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PREFACE

I thank Professor A.G.H. Blakeley for the use of the facilities of the Department of Physiology. Many thanks are due to my supervisor, Dr. N.B. Standen, for his help and guidance and to Professor P.R. Stanfield for much generous assistance. I thank Mr. W. King and Miss M. Moore for expert technical assistance. Financial support was provided by a Medical Research Council Studentship.

I am extremely grateful to my family for their constant support and especially to my husband for his encouragement and infinite patience.

This thesis describes the study of macroscopic and unitary currents of the mouse neuroblastoma cell line N1E115. All experiments described were carried out in the Department of Physiology, University of Leicester, between October, 1984 and September, 1987. The thesis is the result of my own work and includes nothing which is the outcome of work done in collaboration.
Chapter 1

INTRODUCTION

The aim of the work described in this thesis was to investigate some of the electrical currents which flow through the membrane of mouse neuroblastoma cells in certain conditions. Macroscopic and unitary currents were both measured using some of the patch-clamp recording modes which have been developed by Neher, Sakmann and co-workers (Neher and Sakmann, 1976; Neher, Sakmann and Steinbach, 1978; Hamill, Marty, Neher, Sakmann and Sigworth, 1981). The properties of the macroscopic and unitary currents are compared, where this is appropriate, with each other and with currents recorded from the same neuroblastoma cell line by other workers. They are also compared with similar currents recorded from other preparations. In order to make these comparisons the ionic, kinetic and stochastic properties of the currents are described and used as the parameters for the comparisons.

The purpose of this introduction is to describe the use of neuroblastoma cells in other physiological studies and to indicate why they may be a useful model system for studying electrophysiological properties; to summarize the neuronal currents which have been studied using patch-clamp recording techniques so that those currents described later may be categorized in terms of previous findings; and to describe the analytical and statistical techniques which have been developed in order to allow comparison of ionic currents and to derive information about possible working mechanisms of the proteins which allow these currents to pass through the membrane. These experimental and analytical techniques have led to detailed knowledge about the
kinetic properties of the currents and a wealth of possible models for the molecular behaviour of the channel proteins. One property which has been recently discovered in some channels is their ability to take several open states which each have a different conductance. Later it is shown that a potassium channel of mouse neuroblastoma cells probably has this property. I shall therefore also describe, in this introduction, the types of multiple-conductance state channels which have been recorded in an attempt to provide some sort of categorization of these channels and to provide a background against which the properties of the multiple-conductance state channel described later may be considered.

1.1 NEUROBLASTOMA CELLS

The electrophysiological studies described in this thesis were carried out on cells of the mouse neuroblastoma clonal cell line N1E115. This line is derived from the mouse C-1200 neuroblastoma which was a spontaneous tumour of sympathetic origin. Cloned cell lines derived from this tumour serve as a useful system for investigating aspects of nerve cell electrophysiology and differentiation and these cells have been widely used for these purposes.

1.1.1 Neuroblastoma cells as a model system

Many cells from neuroblastoma clones give spikes in response to stimulating current (Nelson, Peacock, Amano and Minna, 1971) and exhibit membrane potential changes in response to iontophoretically applied acetylcholine (Harris and Dennis, 1970; Nelson, Peacock and Amano, 1971; Peacock and Nelson, 1973). Under normal growth conditions, however, many neuroblastoma cells are electrically inexcitable.
(Nelson, Ruffner and Nirenberg, 1969) and those cells which are excitable often have spikes which have much slower rates of rise and repolarization than those evoked from primary neurones of neural crest origin (Hunt and Nelson, 1965). The transition of a culture of neuroblastoma cells from the actively dividing state to the confluent one is characterized by the synthesis of various enzymes involved in neuroblastoma metabolism (Amano, Richelson and Nirenberg, 1972), an enhancement of electrical excitability (Nelson, Ruffner and Nirenberg, 1969) and some degree of process formation. These properties are those which characterize nerve cells in vivo; that is, their unique morphological appearance, the possession of an excitable membrane and a specialized internal biochemical environment.

To achieve a further expression of these properties several chemical agents have been applied to the cells. Peacock, Minna, Nelson and Nirenberg (1972) used the antimetabolite aminopterin to select for neuroblastoma cells with enhanced electrical excitability. This, however, results in the death of a major proportion of the cell population and may interfere with other cellular functions. Chalazonitis and Greene (1974) found that the presence of dibutylyl cyclic adenosine monophosphate (db-cAMP) in the culture medium increases the proportion of electrically excitable cells as well as increasing the rates of rise and amplitudes of invoked spikes. Kimhi, Palfrey, Spector, Barak and Littauer (1976) showed that in the presence of dimethyl sulphoxide, neuroblastoma cells will extend neurites and develop a highly excitable membrane. They found that this method offered advantages over those described above in that the cells appeared to reach a higher level of electrical differentiation and could be maintained in that state for extended periods of time. This was the method used to obtain electrically differentiated cells for the work described in this thesis.
Mouse neuroblastoma cells may be stored in liquid nitrogen and when thawed multiply quickly to give a large stock of cells on which to work. They differentiate, both morphologically and electrically, in certain culture conditions and respond to electrical and chemical (acetylcholine) stimulation in a similar way to sympathetic primary neuronal cells. They therefore provide an appropriate model system in which to study mammalian neuronal currents.

1.1.2 Electrophysiological and differentiation studies on mouse neuroblastoma

Moolenaar and Spector (1978, 1979a, b) used the DMSO differentiation technique to provide differentiated cells in order to study ionic currents under two microelectrode voltage clamp. They recorded a fast sodium current, a delayed potassium current, a calcium current and a calcium-activated potassium current. Since the advent of single channel recording a variety of unitary currents have been described in these cells. Unitary sodium currents have been recorded (for example Aldrich, Corey and Stevens, 1983; Aldrich and Stevens, 1987) and have been shown to exhibit kinetic behaviour which suggests that they may have multiple conductance states (Nagy, Kiss and Hof, 1983; Nagy and Bagany, 1986; Nagy, 1987). Work has also been carried out on the development of the sodium channel protein during DMSO- or hexamethylene-bis-acetamide- (HMBA) induced differentiation. Acetylcholine-activated currents have been recorded (Takeda, Shimahara, Tauc and Berwald-Netter, 1986). A non-selective voltage-activated cation channel was reported by Yellen (1982). Delayed rectifier potassium channels have been recorded (Misler and Falke, 1985; Smith, 1986; Quandt, 1988) and have been shown to have two open conductances (Smith, 1986); and a calcium-activated potassium channel and a slow,
voltage-dependent potassium channel have been described (Quandt, 1988).

The work in this thesis describes macroscopic currents recorded under whole-cell voltage clamp early in the cells' differentiation and compares these with those described by Moolenaar and Spector (1978, 1979a, b) recorded under conventional voltage clamp in cells which had been differentiated for longer periods of time. The properties of the unitary delayed rectifier are described and compared with the macroscopic current properties. The block of these unitary currents by tetraethylammonium ions (TEA\(^+\)) is described and compared with the TEA\(^+\) block of similar currents from other preparations. Some of the properties of the non-selective cation channel and of a calcium-activated potassium channel are also described and compared with those described by other workers in other preparations.

1.2 IONIC CURRENTS

All known physiological electrical signals: action potentials, synaptic potentials and receptor potentials are due to ionic permeability changes. Hermann (1872, 1905) was the first to suggest that electrical conduction along a cell occurred because of the cable properties of that cell, propagation of signals in electrically excitable cells being due to electrical stimulation of unexcited membrane by active regions. Bernstein (1902, 1912) was the first to propose a selective potassium permeability in excitable cell membranes and to suggest that electrical excitability was due to voltage-dependent permeability changes in the cell membrane. Cole and Curtis (1938, 1939), measured the electrical impedance of cell suspensions and proposed an equivalent circuit to represent the membrane, cytoplasm and extracellular medium.
Their results showed that each cell has a high-conductance cytoplasm (around 30 to 60% conductivity of the surrounding saline), a membrane of low conductance and an electrical capacitance of approximately $1\mu$F/cm$^2$. This confirmed the assumptions which were necessary to support Hermann's cable theory of passive spread of electrical excitability. Hodgkin (1937a, b) showed that in a frog sciatic nerve blocked by cold an action potential arrested by the cold block produced a transient depolarization and increase in the electrical excitability of the axon beyond the blocked region. Cole and Curtis (1938, 1939) then found that an action potential was accompanied by a decrease in impedance, the membrane conductance of a squid giant axon rising transiently from less than $1mS/cm^2$ to approximately $40mS/cm^2$. This confirmed Bernstein's theory of a permeability change in the membrane of the cell. Bernstein had, however, proposed that the membrane conductance breaks down. Hodgkin and Huxley (1939, 1945) measured, with intracellular micropipettes, the action potentials of axons and found that the membrane potential of the axon reverses and that the membrane permeability of the membrane does not simply break down but the membrane becomes permeable to sodium ions.

Hodgkin and Huxley (1952a, b, c and d) provided detailed kinetic descriptions of membrane permeability changes without knowledge of the intra-membrane molecules which might be involved. The kinetic parameters which they used to describe the currents still prove to be important in describing currents in a way which allows comparison although it is now known that these do not provide an adequate model of the molecular changes which probably occur in a channel protein in order for it to pass current. Neher and Sakmann (1976), Neher, Sakmann and Steinbach (1978) and Hamill et al (1981) developed techniques for recording unitary currents
Introduction

passing through the membrane of cells. The techniques involve electrically isolating a small area of membrane by forming a tight seal between the tip of a small glass pipette and the cell membrane. This seal has an electrical resistance of the order of gigaohms (GΩ). Using these techniques, which are described in more detail in Chapter 2, electrical currents of less than 0.5 pA which pass through the membrane can be recorded. The use of these techniques has allowed the recording of unitary currents which correspond to ionic currents recorded using conventional voltage-clamp techniques and has also enabled the discovery of currents which could not be recorded or could not be differentiated from other currents using conventional voltage-clamp techniques. They have, for example, allowed the measurement of the unitary currents whose summed activity makes up the potassium current found by Hodgkin and Huxley (1952abd) to repolarize the membrane after an action potential (Conti and Neher, 1981).

1.3 UNITARY IONIC CURRENTS

Since the advent of single channel recording techniques (Neher and Sakmann, 1976) unitary currents have been recorded in a wide range of preparations. These currents have generally been classified by their ionic permeability, by their kinetic properties, by the stimuli which cause them to open and by the actions upon them of pharmacological agents. For example, several types of potassium channel have been recorded, some of which depend mainly on changes in membrane potential to cause them to open while some others depend mainly on calcium-concentrations at the intracellular face of the membrane. Several types of voltage-dependent potassium channel have been identified and classified on the basis of their kinetics of opening and closing in response to a voltage step and by their pharmacological properties. For
example, fast transient potassium channels open and close quickly after a voltage step and are generally blocked by 4-aminopyrididine (4-AP) whereas delayed outward potassium channels open and close more slowly in response to a depolarizing voltage step than do fast transient currents and are generally insensitive or only slightly sensitive to 4-AP but are blocked by TEA⁺ ions.

Ionic channels are believed to be pores rather than carriers. The best evidence in support of this belief is the rate of ionic flux through an ionic channel. The conductance of ionic channels which have been recorded varies from 4 pS for sodium channels of squid giant axon to 300 pS for some chloride channels. Typical currents measured vary from less than 0.5 pA to around 30 pA. 1 pA of current corresponds to an ionic flux of around $6 \times 10^6$ monovalent ions/second. The ionic fluxes through ionic channels are therefore several orders of magnitude higher than the fluxes produced by any known carriers, the fastest of which only go up to $5 \times 10^4$ ions/second (Trinactin, 4 $\times 10^4$ ions/second, Lapointe and Laprade (1982); chloride/chloride exchange, $5 \times 10^4$ ions/second, Brahm (1977); and valinomycin, $3 \times 10^4$ ions/second, Benz and Lauger (1976); in Hille, 1984.). The ionic fluxes through ionic channels carry the electrical currents which are necessary for all physiological electrical signals. The diversity of the channels’ ionic selectivities, kinetics and modulation allows complex changes in the membrane potentials of excitable cells. The section which follows provides a summary of work on some of the main classes of current which have been recorded and have been investigated using patch-clamp techniques. I have not attempted to provide a comprehensive summary but have picked out the work which I consider has contributed greatly to providing an overall picture of the functioning of ionic channels and that which is most relevant to the work described later in this thesis.
1.3.1 Unitary sodium currents

Voltage-dependent sodium channels generate the regenerative depolarization which causes the upstroke of the action potential. They activate and inactivate quickly upon depolarization. Their fast inactivation has lead to problems in generating models which adequately describe the kinetics of the sodium channel. Patlak and Horn (1982) found that application of N-Bromoacetamide (NBA) to the cytoplasmic face of a membrane patch irreversibly removed sodium channel inactivation in rat myotubes and suggested that the normal channel lifetime is primarily determined by the inactivation process. Horn, Vandenbome and Lange (1984) showed removal of sodium channel inactivation in cells of the line GH3 by NBA and indicated that inactivation in these channels is inherently voltage-dependent. They also found a non-random clustering of current traces containing or not containing channel openings, suggesting some form of slow inactivation or "hibernation" and found that NBA made the non-random pattern more apparent. NBA cleaves peptide bonds on the COOH-terminal side of tryptophan, tyrosine, histidine and reduced phenylalanine (Means and Feeny, 1971) and removes the calcium-dependence of calcium-activated potassium channels in rat skeletal muscle (Pallotta, 1985). This specificity of action may therefore be useful in determining which part of the sodium channel protein is responsible for inactivation and which part of the calcium-activated channel protein is responsible for the calcium dependence. The primary structure of the sodium channel of *Electrophorus electricus* has been deduced from cloning and sequence analysis of cDNA (Numa et al, 1984) and a possible transmembrane topology proposed. Direct comparisons cannot be made between the electrophysiological and biochemical studies; when electrophysiological measurements involving specific agents such as
Introduction

NBA are carried out on channels for which the protein sequence can be found there will probably be great steps in understanding the molecular functioning of channels. It is, however, interesting to note that the proposed topology of the *Electrophorus* sodium channel includes a large, negatively charged region and four large positively charged regions extending from the membrane on the intracellular side. The charge on these could confer the voltage dependence seen in activation and inactivation of the channel and the four positively charged regions could confer the cooperativity necessary to obtain a sigmoid activation curve. The single negatively charged region may be involved in inactivation. Although there is no guarantee that the molecular structure of the myotube channel will be similar enough to the *Electrophorus* channel to allow such a comparison, the negatively charged and two of the positively charged regions contain areas which could be sensitive to attack by NBA (phenylalanine residues). NBA would also attack the negative region more quickly, removing inactivation, and attack of the two positive regions might explain the gradual reduction in sigmoidicity seen in the activation of the rat myotube sodium channels.

Aldrich, Corey and Stevens (1983), Aldrich and Stevens (1983), and Aldrich and Stevens (1987) have also studied the inactivation of single sodium channels. Aldrich et al (1983) suggested that the processes of activation and inactivation are not kinetically distinct (the assumption of the Hodgkin and Huxley analysis of sodium current is that these processes are kinetically distinct, activation being slow and inactivation fast). They found that sodium channels of the neuroblastoma cell line N1E115 exhibit a rapid rise and slow decline in activity upon depolarization but that the activation process can be slow and rate limiting while inactivation is rapid. They also concluded that inactivation is not very voltage-dependent. Other work on the
sodium channel of the cell line N1E115 has suggested that there are either two populations of channels or, the favoured explanation by the experimenters, that the channel exhibits several open states of different conductance (Nagy and Hof, 1983; Nagy and Bagany, 1986; Nagy, 1987). These results further complicate kinetic analysis of the channels and may confuse the findings by Aldrich et al (1983) who assumed a single open state.

All voltage-activated sodium channels so far recorded behave in similar ways: they activate and inactivate quickly upon depolarization and are responsible for the depolarizing phase of the action potential. Studies which aim to discover more about the kinetics of the channel are difficult because of the extreme time-dependence of the channels' activity. Agents which remove inactivation help studies on the channels' kinetics but the agents themselves may change other aspects of the channels' behaviour and the activation kinetics seen in these conditions may not be exactly the same as those of an intact channel. Specific agents such as NBA may be more useful when the changes in functionality which they produce are compared with possible physical sites at which they may act.

1.3.2 Unitary calcium currents

Fatt and Ginsborg (1958) discovered that depolarization during the sodium-independent action potential of crab muscle (Fatt and Katz, 1953) is caused by influx of calcium ions. Since then the calcium channel has been found to account for the regenerative electrical excitability in muscle of arthropod, mollusc, nematode and adult tunicate; to coexist with sodium channels and be partially responsible for electrical excitability in smooth and cardiac muscle of vertebrates, in nerve cell bodies
of mollusc, arthropod, amphibian, bird and mammal; and to regulate secretion in eggs, all secretory gland cells and secretory nerve terminals (Hille, 1984).

Calcium channels often exhibit inactivation (Hagiwara and Byerly, 1981; Kostyuk, 1984; Reuter, 1983; Hagiwara, 1983). This inactivation is different from that of sodium channels however, in that it appears to depend partly on membrane potential and partly on the accumulation of calcium ions (Ca$^{2+}$) in the cytoplasm (Eckert and Chad, 1984) since the inactivation is slowed by injection of calcium chelators into the cell and is reduced when barium (Ba$^{2+}$) or strontium (Sr$^{2+}$) are used as the carriers of the current passing through the calcium channels. Standen and Stanfield (1982) and Eckert and Chad (1984) have produced a model for calcium-dependent calcium inactivation by assuming that calcium entering the channel binds to the channel protein and closes the channel.

Calcium channels have been categorized by their voltage-dependence, conductance, ionic selectivity, kinetics and by the pharmacological agents with which they may be blocked. The channels are categorized into three classes in neuronal somata and into two in cardiac muscle. The classes in neuronal somata are:

1) The voltage-dependent calcium channels which are activated by depolarization from the normal resting potential of the cell. These are termed L-type channels and have long open times and a long lasting rise in the probability of the channel being open (P_open) elicited by membrane depolarization;

2) Calcium channels which are inactivated at the resting potential and are primed by first hyperpolarizing the membrane and activated by small depolarizations from the hyperpolarized potentials (Carbone and Lux, 1984). These are termed T-type channels (Nowycky, Fox and Tsien, 1985) and have shorter open times than the L-type
channels. $P_{\text{open}}$ of the T-type channels increases transiently upon depolarization of the membrane and it is thought that the channels may be responsible for controlling the firing of bursts of neuronal action potentials;

3) N-type channels which are inactivated at the normal resting potential and are activated only by a large depolarization from a very negative membrane potential (Nowcky et al., 1985). It is thought that both L- and N-type channels play a part in the generation of the action potentials of dendrites or in transmitter release.

Calcium channels have been reviewed by Hagiwara and Byerly (1981), Hagiwara (1983), Tsien (1983) and Stanfield (1986).

1.3.3 Unitary chloride currents

Chloride channels oppose electrical excitability when they are open since in most cells the equilibrium potential for chloride ions in physiological conditions is either close to the resting potential of the cell or at least very negative. The importance of this stabilisation of the membrane potential is seen in the condition myotonia congenita where the chloride permeability of muscle membranes is low and the muscles are consequently hyperexcitable, cramping when they are exercised (Lipicky, Bryant and Salmon, 1971). Anions have also been shown to play a role in the resting conductance of vertebrate unmyelinated axons (Rang and Ritchie, 1968).

