hu \textit{et al.}, 1989), A-current channels in snail neurons (0.73; Taylor, 1987), and ATP-sensitive potassium channels in frog skeletal muscle (0.76; Spruce, Standen & Stanfield, 1987a) and rat pancreatic \(\beta\) cells (0.73; Ashcroft, Kakai & Kelly, 1989).

Channel conductance, measured as the slope of ramp currents such as those shown in figure 7-1, was 37.1±0.5pS (n=11) for K\(^+\) and 26.1±1.3pS (n=8) for Rb\(^+\) (conductances measured at the reversal potential). Current values measured from ramp currents were used to construct unitary current-voltage relationships (figure 7-2).

Inward Currents Carried by Potassium and Rubidium

Currents carried by K\(^+\) and Rb\(^+\) could also be activated by step changes in membrane potential, as shown in figure 7-3. This figure shows inward currents in two patches activated by 80mV depolarizing pulses, applied from a holding potential of 40mV more negative than the cell resting potential. At this potential, the unitary current carried by K\(^+\)
Figure 7-1  Unitary current-voltage relationships with K* and Rb+ pipette solutions. In each case, the membrane potential was held 40mV more negative than the resting potential, and then depolarised by 180mV, ie to approximately +80mV. The current trace below shows the ensemble current through a single open channel as the membrane potential was then repolarised at a rate of 2.25mV/ms. Note that Rb+ carries much less inward current than K*, and that the reversal potential is shifted to the left, ie to a more hyperpolarised potential. The apparent difference in the point at which the currents are activated is an artefact of the ensembling procedure.
Figure 7-2  Mean unitary current-voltage relationships, constructed from current values measured from ramp currents such as those shown in figure 7-1, for K* (○) and Rb* (●). Current values are the means from 6-11 patches for K*, 5-6 patches for Rb*. The error bar for K* at 10mV represents ± one S.E.M.; for all other points, ± one S.E.M. was smaller than the size of the symbol. Voltages are relative to the resting potential. The slope of the two curves at the reversal potential are 36.4pS for K* and 26.5pS for Rb*, which are in agreement with the mean values calculated from the slope of the individual unitary current ramps and quoted in the text.
(measured from the means of Gaussian curves fitted to amplitude histograms, as described in chapter 2) was -1.59±0.09pA (n=5), compared with -0.81±0.04pA (n=4) for Rb⁺. It can be seen that the currents carried by Rb⁺ are not only smaller in amplitude than those carried by K⁺, but also that channel opening is prolonged in Rb⁺. Open time histograms for these two patches are shown in figure 7-4. Both are fitted with a single exponential function, indicating a single open state of the channel. However, mean open time under these conditions is increased from 2.29±0.38ms (n=4) in K⁺ to 6.01±0.98ms (n=4) in Rb⁺. Rb⁺ has less effect on the distribution of closed times under these circumstances. When either K⁺ or Rb⁺ carry current, the closed time histogram can be fitted by the sum of three exponentials. The shortest two closed times can be identified in figure 7-5; the third always had a time constant in excess of 10ms and represented less than 10% of all closed times, and is not shown. With K⁺, the mean values of the two shortest closed time constants are 0.13±0.02ms and 1.16±0.18ms (n=3); with Rb⁺, they are 0.10±0.01ms and 2.30±0.38ms (n=4). Perhaps more significantly, the proportion of the shortest closed times was increased from 80.0±2.4% of all short closed times in K⁺ to 92.0±1.8% in Rb⁺, suggesting a change in the burst kinetics of the channel. If the shortest component of the closed times is assumed to represent closures within a burst of openings, where the channel is moving rapidly between the open state and a short-lived closed state, and the medium component of closed times represents closures between bursts, or transitions to a longer-lived closed state (see chapter 6), then the change in the relative number of short and medium closed times brought about by Rb⁺ may be due to a change in the number of times a channel opens in each burst, which must be one more than the number of short closings during each burst.

The bursting behaviour of the channel at this potential, when current was carried by K⁺ or Rb⁺, was studied directly by choosing a critical time, $t_c$, to determine if any closing was within a burst or between bursts. For each patch, a value of $t_c$ was chosen to make the proportion of short closings that were misclassified as long equal to the proportion of long closings that were misclassified as short (Colquhoun & Sakmann, 1985), by solving for $t_c$ equation 2-10 of chapter 2, taking $k=3$ and $n=1$. Values of $t_c$ found by this method were between 0.16 and 0.28ms. By analysing bursts in this way, it was found that the mean number of openings per burst at this potential was increased from 2.07±0.13 (n=3) for K⁺ to 4.91±0.85 (n=4) for Rb⁺. As a result of both the increased number of openings per burst and the increased open time, the mean burst length was increased from
Currents carried by K\textsuperscript{*} (A.) and Rb\textsuperscript{*} (B.) through single channels activated by step membrane depolarisations. In each case, the membrane potential was held 40mV more negative than the resting potential, and then depolarised 80mV (i.e. to approximately -20mV) for 400ms, once every 2 seconds. At this potential, both K\textsuperscript{*} and Rb\textsuperscript{*} carry inward current. Below the single channel records are ensemble currents averaged from 200 (in A.) or 201 (in B.) consecutive records, which represent the mean current per trace carried through the channel. Since both patches contain a single channel (see text), the ensemble currents show an increase in open probability when Rb\textsuperscript{*} carries current.
**Figure 7-4** Open time histograms constructed from data from the two patches shown in figure 7-3, where current is carried by $K^+(A.)$ and $Rb^+(B.)$. In each case, the fitted line is to the function $f(t) = A \exp(-t/\tau_{\text{open}})$, where $A$ gives the intercept on the ordinate, and $\tau_{\text{open}}$ is 3.32ms in A. and 7.30ms in B.
Figure 7-5  Closed time histograms constructed from data from the two patches shown in figure 7-3, where current is carried by $K^+$ (A.) and $Rb^+$ (B.). In each case, the fitted line is to the function $f(t) = A \exp(-t/\tau_A) + B \exp(-t/\tau_B)$, where $A$ and $B$ are 1030.5 and 28.1 in A. and 963.9 and 4.6 in B. respectively, and $\tau_A$ and $\tau_B$ are 0.0897ms and 0.830ms in A. and 0.109ms and 2.530ms in B. respectively. A third exponential component to the closed time histograms, with a time constant in excess of 10ms in each case, is not shown.
5.03±0.65ms (n=3) for K⁺ to 30.05±1.68ms (n=4) for Rb⁺. This is illustrated in figure 7-6, which shows the burst time histograms measured directly from the two patches shown in figure 7-3.

Despite the smaller unitary current amplitude in Rb⁺, it can be seen from the ensemble averaged current in figure 7-3 that the average current per sweep carried by Rb⁺ is greater than that carried by K⁺, indicating a higher open probability when Rb⁺ carries current (since both records are from patches containing a single channel, according to the criteria set out in chapter 2). The peak open probability under these conditions is increased from 0.12±0.02 (n=5) for K⁺ to 0.67±0.04 (n=4) for Rb⁺. Since Rb⁺ does not speed up the activation of the channel (see below), and inactivation effectively does not take place at such a negative potential, the increase in open probability is presumably a direct result of the effect of Rb⁺ on channel burst kinetics. The increase in burst duration seen in Rb⁺, caused by both an increase in open time and in the number of openings per burst, means that although Rb⁺ carries less current through an open channel, more charge (given by current multiplied by open time) is passed during each burst of openings. External Rb⁺ also increases the open probability of calcium activated potassium channels from rat skeletal muscle (Demo & Yellen, 1992), an effect which was explained in terms of Rb⁺ interfering with channel closing.

The effect of Rb⁺ on channel activation is shown in figure 7-7. Here the ensemble average currents from figure 7-3 have been superimposed; the current carried by K⁺ has been scaled up by a factor of 2.47, such that the maximum current amplitudes overlie one another. It can be seen that the current approaches this maximum more rapidly when K⁺ carries the current, suggesting that Rb⁺ slows channel activation. However, it must be remembered that the channel open probability is much higher at the peak of the Rb⁺ current; it may take longer for the channel to reach this steady level, where open and closed states are in equilibrium, even if the rates of channel activation are unchanged, because of the slowing of closing rates (see below). Certainly, such a change in activation kinetics is not obvious from studying the first latency histograms for these two patches, which are shown in figure 7-8. Rb⁺ slightly slows the activation of delayed rectifier channels in frog skeletal muscle (Spruce et al., 1989), although in most cases it has been reported to have no effect on activation (Cahalan et al., 1985; Matteson & Swenson, 1986; Plant, 1986; Lucero & Pappone, 1989; Shapiro & DeCoursey, 1991b). Several divalent cations, in particular
Figure 7-6  Burst time histograms constructed from data from the two patches shown in figure 3, where current is carried by K⁺ (A.) and Rb⁺ (B.). These were constructed by grouping together into bursts all openings separated by closed times shorter than a critical time $t_c$, determined as described in the text, and then measuring the duration of these bursts. In each case, the fitted curve is to the function $f(t) = A \exp(-t/\tau_{\text{burst}})$, where $A$ gives the intercept on the ordinate and $\tau_{\text{burst}}$ is 6.15ms in A. and 34.48ms in B.
Figure 7-7 Ensemble average currents for K⁺ and Rb⁺ during a depolarising step, taken directly from figure 7-3. The current carried by K⁺ has been scaled up 2.47 times, so that the rate at which the currents approach maximum activation can be directly compared (see text). The vertical scale bar corresponds to 0.1pA for the current carried by K⁺, and 0.247pA for that carried by Rb⁺.
Figure 7-8 First latency histograms for K⁺ (A.) and Rb⁺ (B.), constructed from the two patches shown in figure 7-3. The form of these histograms gives an index of the activation processes of the channels. The apparent decrease in activation rate in Rb⁺ seen in figure 7-7 is not reflected in a significant change in the distribution of first latencies.
slow delayed rectifier activation kinetics in frog skeletal muscle (Stanfield, 1975),
squid giant axon (Gilly & Armstrong, 1982) and squid neurons (Spires & Begenisich,