Steeply voltage-dependent unitary chloride currents have been recorded from fractionated membrane vesicles from the electric organ of *Torpedo* fused with lipid bilayers (White and Miller, 1979, 1981; Miller, 1982; Tank, Huganir, Greengard and Webb, 1982). This channel has two open state conductances and is discussed in the later section on multiple-conductance channels.
1.3.4 Unitary potassium currents

There are a number of different classes of potassium channel, each with a different role in regulating the excitability of the cell. Whereas neuronal sodium currents are responsible for the rising phase of the action potential and all behave in similar ways (they open with a short (generally less than 1 or 2 msec) delay after a depolarization and inactivate soon afterwards; and they are closed by hyperpolarization) there is a great diversity of voltage dependent potassium channels in neuronal membranes whose functions have been characterized to an advanced degree and which have been shown to have important functional differences and physiological roles. In normal physiological conditions the concentration gradient of potassium across a cell membrane is such that the equilibrium potential of potassium ions, as calculated by the Nernst equation is negative (around -70 to -100 mV). Since action potentials arise from the permeability changes of an excitable cell membrane in response to depolarization (due to the passive spread of electrical currents from already excited regions) this means that when potassium channels are open they tend to bring the potential of the cell membrane to values at which electrical excitability is low. Open potassium channels therefore tend to stabilize the membrane potential at values away from the threshold potentials for electrical excitability. All potassium channels so far discovered have roles which lead to this stabilization: they hold the cell at a negative resting potential; keep fast action potentials short; terminate periods of intense electrical activity; slow the rate of repetitive firing; and in general lower the excitability of the cell.
1.3.4.1 Delayed rectifier

Delayed rectifier channels open with a delay after a depolarizing voltage step. Their opening is slow compared with that of sodium channels upon depolarization and their function is to return the membrane potential to a negative value after the regenerative action of sodium channels which causes the upstroke of the action potential. The unitary currents passing through such channels have been recorded from, for example, squid giant axon (Conti and Neher, 1982), embryonic chick heart (Clapham and DeFelice, 1984), frog skeletal muscle (Standen, Stanfield, Ward and Wilson, 1984; Standen, Stanfield and Ward, 1985), human T lymphocytes (Cahalan, Chandy DeCoursey and Gupta, 1985), from squid and lobster axon inserted into lipid bilayers (Coronado and Bezanilla, 1984; Coronado, Latorre and Mautner, 1984) and from mouse neuroblastoma cell line N1E115 (Mislre and Falke, 1985; Smith, 1986; Quandt, 1988). The channels are probably important in returning the membrane to a negative potential after an action potential in all excitable cells. Channels from different preparations show different degrees of inactivation and are usually blocked by TEA⁺ ions.

1.3.4.2 Transient outward currents

Transient outward currents are inactivated at the normal resting potential of a cell but activate quickly upon depolarization after a period of hyperpolarization. They space repetitive responses because after an action potential the membrane potential of the cell is hyperpolarized by the increased activity of delayed rectifier channels. This hyperpolarization primes the transient currents and removes the activation of the
delayed rectifier channels so that the membrane slowly depolarizes again, opening the transient channels. The current passing through the transient channels will almost cancel out the depolarizing stimulus current so that an action potential does not fire immediately. The transient channels then inactivate and the depolarization reaches the threshold for the regenerative depolarization due to sodium (and calcium) channels and the cell fires again. This makes the interspike interval much longer than could be achieved with delayed rectifier channels alone (Connor, 1978). Transient outward currents are, in general, more sensitive to 4-AP as a blocking agent and less sensitive to TEA⁺ than are delayed rectifier currents. Unitary transient potassium currents have been recorded from rat nodose neurones (Cooper and Shrier, 1985) and from neurones of *Helix aspersa* (Taylor, 1987).

Mutations which selectively modify the transient outward channel have been discovered in the fruit fly *Drosophila melanogaster* (Salkoff, 1984; Salkoff and Wyman, 1981). These mutations are known as *Shaker* mutations and Salkoff concluded that the *Shaker* locus is a structural gene of the transient outward channel. Sequencing of the protein coded for by the *Shaker* locus has since shown that four probable components of a potassium channel are encoded at the *Shaker* locus (Papazian, Schwarz, Tempel, Jan and Jan, 1987; Schwarz, Tempel, Papazian, Jan and Jan, 1988) and messenger RNA (mRNA) coded for by this locus can direct the synthesis of functional transient outward channels in *Xenopus* oocytes (Timpe, Schwarz, Tempel, Papazian, Jan and Jan, 1988). The protein sequence and possible structure of this channel may therefore now be related to its function.
1.3.4.3 Calcium-dependent potassium currents

It has been suggested that the slow bursting rhythm of the membrane potential of cells which show pacemaker activity arises from cyclical variations of intracellular free calcium (Meech and Standen, 1975; Eckert and Lux, 1976; Smith, 1978; Gorman and Thomas, 1978; Gorman, Hermann and Thomas, 1981). Calcium-dependent potassium channels will be activated when the intracellular calcium concentration rises during a train of action potentials and will hyperpolarize the cell and thereby decrease electrical activity, halting the action potentials. The calcium levels inside the cell fall until the calcium-activated channels are no longer active and the cell becomes excitable again. These channels are therefore thought to be involved in the regulation of action potential bursting in cells which exhibit pacemaker activity. Calcium-dependent channels have, however, been recorded in nearly every excitable cell which has been patch-clamped and, since most excitable cells do not exhibit bursting activity, they probably also have another role. It has been suggested that, in neurones, calcium-activated potassium currents which produce an after hyperpolarization contribute to the accommodation of cell firing in response to prolonged depolarizing stimuli (Madison and Nicoll, 1984; Constanti and Sim, 1987). Calcium-activated potassium channels have a high conductance compared with that of delayed rectifier and transient outward currents. Unitary calcium-activated potassium currents have been recorded from cultured rat sympathetic neurones (Smart, 1987) where they have a slope conductance of 200 pS in approximately symmetrical 150mM K⁺. The activity of this channel is later compared with that of the calcium-activated potassium channel recorded from mouse neuroblastoma clone N1E115 since this tissue is also of sympathetic origin. Magleby and Pallotta (1983), have found that the calcium-activated potassium channel of cultured rat muscle exhibits bursting: the
channel has periods of openings separated by short closings, these periods themselves being separated by longer closings. This activity is also compared later to that of the neuroblastoma calcium-activated potassium channel.

1.4 ANALYSIS OF UNITARY CURRENT DATA

When attempting to derive information about the activity of single channel proteins in a cell membrane the raw data consists of a noisy current recording which has rectangular steps indicating periods during which the conductance of the membrane changes. These steps are discrete and are assumed to represent a single open pathway through the membrane. The aim of the analysis of these currents is to provide information to allow a qualitative description of the channel: what ions pass through it, what are the stimuli which cause it to open, what pharmacological agents may be used to block it; and to provide a quantitative description of parameters which may be used to describe its activity in detail and to allow its comparison with other channels, for example: detailed information about its kinetics of opening and closing, its ionic permeability and the dissociation constants of its blocking agents. The nature of the raw data means that detailed analysis must usually be performed on the data even to describe the channel qualitatively. The behaviour of a single channel follows that of a stochastic process where events occur randomly with a certain probability. This means that qualitative conclusions cannot be drawn with any integrity by simply inspecting the data and a large number of events must be recorded to allow the behaviour of the channel to be inferred. Most of the analysis depends on the experimenter being able to produce a probability density function of various properties of the channel in different
conditions in order to provide a model which predicts the channel behaviour. This section does not discuss all of the methods of analysis available for studying unitary currents but summarizes the methods of analysis which have been used later to examine the activity of the unitary currents described in this thesis.

Colquhoun and Hawkes (1977) considered relaxation and fluctuation of membrane currents that flow through drug-operated channels when the potential difference across the membrane was held constant by voltage clamp. They made the assumptions that 1) the opening and closing of a single channel causes a rectangular pulse of current flow and 2) that the instantaneous current-voltage relation is linear (ohmic). They demonstrated that, if there is a single open state, the lifetime is exponentially distributed with mean equal to the time constant of that exponential. They described the behaviour of ion channels in terms of a Markov chain in continuous time. To achieve the objectives of unitary current analysis described above, distributions and their means were derived for the length of the sojourn in any specified subset of states (for example all shut states). These were found in general to depend not only on the state in which the sojourn starts but also on that state immediately following.

In order to describe the channel quantitatively in terms of its open durations, to compare these with a theoretical distribution and ultimately to try to infer a biological mechanism from the result it is necessary to obtain many recordings of the channel activity. For example, the standard deviation of the open time of \( n \) observations when the open time histogram follows a single exponential of time constant \( \tau \) is equal to \( \tau n^{1/2} \) (Colquhoun and Hawkes, 1981) and in order to measure
the mean lifetime with an accuracy of 10% it is necessary to measure in the order of 100 channel lifetimes. For a qualitative description the necessities are less, since it may be shown, for example, that the open time of a channel increases with depolarization over a range of voltages if the estimation of \( \tau \) is less accurate as long as a consistent trend is observed. However, in order to make any such observations the first steps are to record the unitary currents and then to produce an idealized record from the currents recorded.

1.4.1 Production of an idealized record

The idealized record is the basis for any kinetic analysis of channel states. It approximates to the true channel activity and serves as the data set for statistical analysis of the kinetics. It is therefore important that this idealized record be as unbiased as possible. The main problem in creating an accurate idealized record is that the recorded currents have a certain level of noise in the baseline and in the current that flows when a channel is open. This means that sufficiently short channel openings, which will not cause the measured current to change from the closed to the open channel level since the response of the current recording equipment cannot be perfect, are indistinguishable from random noise fluctuations about the baseline and short closings are similarly indistinguishable from fluctuations in the open channel current level. Various methods have been used to obtain idealized current traces. The one used in the experiments described here was the half-amplitude cursor method. This involves the placing of a cursor at an amplitude which is half way between the mean amplitude of the baseline current and the mean amplitude of the open channel current. If the current traverses across this cursor this is assumed to indicate a transition between the
channel being open (passing current) and being closed (passing no current). The cursor may be moved during the course of the analysis to compensate for drifts in the baseline of the recorded current.

1.4.2 Probability density functions

In order to produce quantitative kinetic descriptions of the channels, open and closed times are analysed. Histograms are produced of these times and are approximated to probability density functions. Although the histograms are discontinuous they are constructed from continuous data and the curves which may fitted to the histograms may be specified as probability density functions; that is, functions where the area under the curve represents the probability (or frequency) and so the cumulative form of the distribution has a value of 1. These probability density functions are the sums of a number of exponential components, the number of these components being equal to the number of closed or open states which contribute to the times which have been measured (Colquhoun and Hawkes, 1981). For example, measurements of the open times from a channel with a single open state will yield a histogram which may be fitted with a single exponential distribution with a time constant of $\tau$ which is equal to the mean open time of the channel and also equals $1/\lambda$ where the probability density function is

$$f(t) = \lambda e^{-\lambda t} \quad \text{when } t > 0 \text{ and has dimensions of } \text{sec}^{-1}; \text{ and histograms of open times from a channel with more than one open state may be fitted with a sum of exponential distributions.}$$
1.4.3 First latency distributions.

Measurements of the latency from the start of a depolarizing pulse to the first opening of a voltage dependent channel allow derivation of information about the states which precede the state into which the channel opens (Fukushima, 1981; Patlak and Horn, 1982). If a histogram of these results may be fitted with a single exponential (that is, the distribution peaks at zero time) this indicates that only one closed state precedes the opening of the channel. Patlak and Horn (1982) gave the expression to calculate the waiting time for a voltage dependent channel with two closed states which the channel passes through sequentially before opening

\[ C_2 \xrightleftharpoons[k_2]{k_1} C_1 \xrightleftharpoons[k_2]{k_1} O \]

Assuming that the state transitions fit the restrictions of a Markov process (that is, that the time spent by the channel in any one state is exponentially distributed and that this time is independent of the channel's history) then the probability density function of the distribution of first latencies is given by

\[
f(t) = \frac{R_1R_2 \cdot [\exp(-R_2t)-\exp(-R_1t)]}{R_1-R_2}\]

where \( R_1, R_2 \) are given by

\[ R_1R_2 = \frac{[k_1+k_2+k_2+]}{[(k_1+k_2+k_2)^2-4k_1k_2)]}/2 \]
1.4.4 Runs analysis.

Runs analysis (Swed and Eisenhart, 1943; Gibbons, 1971) allows calculation of the probability that the hypothesis that blank records tend to occur in groups is incorrect. The method has been used in the study of sodium channels by Horn, Vandenberg and Lange (1984) and of delayed rectifier potassium channels by Standen, Stanfield and Ward (1985). If the probability of observing a channel opening within a current trace is $p$ then the expected number of runs (series of blank traces or of traces containing openings) is $2p(1-p)$ where $n$ is the number of traces recorded and must be greater than 40. The distribution of the number of runs is given approximately by

$$Z = \frac{R - 2np(1-p)}{2\sqrt{np(1-p)}}$$

where $Z$ is a standardized random variable with mean $= 0$ and variance $= 1$, and $R$ is the observed number of runs. $Z$ is therefore close to zero if traces are randomly ordered and positive values of $Z$ correspond to grouping of traces with and without openings.

The method of analysis described above allow the discovery or confirmation of qualitative assessments of channel behaviour (for example, clustering of traces with and without openings) and allow detailed kinetic information to be derived from experimental results (for example, the time constants of transitions between states). The methods therefore allow a picture of channel behaviour to be built up, and allow the kinetic properties of different channels to be compared.
1.5 MULTIPLE CONDUCTANCE STATES

The single channel recording technique and the methods of analysis developed to derive information from the data which it yields have shown that channels do not usually have simple kinetics with a single open and a single closed state. Open and closed time analyses have shown that some channels have more than one kinetically distinguishable open state or more than one closed state or both. Studies of channel amplitudes have recently begun to show that some channels possess more than one open state which may be distinguished by their conductances as well as by their kinetics. These studies yield potentially valuable information about the nature of the conformational changes which occur in a membrane channel protein when it undergoes state transitions. They should be more useful when a possible molecular structure can be postulated for a channel which has been shown to have several open states of different conductance. The following provides a possible categorization of the main types of multiple-conductance channel which have been recorded. Such a categorization is possible because some of the channels exhibit different modes of transition; the categorization of multiple-conductance state channels into these different groups may be shown in the future to be totally arbitrary or may have some basis in different molecular behaviours of the different groups of channels.

The behaviour of some of the multiple-conductance channels which have been described using patch-clamp recording techniques suggests that the channels are made up of identical subunits. The acetylcholine receptor channel of *Torpedo* electroplax, reincorporated into lipid bilayers, appears to exist both as a monomer and as a dimer (having twice the conductance of the monomer) (Schindler, Spillecke and...
Newmann, 1984). The conductance of the dimer is approximately equal to that of the channel in vivo, suggesting that in vivo the channel always inserts into the membrane as a dimer whereas reincorporated channels may split into their constituent monomers or may remain associated into dimers. The chloride channel of *Torpedo* electroplax also appears to consist of dimers (Miller, 1982; Hanke and Miller, 1983). The channel shows two conductance states, the larger conductance being equal to twice the conductance of the lower. The channel shows periods of activity where its behaviour suggests that the two conductance levels are made up of two symmetrical subunits, each opening independently, separated by periods where the channel does not open. Miller (1982) and Hanke and Miller (1983) suggest that the substructure of the chloride channel is an expression of the independent opening and closing of two identical chloride diffusion pathways and that the overall channel (both units) may be closed by a separate gate. Hunter and Giebisch (1987) have reported potassium channels in renal tubules of *Amphiuma* which show similar activity. These channels have four subunits with equal conductance which appear to open and close independently from one another, the overall activity of the channel again being controlled by a separate gate which is able to close all four subunits. Matsuda (1988), has recorded the inward rectifier of guinea pig heart and has shown that rectification is conferred upon the channel by a voltage dependent block by magnesium ions. This block also reveals two subconductance states of 1/3 and 2/3 the main conductance; the states are occupied less frequently than is the main conductance state. Matsuda (1988), suggests that this behaviour is because the channel consists of three identical subunits, each of which is blocked independently by magnesium.

Some multiple-conductance channels exhibit two open conductances, the
smaller of these not being equal to half of the larger. It has been suggested (Fox, 1987) that the smaller state may represent a partially blocked form of the larger state although the possibility that there are two subunits of unequal conductance cannot be ruled out. Of these channels, some may open to the open states in any order. Examples of this type of channel are the acetylcholine receptor channel of embryonic muscle (Trautmann, 1982; Takeda and Trautmann, 1984) although Hamill and Sakmann (1981) found that this channel may not open to the smaller of the two states first; and the potassium selective channel from skeletal muscle (Fox, 1985; Tomlins, Williams and Montgomery, 1984; Tomlins and Williams, 1986). Others appear to only reach the larger conductance state after passing through the smaller. The multiple-conductance potassium channel described later may exhibit this behaviour, as does a potassium selective channel of frog skeletal muscle (Labarca and Miller, 1981). The acetyl-choline receptor channel of embryonic muscle recorded by Hamill and Sakmann (1981) appears to only be able to open to the smaller conductance state after opening to the larger although, as mentioned above, this does not agree with findings of other workers for the same channel.

Multiple-conductance states have been recorded in channels which are modulated by excitatory amino acids. Jahr and Stevens (1986) recorded a cation channel in rat hippocampal neurones whose activity depends on which agonist is used to activate the channel. When the channel is activated by glutamate it exhibits several conductance levels between 5 and 60 pS and shows transitions between all of these levels; when activated by N-methyl-D-aspartate (NMDA, a glutamate analogue) conductance levels ranging between 40 and 60 pS are seen; and when quisqualate or kainic acid are used to activate the channel the conductance levels seen are all less than
20 pS. Hamill, Bormann and Sakmann (1983) recorded chloride channels from cultured mouse spinal cord neurones which show two conductance levels when activated with $\gamma$-amino butyric acid (GABA), the most common conductance state having a conductance of 30 pS and the other state having a conductance of 20 pS, whereas when the channel was activated with glycine three conductance levels were seen, the most common being 45 pS and the other states being the 30 pS and 20 pS conductance states seen in the presence of GABA. These observations confirmed the suggestions of Barker and McBumey (1979) who performed fluctuation analysis of GABA- and glycine-activated currents in cultured mouse spinal neurones. Cull-Candy and Usowicz (1987) recorded similar channels from cultured foetal rat cerebellar neurones which were activated by L-glutamate, L-aspartate, NMDA, quisqualate and kainate. They found that all of these agonists opened channels with at least five different conductance states, the largest being approximately 45-50 pS in the conditions used. NMDA predominantly activated conductance levels above 30 pS while quisqualate and kainate mainly activated ones below 20 pS.

There are many other channels in which subconductance states are exhibited rarely. It is unlikely that the presence of multiple conductance states in such channels has any physiological significance since the contribution of the infrequently visited states to the overall membrane conductance will be small. In channels where the openings to a state other than the main conductance state make up a significant proportion of the time which the channel spends open, however, it may be possible that the presence of more than one open conductance state allows for an extra modulation of the membrane conductance. In the case of the channels described above which are regulated by excitatory amino acids (Hamill et al, 1983; Jahr and Stevens, 1986;
Introduction

Cull-Candy and Usowicz, 1987) it is likely that the presence of multiple conductance states has physiological significance and allows complicated modulation of the membrane conductance by those amino-acids. In the case of voltage-activated channels which have multiple conductance states it may also be possible that these allow for a more complicated modulation of the membrane conductance than is possible from a single channel type with one open state conductance.

1.6 SUMMARY

Above, I have summarized previous investigations of the ionic currents of neuroblastoma cells and have briefly described some of the investigations which have been carried out on sodium, calcium, chloride and potassium currents in other preparations. This provides a background against which the work described in this thesis may be viewed. The work which is to be presented below investigates some of the ionic currents of the neuroblastoma cell line N1E115 which may be recorded using patch-clamp recording methods (see section 2.5): sodium currents, non-selective cation currents, calcium-activated potassium currents and delayed rectifier potassium currents are described. The delayed rectifier is described in detail and it is demonstrated (Chapter 4) that the unitary delayed rectifier potassium currents have at least two open states of different conductance. I have therefore provided, in this introduction, a summary of different types of multiple conductance state channels against which the delayed rectifier described later may be compared. The TEA+ block of this current is also described.

In order to characterize the above currents and to compare them with those recorded from other preparations the methods of analysis summarized in section 1.4
have been applied to the recorded currents. There are some basic assumptions which
have been made in order to analyse the currents described here in a way which allows
their properties to be compared with those of other currents. It is assumed that
rectangular changes in recorded current seen under cell-attached patch clamp or
excised patch clamp recording are due to the opening of single channels and that the
instantaneous current-voltage relation of these is linear (ohmic). It is also assumed that
the channel states follow the rules of a Markov process (the dwell time in a single state
is distributed exponentially and does not depend on the channel’s history) so that the
probability density functions for open and closed times and first latencies described
earlier may be applied to the results. It is shown that the ionic currents which may be
recorded from differentiated mouse neuroblastoma N1E115 cells are those which
would be expected in a neuronal cell.
Chapter 2

MATERIALS AND METHODS

2.1 PREPARATION

Cells from the mouse neuroblastoma cell line N1E115 were used exclusively in this work as cells from this line have already been used to study many aspects of neuronal function and differentiation (see Chapter 1). The cells were a gift from Dr. P. Strange, Department of Biochemistry, University of Kent.

Line N1E115 is a clonal cell line derived from the mouse C-1200 neuroblastoma which was a spontaneous tumour of sympathetic origin. The derivation of this line has been described by Amano, Richelson and Nirenberg (1972). The cells are normally grown in a culture medium containing Dulbecco's modified Eagle's medium (DMEM) and foetal calf serum. In the presence of 1-2% dimethyl sulphoxide (DMSO), however, they begin to differentiate morphologically and electrophysiologically. This differentiation continues over a period of at least 18 days, during which time the cells send out processes and divide more slowly than cells maintained in DMSO-free medium (Kimhi, Palfrey, Spector, Barak and Littauer, 1976). They grow larger, develop a larger resting potential and have action potentials with a faster rate of rise than those of undifferentiated cells. The cells used in the experiments described in this thesis were in the early stages of differentiation having been grown in the presence of DMSO for between 1 and 7 days.
2.1.1 Cell storage

Cells were stored in medium of the following composition:

**Storage medium**

- 10% (by volume) x10 DMEM (Gibco)
- 20% (by volume) foetal calf serum
- 10% (by volume) dimethyl sulphoxide (DMSO)
- NaHCO₃ (0.375% by weight; 45 mM)
- 2 mM L-glutamine
- 50 units/ml penicillin
- 50 units/ml streptomycin

made up to 100% with distilled water.

This medium was sterilised by filtration through a 0.2μm pore filter. All media and solutions used were sterilised in this way.

1.5ml aliquots of cells suspended in this medium were placed in sterile plastic vials and cooled slowly by keeping at 0°C for 30 minutes, -20°C for 2 hours and then at -70°C overnight. The vials were then stored at -140°C in liquid nitrogen until cells were required for experiments.
2.1.2 Thawing of cells

When cells were required they were removed from the liquid nitrogen and quickly thawed by placing the vial in an incubator at 37°C for ten minutes. The contents of the vials were then washed out into sterile plastic centrifuge tubes with medium of the following composition:

**Culture medium**

10% x10 DMEM
10% foetal calf serum
NaHCO₃ (0.375% by weight; 45 mM)
2mM L-glutamine
50 units/ml penicillin
50 units/ml streptomycin

made up to 100% with distilled water

When the cell suspension had been placed in sterile plastic centrifuge tubes the total volume in the tube was made up to 10ml with culture medium. The tubes were then centrifuged at 4,000 revolutions per minute for 5 minutes to spin the cells down, separating them from the DMSO contained in the freezing medium. Cells of this line begin to differentiate when placed in medium containing >1% DMSO (Kimhi, Palfrey, Spector, Barak and Littauer, 1976). Once spinning down was
completed the supernatant was removed and discarded. The cell pellet was then resuspended in culture medium and plated out onto plastic tissue culture dishes or flasks. It was important that the culture medium did not contain DMSO since the object of this stage of culturing is to obtain an undifferentiated, dividing stock of cells.

Some of the cells plated out using the above method adhere to the plastic within an hour while some remain in suspension in the medium and others die.

2.1.3 Maintenance of the cell culture

The dishes and flasks containing cells and culture medium were kept at 37°C in a humidified atmosphere in a gas mixture of 5% CO₂ in air. This maintained the pH between 7.3 and 7.4. The medium was replaced every three days and the culture was prevented from becoming confluent by regular seeding of fresh flasks and dishes from ones in which the cells were becoming dense. Cultures could be kept in this way for several weeks.

2.1.4 Cell differentiation

When cells were required for experiments a flask containing undifferentiated cells in the logarithmic growth phase was shaken to detach some of the cells which had adhered to the plastic surface. 0.5 ml of the resultant cell suspension were placed into a plastic dish with at least 4.5 ml of differentiation medium which contains 1.3% DMSO. This gives a final DMSO concentration in the dish of between 1.2 and 1.3%. In these conditions the cells begin to differentiate.
Materials and methods

Differentiation medium

10% x10 DMEM
0.3% foetal calf serum
1.3% DMSO
NaHCO₃ (0.375% by weight; 45 mM)
2mM L-glutamine
50 units/ml penicillin
50 units/ml streptomycin

made up to 100% with distilled water

Cells were allowed to grow in this medium at 37°C in a humidified atmosphere of 5% CO₂ in air for between 1 and 7 days before use in experiments. It was seen that a large number of cells adhered to the bottom of the dishes. This is a useful property of this type of culture as the cells could be used in experiments while remaining on the bottom of the dish. The culture dish could therefore be used as the experimental bath.
2.2 EXPERIMENTAL SOLUTIONS

2.2.1 Bath solutions

The normal "extracellular" solution was solution A (table 1) with an extracellular potassium concentration ([K+]_o) of 5 mM. When it was necessary to reduce the resting potential of the cells [K+]_o was elevated to 120 mM by substituting potassium for sodium in solution A.

Solution A was used as the bath solution in experiments using the cell-attached recording mode and the whole-cell recording mode. For experiments in which I looked at the effect of tetraethylammonium ions (TEA+) on the macroscopic potassium currents TEA chloride was substituted for NaCl in this solution.

For experiments using the ripped-off (excised) patch recording mode solution B, which contained no calcium, was used as the bath solution. This was used in order to minimise the chance of the membrane of the excised patch folding around and sealing into a vesicle on the end of the patch electrode.

2.2.2 Electrode solutions

For cell-attached recording, where the electrode solution is bathing the extracellular face of the membrane patch, solution A was used in the electrode. The pipette potassium concentration, [K]_p, was altered by substituting KCl for NaCl. In experiments which looked at the effect of TEA+ on the single channel currents TEA chloride was substituted for NaCl in the electrode solution.

For ripped off patch recording solution C, a low chloride solution, or D, a zero sodium solution, was used in the pipette. The potassium concentration of solution
C was varied by substituting KCl for NaCl.

For whole-cell recording an "intracellular" solution, solution E, was used in the electrode. This solution had a free calcium concentration ([Ca$^{2+}$]) of $1.5 \times 10^{-8}$ M.

2.2.3 Flow solutions

For experiments using flow pipes in order to change the solution bathing the intracellular side of a membrane patch (see Chapter 6) solutions C, F, and G were used. In some experiments sodium was substituted for potassium in solution F. When it was necessary to change the free calcium concentration in this solution the relative proportions of EGTA and CaEGTA were changed assuming an apparent stability constant for CaEGTA ($K_a$) of $6.645 \times 10^6$ M$^{-1}$ at a pH of 7.2. The ratio of CaEGTA to EGTA required to give a free calcium concentration [Ca$^{2+}$] is given by

\[
\frac{[\text{CaEGTA}]}{[\text{EGTA}]} = [\text{Ca}^{2+}]K_a[\text{EGTA}]
\]

The method of calculating the apparent stability constant of CaEGTA is detailed in appendix III of Plant (1982) and is based on stability constants given in Martell and Smith (1974).

2.2.4 Liquid junction potentials

When the electrode solution is different from the bath solution a liquid junction potential arises between the two solutions because of differences in the ionic mobilities of the ions present in the two solutions. The theoretical liquid junction potential ($E_j$) of the electrode solution relative to the bath solution may be calculated,
for example when the solutions contain different concentrations of sodium and potassium but equal concentrations of chloride, from

$$E_j = \frac{RT \ln \left( \frac{u_{Na^+} [Na^+]_b + u_{K^+} [K^+]_b + v}{F u_{Na^+} [Na^+]_p + u_{K^+} [K^+]_p + v} \right)}{F}$$

where $u$ is the ionic mobility, and $v$ is the ionic mobility of chloride.

Liquid junction potentials between electrode solutions were determined experimentally using a pH electrode as the reference electrode. These values were typically 1 or 2 mV less than the corresponding theoretical liquid junction potential and have been used to correct the commanded values of the electrode potential in an attempt to obtain actual values for the clamping potential.
### Table 1

<table>
<thead>
<tr>
<th></th>
<th>K</th>
<th>Na</th>
<th>Cl</th>
<th>Ca</th>
<th>Mg</th>
<th>EGTA</th>
<th>HEPES</th>
<th>SO$_4^{2-}$</th>
</tr>
</thead>
</table>
| A | 5  | 147| 154.4| 1.8| 0.4|-|5|-
| B | 5  | 147| 154.4| - | 2.2|-|5|-
| C | 5  | 147| 9.4 | - | 2.2|-|5| 72.5 |
| D | 152| -  | 154.4| 1.8| 0.4|-|5|-
| E | 165.5| - | 146 | 1.8x10$^{-6}$| 4.0|11|10|-
| F | 152.3| - | 150 | varied| -|1|5|-
| G | 5  | 147| 154.4| 1.8| 0.4|-|5|-

Table giving mM concentrations (except for the free calcium concentration given for solution E) of ions present in the different experimental solutions used.

The pH of solutions A to D was 7.3 and of E to G was 7.2. Solutions A, B and C contained 5 mM glucose. HEPES was titrated with KOH in solutions D, E and F and with NaOH in solutions A, B, C and G.
2.3 EXPERIMENTAL ARRANGEMENT

Cells were prepared for an experiment by removing the differentiating medium from the culture dish and washing the cells which had adhered to the bottom of the dish once with one of the bath solutions (see section 2.2.1). The temperature of the bathing solutions was first raised to 37°C so that the cells did not experience a sudden temperature change when the solution was changed. Washing was necessary to remove all the DMSO which is known to block some potassium channels (Jourdon, Berwald-Netter and Dubois, 1986). In order to do this approximately 5 ml of bath solution was allowed to stand in the dish for 10 min after which time it was removed and replaced with 1.5 to 3 ml of fresh bath solution in which the experiment was performed.

The experimental bath solution could be exchanged in the course of an experiment through silicone rubber tubes arranged as inlet and outlet pipes. One end of each pipe was held in the experimental bath by a perspex block (figure 2.1) while the other end was attached to a 20 ml syringe. One syringe was used to draw solution out of the bath and the other used to force a new solution into it. This exchange had to be done very slowly so that the recording arrangements between the electrode and the cell were not disrupted. In order to ensure the complete exchange of the bathing solution a volume of new solution ten times that of the solution in the bath was washed through the bath. The efficiency of this method of solution changing was checked by measuring the dilution of dye placed in the bath when washed through with distilled water. A spectrophotometer was used to measure the change in concentration of dye in the bath after completion of the exchange procedure. It was
Materials and methods

Figure 2.1

Schematic diagram of the experimental bath showing the solution-exchanging method.

Two perspex blocks fit onto the bath. Silicone rubber tubes leading from these blocks to 20 ml syringes form the inlet and outlet pipes. The end of the syringe needle which formed the first part of the outlet pipe was placed approximately 3 mm above the bottom of the bath to prevent emptying of the bath during solution exchange.

When a volume of new solution ten times that of the solution in the bath was washed through the bath using this system it was found that 99.4% of the original solution could be replaced.
IN

- diameter approx. 2mm

- syringe needle

OUT

- experimental bath
found that 99.4% of the original solution could be replaced using this method. The solution changing procedure was therefore considered to be adequate.

The experimental bath containing cells which were still adhered to the plastic was placed on the stage of a Zeiss or Nikon inverting microscope. The bath was grounded through an agar bridge between the bath and a pot containing electrode solution, the pot being held at ground by a silver/silver chloride pellet connected by a wire to the circuit ground of the head stage. Illumination through the microscope condenser was provided by a fibre-optic light guide and the cells were viewed under phase-contrast. The microscope was placed on an Ealing vibration isolation system which decreases vibration in the vertical plane making recording with patch-clamp electrodes easier. The whole apparatus was enclosed in a Faraday cage to minimise electrical interference at the recording electrode and head stage.

Recording electrodes were held in a teflon electrode holder (drawn to a scale of 2mm : 1mm in figure 2.2) which plugged into the recording head-stage. The electrodes were moved into contact with a cell using an electrically (fine movement) and manually operated (coarse movement) micromanipulator onto which the recording head stage was mounted. Fine movements of the manipulator were achieved by remotely controlling the manipulator electrically from a control box outside the Faraday cage.

This arrangement was used in the three methods of recording the membrane currents described below.
Materials and methods

Figure 2.2

Electrode holder.

The drawing is to the scale of 2mm : 1mm. The holder was made from teflon. Electrical contact was made between the electrode solution and the head-stage by means of the chloride-coated silver wire. A silicone rubber tube lead to the side-arm so that positive-pressure or suction could be applied to the back of the electrode.
side-arm

O ring

Ag/AgCl wire
2.4 RECORDING ELECTRODES

2.4.1 Pulling

Patch clamp and whole-cell clamp electrodes were formed from borosilicate glass (Clark Electromedical Instruments, GC150-15) with an external diameter of 1.5 mm and an internal diameter of 0.86 mm.

Patch clamp electrodes were made using a vertical microelectrode puller (Kopf, 700D) arranged to give a double pull. The glass was melted by a nichrome heating coil of internal diameter 5 mm and length 5 mm. To produce the first pull a voltage of between 3.27 and 4.30 V was applied across the coil, giving a heater current between approximately 20.5 and 21.0 A. This melted the glass until it stretched and thinned, the elongation being limited to 9 mm by a stop placed beneath the electrode carriage. The heat was then switched off, the glass recentred within the coil and the stop removed. A voltage of between 2.11 and 2.19 V was then applied across the coil, giving a heater current between approximately 13.5 and 15.5 A. This second pull stretched and thinned the glass further until it separated into two electrodes.

An estimate of the resultant tip diameter of the electrodes was gained from a measurement of the pressure which had to be applied to the back of the electrode to cause bubbles to form at its tip. The threshold pressure is termed the "bubble number" and was found by connecting a 10 ml syringe to the back of the electrode with a 10 cm length of polyethylene tubing and then depressing the plunger until bubbles escaped from the tip of the electrode into methanol. The bubble number is taken as the position in the syringe to which the plunger must be depressed for bubbles to escape so a smaller bubble number means that a greater pressure is required to form bubbles.
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After pulling, the most useful patch electrodes had a bubble number between 4.8 and 5.5.

Whole cell clamp electrodes were also formed on a Kopf microelectrode puller. A Kanthal coil of internal diameter 4.6 mm and length 3.8 mm was used with a voltage of between 3.50 and 4.30 V to give a heater current between 8.6 and 9.0 A. The melted glass was stretched over a distance of 7 mm. The second pull, at a voltage between 3.08 and 3.40 V, gave a heater current between 6.4 and 6.8 A. This produced electrodes with a larger tip diameter than that of patch electrodes so that the resistance of the electrodes was low and so that access to the cell could be gained after seal formation by applying suction to the back of the electrode to break the cell membrane within the seal. After pulling the best whole-cell clamp electrodes had a bubble number of between 6.8 and 7.5.

2.4.2 Coating

Electrodes were then coated close to the tip with Sylgard resin using the method described by Corey and Stevens (1983). Partly cured Sylgard was placed onto the electrode using a mounted needle and then completely cured by drawing the pipette into the centre of a heated coil. Coating reduces the capacitance to ground of the electrode since the capacitance between two concentric, cylindrical conductors (the electrode and the bath solution surrounding the electrode) is proportional to the logarithm of the ratio of inside diameter to outside diameter and coating with Sylgard increases the outside diameter by increasing the effective wall thickness of the electrode. Reducing the capacitance of the electrode decreases the background noise of the recording system and reduces the size of the capacitative transient which occurs
2.4.3 Heat polishing

After coating, the tips of the electrodes were heat polished by manipulating the electrode tip close to a glass bead sitting on a hot platinum wire which formed the filament in a heating circuit. A stream of air directed at the filament during polishing decreased any temperature fluctuations caused by extraneous draughts of air. Heat polishing slightly melts and thereby smooths the glass at the tip and burns off any Sylgard which has crept along the glass to the tip. The process was carried out under a compound microscope (magnification 400x) with long working distance objectives. After polishing the tip size, which decreases when the electrode glass near the bead melts, was again tested by bubbling. The most useful patch clamp electrodes had bubble numbers of between 2.5 and 4.5 and whole-cell clamp electrodes bubble numbers of between 5 and 6.5 after heat polishing.

2.5 RECORDING MODES

2.5.1 Cell-attached recording mode

In order to record in the cell-attached recording mode a patch-clamp recording electrode made as described in section 2.4 was placed in the electrode holder and positive pressure (approximately 10 cm H_2O) was applied to the back of the electrode through a pipe attached to the side-arm on the electrode holder (figure 2.2). This helps to prevent any debris from adhering to the tip of the electrode as it is moved through the bath solution. The resistance of the electrodes was estimated by applying
0.5 mV across the electrode tip and measuring the resultant current. Patch clamp electrodes with a resistance between 8 and 26 MΩ were found to be the most useful, while whole-cell electrodes with resistances of between 1 and 4 MΩ were best. The electrode was advanced towards a cell until the resistance of the electrode increased by approximately 50% indicating that the tip had come into contact with the cell surface. Suction (approximately 15 cm H$_2$O), generated by a vacuum pump attached to a water tap, was then applied to the back of the electrode through the side arm of the electrode holder. This caused the apparent resistance of the electrode to increase further as the glass at its tip sealed to the cell membrane. The seal resistance was estimated by measuring the current induced by a voltage pulse of 20-30 mV applied across the electrode tip. The seal resistance of those patches from which current was recorded was in the range of 10-100 GΩ. This method electrically isolates a small patch of membrane (≤1 μm$^2$) which may then be voltage clamped. The high resistance of the seal suggests a very small leakage current between the pipette and the membrane. This current is small enough for trans-membrane patch currents of <0.5 pA to be detected.