Effects of Rb⁺ on tail currents

The effect of Rb⁺ on channel closing was also examined by studying the prolongation
of tail currents seen on repolarization from a depolarized potential. An example is shown
in figure 7-9. This shows tail currents recorded from two patches at a potential of 40mV
negative to the cell resting potential, following a brief depolarizing step to the reversal
potential to activate the channels. It can be seen from the unitary current records that not
only is open time increased in Rb⁺, but also the channel reopens several times before falling
silent. The effects of Rb⁺ are reflected in the prolongation of the ensemble current shown.
In both K⁺ and Rb⁺, the ensemble tail current can be fitted by an exponential decay, as
illustrated in figure 7-10a. The time constant of this exponential decay is dependent on
the holding potential when either K⁺ or Rb⁺ carry the tail current; this voltage dependence
is illustrated in figure 7-10b. Rb⁺ increases this decay time constant by between 6 and 15
told. Other studies have found increases in delayed rectifier tail current decay time
constants with rubidium of 2 fold (Cahalan & Pappone, 1983), 5 fold (Cahalan et al.,
1985), 1.2-2 fold (Lucero & Pappone, 1989), 10 fold (Spruce et al., 1989), 2.5-10 fold
(Sala & Matteson, 1991) and more than 10 fold (Shapiro & DeCoursey, 1991).

Open time histograms for tail currents, like those for currents activated during a
depolarising step, could be fitted with a single exponential function. Averaged over all
voltages, Rb⁺ increased channel open time by a factor of approximately 2.8 during tail
currents, compared with a 2.6 fold increase in mean open time when Rb⁺ carries current
during step changes in voltage (see above). This is very similar to the 2.65 fold increase
in the mean open time of delayed rectifier channels in frog skeletal muscle when Rb⁺
carries current (Spruce et al., 1989). The effect of external Rb⁺ on mean open time is
shown in figure 7-11. At voltages below 80mV more positive than the resting potential,
when current is inward, Rb⁺ has a constant effect on mean open time (see above). However,
above this voltage, when current is outward, the effect of external Rb⁺ is much smaller.
Open time is still increased by external Rb⁺, even though outward currents would be
expected to be carried by K⁺ whatever the ionic composition of the pipette solution.

As can be seen from figure 7-9, as well as increasing open time, Rb⁺ increases the

Zn²⁺, slow delayed rectifier activation kinetics in frog skeletal muscle (Stanfield, 1975),
squid giant axon (Gilly & Armstrong, 1982) and squid neurons (Spires & Begenisich,
Figure 7-9 Unitary tail currents carried by K⁺(A.) and Rb⁺(B.). In each case, the membrane potential is held 40mV more negative than the resting potential, and then given a brief (10ms) depolarising pulse to the reversal potential (in A. the amplitude of the depolarisation was 120mV, in B. it was 100mV) once every second. This pulse activates the channels, but no net current flows until the membrane is repolarised. The inward currents seen on repolarisation are shown, and beneath them are the ensemble currents averaged from 199 (in A.) or 298 (in B.) consecutive records.
A. POTASSIUM

1pA
5ms

B. RUBIDIUM

10pA
5ms
Figure 7-10 Exponential decay of ensemble averaged tail currents. In A., the ensemble tail currents shown in figure 7-9 are fitted with a single exponential decay function of the form $f(t) = A \exp(-t/\tau_{\text{tail}})$, where $A$ gives the peak inward current, and $\tau_{\text{tail}}$ is 0.46ms for $K^+$ and 2.07ms for $Rb^+$. In B., the mean value of $\tau_{\text{tail}}$ from several patches is shown as a function of voltage, for both $K^+ (\bigcirc; n=5-8)$ and $Rb^+ (\bullet; n=3-6)$. Voltages in part B. are relative to the resting potential. Error bars represent ± one S.E.M.
Figure 7-11 Mean open times at different voltages for $K^+$($\bigcirc$) and $Rb^+$($\bullet$) pipette solutions, measured from both step depolarisations and from tail currents. A. shows the mean open time as a function of voltage (relative to the resting potential), B. shows the mean of the reciprocals of open time. Error bars, where shown, represent ± one S.E.M. $n=3$-5 except for $K^+$ at 0mV ($n=2$) and at 140mV ($n=1$), and $Rb^+$ at 100mV ($n=1$). The vertical axis is placed close to the current reversal potential, so that points to the left of this are from inward currents (carried by $K^+$ or $Rb^+$), whilst points to the right are from outward currents, which should be carried by $K^+$ in all cases. Replacing external $K^+$ by $Rb^+$ has a much smaller effect on channel open time when current is outward than when it is inward.
number of times the channel opens during each tail. These openings are grouped into bursts, similar to those which occur at depolarised potentials, which indicates that the channel can return from a second, longer-lived closed state during a tail current. This is reflected by the presence of two exponential functions in the tail current closed time histograms for Rb⁺ at all voltages, and for K⁺ at the cell resting membrane potential (0mV in table 7-1.). At all other voltages, the tail current closed time histogram for K⁺ was fitted by a single exponential function, indicating that the second closed state is absorbing under these conditions. For both Rb⁺ and K⁺, closed times during tail currents appeared independent of voltage. The fast time constant (averaged over all voltages) was 0.21±0.02ms (n=12) for Rb⁺ and 0.20±0.04ms (n=9) for K⁺. The slow time constant seen in the presence of Rb⁺, averaged over all voltages, was 2.30±0.21ms (n=12). Closed time histograms for both K⁺ and Rb⁺ during a tail current at 20mV more negative than the resting potential are shown in figure 7-12.

It was assumed that where the tail current closed time histogram was the sum of two exponential components, the shorter closed times represented closings within a burst of openings, and longer closed times resulted from closings between bursts. The properties of bursts of openings during tail currents were studied in the same way as those occurring during depolarising steps. Values of \( t_e \) calculated from equation 2-10 of chapter 2, were between 0.35 and 0.48ms. The properties of bursts of openings during tails are summarised in table 7-1.

The number of bursts per tail includes only those tails which contained at least one burst, ie it ignores those traces in which no openings occurred following repolarisation, where the channel was presumably closed at the end of the depolarizing pulse. Where the closed time histogram was fitted by a single exponential (ie for K⁺ at voltages other than 0mV), it was assumed that all tails consisted of only one burst. It can be seen that at all voltages studied, Rb⁺ increases both the number of openings in each burst, and the number of bursts occurring in each tail. Both of these effects will contribute to the prolongation of tail currents by Rb⁺.
Figure 7-12 Closed time histograms constructed from tail current data, where tail currents were carried by K⁺ (A.) or Rb⁺ (B.) at a potential 20mV more negative than the cell resting potential. The histogram in A. contains very few events, due to the low probability of the channel reopening at this potential when K⁺ carries current. This histogram has been fitted by a single exponential function of the form \( f(t) = A \exp(-t/\tau_{\text{closed}}) \), where \( A=32.1 \) and \( \tau_{\text{closed}}=0.183 \text{ms} \). More events are included in B., primarily since the channel reopens more readily during a tail when Rb⁺ carries the current. This histogram has been fitted with a double exponential function of the form \( f(t) = A \exp(-t/\tau_A) + B \exp(-t/\tau_B) \), where \( A=306.1 \) and \( B=3.02 \), and the time constants of the two exponential functions are \( \tau_A=0.177 \text{ms} \) and \( \tau_B=2.327 \text{ms} \). The implications of the presence of this second exponential function of the closed time histogram only when Rb⁺ carries current are discussed in the text.


Table 7-1. Properties of Bursts of Channel Openings During Tail Currents

<table>
<thead>
<tr>
<th>Voltage (mV)</th>
<th>Openings per Burst</th>
<th>Bursts per Tail</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K⁺ (n)</td>
<td>K⁺ (n)</td>
</tr>
<tr>
<td></td>
<td>Rb⁺ (n)</td>
<td>Rb⁺ (n)</td>
</tr>
<tr>
<td>0</td>
<td>1.62</td>
<td>2.99±0.22</td>
</tr>
<tr>
<td></td>
<td>(n=2)</td>
<td>(n=3)</td>
</tr>
<tr>
<td>-20</td>
<td>1.51±0.14</td>
<td>2.28±0.10</td>
</tr>
<tr>
<td></td>
<td>(n=3)</td>
<td>(n=3)</td>
</tr>
<tr>
<td>-40</td>
<td>1.18±0.07</td>
<td>2.25±0.19</td>
</tr>
<tr>
<td></td>
<td>(n=5)</td>
<td>(n=4)</td>
</tr>
<tr>
<td>-60</td>
<td>1.17</td>
<td>1.66</td>
</tr>
<tr>
<td></td>
<td>(n=2)</td>
<td>(n=2)</td>
</tr>
</tbody>
</table>

Voltages given are relative to the cell resting membrane potential.

**DISCUSSION**

**Rubidium Permeability**

The observed value of $P_{\text{Rb}⁺}/P_{\text{K}⁺}$ of 0.75 in these channels, which is similar to that reported for other potassium channels (see above), is greater than the ratio of current amplitudes carried by rubidium or potassium (see figure 7-2). This discrepancy, a deviation from the independence relation of Hodgkin & Huxley (1952a; see chapter 1) probably reflects binding of permeant ions to a saturable sites within the pore, which will reduce single channel currents but not affect reversal potentials (Hille & Schwarz, 1978), and indicates that rubidium binds more tightly within the pore than does potassium.

**Rubidium and Channel Gating**

The effects of rubidium on gating were to slow channel closing and to also promote reopening at hyperpolarized potentials, without significantly affecting activation. These effects may be viewed in terms of the linear state scheme constructed to fit the data obtained for this channel using physiological ionic gradients at depolarized potentials (see chapter 6):
The inactivated states have been omitted in this instance.

Since rubidium does not speed activation, it may be assumed that it does not increase any of the forward rate constants \((k_1, k_2, k_3)\). However, the prolongation of open time indicates that the rate constant \(k_3\), which is the reciprocal of mean open time, is slowed by rubidium. A similar scheme has been proposed to explain the effects of rubidium on delayed rectifier channel gating in human lymphocytes (Cahalan \textit{et al.}, 1985) and frog skeletal muscle (Spruce \textit{et al.}, 1989).

The above scheme will, with appropriate transition rate constants, predict the bursting behaviour seen in these channels (see chapter 6). Thus, a burst of openings will consist of a number of successive transitions between states \(C_3\) and \(O\); the burst will be terminated when the channel enters state \(C_2\), which represents a longer-lived closed state. The channel will then, depending on the ratio \(k_2/k_1\), return to \(C_3\), possibly allowing another burst to begin, or move to state \(C_1\), which at the negative voltages at which tail currents were recorded is considered absorbing, and which during depolarising steps represents the longest element of closed times.

According to this scheme, the increase in the number of openings per burst in rubidium must be due to an increase in the ratio \(k_2/k_3\), which determines the probability that a channel in \(C_3\) will move to \(O\) rather than \(C_2\). Since it is assumed that rubidium does not increase the forward rate constant \(k_3\) (see above), the increase in the number of openings per burst must be due solely to a slowing of the rate constant \(k_2\). Similarly, the increase in the number of bursts per tail caused by rubidium implies a decrease in the rate constant \(k_1\). Thus, these results suggest that whilst rubidium affects none of the forward rate constants in the above scheme, it slows all of the backward rate constants \((k_2, k_3, k_3)\), with the slowing effect on each rate constant being similar.

These results do not discriminate between the different models which have previously been suggested for permeant ion effects on channel gating. However, since rubidium can alter the rate constants \(k_1\) and \(k_2\) in the above scheme when the channel is closed, they argue against the "occupancy hypothesis" in its simplest sense, whereby permeant ions

\[
\begin{array}{ccc}
  k_1 & \rightarrow & k_2 \\
  \leftarrow & C_1 & \rightarrow & C_2 \\
  k_{-1} & \leftrightarrow & k_2 \\
  & & \leftrightarrow & C_3 \\
  & & \leftrightarrow & O \\
\end{array}
\]
could only affect closing by binding within an open pore to prevent channel closing. The fact that external rubidium appears to prolong channel open time even when the permeant ion is potassium (i.e. during outward current) similarly argues against a site of action for rubidium within the pore.
CHAPTER EIGHT: BLOCK OF SINGLE DELAYED RECTIFIER CHANNELS IN CA1 NEURONS BY EXTERNAL TETAETHYLAMMONIUM IONS

INTRODUCTION

Tetraethylammonium (TEA) ions are known to block most potassium channels, when applied to either the internal or external face of the membrane (Stanfield, 1983). These effects are generally assumed to occur because the TEA ion has a similar diameter to that of a hydrated potassium ion (Armstrong, 1966a), so that it can enter the wide mouth of the channel but cannot pass through the narrowest part where the potassium ions lose their water of hydration.

TEA was first shown to block potassium channels in squid giant axons, where internal TEA blocks delayed rectifier channels, although external TEA is ineffective (Tasaki & Hagiwara, 1957). In a series of experiments, Armstrong (1966b, 1969, 1971) showed that the delayed rectifier current in squid axons could be activated normally after the axon had been injected with TEA, but that TEA (and other quaternary ammonium compounds) subsequently caused inactivation of the current, which led him to suggest that internal TEA could only interact with open channels. However, the effects of internal TEA on delayed rectifier channels in squid axons have subsequently been described by another model in which TEA block occurs independently of channel gating (Clay, 1985).

Despite the lack of effect on squid axons, TEA also blocks delayed rectifier channels in most preparations when applied from the outside (Stanfield, 1983), acting at a different site than internal TEA. External TEA usually reduces the amplitude of the delayed rectifier current without altering its time course (Hille, 1967; Armstrong & Hille, 1972), which has been explained in terms of TEA block being independent of channel gating. Single channel recording has since shown that the rates of blocking and unblocking by external TEA are much faster than those of delayed rectifier channel opening and closing in frog skeletal muscle (Standen et al., 1985; Spruce, Standen & Stanfield, 1987b) and murine T lymphocytes (DeCoursey et al., 1987). These studies have shown that block is very fast, so that individual blocking events cannot be resolved in the frequency range of the recording, and the filtered current record shows an apparent reduction in unitary amplitude. External TEA does not alter the apparent mean open time (which actually includes time
spent in both open and blocked states) of single delayed rectifier channels in frog skeletal muscle (Spruce et al., 1987b), indicating that these channels can open and close normally whilst blocked by TEA. However, external TEA does prolong the mean open time of ATP-sensitive potassium channels in frog skeletal muscle (Davies et al., 1989), indicating that these channels cannot close whilst blocked by TEA. Block by external TEA also alters the gating of a slowly inactivating voltage dependent potassium channel in human T lymphocytes (Grissmer & Cahalan, 1989a), reducing the peak current but at the same time slowing inactivation, so that the total amount of charge passed by the channel during very long depolarisations is the same. This indicates that these channels cannot enter the inactivated state whilst they are blocked by TEA. A similar effect of external TEA block on slow inactivation is seen in cloned Shaker channels which have been genetically modified to remove the fast inactivation process (Choi et al., 1991).

The delayed rectifier current in a number of mammalian central neurons has been shown to be blocked by external TEA, although no detailed study of its effects has been carried out. Typically, concentrations in the range 10-30mM have been used to block the delayed rectifier current, although the concentration required to block half of the current (the IC$_{50}$) in rat thalamic neurons is only 4mM (Huguenard & Prince, 1991). In a study of single delayed rectifier channels in cultured rat hypothalamic neurons, single channel activity was reversibly abolished by application of 10mM TEA to the external face of an outside-out patch (McLamon, 1989), although the effects of lower concentrations were not reported.

More detailed studies have been made on the effects of TEA on cloned rat and mouse brain potassium channels, which have either delayed rectifier or A-current like properties (see chapter 1). Although these channels are all highly structurally homologous, there is a wide range of sensitivities to block by external TEA. In some cases, IC$_{50}$s below 1mM have been reported (Christie et al., 1989; Yokoyama et al., 1989; T.McCormack et al., 1990; Schröter et al., 1991; Rettig et al., 1992), whilst in others, channels have been found to be virtually insensitive (Stühmer et al., 1989; Christie et al., 1990). These differences in TEA sensitivity have been used to identify those parts of the channel which form the receptor for external TEA, and must therefore lie close to the outer mouth of the channel pore (MacKinnon & Yellen, 1990; Hartmann et al., 1991; Kavanaugh et al., 1991; Tagliatela et al., 1991; see chapter 1). Of particular importance is a tyrosine residue in
the link between the S5 and S6 transmembrane domains (Kavanaugh et al., 1991) which is also present in a cloned Drosophila calcium-activated potassium channel which is highly sensitive to block by external TEA (Adelman et al., 1992). It has been shown that external TEA interacts with one such tyrosine residue from each of the four subunits in a voltage-gated potassium channel (Heginbotham & MacKinnon, 1992; Kavanaugh, Hurst, Yakel, Varnum, Adelman & North, 1992). This interaction appears to be between the cationic TEA molecule and the π electron orbitals of the aromatic rings of the tyrosine residues (Kavanaugh et al., 1991; Heginbotham & MacKinnon, 1992), which is similar to the interactions underlying high-affinity binding of quaternary ammonium ions to synthetic receptors (Dougherty & Stauffer, 1990) and of acetylcholine to acetylcholinesterase (Sussman, Harel, Frolov, Oefner, Goldman, Toker & Silman, 1991). Other nearby parts of the channel have been shown to be involved in the binding of internal TEA to a separate receptor (Hartmann et al., 1991; Taglialatela et al., 1991; Yellen et al., 1991; see chapter 1). The proximity of these two binding sites is indicated by the fact that binding of TEA to one site antagonises binding to the other (Newland, Adelman, Tempel & Almers, 1992).

RESULTS

All experiments were carried out on hippocampal CA1 neurons, grown in culture for between 6 and 14 days, using the on-cell patch clamp technique (see chapter 2). The patch pipette contained low K⁺ patch solution, to which small volumes of a TEA stock solution were added as required (see chapter 2). Current signals were simultaneously recorded onto the hard disk of the Dell System 210 computer (via the CED 1401 interface) and onto video tape (via the digital audio processor), as described in chapter 2. Current records written directly onto hard disk were filtered on-line using an 8-pole Bessel filter set at 2kHz (-3dB filter rate) and digitised at 10kHz; those recorded on video tape bypassed this filter, but were still filtered by the 10kHz 3-pole Bessel filter inside the EPC-7. Measurement of unitary current amplitudes, construction of ensemble average currents and measurement of open and closed times were all carried out on the Dell computer using CED “Patch” software, as described in chapter 2. Open and closed times were not corrected for missed events (see chapter 2), except where indicated. Nor was a minimum resolution routinely applied to the data.