In the cell-attached recording mode the electrically isolated membrane patch is allowed to remain attached to the cell so that the intracellular membrane surface of the patch remains in contact with the intracellular solution. Cell-attached patch clamped potentials are therefore measured relative to the membrane potential of the cell.
Materials and methods

Figure 2.3

Schematic diagram of the flow restrictor.

In order to change the solution bathing the intracellular surface of a ripped-off patch the recording electrode was moved between the mouths of a series of glass flow pipes with an approximate tip diameter of 400 μm. Solutions flowed through these pipes from reservoirs (20 ml syringes) through flow restrictors. The flow restrictors were formed from conventional microelectrodes the tips of which were broken off. These were cemented into the end of glass tubes with epoxy resin.
20 ml syringe

patch electrode

flow pipe

tip diameter approx. 400µm

epoxy resin
2.5.2 Ripped-off patch recording mode

The ripped-off or excised patch recording mode was achieved by first establishing a cell-attached patch as described above and then pulling the electrode back from the cell. This pulls a patch of membrane away from the cell, exposing its intracellular surface to the bath solution. This surface of the membrane may then be exposed to a number of different solutions. The solutions were changed using a method slightly modified from that of Spruce, Standen and Stanfield (1985) which was derived from the method of Yellen (1982). The recording pipette was moved between the mouths of a series of glass flow pipes with an approximate tip diameter of 400 μm. Solutions flowed through these pipes from reservoirs (figure 2.3) through flow restrictors which reduce the sensitivity of the flow rate to the head height. With this arrangement and a head of 2-5 cm flow rates of 0.3 to 0.6 ml/h, which are unlikely to disrupt the seal between the electrode and the membrane patch, could be achieved (Spruce, 1987).

2.5.3 Whole-cell recording mode

The whole-cell recording mode was established by using whole-cell clamp electrodes to form a seal as described above. After the seal had been made a further and sudden suction was applied to the pipe attached to the arm of the electrode-holder, either by mouth or by using a 10 ml syringe. This causes rupture of the cell membrane within the area of the seal to form a low resistance pathway into the cell. In this situation the contents of the recording electrode are able to exchange freely with the contents of the cell. As the volume of the cell was negligible in comparison to that of the electrode, the concentration of ions in the new intracellular fluid was assumed to
be the same as that in the electrode filling solution. Exchange of ions such as Na\(^+\) and K\(^+\), which are free in the cytoplasm, is complete after approximately 15 seconds (Marty and Neher, 1983) although, because the speed of diffusion of cytoplasmic constituents depends on their size, a globular protein will have a time constant for exchange of around 3 minutes. Ions such as Ca\(^{2+}\), which are buffered by cell organelles, will also exchange slowly so a high concentration of EGTA (11 mM) is included in the pipette solution to give a high calcium-buffering capacity. The electrodes used for whole-cell recording have a larger tip than those used for patch-clamping (see section 2.4.1) and have a correspondingly lower resistance (1 to 4 M\(\Omega\)). The resistance of the electrode must be low since the access or series resistance of the recording is partly determined by the electrode resistance and partly by the degree of membrane disruption achieved by the suction. It is important to keep this series resistance low so that the voltage drop which occurs across the series resistance when current flows through the pipette tip is small and so that the time constant of the voltage clamp is short. In practice electrodes giving series resistances of 9 to 35 M\(\Omega\) (rarely above 20 M\(\Omega\)) with 40-90% series resistance compensation were used.

The series resistance \(R_s\) was always measured after disruption of the membrane, and if possible later in the experiment, by applying a 10 mV pulse to the cell and recording the resultant capacitative transient. This was done without any capacity or series resistance compensation (such compensation is described in section 2.6.4). The decline of the capacitative transient was fitted to a single exponential using a least-squares fitting method (Marquardt, 1963) and the time constant for the decay \(\tau\) was measured. A transient capacitative current recorded from a preparation which had a series resistance of 20 M\(\Omega\) is shown in figure 2.4 A.
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Figure 2.4

Transient capacitative current recorded under whole-cell clamp without capacity and series resistance compensation.

The current was recorded from a preparation which had a series resistance of 20 MΩ. The current shown in A is repeated in B fitted with a single exponential of time constant 1.82 msec.
The same current fitted with a single exponential with a time constant of 1.82 msec is shown in figure 2.4 B. Measurement of the area under the capacitative transient gave the charge moved by the voltage step (Q). The capacity, C, was calculated using the relationship $C = Q/V$ where V is the voltage pulse. This value of C was then used to determine the series resistance from the relationship $R_s = \frac{V}{Q}$. FORTRAN programs were written to find both the single exponential time constant and the area under the capacitative transient and to calculate the series resistance.

A proportion of the series resistance could be electrically compensated for using the method described in section 2.6.4. The current necessary to clamp the membrane voltage at the command voltage was measured and the voltage drop which this current produced across the series resistance (after compensation) was calculated using Ohm's law. Using the criterion of Marty and Neher (1983) the results were discarded if the current necessary to clamp the voltage of the cell membrane was found to produce a voltage drop across the series resistance of > 3 mV. Small cells (< 30 μm) were chosen so that the membrane currents elicited by a voltage step were small and the current which flowed through the pipette to maintain the membrane voltage at the command voltage was also small, leading to a small voltage drop across the series resistance.
Figure 2.5

Block diagram of the electrical apparatus.

The pipette current is converted to a voltage $i_p R_f$ in the head stage. $V_c$ is the voltage commanded by the voltage pulse-producing apparatus. $V_{\text{ref}}$ is the voltage after modifications for capacity and series resistance compensation. Recorded currents were stored either on FM tape or on floppy disk.
2.6 VOLTAGE CONTROL AND CURRENT RECORDING

2.6.1 Electronic apparatus

A block diagram of the apparatus used in these experiments is shown in figure 2.5. The apparatus is arranged to allow currents to be passed through the electrode in order to control the membrane potential of a patch and to allow sampling and recording of these currents. The diagram shows the pathway through the apparatus which produces the command voltage controlling the potential of the electrode (from the BBC microcomputer to the electrode), the pathway of the measured pipette voltage (from the patch clamp amplifier through a variable gain to the analog-to-digital converter or the oscilloscope) and the pathway taken by the recorded current (from the electrode to the BBC). The BBC microcomputer linked to an intelligent analog-to-digital converter (Cambridge Electronic Design; 1401) was used to produce changes in the command voltage and to record the membrane currents.

The head stage was connected to the electrolyte inside the electrode with a silver/silver chloride wire.

2.6.2 Control of holding potential

The membrane voltage was clamped to a holding potential \( V_{\text{ref}} \) commanded by the patch clamp amplifier (List electronic; EPC7). In the cell-attached recording mode this holding potential is set relative to the membrane potential of the cell, whereas in excised patch or whole-cell recording mode the potential is set relative to the bath potential which is at ground. The reference voltage \( V_{\text{ref}} \) is set by the patch clamp amplifier in response to stimulus inputs. This reference voltage is
applied to the non-inverting input of an operational amplifier in the head stage as shown in figure 2.6. The inverting input of the op-amp is at the pipette potential \( V_p \). If \( V_p \) is not equal to \( V_{\text{ref}} \) then the output of the op-amp has a voltage \( (V_B) \) which causes a current to flow through the 10 G\( \Omega \) resistor until \( V_p = V_{\text{ref}} \). The op-amp has a high input impedance and therefore draws no current.

2.6.3 Changes in voltage

1. Voltage steps

Step-wise changes in the command voltage were produced using a BASIC program written for the BBC microcomputer (Quayle, Smith and Ward, 1985). This program enables the BBC to be used to set up arrays of pulses which are defined by the start time, duration and amplitude. These arrays are then passed to an analog-to-digital converter (ADC) which gives corresponding voltage pulses at its digital-to-analog ports.
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Figure 2.6

Head-stage circuit.

$V_p$ is the pipette voltage, $V_{\text{ref}}$ the commanded clamping voltage and $V_B$ the difference between the two. $R_f = 10 \, \text{G}\Omega$. $i_p$ is the clamping current.
A diagram showing a circuit with an operational amplifier and a differential amplifier. The operational amplifier is connected to a resistor $R_f$ and a reference voltage $V_{\text{ref}}$. The differential amplifier produces a current $i_p R_f$. The diagram includes labels for $V_p$, $V_B$, and $i_p R_f$. The circuit represents an operational amplifier and a differential amplifier.
Materials and methods

These pulses are amplified in pulse divider A (figure 2.5 A) to give a voltage which is ten times that required at the pipette and then pass to the stimulus input of the patch clamp amplifier in which they are divided by ten before being applied to the head stage.

2. Ramped voltages

In a number of experiments it was necessary to produce ramped changes in the command voltage. To produce these changes the ADC triggered a digitimer to give two pulses of variable duration. One pulse passed to a pulse divider giving a voltage step of the form shown below:

![Voltage step diagram](image)

The second pulse was delayed by 20 msec with respect to the first and triggered a voltage ramp generator (appendix I). This generator produced a voltage ramped linearly at a pre-determined rate. This ramped voltage was added to the voltage step using a summing amplifier so that the voltage at the stimulus input of the patch clamp amplifier had the general form

![Ramped voltage diagram](image)
2.6.4 Data collection

The clamping current causes a voltage drop across the 10 GΩ resistor, $R_f$, in the head stage circuit (figure 2.6). This voltage drop, $i_p R$, which is equal to the difference between $V_B$ and $V_{ref}$, is measured by the differential amplifier. This voltage is amplified further in the patch clamp amplifier to give a maximum gain equivalent to 1 V/pA. The equipment is recording this voltage which is proportional to the current flowing at the pipette tip. The recordings are, however, always referred to as current recordings.

Because of the high resistances used in these circuits their gains can alter with changes in humidity and temperature. The gain of the system was therefore calibrated at the start of each experiment by measuring the current produced by a 5 mV pulse applied across a 10 MΩ resistor. This value for the input gain was then used in analysis of currents to correct the measured current amplitudes.

A complex transient capacitative current occurs when the voltage of the electrode is stepped. This current arises because of the capacitance of the pipette ($C_p$) and the stray capacitance of the head stage ($C_s$) (see figure 2.7). It is important to cancel at least a proportion of the capacitative transient since its amplitude is large enough to saturate the ADC and prevent measurement of single channel current amplitudes early in the pulse. If the capacitances involved are large their charging may also require so much current from the I-V converter in the head stage that the op-amp is saturated and control of the pipette voltage is lost.
Materials and Methods

Figure 2.7

Diagram of the circuit which charges the pipette and head stage capacitances.

The patch clamp amplifier generates capacitative currents with variable time courses and amplitudes the values of which were selected during an experiment in order to minimise the current required from the I-V converter. Without this compensation the charging of the pipette and head stage capacitances may require so much current from the I-V converter that the op-amp is saturated and control of the pipette voltage is lost.
The capacitative currents were cancelled as much as possible by charging the pipette and head stage capacitances by a separate pathway, shown in figure 2.7, from that clamping the voltage. This facility is provided by the patch clamp amplifier which generates capacitative currents with variable amplitudes and time constants. These currents are of opposite sign to those arising in the pipette and head stage and are used to charge these capacitances. Their amplitudes and time courses were selected in order to minimise the current required from the I-V converter.

In whole-cell recording mode it is also necessary to cancel a slow capacitative current arising in the cell membrane. A proportion of the series resistance which is present in whole-cell recording mode was also compensated for. This was achieved in the patch clamp amplifier by electronically estimating the voltage error caused by the series resistance and making a correction to the voltage applied to the pipette (figure 2.8). The current monitor signal is scaled by a variable factor and added to $V_{\text{ref}}$ with a polarity corresponding to positive feedback.

In order to prevent saturation of the ADC the leakage current was subtracted from the measured currents. This leak subtraction was not supplied by the patch clamp amplifier but was achieved by adding the inverted command voltage to the recorded current in the stage marked leak subtraction in figure 2.5. The gain of this stage was varied until a voltage step applied across the patch appeared to produce no leak in the recorded current.
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Figure 2.8

Series resistance compensation.

The current monitor signal, $i_p R_f$, is scaled by a variable factor and added to $V_c$ with a polarity corresponding to positive feedback in order to compensate for the voltage error caused by the series resistance.
summing amplifier

$V_c$

$i_p R_f$
The recorded currents were filtered with a low pass eight-pole Bessel filter set to reduce the signal by 3 dB at 1 kHz. These currents then passed to the analog-to-digital ports of the ADC to be digitized. The currents were digitized using a sampling procedure within the BASIC program for pulse production and data capture.

Segments of the current were digitized by using this program to define the duration, in msec, of the sampled current segment (trace), the interval between traces and the interval between sample points within a trace. These parameters are given for each experimental situation in the results. Usually a trace length of 102.4 msec was chosen with an interval of 2 or 4 sec between traces and a 0.2 msec interval between sample points giving a sample rate of 5 kHz. Command voltage pulses were delivered at selected times during sampling of a trace. Up to five command pulses could be produced by the digital-to-analog converter during the sampling of one current trace.

Traces were sampled in one of three ways:

1) Sampled continuously. In this mode the sampled current traces were displayed on the digital display but not digitally stored.

2) Sampled and recorded continuously. Here 1 to n current traces (where n is determined by the space left on the floppy disk) were sampled. The digitized traces were stored as arrays within the memory of the ADC and written to disk.

3) Recorded and stacked in the ADC memory until ten traces had been recorded. It was then possible to write the ten traces to disk or to discard them so that space on the disk was not wasted. In this manner 700 traces, 102.4 msec long and sampled at a rate of 5 kHz could be stored on one floppy disk.
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In those experiments which did not employ command voltage pulses the membrane potential was held at a constant value for several minutes whilst channel activity was recorded onto FM tape using a Racal tape recorder. These currents were unfiltered and were recorded at a tape speed of 7.5 in/sec giving a bandwidth of 5 kHz.
2.7 DATA ANALYSIS

Currents initially recorded onto floppy disk were transferred to a PDP 11/23 minicomputer for analysis. This transfer was achieved by reading the currents from the disk into BBC memory and from there into arrays in the ADC (1401) memory. These arrays were then played out of the digital outputs of the 1401 down a data line to the digital inputs of an ADC (Cambridge Electronic Design; 502) connected to the PDP 11/23. They were then read into the PDP memory and written to hard disk (RL02).

Currents which had been recorded onto tape were later digitized by playing them back through the ADC attached to the PDP 11/23 and recording the digitized currents onto hard disk.

All analysis was done using the PDP 11/23. The purpose of the analysis was to investigate certain characteristics of the macroscopic and single-channel currents of the membrane. For macroscopic currents, activated by a step change in voltage, the characteristics investigated were current amplitude and the activation and deactivation time courses. For single-channel currents the characteristics under investigation were amplitude and open and closed times. For channels activated by a step change in voltage, the time to first opening after the voltage pulse and the activation and deactivation time courses were also studied. In order to study these activation and deactivation time courses the single-channel current traces were added together to form a pseudo-macroscopic current which could then be compared with the actual macroscopic current.
2.8 ANALYSIS OF MACROSCOPIC CURRENTS

2.8.1 Cleaning of current traces

Before analysis, any leakage current remaining in the traces was subtracted from the macroscopic currents recorded using the whole-cell recording technique. To estimate the size of the leakage current, 'leakage files' containing ten traces of currents elicited by a small voltage pulse (usually 10 mV) from the holding potential were formed. This pulse was small enough to produce a leakage current without eliciting any voltage-activated currents. A leakage file was formed before each set of ten test pulses (constituting a test file) during an experiment in case the leakage current was changing with time. A FORTRAN program was written to average these ten leakage traces and to scale the result by a certain factor. This factor was the ratio of the test pulse amplitude to the amplitude of the pulse used to measure the leakage and thus the scaling assumed that the leak behaved in an Ohmic fashion. The resultant scaled current was then subtracted from the test currents and the results were written to disk. This process is termed cleaning and will also remove any linear capacitative current that may remain after analogue capacity compensation.

2.8.2 Analysis of the time course of the currents

Macroscopic potassium currents were fitted with a curve described by the equation derived by Hodgkin and Huxley (1952d) to describe the time course of voltage-activated potassium currents in the squid giant axon. A FORTRAN program modified from one written by Dr. P.R. Stanfield to fit summed single channel potassium currents of frog skeletal muscle was used to fit the macroscopic potassium
currents described here. This enabled macroscopic current traces to be analysed in terms of their maximum current amplitude and activation time constant ($\tau_a$).

Tail currents (those currents recorded when the membrane voltage is changed from one which activates the channels to one at which channels close) were fitted with a single exponential using a FORTRAN program modified from a histogram-fitting program written by Dr. N.B. Standen.

2.9 ANALYSIS OF SINGLE CHANNEL CURRENTS

2.9.1 Cleaning of current traces

Before analysis patch clamp current traces must also be cleaned to remove incompletely compensated capacitative and leakage currents. This was achieved by forming an array containing averaged baseline currents. These were chosen from traces containing no channel openings or from traces containing channel openings using cursors to select areas of baseline (Standen, Stanfield and Ward, 1985; Ward, 1986). This average was then subtracted from all traces.

Currents which were not elicited by step changes in voltage but by steady holding potentials did not require cleaning before analysis.

2.9.2 Amplitude histograms

When the traces have been cleaned the amplitude of single channel openings may be found. Data points from areas of traces containing channel openings and areas containing empty baseline were accepted into an array of amplitude bins. From this a histogram of the number of data points within each amplitude bin was
constructed (see for example figure 4.3). The current trace has a certain level of random noise about the baseline and open channel levels. Amplitude histograms of this noise form normal distributions and histograms constructed in the above way may therefore be fitted with Gaussian curves. Using such analysis the amplitude of a channel current was taken as the mean of the Gaussian which fits the open channel noise minus the mean of the Gaussian which fits the baseline noise. The distribution of the noise around these two levels did not overlap significantly at any of the amplitudes measured. For traces containing channels which had more than one open level, the noise of the two open levels did overlap and the histogram fitting programme had to be modified to fit the data to the sum of two Gaussians (see figure 4.3).

2.9.3 Open and closed time and first latency measurements

First latency (i.e. the time from the start of the pulse to the first appearance of channel activity during a voltage pulse) as well as open and closed times were measured from data displayed on a digital monitor. To make these measurements a cursor, whose position could be controlled from the computer keyboard, was placed in a position equal to half the open channel amplitude. Transitions of the current trace across this cursor were registered as openings and closings of the channel. Each transition was either accepted or rejected by eye. The cursor could also be repositioned manually so that obvious artefacts or drifts in the baseline were not registered as channel openings. These procedures produced an idealised record from which histograms of first latency (for voltage-activated channels, see for example figure 4.15) and open (for example figure 4.6) and closed (for example figure 4.12) times could be constructed. A single channel current record is shown in figure 2.9 along with an
idealised record produced using the half-amplitude cursor method. The arrow shows the position of the cursor. These histograms could then be fitted to one exponential or to the sum of several exponentials using FORTRAN programs written by Dr. N.B. Standen. The significance of these fits is explained in appropriate sections in the results.

For some of the measurements of open and closed times and first latencies the data points were interpolated using a cubic spline function. This reconstructs the current trace from the digitized data with a peak error of around 2% when Bessel-filtered data has been sampled at a rate which is five times the filter frequency (Colquhoun and Sigworth, 1983). Data points were interpolated using the subroutine SPLINE of Colquhoun and Sigworth (1983). This procedure was also carried out on all data which was to be displayed using a Hewlett-Packard plotter (7225B).
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Figure 2.9

Single channel current and idealised current trace.

Transitions of the single-channel current across a cursor placed at the amplitude of the arrow were registered as openings or closings of the channel to give the idealised record shown.
2.9.4 Summed and averaged currents

Pseudo-macroscopic currents were reconstructed by adding together individual current traces containing openings of voltage-activated channels. The resultant current represents an averaged behaviour of the single channels and has the same shape and time course as the macroscopic current. It was fitted to Hodgkin and Huxley kinetics in the same way as described for the macroscopic currents.

2.9.5 Probability of being open

The probability of being open ($P_{\text{open}}$) was calculated for channels which were not activated by voltage pulses. Activity of these channels was recorded continuously (see above) and durations were measured by using a half amplitude cursor as described above. $P_{\text{open}}$ is given by

$$2.3 \quad P_{\text{open}} = \frac{\sum_{i=1}^{N} t_i}{T \cdot N}$$

where $t_j$ is the time spent at level $j$, $T$ is the duration of the recording and $N$ is the number of channels active in the patch. $N$ is estimated from the maximum number of overlapping openings seen.

2.10 SCANNING ELECTRON MICROSCOPY

Scanning electron micrographs were taken of cells which had been dried using the critical point drying technique. This involves first replacing the water in the cells with acetone. Cells were grown for 1 day in differentiation medium on
Materials and Methods

poly-lysine coated coverslips. The cells were fixed in Karnowskii’s fixative (see Appendix II) for 1 hour and then in 2% osmium tetroxide in distilled water for 1 hour. The purpose of fixatives is to cross-link the proteins in the cell so that the structure does not disappear when the cells are dehydrated. The coverslips were then sequentially placed in solutions of 50%, 70%, 90%, 100% and absolute ethanol and then in acetone for 15 minutes in each solution. The cells were then placed in a critical point drier which consists of a pressure vessel and water jacket for heating and cooling. Once the cells had been placed in the vessel it was flushed through three times with liquid carbon dioxide, ensuring that the cells remained below the level of the liquid, to replace the acetone with carbon dioxide. The cells were then left in the vessel, in liquid carbon dioxide, for 1 hour. The temperature of the vessel was then brought up to 32°C by flowing warm water through the water jacket. This brought the pressure in the chamber to approximately 1200 p.s.i. This is the critical point of carbon dioxide where the transition from liquid to gas takes place without an interface because the densities of the liquid and gas are equal at this temperature and pressure. The temperature was then raised to approximately 35°C to prevent recondensation and the gas was vented. This method of drying tissue produces much less damage than the alternative method of drying under a vacuum (which was also attempted) since the latter involves the evaporation of liquid from a liquid/gas interface. As the tissue dries this meniscus travels through the cells so that large surface tension forces are created, collapsing cavities which may be present in the tissue.

The cells were then sputter coated with gold by Mr. George McTurk and were viewed under a scanning electron microscope.
Chapter 3

MACROSCOPIC CURRENTS RECORDED USING THE WHOLE-CELL RECORDING TECHNIQUE

3.1 INTRODUCTION

Moolenaar and Spector (1978) recorded voltage-activated fast inward and delayed outward currents from cells of the mouse neuroblastoma clone N1E115. They recorded currents with the two microelectrode voltage-clamp from cells which had been differentiated for 1 to 3 weeks in a culture medium containing 1 or 2% DMSO.

The fast inward and delayed outward currents described in this chapter were recorded from cells of the same line which had been differentiated for only 1 to 7 days in a culture medium containing 1.2 to 1.3% DMSO. These cells were smaller and had fewer processes than those recorded from by Moolenaar and Spector. The voltage dependence of the time course and amplitude of the currents was investigated, as was their ionic nature. These properties of the currents were then compared with those of the currents described by Moolenaar and Spector.
3.2 RESULTS

Using cells which had been allowed to differentiate for 1 to 7 days, macroscopic currents, i.e. whole membrane currents, were recorded with the method described in section 2.5.3. Access to the interior of the cell was gained with either the membrane potential clamped to -34 mV or with the amplifier set to the search mode, when the membrane potential was not held. The electrode solution was solution E (table 1: [K\(^+\)] = 165.5 mM, [Na\(^+\)] = 0). In the search mode, the resting potential of the cells was measured. The mean resting potential of cells which had been differentiated for 1 to 2 days was -26.8 +/- 3.6 mV (17 cells). The potential of the cell was then clamped to -54 or -64 mV.

Since two cells of different size with the same membrane current density will have different current amplitudes in the same conditions it is necessary to correct for cell size when measuring membrane currents. All whole-cell currents are therefore expressed as amps per Farad (A/F): a constant capacity per unit area of the cell membrane was assumed and the capacity of each cell was measured using the method described in section 2.5.3. The data collected to measure cell capacity were also used to calculate the series resistance of the preparation as described in the same section.

The mean membrane capacity was found to be 4.8 +/- 0.34 μF/cm\(^2\) (24 cells) when the area of cell membrane was calculated, assuming the cell to be a smooth sphere, from measurements of cell diameter made using a graticule in the eyepiece of the microscope. This value is higher than the usual values of around 1 μF/cm\(^2\) found for most cell membranes (Hille, 1984).
Scanning electron-micrographs of a cell which had been grown for 1 day in differentiation medium.

The cells chosen for the electron-microscopy had the same appearance under the light microscope (small and round) as those chosen for electrical recording. All of the cells which had this appearance under the light microscope and which were viewed under the scanning electron-microscope had the appearance of that shown in the figure. Scale bars represent 5 μm in the upper micrograph and 1 μm in the lower.

Cells were prepared for the microscopy by the critical-point drying technique (see section 2.10). The cell shown appears to have both extensive infolding of the membrane and short processes.
Macrosopic currents

This is probably because the cells, even when they have been differentiated for only one or two days, have extensive invaginations and have some short processes which cause the membrane area to be greater than that estimated from calculations based on cell diameter. Figure 3.1 shows scanning electron micrographs from cells which had been grown for 1 day in differentiation medium. Small, round cells were chosen both for the electrical recording and for the electron-microscopy. It can be seen that the cells have short processes (the thicker process in the top left of the upper micrograph of figure 3.1 belongs to a cell which is out of the field of view) and that the membrane is highly folded. The infolding, however, may be partly due to the drying technique which was used to prepare the cells for scanning electron-microscopy and which causes a certain amount of shrinkage in cell volume. The extent of the infolding seen in these cells is greater than that seen in certain other cells prepared in the same way (G. McTurk, unpublished observations). The approximate diameters of the cells shown are 14 µm (upper) and 16 µm (lower).

When cells such as these were placed in solution A (table 1: \([K^+] = 5 \text{ mM}, [\text{Na}^+] = 147 \text{ mM}\)) depolarized with solution E (table 1: \([K^+] = 165.5 \text{ mM}, [\text{Na}^+] = 0\)) as the electrode solution, a fast inward current followed by a delayed outward current could be seen. These are shown in figure 3.2 where a cell held at -54 mV was depolarized to +26 mV for 90 msec. In this figure an incompletely cancelled capacitative transient can be seen corresponding to the beginning of the depolarization. This is followed by a fast inward current and a delayed outward current which reaches a maximum approximately 55 msec after the onset of the depolarization and does not decline. Another capacitative transient occurs at the end of the depolarization.

With 3 µM tetrodotoxin (TTX) in the electrode solution the inward current...
disappeared to leave the delayed outward current. A recording of a delayed outward current is shown in figure 3.3 A. The current illustrated was recorded from a cell which had been grown in differentiation medium for 1 day. It reaches a maximum of approximately 260 pA and does not decrease again during a 90 msec depolarization from -54 to +36 mV. Conversely, with 25 mM tetraethylammonium ion (TEA\(^+\)) in the electrode the delayed outward current was abolished leaving the inward current intact. This is shown in figure 3.3 B where a cell, cultured in differentiation medium for 1 day, was depolarized from -64 to -24 mV for 90 msec. The fast inward current illustrated in this figure reaches a maximum of approximately -320 pA and decays back to zero within 25 msec of the onset of the voltage pulse. Moolenaar and Spector (1978) showed that the delayed outward potassium current of these cells is abolished by 15 mM TEA\(^+\).

TTX and TEA therefore allowed the macroscopic current to be partitioned into two currents which could be studied separately.
Macroscopic currents

Figure 3.2.

Macroscopic currents.

Currents were recorded from a cell which had been cultured in differentiating medium for 2 days. The electrode contained solution E (table 1: $[K^+] = 165.5$ mM, $[Na^+] = 0$ mM) and the bath solution contained solution A (table 1: $[K^+] = 5$ mM, $[Na^+] = 147$ mM). The cell was held at -54 mV and pulsed to +26 mV for 90 msec. Currents were filtered at 1kHz and digitized at 5kHz. Temperature = 19°C.

Incompletely cancelled capacitative transients can be seen corresponding to the beginning and end of the voltage pulse. A fast inward current and a delayed outward current which reaches a maximum approximately 55 msec after the beginning of the voltage pulse and does not decline are elicited by this pulse.
Figure 3.3.

Delayed outward and fast inward currents.

Currents were recorded from cells which had been cultured in differentiating medium for 1 day. In A the electrode contained solution E containing 3μM TTX and the cell was held at -54 mV and pulsed to +36 mV while in B the electrode solution contained 25 mM TEA⁺ chloride and 35 mM NaCl substituted for KCl in solution E and the cell was held at -64 mV and pulsed to -24 mV for 90 msec. Currents were filtered at 1 kHz and digitized at 5 kHz. Temperatures = 22 and 19°C.

A.

Incompletely cancelled capacitative transients can be seen corresponding to the beginning and end of the voltage pulse. No inward current can be seen in this current trace but a delayed outward current, which reaches a maximum at approximately 55 msec and does not decay, can be seen.

B.

Again capacitative transients can be seen. The fast inward current, which reaches a maximum approximately 5 msec after onset of the depolarization and has decayed to zero 20 msec later, is not followed by a delayed outward current.
3.3 THE DELAYED OUTWARD CURRENT

With 3 μM TTX in the electrode solution (solution E, table 1), depolarization of the cells from the holding potential elicited a delayed outward current which was recorded uncontaminated by inward current. The time course and amplitude of the outward current was studied when the cell was depolarized from a holding potential of -54 mV to potentials more positive than -14 mV with voltage pulses 90 msec long. During these pulses the current activated slowly and no sign of inactivation (i.e. decline in current amplitude) was seen (figure 3.3 A).

3.3.1 The current-voltage relation of the delayed outward current

The amplitude of the delayed outward current varied directly with the size of the depolarization as shown in figure 3.4. 4 to 10 current traces were recorded and averaged to produce each trace shown in the figure.

The maximum current reached at the steady-state was divided by the measured cell capacity and the resultant current density was plotted as amps/Farad (A/F) against membrane potential. This relationship is illustrated in figure 3.5 which suggests that, within the range of potentials studied, the steady-state current elicited by a voltage pulse increases linearly with increasing depolarization. When the membrane potential was stepped from -54 mV to -14 mV the maximum delayed outward current reached during a 90 msec depolarization was 1.17 +/- 0.28 A/F (4 cells) whereas when the membrane potential was stepped to +36 mV the current increased to 9.78 +/- 1.51 A/F (5 cells).
Figure 3.4.

Delayed outward currents recorded at different membrane potentials.

Currents were recorded from cells which had been cultured for 1 day in differentiating medium and were bathed in solution A ($[\text{K}^+]_b = 5 \text{ mM}$). The electrode contained solution E + 3 $\mu$M TTX. Cells were held at -54 mV and pulsed to -4 mV (A), +26 mV (B) and +36 mV (C) for 90 msec. 4 to 10 individual current traces were recorded and averaged to produce the current traces shown.

The figure shows that the activation time course and maximum amplitude of the delayed outward current are voltage dependent. Currents have been fitted with the equation $G_K = G_K^n$ which produced the smooth curves plotted over the current traces. The maximum amplitude and time course of activation ($\tau_a$) estimated by these fits are: 171.1 pA and 23.3 msec (A); 551.1 pA and 15.6 msec (B); and 697.2 pA and 12.6 msec (C).
A

- 4 mV

B

16 mV

C

36 mV

pA

0

200

400

600

800

1000

-200

0

20

40

60

80

100

120

msec
Macroscopic currents

Figure 3.5.

Current-voltage relation for the delayed outward current at steady-state.

Cells were depolarized from a holding potential of -54 mV. 4 to 10 currents elicited by depolarizations to the same membrane potential in the same cell were averaged to give a mean current at each potential for each cell. This mean was then divided by the capacitance of the cell to give a mean current density (A/F). The mean current densities from four or more cells depolarized to the same potential were then averaged and are plotted, with standard errors, against membrane potential.

This figure indicates that the steady-state delayed outward current increases with increasing depolarization.
Figure 3.6 shows a semi-logarithmic plot of conductance (calculated from the data used to plot figure 3.5 and expressed as Siemens/Farad) against membrane potential. It can be seen that the conductance is asymptotic to approximately $8.2 \times 10^{-5}$ S/F.

The maximum current density reached during a depolarization is seen also to depend upon the holding potential. No current could be activated in cells which were held at potentials more positive than -34 mV. When cells were held at -64 mV a voltage pulse to +36 mV elicited a maximum current of $8.42 \pm 1.9$ A/F (4 cells) and at +36 mV a maximum current of 12.7 A/F (one cell) was elicited whereas when cells were held at -54 mV, pulses to +36 mV elicited a maximum current of $5.99 \pm 0.41$ A/F (9 cells) and to +36 mV gave a maximum of $9.78 \pm 1.51$ A/F (5 cells).

3.3.2 The time course of activation

Figure 3.4 also shows that the time taken to reach steady-state is decreased by increasing depolarization. This time course may be described by using the equation which was derived by Hodgkin and Huxley (1952d) to describe the rise in the delayed potassium conductance of squid giant axon following a step depolarization.

The potassium current, $I_K$, is given by

$$I_K = g_K (V - E_K)$$

where $V$ is the membrane potential, $E_K$ the equilibrium potential for potassium ions, and $g_K$ the potassium conductance. The linearity of the current-voltage relation (figure 3.10) justifies the use of this relationship.
Macroscopic currents

Figure 3.6

Semi-logarithmic plot of membrane conductance against membrane potential.

Membrane conductance was calculated for the data illustrated in figure 3.5.

The membrane conductance is asymptotic to approximately $8.2 \times 10^{-5}$ Siemens/Farad.
Hodgkin and Huxley described the rise in potassium conductance of squid giant axon after a step depolarization by

\[ g_K = g_K^n \]

where \( g_K \) is the maximum potassium conductance, \( n \) is an integer and \( n \) is the dimensionless activation parameter which varies with time after depolarization and has a value between 0 and 1.

At a certain voltage \( n_{\text{ls}} \) is given by

\[ n_{\text{ls}} = \frac{\alpha_n}{(\alpha_n + \beta_n)} \]

where \( \alpha_n \) and \( \beta_n \) are the forward and backward rate constants for the change in value of \( n \) on depolarization and are voltage dependent.

The rate of change of the value of \( n \) with time after a depolarizing voltage pulse is described by the equation

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

Hodgkin and Huxley solved equation 3.4 for the boundary conditions \( n = n_o \) at \( t = 0 \) and \( n = n_\infty \) at \( t = \infty \), so that

\[ n = n_\infty - (n_\infty - n_o) \cdot \exp(-t/\tau_n) \]
where the time constant of activation, $\tau_n$, is given by

$$\tau_n = 1/(\alpha_n + \beta_n).$$

These may then be substituted in equation 3.2 for the condition where the initial potential is negative enough for $n_o$ to be zero, so that for $i = 2$

$$g_K = \left\{ \left( g_{Koo} \right)^{1/2} - \left[ \left( g_{Koo} \right)^{1/2} - \left( g_{Ko} \right)^{1/2} \right] \exp(-t/\tau_n) \right\}^2$$

If current is substituted for conductance in this equation then it may be used to fit the time course of currents recorded during a step depolarization.

Such fits are illustrated in figure 3.3 (smooth curves) where $i$ (equation 3.2) equals 2. Setting $i$ to 1 does not produce a delay and produces a more rapid rate of rise in the theoretical curve than that seen experimentally whereas when $i$ equals 3 the delay is too long and the rate of rise of the theoretical curve is too slow.

When the membrane potential is made more negative after a depolarization equation 3.7 predicts an exponential decline in current to a new resting value. The time constant of this exponential (or tail current), $\tau_t = \tau_n/2$.

Such tail currents have been fitted with a single exponential and are illustrated in figure 3.7. The currents were activated by a depolarizing step from -54 mV to +26 mV for 50 msec. The membrane potential was then reduced to a more negative value and the current decayed to a new steady-state (which was zero by 40 msec after a voltage step to potentials more negative than -34 mV). The time constant of this exponential, $\tau_t$, is equal to $\tau_n/2$ and $2\tau_t$ is therefore plotted against voltage in
Macroscopic currents

Figure 3.7.

Tail currents.

Currents were recorded when cells held at -54 mV were depolarized to +24 mV for 50 msec and the membrane potential then stepped back (at 5 msec in the figure) to -24 mV (A) and -54 mV (B).

The currents have been fitted with single exponential distributions with time constants of 11.36 msec (A) and 3.0 msec (B). The current in A decays back to 33 pA and that in B to 0 pA.
figure 3.8 along with the values for \( \tau_n \) obtained at more positive voltages by fitting the depolarization-induced activation of the current with equation 3.7.

The figure shows that \( \tau_n \) first increases and then decreases as the membrane potential is increased. At more negative voltages \( \beta_n \) is small and at more positive voltages \( \beta_n \) is small, at intermediate voltages both are small. \( \tau_n = \beta_n(\alpha_n + \beta_n) \) so at intermediate voltages \( \tau_n \) is large and at the voltage extremes \( \tau_n \) is small.

Values of \( n_\infty \) at different membrane potentials have been obtained from the relation \( n = (g_{\infty_0}/g_{\infty})^{1/2} \), so that \( n_\infty = 1 \) where \( g_{\infty_0} = g_e \). These values are plotted in figure 3.9.

### 3.3.3 The main charge carrier is potassium

The value for the reversal potential of the current was estimated from a plot of the instantaneous current-voltage relationship. This is shown in figure 3.10. In this figure, the instantaneous current-voltage relationship is plotted by taking the value of the exponential, used to fit the tail current, at zero time after the initiation of the voltage change and plotting it against membrane potential. The figure shows that the current reverses around -70 mV. This value is near to the theoretical reversal potential of -88.5 mV for a potassium current at 20°C. Using the Goldman-Hodgkin-Katz voltage equation (Goldman, 1943; Hodgkin and Katz, 1949) with \([K]_o = 5 \text{ mM}; [K]_i = 140 \text{ mM}; [Na]_o = 145\text{ mM}; \text{ and } [Na]_i = 0 \text{ mM and assuming that the chloride permeability is negligible:}

\[
3.8 \quad E_{\text{rev}} = \frac{RT}{F} \ln \frac{[K]_o + P[Na]_o}{[K]_i + P[Na]_i}
\]
Macroscopic currents

Figure 3.8.

Voltage-dependence of the activation time constant.

Cells which had been grown in differentiating medium for 1 to 2 days were bathed in solution A \([K^+_{p}] = 5\) mM and were recorded from with solution E \([K^+_{p}] = 165.5\) mM plus 3 \(\mu\)M TTX in the electrode. Cells were depolarized from -54 mV to different potentials and mean currents were constructed for each cell at each potential as described for figure 3.4.