Current signals recorded onto video tape were used for amplitude distribution
analysis and analysis of excess current variance (see chapter 2). For amplitude distribution analysis, these current records were played back through an 8-pole Bessel filter set at 1kHz (-3dB filter rate; this gives an approximate overall low-pass filter frequency, \( f \), given by
\[
\frac{1}{f} = \frac{1}{f_1} + \frac{1}{f_2}
\]
where \( f_1 \) is this 1kHz filter and \( f_2 \) is the 10kHz filter of the EPC-7, of 0.995kHz)

and digitised at 5kHz onto the hard disk of a Dell System 325 or 450DE computer for analysis using “Tracan” software (see chapter 2). For analysing excess current variance, these same current records were played back through an 8-pole Butterworth filter set at 4kHz (-3dB filter rate; overall low-pass filter frequency 3.71kHz) and digitised at 8192Hz onto the hard disk of the PDP11-73 computer for analysis using “Noise” software (see chapter 2).

**External TEA Blocks Delayed Rectifier Channels**

Single channel currents recorded during depolarising steps to 100mV more positive than the cell resting membrane potential are shown in figure 8-1, in the absence of external TEA and when the pipette contained 30\( \mu \)M or 100\( \mu \)M TEA. It can be seen that external TEA has two effects on these currents. First, it reduces the amplitude of the currents and increases open level noise, as illustrated by the amplitude histograms shown in figure 8-2. This is presumably due to a “flickery” block of the channel, where the individual blocking and unblocking events are too fast to be resolved at the low-pass filter frequency used (see chapter 2). Secondly, TEA causes brief interruptions of the current, so that mean open time is reduced, as shown in figure 8-3. This indicates a distinct, kinetically slower block. Because of the differences in blocking kinetics, these two components of the block will be termed the “fast block” and ”slow block” respectively.

**Properties of the Fast Block**

The concentration dependence of the fast block was considered in terms of a simple blocking mechanism:

\[
\begin{align*}
&k_+ &\beta[\text{TEA}] \\
&\text{C} &\rightarrow &\text{O} &\rightarrow &\text{B} &\text{(8 - 1)} \\
&k_- &\alpha &
\end{align*}
\]
Figure 8-1  Block of single delayed rectifier channel currents by low concentrations of external TEA. In each case, the currents were elicited by depolarising steps to a potential 100mV positive to the resting potential. It can be seen that the presence of TEA (30 or 100µM) in the pipette causes both a reduction in the unitary amplitude and a reduction in the duration of each channel opening.
Control

30µM TEA

100µM TEA
Figure 8-2  Amplitude histograms formed from the patches illustrated in figure 1. Each histogram has been fitted by two Gaussian curves, one to the open level current and one to the baseline current. The mean (± one standard deviation) of the Gaussian fit to the open level current was 1.505±0.240pA in control, 1.121±0.274pA in 30μM TEA and 0.610±0.228pA in 100μM TEA. This reduction in mean unitary amplitude was assumed to be due to a fast block of the channel by TEA. The standard deviations of the Gaussians fit to the closed levels (which all had amplitudes of 0pA) were 0.137, 0.116 and 0.154pA respectively. In the presence of TEA, a Gaussian curve gives a less good fit to the open level current, which is skewed towards the closed level; this effect is taken advantage of in the fitting of β-distributions to the amplitude histograms (see text), although it is due not only to the fast block, but also partially to the slow block and to normal channel gating.
Figure 8-3. Open time histograms formed from the patches illustrated in figure 1. In each case, the histogram has been fitted with a single exponential function (indicating the presence of a single open state) of the form \( f(t) = A \exp(-t/\tau_{\text{open}}) \) where \( A \) gives the intercept on the ordinate and \( \tau_{\text{open}} \) is 5.04 ms in control, 1.36 ms in 30 \( \mu \)M TEA, and 0.57 ms in 100 \( \mu \)M TEA.
where $C$ represents at least three closed states (see chapter 6), $O$ the open state and $B$ the blocked state. $k_i$ and $k_j$ are the rate constants for channel opening and closing, whilst $\beta$ and $\alpha$ are the rate constants for blocking and unblocking, such that the dissociation constant $K_d$ is equal to $\alpha/\beta$. It is not necessary at this stage to consider whether or not block is independent of gating, i.e., whether or not blocked channels can close. According to this scheme, the openings of reduced amplitude recorded in the presence of TEA actually represent time spent flickering rapidly between states $O$ and $B$. Obviously, the amplitude of the filtered current will be less the larger proportion of this time is spent in state $B$, which is given by:

$$\frac{T(B)}{T(B) + T(O)} = \frac{\beta[TEA]}{\alpha + \beta[TEA]} \quad (8-2)$$

where $T(B)$ is the total time spent in state $B$, and $T(O)$ the total time spent in state $O$. This can be rearranged to give the relationship between the fractional current amplitude (unitary amplitude in the presence of TEA as a fraction of control) and TEA concentration:

$$\frac{m_i}{i} = \frac{1}{1 + ([TEA]/K_d)} \quad (8-3)$$

where $m_i$ is the mean current amplitude in the presence of TEA, and $i$ is the control current amplitude.

In spite of the reduction in mean open time caused by the slow block by TEA (see below), and also in spite of the poor fit of Gaussian curves to the open level amplitude histograms in the presence of TEA (see figure 8-2), it was possible to measure $m_i$ in the same way as $i$, that is from amplitude histograms such as those shown in figure 8-2. $m_i$ and $i$ were also measured directly from unitary ensemble ramp currents, such as those shown in figure 8-4. Values of $m_i$ and $i$ measured by these two methods were in close agreement, and were pooled to construct unitary current-voltage relationships for control and the different TEA concentrations used (see figure 8-5). This figure shows that the fast block is practically voltage independent for all TEA concentrations studied. For this reason, fractional currents ($m/i$) measured at different voltages are pooled in figure 8-6, which illustrates the concentration dependence of the fast block. The points in figure 8-6 have been fitted by equation 8-3, with a $K_d$ of $53.4\mu$M; this is consistent with 1:1 binding between TEA molecules and the channel.
Figure 8-4. Reduction in unitary amplitude caused by external TEA, as illustrated by ramp currents. Each current trace represents the ensemble current through a single delayed rectifier channel during a ramp change in membrane potential, formed as described in chapter 2, when the patch pipette contained (from top to bottom) 0, 10, 30 or 60μM TEA. The effects of 100μM TEA were not investigated using this ramp protocol. Each patch illustrated here was held 20mV negative to the resting potential before giving a 160mV depolarising step to activate the channels; the membrane potential was then returned to the holding potential linearly with time over the next 100ms, ie at a rate of 1.6mV/ms. It can be seen that TEA reduces the amplitude of the current without changing the shape of the ensemble current, suggesting that the degree of block is the same at all membrane potentials.
Figure 8-5  Unitary current-voltage relationships in the presence of different external concentrations of TEA, formed using data from amplitude histograms (figure 2) and ensemble ramp currents (figure 4). Voltage here is relative to the resting potential. Each point represents the mean ± one S.E.M. (where this is larger than the symbol) from 8-16 patches in control (●), 9-10 patches in 10μM TEA (○), 6-12 patches in 30μM TEA (■), 3-5 patches in 60μM TEA (□) and 3-5 patches in 100μM TEA (▼). As with figure 4, the form of the current-voltage relationship suggests that the fast block is voltage independent.
Figure 8-6 Concentration dependence of the block by external TEA. Each point represents the mean unitary current in the presence of different concentrations of TEA, as a fraction of the control current, averaged over all voltages studied. Each point is the mean of 5-9 patches; at each concentration, ± one S.E.M. is smaller than the size of the symbol, hence no error bars are shown. The fitted line is to equation 3, and gives a $K_d$ for the fast block of 53.4μM.
The kinetics of the fast block were studied by two methods: amplitude distribution analysis (Yellen, 1984; see chapter 2) and analysis of excess current variance (Ogden & Colquhoun, 1985; see chapter 2). Amplitude histograms for currents recorded at 100mV positive to the cell resting potential, when the pipette contained 0, 30, or 100μM TEA, are shown in figure 8-7. In each case, the closed level histogram has been fitted by a Gaussian curve, as described in chapter 2. For control, the open level histogram has also been fitted by a Gaussian curve, whilst in the presence of TEA the open level histograms have been fitted by β functions of the kind described in chapter 2. Amplitude distribution analysis was not used to study the kinetics of the block caused by 10μM TEA, as the open level histogram under these conditions was well fitted by a Gaussian distribution, even when the Bessel filter rate was reduced to 250Hz. The blocking and unblocking rates used to generate the β distributions in figure 8-7 (which are given by β[TEA] and α respectively in equation 8-1) were 11.4ms⁻¹ and 19.7ms⁻¹ for 30μM TEA and 36.8ms⁻¹ and 20.0ms⁻¹ for 100μM TEA. These values correspond to Kₛ (given by α/β) of 51.8μM and 54.3μM respectively, in close agreement to those calculated by the reduction in unitary amplitude (53.4μM; see figure 8-6).