These mean currents were fitted with equation 3.7 (potentials -4 to +46 mV). Tail currents recorded as described for figure 3.7 were measured at potentials between -104 and -14 mV were fitted with a single exponential. The values of \(\tau_n\) estimated from these fits for four or more cells at the same membrane potential were averaged and plotted, with standard errors, against membrane potential. Values were obtained for only two cells at -104 mV and only one cell at -14 mV.
Macroscopic currents

Figure 3.9.

Values of $n_\infty$ plotted against membrane potential.

Values of $n_\infty$ were calculated from $n_\infty = (g_{K\infty}/g_K)^{1/2}$ where $g_{K\infty}$ was assumed to be approximately $8.2 \times 10^{-5}$ S/F from figure 3.6.
Macroscopic currents

Figure 3.10.

Instantaneous current-voltage relation.

Currents were activated by stepping the membrane potential of the cell from -54 mV to +24 mV for 50 msec. The membrane potential was then stepped back and the value of the exponential curve fitted to the tail currents was taken at zero time from this step. This value is plotted against membrane potential.

The instantaneous current-voltage relation is linear and reverses at approximately -74 mV. The theoretical reversal potential for a potassium current under these conditions ([K]_o = 5 mM; [K]_i = 165.5 mM) is -88.5 mV at 20°C.
where $P$ is the permeability of the current pathway to sodium ions divided by the permeability to potassium ions.

This reversal potential suggests a permeability ratio of sodium to potassium of 0.037 for these currents.

The conclusion that the main charge carrier of the delayed outward current is potassium is supported by the evidence that TEA, applied either externally (see below) or internally (see figure 3.2 B), effectively blocks the current (Stanfield, 1983).

### 3.3.4 Block by TEA$^+$

A block of the delayed outward current by externally applied TEA$^+$ was demonstrated by recording currents from cells bathed in solution A (table 1; $[K^+] = 5$ mM, $[TEA^+] = 0$) and then exchanging the bath solution for one containing TEA. Currents were recorded after TEA application only from cells whose leakage current did not change significantly on exchange of the bath solution. The effect of 0.5 mM TEA was investigated on two preparations where it reduced the current elicited by a change in membrane potential from -54 to +26 mV. The current was reduced from 5.97 to 3.56 A/F in one cell and from 5.15 to 3.37 A/F in the other. The effect of 1.0 mM TEA was investigated only on one cell where it caused a reduction in the current from 3.41 to 1.15 A/F. Assuming that TEA blocks the current when it is bound to a receptor and that binding of the TEA molecule with the receptor site, to produce a block, is 1:1, then the dissociation constant ($K_d$) suggested by the above is 0.73 +/- 0.22 mM (where the fractional current, $f = 1/(1 + [TEA^+]/K_d)$ and the fractional current is the current recorded in the presence of TEA divided by the current recorded at the same potential in the absence of TEA). The significance of this dissociation constant and support for
the assumption that binding is 1:1 is discussed in Chapter 5 where TEA block of the single channel currents corresponding to the delayed outward current is discussed.

3.3.5 The dependence of the current upon time in differentiating medium

Some data were recorded which suggest that the length of time which the cells spend in differentiation medium has an effect on the current density of the cells. Currents were recorded at +26 mV (stepped from a holding potential of -54 mV) from cells which had been differentiated for different times. A cell which had been differentiated for 5 hours had a steady-state delayed outward current of 3.6 A/F, cells which had been differentiated for between 16 and 24 hours had a steady-state current of 5.8 +/- 0.37 A/F (6 cells) and two cells which had been differentiated for approximately 96 hours had steady-state currents of 12.7 and 14.0 A/F. More data would be necessary in order to draw conclusions but it is possible that, over the time scale investigated, the steady-state current reached at this potential may increase with increasing time in DMSO, possibly reflecting an increase in potassium channel density.

These currents were fitted with equation 3.7 and the resultant time constants were 7.5 msec for the cell differentiated for 5 hours; 12.5 +/- 0.5 msec for the cells differentiated for between 16 and 24 hours and 13.21 and 13.99 msec for the cells differentiated for approximately 96 hours. This analysis therefore provides no evidence for a dependence of $\tau_n$ on the time spent by the cells in differentiating medium.
3.4 THE FAST INWARD CURRENT

The fast inward current was recorded from cells when 25 mM TEA chloride and 35 mM NaCl were substituted for KCl in solution E (electrode solution). The cells had been grown for between 1 and 2 days in differentiation medium. Currents were free from contamination by the delayed outward current since the high concentration of TEA abolished this current completely (see for example figure 3.3). The membrane potential of the cells was held at -54 or -64 mV and the current activated and inactivated quickly when the potential was raised above -36 mV. The voltage pulses which were used to step the membrane potential were 90 msec long and the time between successive pulses was 5 sec to give time for the current to recover from inactivation.

3.4.1 The amplitude of the fast inward current

The maximum amplitude of the fast inward current, expressed as amps per Farad of membrane capacity, is plotted against membrane potential in figure 3.10. These data were recorded from one cell which was held at -64 mV and had been differentiated for 1 day. When the current is extrapolated it appears to reverse around +28 mV. This is reasonably close to the theoretical reversal potential for sodium ions (at 20°C) of +36.3 mV calculated from the Nernst equation with \([\text{Na}]_o = 147 \text{ mM}\) and \([\text{Na}]) = 35 \text{ mM}\) (assumed to be equal to the pipette sodium concentration since there should have been free exchange of the ions in the pipette solution with the ions in the intracellular solution (see section 2.5.3)) and suggests that the current is carried primarily by sodium ions.
Figure 3.11.

Amplitude of the fast inward current plotted against membrane potential.

Currents were recorded from one cell which had been grown in differentiation medium for 1 day. The membrane was stepped to the potentials shown from a holding potential of -64 mV.

The current first increased and then decreased with increasing depolarization. No current was activated at potentials more negative than -34 mV. When the current is extrapolated it appears to reverse around +28 mV. The theoretical reversal potential for sodium ions at 20°C is +36.3 mV.
3.4.2 Block of the fast inward current by tetrodotoxin

When 3 μM TTX was present in the electrode solution (see for example figure 3.3 A) the fast inward current was abolished. Block of the fast inward current by externally applied TTX was also demonstrated. Cells were held at -54 or -64 mV and depolarized to activate the sodium current. TTX was then added to the bath solution to give a concentration of approximately 3 μM and several minutes were left before resumption of current recording to allow time for the TTX to mix with the bath solution. Figure 3.12 shows currents recorded from a cell held at -54 mV and pulsed to -34 mV. 3.12 A shows the fast inward current, recorded before application of TTX to the bath and 3.12 B current recorded from the same cell at the same potential after application of TTX.

Block of the current by TTX also suggests that it is primarily carried by sodium ions since it has been shown to be a highly selective blocker of sodium currents in, for example, lobster giant axons (Narahashi, Moore and Scott, 1964), squid giant axons (Nakamura, Nakajima and Grundfest, 1965), *Electrophorus electricus* electric organ (Nakamura, Nakajima and Grundfest, 1965b) and frog myelinated axons (Hille, 1966, 1967a, 1968a).

3.4.3 Kinetic parameters of the fast inward current

Fast inward currents recorded using the whole-cell recording technique were cleaned using the method described in section 2.5.3. The cleaned currents were then averaged and the average (4 to 10 traces at one membrane potential) was fitted by the equation derived by Hodgkin and Huxley (1952d) to describe the sodium current of squid giant axon. The equation is similar to that used to describe the time
Macroscopic currents

Figure 3.12.

Fast inward currents recorded before and after the application of TTX to the bath solution.

Currents were recorded from a cell which had been differentiated for 2 days. The electrode solution was solution E with 35 mM NaCl and 25 mM TEA chloride substituted for KCl. The membrane potential was held at -54 mV and stepped to -34 mV for 90 msec.

In A the fast inward current can be seen; in B, TTX was added to the bath solution to bring the concentration to approximately 3 μM. It can be seen that this abolished the inward current.
Figure A: Graph showing pA (picoamperes) vs. time (msec) for 0 pA to 200 pA.

Figure B: Graph showing pA vs. time for 0 pA to 200 pA.

3μM TTX
course of the potassium conductance except that it must describe the time course of inactivation as well as activation.

The current, $I_{Na}$, is given by

$$I_{Na} = g_{Na}(V-E_{Na})$$

where $g_{Na}$ is the sodium conductance, $V$ the membrane potential and $E_{Na}$ the Nernst potential for sodium ions. Hodgkin and Huxley (1952b) described the time course of $g_{Na}$ during a step depolarization by

$$g_{Na} = g_{Na}^m m^3 h$$

where $g_{Na}$ is the maximum sodium conductance and $m$ and $h$ dimensionless activation and inactivation parameters respectively.

The values of $m$ and $h$ change with time according to the equations

$$\frac{dm}{dt} = \alpha_m(1-m) - \beta_m m$$

and

$$\frac{dh}{dt} = \alpha_h(1-h) - \beta_h h$$

where, as for the potassium activation parameter $n$, the $\alpha$'s and $\beta$'s are the rate constants for the change in value of $m$ and $h$ after a depolarization and are voltage
but not time dependent. The solutions of the equations which satisfy the boundary conditions \( m = m_0 \) and \( h = h_0 \) at \( t = 0 \) are

\[
3.13 \quad m = m_\infty - (m_\infty - m_0) \exp(-t/\tau_m)
\]

and

\[
3.14 \quad h = h_\infty - (h_\infty - h_0) \exp(-t/\tau_h)
\]

where \( m_\infty = \frac{\alpha_m}{(\alpha_m + \beta_m)} \), \( \tau_m = \frac{1}{(\alpha_m + \beta_m)} \), \( h_\infty = \frac{\alpha_h}{(\alpha_h + \beta_h)} \) and \( \tau_h = \frac{1}{(\alpha_h + \beta_h)} \).

In the resting state the sodium conductance is very small compared with that attained during a large depolarization so they assume that for depolarizations greater than 30 mV \( m_0 \) is negligible. Also, if the initial voltage is less than -30 mV they assume that \( h_\infty \) may be neglected. In the preparation described here there \( m_0 \) also appears to be negligible for depolarizations greater than 20 mV from -64 mV (the holding potential) and \( h_\infty \) may be neglected if the holding potential is less than approximately -35 to -45 mV. In these conditions the expression for the sodium conductance becomes

\[
3.15 \quad g_{Na} = g_{Na00} [1 - \exp(-t/\tau_m)]^3 \exp(-t/\tau_h)
\]

where \( g_{Na00} = g_{Na} m_\infty^3 h_0 \) and is the value which the sodium conductance
would attain if \( h \) remained at its resting level.

With current substituted for conductance this equation was used to fit currents recorded at different potentials. 4 to 10 current traces were averaged before fitting and examples of the fits are shown in figure 3.13 (smooth curves).

It can be seen from the traces that the time course of the currents and the maximum current reached during a depolarization vary with membrane potential. It can also be seen that the theoretical curves fit the recorded current well. The currents were fitted with \( m \) raised to the power of 3 so that the kinetic parameters could be compared with those measured by Moolenaar and Spector (1978) who fitted the sodium currents in cells of the line N1E115 which had differentiated for up to 3 weeks with equation 3.15. The maximum of the theoretical current differed from the data recorded using the whole-cell recording technique by very little. Moolenaar and Spector reported deviations of up to 20%.

The values of the time constants \( \tau_m \) and \( \tau_h \) which gave the fits shown in figure 3.13 are plotted against membrane potential in figure 3.14. These values are compared in the discussion with those obtained by Moolenaar and Spector (1978).
Macroscopic currents

Figure 3.13.

Fast inward currents recorded at different membrane potentials.

Currents were recorded from cells which had been differentiated for 1 day. The electrode contained solution E with $[\text{Na}^+]_p = 35 \text{ mM}$ and $[\text{TEA}^+]_p = 25 \text{ mM}$. The membrane potential of the cell was held at -54 mV and stepped to -24 mV (A), -14 mV (B) and -4 mV (C).

Currents have been fitted with equation 3.15. It can be seen that this fits well but deviates from the recorded currents at the baseline, probably due to leakage currents which have been incompletely subtracted from the recorded currents. Moolenaar and Spector (1978) found deviations of up to 20% between the measured and theoretical sodium currents in cells differentiated for between 1 and 3 weeks. In A $\tau_m = 4.66$ and $\tau_h = 3.67$, in B $\tau_m = 3.47$ and $\tau_h = 2.88$ and in C $\tau_m = 3.16$ and $\tau_h = 2.25$. 
Macroscopic currents

Figure 3.14.

Values of $\tau_m$ and $\tau_h$ plotted against membrane potential.

These values are compared with those obtained by Moolenaar and Spector (1978) in the discussion.
3.5 DISCUSSION

The electrophysiological properties described above, of cells of the neuroblastoma cell line N1E115, are compared here with properties of the same cell line described by Kimhi, Palfrey, Spector, Barak, and Littauer, (1976) and by Moolenaar and Spector, (1978).

The electrophysiological recordings described in this chapter were made from cells which had been differentiated in medium containing between 1.2 and 1.3% DMSO. The recordings described in section 3.3.5 were made from cells which had been differentiated for between 5 hours and 4 days while all other recordings were from cells which had been differentiated for between 1 and 2 days. Cells of this age had the appearance, under a scanning electron microscope, of the cell shown in figure 3.1. Cells which had been differentiated for longer periods of time grew larger and flattened out. They also grew more and longer processes, and had much less extensive infolding of the membrane, than the less differentiated cells which can be seen in the figure.

Cells of the adrenergic mouse neuroblastoma clone N1E115 and other clonal cell lines derived from the C-1300 mouse neuroblastoma have been used extensively for the study of various aspects of neuronal function and differentiation (see Introduction). In this discussion the electrophysiological properties of cells of the clone N1E115 which have been described in this chapter are compared with previous work on the electrophysiological properties of cells of the same line and particularly with the work of Moolenaar and Spector (1978) who recorded from cells of this line which had been differentiated for between 1 and 3 weeks in medium containing between 1 and
Kimhi et al. (1976) found that the resting potential of cells from the clone N1E115 which had been cultured for 2 days in 1% DMSO was -28.5 +/- 2.5 mV. This compares with the value reported earlier in this chapter of -26.8 +/- 3.6 mV (17 cells) for cells differentiated for between 1 and 2 days in 1.2 to 1.3% DMSO. Both of these values are lower than the control value reported by Kimhi et al. (1976) of -37.8 +/- 1.8 for cells subcultured for 2 days in culture medium without DMSO. They report, however, that DMSO proceeds to increase the resting potential so that after 8 days in 1% DMSO the cells have a resting potential of -45.6 +/- 2.3 mV compared with a control value of -39.3 +/- 1.9 and after 18 days the cells in DMSO have a resting potential of -37.7 +/- 1.6 mV compared with -30.4 +/- 6.0 mV in the control solution.

When recording from cells differentiated for between 1 and 2 days I found that the membrane capacity was 4.8 +/- 0.34 μF/cm² when the membrane area was calculated by measuring the diameter of the cell and assuming that the cell was a smooth sphere. Moolenaar and Spector (1978) estimated the membrane capacity of cells differentiated for between 1 and 3 weeks as roughly 2 μF/cm², again assuming that the cell is a smooth sphere. The expected value of membrane capacity for a biological membrane is 1 μF/cm² (see for example Hille, 1984). It appears from these results, therefore, that the assumption that the cell membrane is smooth is incorrect and that the extensive infoldings in the membrane of cells differentiated for a short time result in a membrane area of approximately 5 times that expected from assuming a smooth sphere and the infoldings in cells differentiated for longer times result in a membrane area equal to approximately twice that expected.

Kimhi et al. (1976) found that several morphological and
electrophysiological properties of the N1E115 cells changed with the concentration of DMSO present in the culture medium and with the time spent by the cells in DMSO. They found that addition of 1 or 2% DMSO to the cells induced morphological differentiation with about 75% of the cells extending long neurites. They measured the resting potential and the rate of rise of action potential of the cells to give a measure of the effect of DMSO on the electrophysiological properties of the cells. The effect of DMSO on the resting potential of the cells has already been mentioned. They found that 1 or 2% DMSO caused a marked enhancement in the electrical excitability of the cells. Table 3.1 shows the results obtained by Kimhi et al (1976) for the rate of rise (in volts/second) of the action potential after 2, 8 and 18 days in culture medium containing 1, 2 and 4% DMSO.
### Table 3.1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Days in culture</th>
</tr>
</thead>
<tbody>
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<td></td>
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</tr>
<tr>
<td>Control</td>
<td>74.3 +/- 9.7</td>
</tr>
<tr>
<td>1% DMSO</td>
<td>71.6 +/- 10.0</td>
</tr>
<tr>
<td>2% DMSO</td>
<td>65.0 +/- 8.2</td>
</tr>
<tr>
<td>4% DMSO</td>
<td>32.7 +/- 9.6</td>
</tr>
</tbody>
</table>

Maximum rate of rise of action potential, measured in volts/second, evoked from a resting potential of -90 mV at various times after sub-culture into DMSO-containing medium. From Kimhi, Palfrey, Spector, Barak, and Littauer, (1976).

If it is assumed that these results give an indication of the sodium channel density then it appears that 1% DMSO may first slightly decrease and then increase the sodium channel density of the cells. Current densities of potassium currents recorded, in the presence of 3 μM TTX to block sodium currents, from cells whose membrane potential was held at -54 mV and stepped to +26 mV were reported earlier in this chapter. These were 3.6 A/F for a cell which had been differentiated for 5 hours in DMSO, 5.8 +/- 0.37 A/F (6 cells) for cells which had been differentiated for between 16 and 24 hours and 12.7 and 14.0 A/F for 2 cells which had been differentiated for 4 days. (Currents are given as A/F so that current densities are compared, assuming that
Macroscopic currents

the membrane area is directly proportional to the membrane capacity.) This suggests that after 4 days in DMSO the potassium current density has been increased.

It is possible, therefore, that once differentiation has been induced by placing the cells in medium containing approximately 1% DMSO the area of the membrane increases before the ionic channel density increases. This would explain the measurements of resting potential and rate of rise of action potential made by Kimhi et al (1976) where, in 1% DMSO, both the resting potential and the rate of rise of action potential were lower than the control values after 2 days of differentiation but had increased above the control values by the eighth day of differentiation. Baumgold and Spector (1987) studied development of the sodium channel protein of N1E115 cells using $[^3]$Hsaxitoxin binding, $[^{125}]$scorpion toxin binding and $^{22}$Na uptake techniques. They found that when differentiation has been induced using DMSO or hexamethylene-bis-acetamide (HMBA) the cells differentiate morphologically within 3 days but the toxin binding and sodium uptake values are similar to those of undifferentiated cells whereas the binding and uptake levels in cells differentiated for 6 to 9 days are significantly elevated above the control levels.

The activation kinetics of the potassium currents and activation and inactivation kinetics of the sodium currents were fitted with Hodgkin and Huxley kinetics as described in section 3.4.2 (potassium) and 3.5.3 (sodium). These fits were performed so that the time courses of the currents could be compared with those of the currents recorded by Moolenaar and Spector (1978) since they fitted the time courses of these with Hodgkin and Huxley kinetics. It was found that the potassium currents were fitted well with equation 3.7, derived from equation 3.2 with $i = 2$. Equation 3.7 was also used by Moolenaar and Spector (1978). The voltage dependence of the two
Macrosopic currents

currents is similar with the steady-state value of \( n = 0.5 \) at approximately -8 mV in the currents described here and for the currents described by Moolenaar and Spector (1978) equal to 0.5 at approximately -9 mV. Moolenaar and Spector found, however, that values of the activation time constant, \( \tau_a \), determined from equation 3.7 varied from 20 msec at a membrane potential of -10 mV to approximately 0.75 msec at +40 mV. These values for the activation time constant are much smaller than those for the currents described in this chapter indicating that the currents described here activate more slowly than those described by Moolenaar and Spector (1978). For example, for the currents described earlier in this chapter \( \tau_a = 23.5 \) msec (one cell) at -14 mV and at +46 mV \( \tau_a = 6.3 \) mV (one cell). The values of \( \tau_a \) for the potassium currents were plotted against membrane potential in figure 3.8. It is unlikely that these values for \( \tau_a \) are made artificially slow by poor voltage clamping for several reasons:

1. Cells with few processes were chosen since the membrane potential of voltage clamped cells with one or more long neurites is not usually spatially uniform under volatge clamp which may result in anomalous current patterns under voltage clamp (for example Hagiwara and Saito, 1959; Standen, 1975);

2. Small cells were chosen and results which on subsequent analysis showed that the voltage drop across the series resistance was greater than 3 mV were rejected (see section 2.5.3 the series resistance was measured at least once for each preparation); and

3. The time course of the currents recorded under whole-cell clamp agrees closely with the time course of potassium currents recorded under patch-clamp (see Chapter 4).

It should therefore be a reasonable assumption that the differences between
the currents described in this chapter, which were recorded from cells differentiated for between 1 and 2 days, and those recorded by Moolenaar and Spector (1978) are due to differences between the two preparations or the voltage pulse protocol and not due to poor voltage clamp of the currents described here.

Moolenaar and Spector recorded currents from cells the membrane potential of which was held at -85 mV while the currents described here were recorded from cells which were held at -54 mV. It is possible, therefore, that there is a fast transient outward current which is present in the recordings by Moolenaar and Spector but which is not present in the currents described here. Fast transient or A- current is often inactivated at less depolarized holding potentials (Hille, 1984) and would therefore probably be inactivated in the cells whose membrane potential was held at -54 mV. For example, Connor and Stevens (1971a and b) found that the only potassium current which could be recorded from neurones of the nudibranch *Anisodoris* which were depolarized from a holding potential of -40 mV was a delayed rectifier potassium current whereas if the membrane potential of the cell was depolarized from -80 mV fast transient currents were elicited as well as the delayed rectifier currents; and Cooper and Shrier (1985) recorded unitary fast transient potassium currents from rat nodose neurones when the membrane potential was depolarized from -90 or -100 mV but the currents were largely inactivated at a holding potential of -65 mV.

A similar difference would be seen if a fast transient current was present in cells which had been differentiated for between 1 and 3 weeks but had not yet developed in cells which had only been differentiated for 1 or 2 days. Differences in the development times of different neuronal currents have been seen in the flight muscles of the fruit fly *Drosophila melanogaster* (Salkoff, 1983; Salkoff and Wyman,
In this case the fast transient current is mature by 72 hours of development of the pupa but the delayed outward current is not mature until 90 hours of development.

A further difference between the results described in this chapter and those reported by Moolenaar and Spector is the absence of fast inactivation in the former. Moolenaar and Spector (1978) report that during maintained depolarizations the outward current declined slowly from a maximum value to a lower steady-state value. They give examples from two cells: in one cell outward current elicited by a depolarization to +50 mV from a holding potential of -85 mV increases to a maximum quickly (the time to maximum cannot be read from the figure) and decreases by approximately 14% within 100 msec; in the other the current reaches its maximum by approximately 50 msec after the start of the depolarization and decreases slowly, not decreasing significantly within 100 msec. The latter results are comparable with those described in this chapter (see figures 3.3 and 3.4). Fast transient currents (A currents) such as those mentioned above show a fast inactivation and it is possible, therefore, that the currents in the latter cell were predominantly delayed rectifier while fast transient currents played a significant part in the former. The slow inactivation seen in the second of these two cells reported by Moolenaar and Spector may be the macroscopic current correlate of the slow inactivation of single channel currents described in Chapter 4.
Chapter 4

UNITARY DELAYED RECTIFIER POTASSIUM CURRENTS

4.1 INTRODUCTION

In Chapter 3 a voltage-activated delayed outward current present in cells of the neuroblastoma line N1E115 which have been allowed to differentiate for 1 to 4 days was described. The activation kinetics of the current were investigated and it was shown that the current is probably carried by potassium ions. In this chapter single channel potassium currents in membrane patches attached to these cells are described and it is shown that these are probably the unitary current correlate of the macroscopic current described in Chapter 3. Misler and Falke (1985) briefly reported such single channel currents.

It is also shown in this chapter that the channels have two open states of different conductance.
4.2 RESULTS

Cell-attached patches (see section 2.5.1) were formed with the electrode containing solution A ([K⁺]₀ = 5 mM) on cells which had been allowed to differentiate for 1 to 2 days. In many of the experiments described in this chapter these cells were bathed in solution A with [K⁺]₀ = 120 mM in order to make the resting membrane potential small (between -3.9 and -5.6 mV, calculated using the Nernst equation, assuming the intracellular potassium ion concentration, [K⁺]ᵢ, is between 140 and 150 mM (Palfrey, 1976)). When membrane patches on cells in these conditions were depolarized by 70 mV or more from a holding potential of -82 mV relative to the resting membrane potential then stepwise changes were seen in the current recorded from some of the patches. Some of these steps in current were negative or inward currents, corresponding to positively charged ions flowing from the extracellular side to the intracellular side of the membrane patch (or negatively charged ions moving in the opposite direction), and others were positive or outward.

Figure 4.1 shows inward currents (represented as downward deflections in the current trace) recorded when a cell-attached patch was held at -82 mV and pulsed to -2 mV relative to the resting membrane potential for 80 msec. The baseline current level is marked by C and 1, 2 and 3 represent the current levels of one, two and three open channels. All currents discussed in this chapter were filtered at 1 kHz and digitized at 5 kHz unless otherwise stated. At this membrane potential (approximately -7 mV since the cell resting potential is assumed to be approximately -5 mV and the cell-attached patch was held at -2 mV relative to this) and with the concentrations of sodium present ([Na⁺]ᵢ = 25 mM (Palfrey, 1976) and [Na⁺]₀ = 147 mM) sodium
Figure 4.1

Unitary inward currents.

Currents were recorded from a cell-attached patch on a cell which had been differentiated for 2 days. The bath solution was solution A but with \([K^+]_b = 120 \text{ mM}\). The electrode solution was solution A (\([Na^+]_p = 147 \text{ mM}\)). The membrane potential of the patch was held at -82 mV and stepped to -2 mV relative to the resting membrane potential of the cell.

The baseline current level is marked by C and 1, 2 and 3 represent the current levels at which it is thought that one, two and three channels are open. All currents shown in this chapter were filtered at 1 kHz and digitized at 5 kHz unless otherwise stated.
currents would be inward. Calcium currents would also be inward ([Ca\(^{2+}\)]_o = 1.8 mM) but it would be unlikely that currents passing through single calcium channels could be recorded under these conditions; unitary calcium currents are often too small to record easily when calcium is the charge carrier and have usually been recorded with barium as the charge carrier which increases the single channel conductance (for example Hess, Lansman and Tsien, 1984). The currents illustrated in figure 4.1 are therefore probably passing through sodium channels.

When 3 μM TTX was present in the electrode solution the inward currents disappeared (supporting the assumption that these are passing through sodium channels) leaving the outward currents intact. This is shown in figure 4.2 where outward currents were recorded, with solution A ([K\(^+\)]_o = 5 mM) plus 3 μM TTX in the electrode, from cell-attached patches held at -82 mV and stepped by +80 mV for 90 msec. C again denotes the baseline current level while I and II are two distinct levels which could be seen in the open channel current. [K\(^+\)]_o was again 120 mM. These currents were thought to be the unitary correlate of the macroscopic delayed rectifier currents described in the previous chapter and their kinetics and the ionic nature of the current carried through them are described below.
Unitary potassium currents

Figure 4.2

Unitary outward currents.

Currents were recorded from a cell-attached patch on a cell which had been differentiated for 4 days in differentiation medium. The bath solution was solution A but with $[K^+]_b = 120$ mM and the electrode solution was solution A with $[K^+]_p = 5$ mM and with 3 uM TTX. The membrane potential was stepped by +80 mV from a holding potential of -82 mV relative to the resting membrane potential of the cell (approximately -5 mV).

C again denotes the baseline current level while I and II represent two distinct levels which could be seen in the open channel current. These are thought to be two open states of different conductance.
4.3 THE CHANNEL HAS TWO OPEN STATES OF DIFFERENT CONDUCTANCE

Two distinct open levels can be seen in recordings of these currents at all potentials where single channel currents could be detected. These are indicated by I and II in figure 4.2 and, since both I and II are always seen in recordings, suggest two open states of different conductance. These two levels were seen at all potentials at which current could be detected and were also seen when the currents were recorded without TTX in the electrode, when they were recorded from undifferentiated cells and when currents were recorded from cells which remained in a solution with $[K^+]_b = 5$ mM for the duration of an experiment.

The amplitude of these currents was analysed to produce amplitude histograms. These were constructed by plotting the distribution of all the digitized data points and were fitted by computer to Gaussian relationships using a least squares fitting algorithm (Marquardt, 1963). Data analysed in this way is shown in figure 4.3. This shows an amplitude histogram constructed from data recorded from a membrane patch the potential of which was held at -82 mV relative to the resting potential and pulsed by +80 mV. (Single current traces recorded from the same patch were illustrated in figure 4.2 ($[K^+]_b = 120$ mM; $[K^+]_p = 5$ mM).) The single peak, close to 0 pA, corresponds to the amplitude of the baseline current and has been fitted with a single Gaussian distribution (smooth curve) with mean = 0.017 pA. The other two peaks represent the amplitudes of levels I and II seen in figure 4.2. These peaks are shown fitted by the sum of two Gaussian distributions (smooth curve). The summed distribution is defined by the area, mean and standard deviation of the two distributions (I and II) which make up the sum. Distribution I has a mean = 0.847 pA and
Unitary potassium currents

Figure 4.3

Amplitude histogram.

The histogram was constructed by plotting the distribution of all the digitized data points from currents recorded from a cell-attached patch with \([K^+]_b = 120 \text{ mM}, [K^+]_p = 5 \text{ mM}\) and with 3 \(\mu\text{M TTX}\) in the pipette. Some of the data which was used to form this histogram are shown in figure 4.2. The membrane potential of the patch was stepped by +80 mV from a holding potential of -82 mV.

The single peak close to 0 pA corresponds to the baseline current level and has been fitted with a single Gaussian distribution (smooth curve). This has an area = 124.11, mean = 0.017 pA and standard deviation = 0.069 pA. The other two peaks represent the amplitudes of levels I and II seen in figure 4.2 and have been fitted with the sum of two Gaussian distributions (I and II). Distribution I has area = 159.269, mean = 0.847 pA and standard deviation = 0.176 pA and distribution II has area = 73.132, mean = 1.169 pA and standard deviation = 0.105 pA.
distribution II has a mean = 1.169 pA.

When the patches were pulsed to -2 mV relative to the resting membrane potential the means of the two Gaussian curves suggest channel currents for levels I and II of 0.86 +/- 0.14 pA and 1.27 +/- 0.12 pA (6 patches). The chord conductance of these two levels at this potential (i.e. where chord conductance, \( g_r = \frac{i}{(V-E_K)} \) and \( i \) is the single channel current, \( V \) the membrane potential of the patch (assumed to be -7 mV) and \( E_K \) is -84.3 mV at 20°C) is 11.1 +/- 1.8 pS for level I and 16.4 +/- 1.6 pS for level II.

Cell-attached patches on cells differentiated for between 1 and 4 days were stepped by different voltages from a holding potential of -32 mV relative to the resting membrane potential of the cell. Cells were bathed in solution A ([\( K^+ \])_b = 5 mM) giving a resting potential of -26.8 +/- 3.6 mV (17 cells; see section 3.2). [\( K^+ \])_p was also 5 mM. Amplitude histograms from the resultant currents were fitted with the sum of two Gaussian distributions and the means of these Gaussians are plotted against membrane potential in figure 4.4. It can be seen that the current-voltage relation is linear for both current levels over this range of voltages. If the currents are extrapolated linearly they reverse at approximately -45 mV relative to the resting membrane potential of the cell. Assuming a mean resting potential of 27 mV the currents reverse at approximately -72 mV. (It is likely, however, that the currents would show some rectification as they approached zero.) The theoretical reversal potential for potassium currents under these conditions is -85.5 mV.
Unitary potassium currents

Figure 4.4

Current-voltage relation for the two unitary current levels.

Amplitude histograms were constructed from currents recorded from cells bathed in solution A ($[K^+]_b = 5$ mM). Solution A was also the electrode solution. The means of the two Gaussian distributions used to fit the histograms are plotted against membrane potential for between 4 and 7 cells at each membrane potential except 18 mV and +98 mV where only one result is plotted.

The current-voltage relation is linear with the currents reversing at approximately $-45$ mV (estimated by linear extrapolation; the current may rectify in these solutions) relative to the resting membrane potential of the cells (approximately $-27$ mV in these conditions).
4.4 SINGLE CHANNEL KINETICS

4.4.1 Patterns of opening to the two levels

The two current levels seen in figure 4.2 could be interpreted in terms of either two different channels or a single channel with two open states of different conductance. The patterns of opening seen in all patches suggest that the two open levels (I and II) represent two open states of the same channel. The patterns of opening which have been observed are shown in figure 4.4 A. The interruptions marked by short parallel lines in the figure indicate that at these points the channel may move several times between levels I and II. All of these patterns can be seen in figure 4.2. If the levels I and II represented the amplitude of two different channels it would be expected that an amplitude corresponding to I + II (shown in figure 4.5 B) might be seen but this level has never been recorded in this study. Alternatively, if state II corresponded to a small channel opening on top of state I then a level corresponding to II - I might be expected (also shown in figure 4.5 B). This level also has never been recorded so it has been assumed that the levels I and II correspond to open open states of different conductance. This has been further supported by the finding that the two levels do not behave independently of one another:
Unitary potassium currents

Figure 4.5

Patterns of opening to levels I and II.

The transitions illustrated in A were all regularly seen in patch clamp current recordings. Those possible levels illustrated in B were never seen but might have been expected if the two current levels did not represent two open states of the same channel. Level II-I would be expected if level II represented a small channel opening on top of level I and level II+I would be expected if levels I and II represented the amplitudes of two different channels.
4.4.2 Openings to the two levels do not behave independently

The probability of a channel opening to one or other of the open states during a voltage pulse may be calculated by counting the number of traces recorded from one patch at a given voltage which exhibit openings to state I and the number which exhibit openings to state II. These probabilities may then be multiplied together to give the expected probability of both states occurring during the same voltage pulse if the two states behave independently. The theory assumes that if, for example, 100 pulses are applied to a patch containing a single channel but only 20 elicit openings to state I only, 5 to state II only and 50 have openings to both states then the probability of a pulse eliciting an opening to state I is 0.7 and to state II is 0.55. The probability of seeing state I and state II during the same pulse is therefore 0.385. In this example one would expect 39 of the pulses to give openings to both states. The results of these calculations are shown in table 4.1 for ten different patches.

The $\chi^2$ test was performed on the results given in table 4.1 to test the null hypothesis that the actual number of traces containing openings to both levels was equal to the number expected if the states behave independently. The probability that the null hypothesis is correct is <0.1%. This suggests that levels I and II occur together during one voltage pulse more often than would be expected if they behaved independently and agrees with the assumption that the two levels do not represent two separate, independent channels.
<table>
<thead>
<tr>
<th>Patch</th>
<th>n</th>
<th>nI</th>
<th>nII</th>
<th>P(I + II)</th>
<th>predicted no.</th>
<th>actual no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>105</td>
<td>65</td>
<td>93</td>
<td>0.59</td>
<td>62</td>
<td>69</td>
</tr>
<tr>
<td>2</td>
<td>130</td>
<td>53</td>
<td>50</td>
<td>0.16</td>
<td>21</td>
<td>45</td>
</tr>
<tr>
<td>3</td>
<td>112</td>
<td>23</td>
<td>22</td>
<td>0.04</td>
<td>5</td>
<td>17</td>
</tr>
<tr>
<td>4</td>
<td>60</td>
<td>5</td>
<td>6</td>
<td>0.008</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>5</td>
<td>40</td>
<td>11</td>
<td>6</td>
<td>0.04</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>6</td>
<td>79</td>
<td>35</td>
<td>28</td>
<td>0.15</td>
<td>12</td>
<td>27</td>
</tr>
<tr>
<td>7</td>
<td>68</td>
<td>18</td>
<td>17</td>
<td>0.07</td>
<td>5</td>
<td>13</td>
</tr>
<tr>
<td>8</td>
<td>111</td>
<td>38</td>
<td>30</td>
<td>0.09</td>
<td>10</td>
<td>29</td>
</tr>
<tr>
<td>9</td>
<td>187</td>
<td>132</td>
<td>112</td>
<td>0.43</td>
<td>80</td>
<td>99</td>
</tr>
<tr>
<td>10</td>
<td>60</td>
<td>47</td>
<td>46</td>
<td>0.6</td>
<td>36</td>
<td>46</td>
</tr>
</tbody>
</table>

Table showing the number of traces containing openings to state I (nI), state II (nII) and the total number of traces in the set of recordings (n). From these the probability of seeing traces containing openings to both levels was calculated ($P(I + II)$) and this was used to predict the number of traces containing openings to both levels. This was compared with the actual number using the $\chi^2$ test.
4.4.3 Transitions between states

From an inspection of the traces, for example those seen in figure 4.2 B, it appears that the channel opens to and closes from each of the open states and that it alternates between open states. Sometimes, however, the recorded current may appear to make a certain transition between states when the channel has actually passed through an intermediate state too quickly for it to be detected by the recording system. For example, when the channel appears to move from the closed state (C) into state II it may have moved from the closed state to a brief opening, too short to be detected by the recording system, in state I before moving into state II.

For each possible transition the probability of missing an intermediate state may be calculated using the method of Labarca and Miller (1981). This method calculates the probability ($P_k^*$) of missing an intermediate state ($k$) from

$$4.1 \quad P_k^* = 1 - \exp(-t/\tau_k)$$

where $t$ is the time response of the recording system and $\tau_k$ the mean open time of the intermediate state $k$. The shorter the mean open time of the possible intermediate state, therefore, the greater the probability that it could have occurred but not been detected. By shortening the duration of a constant amplitude pulse fed into the recording system until the resultant output would not have been accepted as an opening by the half-amplitude cursor method (see section 2.9.3), $t$, the response time of the recording system, was measured. The minimum duration of a state for it to be recognised by the recording system was found to be 280 μsec.

The probability of missing an intermediate state when a certain transition
Unitary potassium currents

appears to occur may then be compared with the number of times that particular transition is seen. If the probability of there being an intermediate state which has been missed comes close to the probability of seeing the transition in question then it is not possible to say whether that transition ever actually takes place or whether it just appears to take place because a short intermediate state has always been missed.

The probability of seeing a certain transition is found experimentally by looking at all the transitions which occur in the current recorded from one membrane patch at one potential. There are six possible transitions: C-I, I-C, C-II, II-C, I-II and II-I. The probability of seeing a certain transition between two states \(i\) and \(j\) is given by

\[
P_{ij} = \frac{n_{(i,j)}}{n_t}
\]

where \(n_{(i,j)}\) is the number of transitions from state \(i\) to state \(j\) which can be seen in the recorded current and \(n_t\) is the total number of state transitions detected.