Spectral density functions of the excess variance associated with an open channel at 100mV positive to the cell resting potential are shown in figure 8-8, where the patch contained 0, 30 or 100μM TEA. These are difference spectra, formed by subtracting the spectrum for the baseline noise from that for a single open channel as described in chapter 2. Each spectrum in figure 8-8 has been fitted by the sum of two Lorentzian functions of the kind described in chapter 2. For all difference spectra formed, over all TEA concentrations, the low-frequency component had a mean cut-off frequency, fₑ, of 182±10Hz (n=56), corresponding to a time constant (given by 1/2πfₑ) of 0.87ms; this component probably represents the fastest component of channel gating, since the briefest closed times could not be excluded from the open level currents used to form the power spectra. Slow block by TEA also probably contributed to this component of the noise; however, this would have only a very small effect on the total current variance, which is dominated by the higher frequency component of the noise. This high frequency component had a mean fₑ of 3510±110Hz (n=56), close to the cut-off frequency associated with the low-pass filtering of the current. The total current noise variance of each spectrum, σ², was calculated according to the equation (see equation 2-30, chapter 2):

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Figure 8-7 Amplitude histograms, formed using “Tracan” software as described in the text. In control, the open level current has been fitted with a Gaussian curve with a mean of 1.424pA and a standard deviation of 0.195pA. In both 30 and 100μM TEA, the open level current has been fitted with a β-distribution of the kind described in chapter 2. The β-distributions have been formed assuming blocking and unblocking rates of 11.4ms⁻¹ and 19.7ms⁻¹ for 30μM TEA and 36.8ms⁻¹ and 20.0ms⁻¹ for 100μM TEA respectively. For each histogram, the baseline current has been fitted with a Gaussian curve with a mean of zero and standard deviations of 0.111pA (control), 0.083pA (30μM TEA) and 0.119pA (100μM TEA).
Control

Amplitude (pA)

Number of Points

30μM TEA

Amplitude (pA)

Number of Points

100μM TEA

Amplitude (pA)

Number of Points
Figure 8-8  Spectral density functions of the excess noise associated with a single open channel in the presence of different concentrations of TEA. These difference spectra were formed using “noise” software, as described in the text. Each has been fitted by the sum of two Lorentzian curves, such that the total excess variance in each case (equal to the area under the curve) is given by equation 4 of the text. In control, the fitted Lorentzian curves have $S(0)_1 = 5.2 \times 10^{-5} \text{ pA s}$, $\nu_1 = 300\text{ Hz}$, $S(0)_2 = 2.05 \times 10^{-4} \text{ pA s}$ and $\nu_2 = 3700\text{ Hz}$, giving a total variance (from equation 4) of 0.144$\text{ pA}^2$; for 30$\mu$M TEA, $S(0)_1 = 2.8 \times 10^{-5} \text{ pA s}$, $\nu_1 = 320\text{ Hz}$, $S(0)_2 = 7.4 \times 10^{-5} \text{ pA s}$, $\nu_2 = 2800\text{ Hz}$ and total variance 0.340$\text{ pA}^2$; and for 100$\mu$M TEA, $S(0)_1 = 2.2 \times 10^{-5} \text{ pA s}$, $\nu_1 = 240\text{ Hz}$, $S(0)_2 = 3.0 \times 10^{-5} \text{ pA s}$, $\nu_2 = 4900\text{ Hz}$ and total variance 0.239$\text{ pA}^2$. It can be seen by inspection of the spectra that the total variance (the area under the fitted curve) increases as TEA concentration is increased, but begins to decrease again at very high TEA concentrations; the binomial theorem predicts that the variance will be greatest when the fractional current is 0.5, ie when the TEA concentration is equal to the $K_d$ of the fast blocking reaction (approximately 53.4$\mu$M, from figure 6).
\[
\sigma^2 = \frac{S(0)_1 \pi f_{c1}}{2} + \frac{S(0)_2 \pi f_{c2}}{2}
\]  \hspace{1cm} (8-4)

where \(S(0)_1\) is the spectral density of each Lorentzian component at zero frequency, and \(f_c\) is the cut-off frequency of each Lorentzian. The excess variance caused by fast TEA block was calculated as the difference between that in the presence of TEA and that in control under the same conditions. The ratio of this excess variance to the theoretical excess variance calculated from the binomial theorem (see equation 2-28, chapter 2) was used to calculate the original cut-off frequency of the noise due to fast TEA block. This ratio, given by \(R\) in equation 2-31 of chapter 2, was always less than 2.5 for experiments with 10\(\mu\)M TEA, so that this method could not be used to accurately estimate the blocking kinetics of this concentration of TEA (in fact, often no excess current variance could be detected in the presence of 10\(\mu\)M TEA). For all other concentrations, \(R\) was greater than 2.5, so that the estimate of the cut-off frequency of the noise due to fast TEA block could be calculated to within 5\% accuracy (see chapter 2). This cut-off frequency, together with the \(K_d\) value calculated from the reduction in unitary amplitude (53.4\(\mu\)M; see figure 8-6), was used to calculate the blocking and unblocking rates (\(\beta\) [TEA] and \(\alpha\), from equation 8-1) as described by equations 2-32 and 2-34 in chapter 2. For example, the patches shown in figure 8-8 for 30 and 100\(\mu\)M TEA gave excess variances of 0.214\(p\)A\(^2\) and 0.114\(p\)A\(^2\), compared with predicted values (according to equation 2-28 of chapter 2) of 0.573\(p\)A\(^2\) and 0.559\(p\)A\(^2\). With an overall filter cut-off frequency of 2.86kHz (see above), this gives a cut-off frequency associated with the fast TEA block of 6.36kHz (for 30\(\mu\)M TEA) and 11.7kHz (for 100\(\mu\)M TEA). Assuming a \(K_d\) of 53.4\(\mu\)M (from figure 8-6), this gives a blocking rate of 14.4\(ms^{-1}\) for 30\(\mu\)M TEA and 47.9\(ms^{-1}\) for 100\(\mu\)M TEA, and unblocking rates of 25.6\(ms^{-1}\) for both 30\(\mu\)M TEA and 100\(\mu\)M TEA.

The concentration dependence of the blocking and unblocking rates calculated by both these methods is shown in figure 8-9. It can be seen that the values of both the blocking rates (figure 8-9A) and the unblocking rates (figure 8-9B) calculated by the two methods are in good agreement. The blocking rate is linearly related to TEA concentration, such that the underlying rate constant \(\beta\) is the same at all TEA concentrations. The unblocking rate constant, \(\alpha\), is similarly concentration independent. \(\alpha\) and \(\beta\) were also independent of membrane potential, as illustrated in figure 8-10. The mean results shown in figure 8-9 suggest a mean open time of 89.4\(\mu\)s in 30\(\mu\)M TEA, 38.5\(\mu\)s in 60\(\mu\)M TEA.
Figure 8-9  Concentration dependence of the blocking (A.) and unblocking (B.) rates of the fast block, as calculated by amplitude distribution analysis (●) and by analysis of excess current variance (○). Each point represents the mean ± one S.E.M. of the results from 5-14 experiments (amplitude distribution analysis) or 3-7 experiments (analysis of excess current variance). The dashed line in A. is a regression fit to both sets of data, showing that the blocking rate is linearly related to the TEA concentration. The unblocking rate (B.) appears independent of TEA concentration.
Figure 8-10 Voltage dependence of the blocking rate constant $\beta$ (A.) and the unblocking rate constant $\alpha$ (B.) of the fast block, as calculated by amplitude distribution analysis (●) and by analysis of excess current variance (○). Each point represents the mean ± one S.E.M. of the results from 3-11 experiments (amplitude distribution analysis) or 3-6 experiments (analysis of excess current variance). Both $\beta$ and $\alpha$ show little apparent voltage dependence. Voltage here is relative to the resting potential.
and 25.2\textmu s in 100\textmu M TEA, and mean fast blocked times of 48.6\textmu s, 43.3\textmu s and 45.0\textmu s at the same three TEA concentrations, assuming the blocking scheme shown in figure 8-1. In fact, the mean open time will be less than calculated here because of the slow blocking process (see below).

**Properties of the Slow Block**

The properties of the slow block were studied by examining the effects of external TEA on channel open and closed times. As illustrated in figure 8-3, TEA causes a reduction in the mean open time due to a fully resolved block. In order to estimate the rate of the slow channel blocking reaction it was first necessary to consider whether the fast block would also affect open time. According to the scheme of equation 8-1, channels cannot close while they are fast blocked, but must first become unblocked. Adapting this scheme to include a slow blocked state, which is independent from the fast blocked state, gives the scheme:

\[
\begin{align*}
  & \text{C} \quad \xrightarrow{k_{\text{i}}} \quad \text{O} \quad \xrightarrow{\beta_{f}[\text{TEA}]} \quad \text{B}_{(f)} \quad \xleftarrow{\alpha_{f}} \\
  & \downarrow \alpha_{s} \quad \uparrow \downarrow \beta_{s}[\text{TEA}] \quad \downarrow \quad \text{B}_{(s)}
\end{align*}
\]

where \(B_{(f)}\) is the fast blocked state and \(B_{(s)}\) the slow blocked state, \(\beta_{f}\) and \(\alpha_{f}\) are the blocking and unblocking rates for the fast blocking reaction (see above), and \(\beta_{s}\) and \(\alpha_{s}\) are the blocking and unblocking rates for the slow blocking reaction. According to this scheme, each apparent opening in the presence of TEA will actually be a burst of openings separated by very brief, unresolved blockings (visits to state \(B_{(f)}\)). Increasing the TEA concentration will increase this burst length, whilst the total open time per burst \(t_{o}\) remains the constant and is equal to the inverse of the sum of the rate constants for leaving the open state and entering a resolvable “closed” state (actually either C or \(B_{(s)}\)) (from equation 3.32 of Colquhoun & Hawkes, 1982):

\[
t_{o} = \frac{1}{(k_{-1} + \beta_{s}[\text{TEA}])} \quad (8-6)
\]
The mean burst length, which will be the apparent mean open time, is given by (from equation 3.20 of Colquhoun and Hawkes, 1982):

\[ \tau_b = \frac{1 + ([\text{TEA}]/K_d)}{k_{-1} + \beta_i[\text{TEA}]} \]  

(8 - 7)

Where \( K_d \) is the dissociation constant of the fast block, given by \( \alpha_f/\beta_f \).

However, it may be that channels which are fast blocked do not have to unblock before closing:

\[
\begin{align*}
\text{CB} & \quad \rightarrow \quad \text{B}(0) \\
\uparrow \downarrow & \quad \alpha_f \quad \uparrow \downarrow \quad \beta_i[\text{TEA}] \\
& \quad k_{+1} \\
\text{C} & \quad \rightarrow \quad \text{O} \\
& \quad k_{-1} \\
\quad \alpha_s \quad \uparrow \downarrow \quad \beta_i[\text{TEA}] \\
& \quad \text{B}(0)
\end{align*}
\]

(8 - 8)

Where CB is a closed, blocked state. In this case, the fast blocking reaction will not influence the measured mean open time, which will be given by:

\[ \tau_o = 1/(k_{-1} + \beta_s[\text{TEA}]) \]  

(8 - 9)

This is the same as the total open time per burst in the scheme of equation 8-5, which is given by equation 8-6, since this value should be unchanged by any fast blocking reaction (Neher & Steinbach, 1978; Colquhoun & Hawkes, 1982). From equation 8-9, it can be seen that \( 1/\tau_o \) should be a linear function of TEA concentration, with a slope of \( \beta_s \) and an intercept at zero concentration of \( k_{-1} \).

The effect of TEA on mean open time at a potential 100mV positive to the resting potential is shown in figure 8-11. The data appear to be better fit by a straight line than by equation 8-7 (dashed line), favouring the scheme of equation 8-8 over that of equation...
Figure 8-11 Concentration dependence of mean channel open time at a potential 100mV positive to the resting potential. Each point represents the mean ± one S.E.M. (where this is larger than the size of the symbol) from 3-4 patches. The straight line is a regression fit to the data, with a slope of 13.3mM"ms"; this is considered a better fit to the data than the dashed line, which is fit by equation 7 of the text, assuming $K_{eq} = 53$µM, $k_1 = 0.18$ms" and $\beta_2 = 37.95$mM"ms". For this reason, it is most likely that the kinetic scheme of equation 8, where fast blocked channels are assumed to be able to close normally without having to become unblocked first, more closely represents the true situation than does the scheme of equation 5, which assumes fast blocked channels cannot close.
8-5. The slope of this straight line gives a value of $\beta_p$ of 13.3mM$^{-1}$ms$^{-1}$. Similar plots at other voltages suggested that $\beta_p$ was virtually voltage independent; it was estimated to be 11.3mM$^{-1}$ms$^{-1}$ at 60mV positive to the resting potential, 12.2mM$^{-1}$ms$^{-1}$ 80mV positive to the resting potential, and 14.8mM$^{-1}$ms$^{-1}$ 120mV positive to the resting potential.

From the slow blocking reaction illustrated in figure 8-8, it should be possible to calculate the unblocking rate, $\alpha_u$, from the mean lifetime of the slow blocked state, which should be equal to $1/\alpha_u$. In the presence of TEA, the closed time histogram should show an extra exponential component not seen in the absence of TEA, due to the blocking events; the time constant of this component should be equal to $1/\alpha_u$. Examples of closed time histograms in the presence of 0, 30 and 100µM TEA are shown in figure 8-12. Each has been fitted by the sum of two exponential components, indicating two resolvable closed states; a third, longer component of closed times (which actually represents visits to an inactivated state; see chapter 6) has been omitted here. The shortest component of the closed times probably represents two separate components in control (see chapter 6) and three components in the presence of TEA, since the proportion of all closed times which were of this shortest component increased with increasing TEA concentration. In the absence of TEA, this component accounted for 91.7±0.5% (n=3) of all closed times at 100mV positive to the resting potential; this increased to 92.2±1.2% (n=3) with 10µM TEA, 96.6±1.1% (n=4) with 30µM TEA, 97.5±0.7% (n=3) with 60µM TEA, and 97.6±0.4% (n=3) with 100µM TEA. A similar increase was seen at other potentials. This suggests that the lifetime of the slow blocked state is similar to the shortest component of closed times seen in the absence of TEA, such that in the presence of TEA short closures and blockings are indistinguishable at the filter rate used. If the unblocking rate of the slow reaction was assumed to be equal to the reciprocal of the time constant of the shortest component of the closed time histogram (although this is clearly not the case, as this time constant is determined mainly by normal channel gating at all TEA concentrations studied), then from figure 8-12 $\alpha_u$ would be 8.0ms$^{-1}$ for 30µM TEA and 5.6ms$^{-1}$ for 100µM TEA, giving $K_u$ values (given by $\alpha_u/\beta_p$ and using the mean $\beta_p$ values given above) of 600µM and 420µM respectively. It can be seen from figure 8-12 that the time constant of the shortest component of closed times increases with increasing TEA concentration, suggesting that the lifetime of the slow blocked state may be slightly longer than the shortest component of normal closed times, since increasing the TEA concentration will presumably increase
Figure 8-12. Closed time histograms formed from patches at a potential 100mV positive to the resting potential, in the presence of different concentrations of TEA. Each histogram has been fitted by a double exponential function of the form $f(t) = A \exp(-t/\tau_a) + B \exp(-t/\tau_b)$, where $A$ and $B$ represent the intercept of each exponential with the ordinate, and $\tau_a$ and $\tau_b$ are the time constants of each exponential. In the absence of TEA, these two exponentials represent the two shortest components of all closed times, whilst in the presence of TEA the shorter of the two exponentials probably represents a mixture of the shortest component of closed times and time spent in the slow blocked state (see text). In control, $\tau_a = 0.109\text{ms}$ and $\tau_b = 2.22\text{ms}$; 90.88% of all events are of the briefer time constant. In 30µM TEA, $\tau_a = 0.125\text{ms}$ and $\tau_b = 1.87\text{ms}$; here 97.74% of all events are of the briefer kind. In 100µM TEA, $\tau_a = 0.179\text{ms}$ and $\tau_b = 2.57\text{ms}$; here, the briefer component accounts for 97.73% of all events.
the proportion of apparent brief closures which are actually blocking events. The unblocking rate will therefore be slower than calculated from the time constant of the shortest component of the closed time histogram, such that the $K_d$ of the slow blocking reaction may be less than suggested above.

It should also be possible to obtain an estimate of the $K_d$ of the slow block uncontaminated by the fast block by measuring the reduction in channel open probability, $P_o$, in the presence of TEA. Open probability was calculated according to equation 2-9 of chapter 2. Traces during which no channel openings occurred were excluded from the ensemble average current used to calculate the open probability, in order to exclude any effect of slow inactivation (see chapter 6) which would have a potentially greater effect on open probability than would the slow block by TEA. Comparing open probabilities in different TEA concentrations under conditions where the control open probability is high also minimises any possible effects which may be caused by blocked channels being unable to close (see Davies et al., 1989). Open probabilities in figure 8-13 are plotted as a fraction of the open probability in the absence of TEA (which was very close to one under these conditions). The fitted line is to the equation:

$$\frac{P_o'}{P_o} = \frac{1}{1 + ([\text{TEA}]/K_d)}$$  

(8 - 10)

where $P_o'$/$P_o$ is the fractional open probability and $K_d$ is 748µM. However, the scatter of data points makes this method of estimating $K_d$ for the slow blocking reaction just as unreliable as studying the distribution of open and closed times (see above). To obtain a more reliable fit in this case, it would be necessary to study a wider range of TEA concentrations; however, the presence of the higher-affinity fast block makes it impossible to study the effects of higher concentrations accurately. For example, at a TEA concentration of 700µM, $m/i$, given by equation 8-3 and assuming a $K_a$ of the fast block of 53.4µM (see figure 8-6), would be approximately 0.07, such that the peak unitary current (see figure 8-5) would be around 0.11pA.
Figure 8-13 Reduction in mean channel open probability caused by slow block by TEA. Open probability, calculated at different TEA concentrations as described in the text, is plotted as a fraction of control open probability. Each point is the mean value from 3-4 patches, ± one S.E.M. The points have been fitted by equation 8-10 of the text, giving a value of $K_a$ for the slow block of 748μM.
DISCUSSION

Affinities of the Blocking Reactions

External TEA blocks delayed rectifier channels in these neurons at two different sites, each of which has a high affinity for TEA; the $K_d$ of the fast block is approximately 53.4$\mu$M, whilst that of the slower block, which was more difficult to calculate accurately, was probably in the range 400-800$\mu$M. Both blocking reactions were also effectively voltage independent. These affinities are much greater than have been reported from single channel recordings of delayed rectifier channels from frog skeletal muscle ($K_d$ 5.8mM at -3mV; Spruce et al., 1987b), but are more similar to that in murine T lymphocytes (50-100$\mu$M; DeCoursey et al., 1987). These affinities are also far greater than would have been predicted from any previous whole-cell recordings from mammalian central neurons (see above and chapter 1). Although TEA blocks most types of potassium channels, it is usually considered to have the highest affinity for large conductance calcium activated potassium channels. However, the affinity of the fast block seen here is considerably greater than the affinity of external TEA for large conductance calcium activated potassium channels from rat skeletal muscle (300$\mu$M, Blatz & Magleby, 1984; 290$\mu$M, Vergara, Moczydlowski & Latorre, 1984; 120$\mu$M, Villarroel, Alvarez, Oberhauser & Latorre, 1988), bovine chromaffin cells (200$\mu$M, Yellen, 1984) and the GH$_3$ pituitary cell line (260$\mu$M, Lang & Ritchie, 1990).

The $K_d$ of the fast block is also less than the reported IC$_{50}$ for external TEA block (measured from whole cell or single channel recordings) of any cloned mammalian brain potassium channel. A number of cloned mammalian channels have been reported to have IC$_{50}$s between 100 and 300$\mu$M (Christie et al., 1989; Yokoyama et al., 1989; T.McCormack et al., 1990; Schröter et al., 1991; Rettig et al., 1992). Interestingly, only one of these (Kv1.1; Christie et al., 1989; Kavanaugh et al., 1991) was not from the Kv3 (Shaw-like) subfamily. The Kv3 subfamily expresses both delayed rectifier like (Yokoyama et al., 1989; T.McCormack et al., 1990; Rettig et al., 1992) and A-current like channels (Schröter et al., 1991; Rettig et al., 1992). Shaw channels themselves show a relatively low sensitivity to external TEA compared with other cloned Drosophila potassium channels (Wei et al., 1990).
Kinetics of the Blocking Reactions

The kinetics of the two blocking reactions were very different, one being too rapid for individual blocking events to be resolved at the filter frequency used. For this reason different methods needed to be used to calculate the blocking and unblocking rates of the two reactions.

For the fast blocking reaction, both amplitude distribution analysis (Yellen, 1984) and analysis of excess current variance (Ogden & Colquhoun, 1985) were used. In practice, both methods gave very similar results (see figures 8-9 and 8-10), as was the case when both were used to study proton block of calcium channels (Prod'hom, Pietrobon & Hess, 1987; Pietrobon, Prod'hom & Hess, 1989), caesium block of ATP-sensitive potassium channels (Quayle, Standen & Stanfield, 1988) and iron and cobalt block of calcium channels (Winegar, Kelly & Lansman, 1991). The relative merits of these two methods have been discussed by Ogden & Colquhoun (1985) and by Quayle et al. (1988). Briefly, analysis of excess current variance has three major advantages. First, components of the noise not associated with block, such as that due to channel gating, are more easily removed because the excess variance caused by the blocker is calculated by subtracting the total current variance in the absence of blocker from that in the presence of blocker; components of the noise not associated with block should be the same in each case. With the amplitude distribution method, such other components of the noise cannot easily be excluded from the amplitude histogram. Secondly, the effects of filtering using any kind of filter on the excess current variance has been given by Ogden & Colquhoun (1985), whereas the amplitude distribution method assumes a first order filter is used, so that an empirical correction must be made to the data where any other filter is used (see equation 2-27 of chapter 2). Thirdly, background noise is effectively removed by forming a difference spectrum from two different spectra where the channel is open or closed, whereas for amplitude distribution analysis a Gaussian curve fit to the baseline noise must be convolved with the theoretical open level β-distribution. On the other hand, amplitude distribution analysis does give values for the blocking and unblocking rates independently, whereas the analysis of excess current variance gives the cut-off frequency of the noise associated with the blocking reaction, such that the $K_d$ of the block must be known in order to separate the blocking and unblocking rates.

However, with both these methods it was not possible to study the fast blocking
reaction in complete isolation from the slower block. The reduction in mean open time caused by the slow block means that fewer events will reach full amplitude at higher concentrations of TEA, such that the open level current may be underestimated, possibly leading to an overestimate of the $K_d$ of the fast block. Slow blocking events will also contribute to the area of the amplitude histogram between open and closed levels, possibly distorting the $\beta$-distribution fit to the open level current. These events will also add to the low frequency noise, causing an increase in the total current variance which is not associated with the fast blocking reaction.

Since the kinetics of the slow block were examined by studying channel open and closed time distributions, and also changes in channel open probability, they were less likely to be distorted by the presence of the fast block. However, because the fast block was of a much higher affinity, the effects of TEA concentrations only much less than the $K_d$ of the slow block could be studied. Problems also arose due to the similarity in the duration of individual blockings and the shortest component of closed times (see above).

For the fast blocking reaction, both blocking and unblocking rate constants ($\beta$ and $\alpha$) appeared virtually independent of voltage. Mean values of $\beta$ and $\alpha$, averaged over all voltages and TEA concentrations, were $392\pm14\text{mM}^{-1}\text{ms}^{-1}$ ($n=42$) and $21.6\pm0.9\text{ms}^{-1}$ ($n=42$). The mean value of $\beta$ for the slow block was $12.9\pm0.8\text{mM}^{-1}\text{ms}^{-1}$ ($n=4$ different voltages, measured as the slope of graphs such as that shown in figure 8-11). $\alpha$ was more difficult to evaluate for the slow block, due to the similarity between the lifetime of the slow blocked state and the fastest component of normal channel closed times (see above); however, values somewhere in the range 5-10ms$^{-1}$ might be expected (see above). Single channel experiments have previously described only “fast” block by external TEA of delayed rectifier (DeCoursey et al., 1987; Spruce et al., 1987b), ATP-sensitive (Davies et al., 1989) and calcium activated potassium channels (Blatz & Magleby, 1984; Vergara et al., 1984; Yellen, 1984; Benham, Bolton, Lang & Takawaki, 1985; Villarroel et al., 1988; Lang & Ritchie, 1990). In all of these cases, block by external TEA was either independent or only very weakly dependent on membrane potential, as was the case for both fast and slow blocks here, suggesting that a common structural feature of different classes of potassium channels is a TEA binding site accessible to the extracellular solution which is located close to the outer mouth of the channel pore, only superficially within the transmembrane electric field. From mutation experiments on cloned Shaker potassium channels, it has
been shown that the high affinity TEA block seen when the channel contains a key tyrosine residue (see above) is less voltage dependent than the much lower affinity block seen when this tyrosine residue is absent (Heginbotham & MacKinnon, 1992). It was suggested that this tyrosine residue forms a TEA receptor located near the outer mouth of the channel pore, and that when it is absent TEA is able to enter further into the channel before encountering a lower affinity binding site, perhaps simply a region too narrow for it to pass through.

**Presence of Two TEA Binding Sites**

The properties of the fast and slow blocks suggest that this channel has two external binding sites for TEA, both located only quite superficially within the channel pore, and that binding to each site is independent of the other. As pointed out above, previous reports of external TEA block of several different types of potassium channels have described only one binding site, with fast blocking kinetics. However, in both ATP-sensitive potassium channels from frog skeletal muscle (Davies *et al.*, 1989) and delayed rectifier channels from Drosophila neurons (Yamamoto & Suzuki, 1989), internal TEA causes both a fast and a slow block.
CHAPTER NINE: SUMMARY

The results presented in this thesis describe some of the properties of two types of voltage-gated potassium channel, A-current channels and delayed rectifier channels, in the rat brain. These channels were investigated in cultured neurons prepared from two areas of the rat brain, the locus coeruleus and the CA1 region of the hippocampus. Whole cell patch clamp recording showed that neurons from both these areas of the rat brain exhibited both a rapidly inactivating A-current and a slowly inactivating delayed rectifier current (see chapter 4). It was shown that the electrophysiological and pharmacological properties of these currents were broadly the same as those previously described in a large number of studies on both cultured and acutely isolated mammalian central neurons. However, very little work has been carried out on the unitary properties of these channels in the CNS, and those studies which have been reported have not described these properties in great detail (e.g. Gähwiler & Llano, 1989; McLarnon, 1989; Lynch & Barry, 1991). In fact, more is currently known about the unitary properties of cloned voltage-gated potassium channels which have been isolated from the rat brain (Stühmer et al., 1988, 1989; Grupe et al., 1990; Ruppersberg et al., 1990; Baldwin et al., 1991; Kirsch et al., 1991; Schröter et al., 1991; Taglialatela et al., 1991; Rettig et al., 1992). Since it is not known which cloned subunits go together to form functional potassium channels in the rat brain (see chapter 4), more in depth studies of the properties of native neuronal channels are needed so that these properties can be compared with those of the cloned channels.

The unitary properties of A-current channels in cultured locus coeruleus neurons are described in chapter 5. The conductance (14.8pS with a physiological potassium concentration gradient, and 39.0pS when the extracellular potassium concentration was raised to the presumed intracellular concentration) and voltage dependence of activation and inactivation were similar to those previously described for A-current channels in non-CNS cells, and also showed some interesting similarities and differences to those of certain cloned rat brain A-type channels. Rectification of the unitary current-voltage relationship of this channel at positive potentials, which is similar to that seen in some other A-current channels (e.g. Cooper & Shrier, 1985; Schröter et al., 1991; Rettig et al., 1992), was shown to be due to a voltage dependent block of the channel by internal Mg$^{2+}$ and Na$^+$ ions. These ions had previously been shown to block both inward rectifier
(Matsuda et al., 1987; Vandenberg, 1987; Matsuda, 1988, 1992) and ATP-sensitive potassium channels (Horie et al., 1987; Quayle & Stanfield, 1989) from inside the cell.

The conductance of delayed rectifier channels in cultured hippocampal CA1 neurons (18.4pS with a physiological potassium concentration gradient, and 36.4pS in symmetrical potassium; see chapter 6) was less than previously described in other rat central neurons (Gähwiler & Llano, 1989; McLarnon, 1989), but similar to that of the A-current in locus coeruleus neurons (see above) and also to that previously described in a number of non-neuronal cell types. The voltage dependent gating of this channel is described in detail in chapter 6, and can be explained in terms of a model where the channel has a single open state, at least four closed states and at least three inactivated states. Gating could not be explained by a Hodgkin-Huxley type model (Hodgkin & Huxley, 1952d), but instead favoured a model whereby there was cooperativity between the “gates” controlling channel activation, with opening of one “gate” increasing the rate at which the other “gates” opened. This is consistent with the theory of each subunit of a tetrameric potassium channel gating in a cooperative way (Tytgat & Hess, 1992).

Another aspect of the gating of single delayed rectifier channels in CA1 neurons was investigated in chapter 7. It was shown that when the permeant ion was changed from potassium to rubidium, gating of the channel was altered profoundly. This was to be expected from previous reports of permeant ion effects on potassium channel gating (see chapter 7), but is the first example of this phenomenon in a CNS potassium channel. The results could be explained in terms of rubidium ions slowing all channel closing rates, with the effect on each rate being about the same, with no detectable effect on opening rates. This was consistent with the theory that a permeant ion binding site on the extracellular part of the channel, rather than actually within the pore, might cause these permeant ion effects (Adams et al., 1981; Clay, 1988; Shapiro & Decoursey, 1991b). It was also shown that the permeability of the channel to rubidium relative to its potassium permeability, $P_{RB}/P_{K}$, was 0.75, similar to that previously reported for potassium channels in non-neuronal cells.

The effects of a channel blocker, TEA, on currents through single delayed rectifier channels in CA1 neurons are described in chapter 8. The affinity of the blocking reaction was surprisingly high when compared with previous experiments with this blocker. External TEA had two effects on single channel currents, causing a reduction in mean
unitary amplitude (due to a kinetically fast block) and a reduction in mean open time (due to a separate, kinetically slower block). The $K_d$ of the fast block was estimated to be 53.4\textmu M, lower than that previously reported for either native or cloned potassium channels (see chapter 8). The blocking rate was estimated to be 392mM$^{-1}$ms$^{-1}$, and the unblocking rate 21.6ms$^{-1}$. The results were consistent with "fast blocked" channels being able to close normally. The $K_d$ of the slower block was more difficult to estimate, since it was around an order of magnitude higher than that of the fast block; it was probably between 400 and 800\textmu M. Even this is unusually high affinity for a neuronal delayed rectifier channel. The blocking rate was much slower than for the fast block, around 12.9mM$^{-1}$ms$^{-1}$. The high affinity of the two blocks could not have been predicted from the effects of external TEA on the whole cell delayed rectifier current in these cells, where 30mM TEA caused only a 73.9% reduction in current amplitude (see chapter 4). This discrepancy is most likely a result of the use of a puffer pipette to administer the TEA during whole cell experiments; it is possible that the concentration of TEA experienced by the channels was far less than that in the pipette, due to the effects of dilution and diffusion once it was applied. Also, any current due to delayed rectifier channels present on the neurites may have been unaffected (since the TEA was applied locally to the soma). The apparently anomalous effects of TEA on single channel and whole cell delayed rectifier currents show the advantage of carrying out this kind of experiment at the single channel level.

Neither the fast nor the slow channel block by TEA appeared dependent on voltage, implying that both binding sites were outside the transmembrane electric field. In cloned potassium channels, it has been shown that high affinity, voltage independent block by external TEA is due to simultaneous interaction with four tyrosine residues in the channel (Kavanaugh et al., 1991, 1992; Heginbotham & MacKinnon, 1992). It is therefore an unexpected finding that this channel has two high affinity, voltage independent binding sites for TEA, and also that the affinity of one site is apparently greater than that so far reported for any cloned potassium channel in which all four of these tyrosine residues are present.

The two channels described here, the A-current channel of locus coeruleus neurons and the delayed rectifier channel of hippocampal CA1 neurons, underlie whole cell currents which are similar to those previously reported in a number of different mammalian central neurons (see above, and chapter 4). It is therefore possible that the single channel properties
described for these channels may be similar to those in other parts of the rat brain. However, many different genes encoding subunits are expressed in the rat brain, with different spatial, temporal and activity-dependent expression patterns (Beckh & Pongs, 1990; Drewe et al., 1992; Rettig et al., 1992; Sheng et al., 1992; Tsaur et al., 1992), such that very slight differences in channel properties may exist in different central neurons. Interestingly, despite the similarity of the whole cell potassium currents recorded from locus coeruleus and CA1 neurons to those previously reported in central neurons, the whole cell and single channel properties of the A-current and delayed rectifier described here are significantly different from those of any reported cloned rat brain potassium channel. As is discussed in chapter 4, this difficulty in matching the properties of native and cloned rat brain potassium channels stems from a lack of understanding of how functional neuronal potassium channels are formed from individual gene products in vivo.
APPENDIX: SOLUTIONS USED FOR CELL CULTURE

All solutions used for tissue culture were sterilised by passing through a 0.22μm filter under vacuum pressure, and stored at 4°C. Some serum solutions have been supplemented with putrescine, selenium dioxide, progesterone, transferrin and insulin, constituents of the “N2” solution introduced by Bottenstein & Sato (1979). Where N2 has been used, it was made up as a stock solution before being added to the serum. Glucose, sodium bicarbonate, sodium pyruvate and ascorbic acid was also made up as a stock solution before being added to some media.

Minimum essential medium (MEM)

Minimum essential medium with Earle’s salts (Gibco 072-01700A):
- 116mM sodium chloride
- 5.4mM potassium chloride
- 1.8mM calcium chloride
- 1.0mM sodium dihydrogen phosphate
- 0.81mM magnesium sulphate
- 5.5mM D-glucose
- 0.93mM sodium succinate
- 0.64mM succinic acid
- 0.60mM L-arginine
- 0.50mM L-tryptophan
- 0.40mM L-isoleucine
- 0.40mM L-leucine
- 0.40mM L-lysine
- 0.40mM L-threonine
- 0.40mM L-valine
- 0.20mM L-histidine
- 0.20mM L-phenylalanine
- 0.20mM L-tyrosine
- 0.10mM L-cysteine
- 0.10mM L-methionine
- 11μM i-inositol
- 8.2μM nicotinamide
7.1μM choline bitartrate
4.9μM pyridoxal hydrochloride
4.2μM calcium D-pantothenate
3.0μM thiamine
2.3μM folic acid
0.27μM riboflavin

To which was added:
26mM sodium bicarbonate (Sigma)

D1 Solution
137mM sodium chloride (Fisons)
22mM sucrose (Sigma)
17mM glucose (Sigma)
5mM potassium chloride (Fisons)
0.7mM disodium phosphate (Fisons)
0.2mM monopotassium phosphate (Fisons)
10mM HEPES (Sigma)
1.2μg/l phenol red (Sigma P5530)
50 units/ml penicillin (Gibco 043-0507H)
50μg/ml streptomycin (Gibco 043-0507H)

Phosphate buffered saline (PBS)
137mM sodium chloride (Fisons)
26mM sodium hydrogen phosphate (Fisons)
in “MilliQ” sterile water
brought to pH 7.4 with hydrochloric acid (Fisons)

Serum free medium
MEM (see above), plus:
33mM glucose (Sigma)
26mM sodium bicarbonate (Sigma)
2mM glutamine (Gibco 043-05030H)
1mM sodium pyruvate (Sigma)
60μM ascorbic acid (Sigma)
100μM putrescine (Sigma P7505)
30nM selenium dioxide (Sigma S9379)
20nM progesterone (Sigma P01301)
100μg/ml transferrin (Sigma T8027)
5μg/ml insulin (Sigma I5500)
1% ovalbumin (Sigma A5503)

10% foetal calf serum (FCS)
MEM (see above), plus:
10% foetal calf serum (Applied Protein Products AP1167)
33mM glucose (Sigma)
26mM sodium bicarbonate (Sigma)
2mM glutamine (Gibco 043-05030H)
1mM sodium pyruvate (Sigma)
60μM ascorbic acid (Sigma)
50 units/ml penicillin (Gibco 043-0507H)
50μg/ml streptomycin (Gibco 043-0507H)

10% horse serum (10HS)
MEM (see above), plus:
10% heat inactivated donor horse serum (Applied Protein Products ADH 1166)
33mM glucose (Sigma)
26mM sodium bicarbonate (Sigma)
2mM glutamine (Gibco 043-05030H)
1mM sodium pyruvate (Sigma)
60μM ascorbic acid (Sigma)

5% horse serum (5HS)
MEM (see above), plus:
5% heat inactivated donor horse serum (Applied Protein Products ADH 1166)
33mM glucose (Sigma)
26mM sodium bicarbonate (Sigma)
2mM glutamine (Gibco 043-05030H)
1mM sodium pyruvate (Sigma)
60μM ascorbic acid (Sigma)
50μM putrescine (Sigma P7505)
15nM selenium dioxide (Sigma S9379)
10nM progesterone (Sigma P01301)
50μg/ml transferrin (Sigma T8027)
2.5μg/ml insulin (Sigma I5500)
0.5% ovalbumin (Sigma A5503)

5-fluoro-2'-deoxyuridine solution (FdUr)
MEM (see above), plus:
2mg/ml 5-fluoro-2'-deoxyuridine (Sigma F0503)
5mg/ml uridine (Sigma U3003)

Trypsin solution
0.25% trypsin (Gibco 043-05090H) in D1 solution (see above)

Collagen solution
0.5mg/ml collagen (Sigma C7661)
0.1% glacial acetic acid
in “MilliQ” sterile water

Poly-L-lysine solution
50mM boric acid (Sigma)
24mM borax (sodium tetraborate; Sigma)
10μg/ml poly-L-lysine (Sigma P2636)
in “MilliQ” sterile water
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