Counting of state transitions was performed on data which had been interpolated using the method described in section 2.9.3 and quiet current traces were chosen. These were chosen in order that errors generated by random noise were kept to a minimum; most of the data recorded were too noisy to allow this type of analysis to be performed. This can be seen from the amplitude histograms (figure 4.3) where the two Gaussian distributions corresponding to the open channel noise of state I and state II overlap. This means that it is often difficult to distinguish between noise in the current trace and true transitions between states.

\(\tau_k\), the mean open time of state \(k\), is given by the time constant of an
exponential fitted to the dwell-time histogram constructed for each open state. Dwell-time histograms for open states I and II are shown in figure 4.6 for a cell-attached patch \([K^+]_b = 120 \text{ mM}, [K^+]_p = 5 \text{ mM}\) stepped by +80 mV from a holding potential of -82 mV. The histograms have been fitted with single exponentials the time constants of which are 1.54 msec (state I) and 2.73 msec (state II).

The closed time histogram may not be satisfactorily fitted by a single exponential (see section 4.4.6) and so the mean closed time was taken as the total time which the channel spent closed divided by the number of closings.

The results are summarized in table 4.2 which lists the experimentally determined probability of seeing a transition between two states i and j \((P_{ij})\) and the probability that an intermediate state \((k)\) has been missed \((P^k)\). It can be seen that the probability of missing an intermediate state I is greater than that of seeing a transition between the closed state and state II. However, the probability of missing state II is smaller than the probability of seeing a transition between state I and the closed state and the probability of missing the closed state is smaller than that of seeing a transition between state I and state II. It therefore seems likely that transitions do occur between the closed state and state I and between state I and state II but it is possible that no transitions occur between the closed state and state II.

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Open time histograms of state I and state II.

Open time histograms were constructed from quiet current traces in order that errors generated by random noise were kept to a minimum. The data were recorded from a cell-attached patch ([K]_p = 120 mM, [K]_p = 5 mM) stepped by +80 mV from a holding potential of -82 mV relative to the resting membrane potential of the cell.

The histogram constructed from the open times of state I has been fitted with a single exponential distribution with a time constant of 1.54 msec while that constructed from the open times of state II was best fitted with a single exponential distribution with a time constant of 2.73 msec.
## Table 4.2

<table>
<thead>
<tr>
<th>Transition i-j</th>
<th>$P_{ij}$</th>
<th>$k$</th>
<th>$P_k$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Patch 1</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-I</td>
<td>0.166</td>
<td>II</td>
<td>0.098</td>
</tr>
<tr>
<td>I-C</td>
<td>0.114</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-II</td>
<td>0.146</td>
<td>I</td>
<td>0.166</td>
</tr>
<tr>
<td>II-C</td>
<td>0.16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I-II</td>
<td>0.191</td>
<td>C</td>
<td>0.129</td>
</tr>
<tr>
<td>II-I</td>
<td>0.223</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Patch 2</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>C-I</td>
<td>0.129</td>
<td>II</td>
<td>0.058</td>
</tr>
<tr>
<td>I-C</td>
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<td></td>
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<tr>
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<td>II-C</td>
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</tr>
<tr>
<td>I-II</td>
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</tr>
<tr>
<td>II-I</td>
<td>0.211</td>
<td></td>
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</tr>
</tbody>
</table>
4.4.4 Open time distributions

Although the amplitude analysis showed that the channel opened to two distinct levels, open-time histograms for the separate levels could not be constructed for most data files because the amplitude difference between the two levels is small in comparison to the random noise level in the open-channel current. (Open-time analysis for the separate states is described in section 4.4.3 for data from two membrane patches.) Combined-state open time histograms, however, could be constructed even for noisier current traces by using a cursor placed at half the amplitude of state I and thereby only registering transitions to or from the closed level. Figure 4.7 shows open time histograms calculated from results recorded from a cell-attached patch with $[K^+]_p = 5$ mM and $[K^+]_b = 120$ mM. The patch was pulsed to -2 mV relative to the resting membrane potential from a holding potential of -82 mV. Any openings which were terminated by the end of the voltage pulse were excluded from the results. This tends to bias the results in favour of short openings since a long opening is more likely to be terminated by the end of the voltage pulse. In the figure a single exponential probability density function (A) with a time constant of 2.25 msec, fitted by the least-squares method, gives a poorer fit to the data than a line constructed using a least-squares fit to the sum of two exponentials with time constants of 0.53 and 7.61 msec (B). The observation that the data are fitted more accurately by the sum of two exponentials adds support to the assumption, inferred from the appearance of the records, that the channel exhibits two open states (Colquhoun and Hawkes, 1981).
The histogram was constructed by placing a cursor at half the amplitude of state I and thereby only registering transitions to and from the closed state. The histogram was constructed from data recorded from a cell-attached patch with \([K^+]_o = 120 \text{ mM}\) and \([K^+]_p = 5 \text{ mM}\). The membrane potential of the patch was stepped by +80 mV from a holding potential of -82 mV.

In A the histogram has been fitted with a single exponential distribution with a time constant of 2.25 msec while in B it has been fitted by the sum of two exponential distributions with time constants of 0.53 and 7.61 msec. It can be seen that the data is fitted better by the sum of two exponential distributions than by a single exponential.
4.4.5 The open time distribution is voltage-dependent

The voltage-dependence of the open time of the channel was investigated using combined state open-time histograms as described above since single-state open-time histograms could not be constructed for most patches. Open time histograms formed from cell-attached patches with $[K^+]_p = 5$ mM and $[K^+]_b = 120$ mM, when patches were held at -82 mV and stepped by different voltages are shown in figure 4.9. The time constants of the exponentials used to fit these histograms and other histograms formed in the same way are plotted against voltage in figure 4.8. It can be seen that, while the time constant of the first (faster) exponential (♦) may not be voltage dependent, the time constant of the second exponential (●) increases with increasing depolarization, being 7.11 +/- 0.73 msec (4 patches) at -2 mV, 16.13 +/- 3.09 msec (4 patches) at +18 mV and 17.35 (one patch) at +38 mV. This voltage-dependence of the second time constant is paralleled by a change in the amplitude distributions used to fit the single channel currents. Figure 4.10 shows amplitude distributions taken from the same data which was used to form the open time distributions in figure 4.8 A and B. The relative times spent in the two open states may be determined by comparing the areas of the two Gaussian distributions used to fit the open channel current. The area under the distribution is proportional to the time spent in that state. The amplitude histogram shown in figure 4.10 (A) was formed from currents recorded from a patch whose membrane potential was stepped from -82 mV to -2 mV relative to the resting potential of the cell ($[K]_b = 120$ mM) and the channel spent 69% of its open time in state I while the histogram in B was formed from currents recorded from a patch stepped from -82 mV to +18 mV relative to the resting membrane potential of the cell and the channel spends 51% of time in state I.
Unitary potassium currents

Figure 4.8

Open time histograms constructed from data recorded at different membrane potentials.

Combined state open time histograms were constructed from cell-attached patches stepped to -2 mV (A), +18 mV (B) and +38 mV (C) from a holding potential of -82 mV relative to the resting membrane potential of the cell ([K$^+$]$_b$ = 120 mM, [K$^+$]$_p$ = 5 mM).

The histograms have been fitted with the sum of two exponential distributions with time constants of 0.53 and 7.61 msec (A), 3.88 and 13.9 msec (B) and 0.85 and 17.35 msec (C).
Unitary potassium currents

Figure 4.9

Open time histogram time constants plotted against membrane potential (relative to the resting membrane potential of the cell).

The time constants of the faster and the slower exponential distributions used to fit the combined state histograms have been plotted against the membrane potential of the patch relative to the resting membrane potential of the cell ([K⁺]₀ = 120 mM, [K⁺]ᵢₚ = 5 mM). The faster time constant is represented by circles and the slower by diamonds.

It can be seen that, while the time constant of the faster exponential may not be significantly voltage dependent, the time constant of the slower exponential increases with increasing depolarization.
Amplitude histograms.

Histograms were constructed from a patch held at -82 mV and pulsed to -2 mV (A) and +18 mV (B).

The area under distribution I is 69% (A) and 51% (B) of the total area under distributions I and II. The figure therefore suggests that the amplitude distribution of the two states may be voltage dependent. The time which the channel spends in state I as plotted as a proportion of the total open time of the channel in figure 4.10.
This suggests that the channels may spend more time in state II at more positive potentials. The area of the distribution used to describe the current flowing through state I is plotted in figure 4.11 as a proportion of the total area of the two distributions used to describe the current flowing through state I and state II. It can be seen that the amplitude distribution does have a voltage dependence and that the channel spends more time in state II at the more depolarized potentials as implied by figure 4.10. At -2 mV relative to the resting membrane potential the channel spends 54 +/- 3% (4 patches) of its open time in state I, at +18 mV it spends 48 +/- 1% (4 patches) in state I and at +38 mV 38% (one patch) of its open time is spent in state I. This voltage-dependence of the time spent in each state is similar to that of the open-time distribution.

4.4.6 Closed time distributions

Closed time histograms were constructed from results recorded from cell-attached patches on cells bathed in solution A but with $[K^+]_b = 120$ mM and with $[K^+]_p = 5$ mM. Histograms were formed using the half amplitude cursor method (see section 2.9.3) with the cursor placed at half the amplitude of state I. Such a histogram, constructed from data recorded from a patch whose membrane potential was stepped by +80 mV from a holding potential of -82 mV, is shown in figure 4.12. Attempts were made to fit the data with a single exponential and these are shown in A. The data were not satisfactorily fitted with a single exponential which implies that the channel possesses more than one closed state (Colquhoun and Hawkes, 1981). The same histogram is shown fitted with the sum of two exponential distributions with time constants of 0.45 and 3.63 msec in B.
Figure 4.11

Area under distribution I in the amplitude histogram plotted as a percentage of the areas under distributions I and II (A_p), against membrane potential.

Membrane potential is relative to the resting membrane potential of the cell ([K^+]_o = 120 mM). The values shown are means of the results from 4 patches at -2 mV and +18 mV; at +38 mV the result from one patch is shown. Area is proportional to time in the amplitude distribution. The plot therefore shows that the time which the channel spends in state I decreases with increasing depolarization.
The closed time histogram was constructed from currents recorded from cell-attached patches with \([K^+]_o = 120\) mM and \([K^+]_p = 5\) mM. The membrane potential was stepped by +80 mV from a holding potential of -82 mV.

The histogram has been fitted with a single exponential distribution (A) which does not fit the data well and with the sum of two exponential distributions which gives a much better fit. The time constants of the two distributions (1 and 2) are 0.45 msec (\(\tau_1\)) and 3.63 msec (\(\tau_2\)).
The closed time distributions show no voltage-dependence, at -2 mV $\tau_1 = 0.87 +/- 0.05$ and $\tau_2 = 2.54 +/- 0.49$ (4 patches), at +18 mV $\tau_1 = 0.84 +/- 0.18$ and $\tau_2 = 3.28 +/- 1.45$ (4 patches) and at +38 mV $\tau_1 = 0.88$ and $\tau_2 = 2.686$ (one patch).

4.4.7 Activation kinetics

The activation kinetics of the single potassium channel described above can be compared to those of the macroscopic delayed outward current described in Chapter 3 by calculating the activation time constant $\tau_a$ from the Hodgkin-Huxley equation given in section 3.3.2. The macroscopic current has been shown, in Chapter 3, to be well described by this equation with $i = 2$.

To fit the single channel data to this equation the single channel currents recorded at each membrane voltage were summed together to produce the equivalent of a macroscopic current. The magnitude of the current at any time after the depolarization is reasonably accurately predicted only if $i = 2$. If $i$ is set to 1 there is no delay in the rise of the theoretical curve and it rises faster than the trace derived from the sum of the single channel currents while if $i = 3$ the delay is too long and the rise time too slow. This agrees with the macroscopic data described in Chapter 3 (see section 4.6).

Summed single channel currents, from cell-attached patches on cells with $[K^+]= 120$ mM, in response to voltage steps of +70 and +90 mV from a holding potential of -82 mV relative to the resting membrane potential of the cell are shown in figure 4.13 alongside the whole-cell delayed outward currents recorded at +16 and +36 mV (A and B in the figure) from TTX poisoned cells and compared to the prediction of the Hodgkin-Huxley equation with $i = 2$. 

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Unitary potassium currents

Figure 4.13

Macroscopic delayed outward currents (A and B) and summed single channel currents (C and D).

Currents in A and B were recorded from cells which had been differentiated for 1 day. The membrane potential was stepped from a holding potential of -54 mV to +16 mV (A) and +36 mV (B) for 90 msec. Currents in C and D were recorded from cell-attached patches ([K$^+$]$_b$ = 120 mM, [K$^+$]$_p$ = 5 mM). The membrane potential was stepped from a holding potential of -82 mV to +18 mV (C) and +38 mV (D) relative to the resting membrane potential of the cell.

Currents have been fitted with equation 3.7. In A and B the time constants of activation ($\tau_a$) which gave the best fit to the data were 15.6 msec and 12.6 msec respectively whilst in C and D $\tau_a$ was estimated as 13.9 msec and 12.0 msec respectively.
Unitary potassium currents

It can be seen that the time course of the single channel and whole cell currents agree well with each other and with the predicted curve.

The activation time constant, $\tau_n$, determined from these fits is voltage dependent and decreases with increasing depolarization over the range of potentials studied. In Chapter 3, figure 3.8, the values of $\tau_n$ determined from Hodgkin-Huxley fits of macroscopic potassium currents were plotted against membrane potential. This figure is repeated in figure 4.14 along with the values of $\tau_n$ obtained from fits of the summed single channel data. This shows that $\tau_n$ shows the same voltage dependence in both cases. $\tau_n$ for the summed single channel currents decreases from 30.0 +/- 4.9 msec (4 patches) at -2 mV to 12.8 msec (one patch) at +38 mV and $\tau_n$ for the whole cell currents decreases from 36.6 +/- 8.6 msec (4 cells) at 0 mV to 10.2 +/- 0.6 msec at +40 mV (one cell).

4.4.8 Latency to first event

If the time to first event is measured using the half amplitude cursor method and histograms are constructed of the resultant values then it can be seen that these histograms peak at a time greater than zero and then fall. This is shown in figure 4.15 where such histograms have been fitted with equation 4.3 where the probability density function for the distribution of first latencies is described for the linear state diagram

\[
\begin{align*}
C_2 & \xrightarrow{k_2} C_1 & C_1 & \xrightarrow{k_1} O_1 & \xrightarrow{k_{-1}} O_2 \\
& \xleftarrow{k_{-2}} & & \xleftarrow{k} & \end{align*}
\]
Unitary potassium currents

Figure 4.14

\( \tau_n \) plotted against membrane potential.

Figure 3.8 (\( \tau_n \) for the whole cell currents plotted against membrane potential) is repeated (●) along with values of \( \tau_n \) for single channel data plotted against membrane potential relative to the resting membrane potential of the cell (◆).
Unitary potassium currents

Figure 4.15

First latency histogram.

The histogram is constructed from data recorded from a cell attached patch with $[K^+]_p = 5$ mM and $[K^+]_b = 120$ mM. The membrane potential was held at -52 mV and stepped by +60 mV.

The histogram has been fitted with equation 4.3 with an area of 88.5, $R_1 = 0.076$ ms$^{-1}$ and $R_2 = 0.139$ ms$^{-1}$. This gives a satisfactory fit to the data suggesting that the channel passes through two kinetically distinguishable closed states before it opens.
The probability density function $f(t)$ for the first latency distribution arising from above scheme is given by

4.3 $f(t) = \frac{R_1 R_2 [\exp(-R_2 t) - \exp(-R_1 t)]}{R_1 R_2}$

where $R_1$ and $R_2$ are related to the rate constants for transitions between states by

4.4 $R_1 = \frac{[k_1 + k_2 + k_2^2 + ((k_1 + k_2 + k_2^2)^2 - 4k_1 k_2)]}{2}$

and

4.5 $R_2 = \frac{[k_1 + k_2 + k_2^2 - ((k_1 + k_2 + k_2^2)^2 - 4k_1 k_2)]}{2}$

(from Patlak and Horn, 1982; and Standen, Stanfield and Ward, 1985). Equation 4.3 gives a satisfactory fit to the data and suggests that the channel passes through at least two closed states prior to an opening induced by a depolarization. The mean first latency is voltage-dependent, decreasing from 35.44 +/- 8.3 msec (4 patches) at 0 mV relative to the resting membrane potential to 19.35 msec (one patch) at +40 mV.

Both the summed currents and the measurements of first latency show that activation of the channel is voltage dependent and the first latency measurements also show that the channel passes through at least two closed states prior to opening. These are related to the information gained from the closed-time histograms (which also
suggest at least two closed states but which are not voltage-dependent) in the discussion.

4.4.9 Channel inactivation

Figure 4.13 shows that neither the summed single channel currents nor the macroscopic currents decline during a 90 msec depolarization. Such a decline was seen in a very small proportion of the patch clamp recordings made and in all but one patch was only slight (between 0 and approximately 10% decline in current in 90 msec). In the one patch in which the decline was significant it also proceeded more quickly, and the activation time course of the channel was faster, than in all other patches. The results from this patch have therefore been excluded from the results and it is thought that this may have been an early outward current channel such as those recorded by Cooper and Shrier (1985).

If a decline in current had been evident in more patches it would have indicated a process of fast inactivation as seen in delayed rectifier channels of frog skeletal muscle (Standen, Stanfield and Ward, 1985). It is concluded from the results (as illustrated in figure 4.13) that fast inactivation does not take place in these channels to any significant degree during a 90 msec voltage pulse. However, single channel recording suggests some form of slow inactivation. This suggestion comes from the observation that when depolarizing pulses are repeated at an interval of 2 sec the records which do not contain any channel openings appear to cluster together. A possible macroscopic current correlate of this observation is the decline in outward current which can be seen if cells are subjected to step depolarizations at a rate greater than 1 every 4 msec. To test this hypothesis the degree of randomness in the occurrence
of traces with or without channel openings was studied using the method of runs analysis (Swed and Eisenhart (1943); Gibbons (1971)). This method has been used to demonstrate nonrandom clustering of blank traces for sodium channels (Horn, Vandenberg and Lange, 1984) and for the delayed rectifier potassium channel of frog skeletal muscle (Standen, Stanfield and Ward, 1985).

In this analysis the expected number of runs (series of records with or without openings) is predicted as $2np(1-p)$ for $n$ trials (where $n>40$) with a probability, $p$, of observing an event (at least one channel opening in a trace). The distribution of the number of runs is given approximately by

$$Z = \frac{R - 2np(1-p)}{\sqrt{np(1-p)}}$$

where $Z$ is a standardized random variable with mean = 0 and variance = 1, and $R$ is the observed number of runs. $Z$, therefore, is close to zero if events are randomly ordered and positive values of $Z$ indicate nonrandom grouping.

Table 4.3 shows that randomness could not be demonstrated in nine out of ten patches analysed in this way. Such a conclusion may suggest that the channel moves slowly between a closed state in which it is available for activation and one in which it is inactivated.
Table 4.3

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<td>0.0222</td>
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</tr>
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</table>

Table showing the results of runs analysis performed to investigate clustering of traces with and without openings.
4.5 THE IONIC NATURE OF THE CURRENT

4.5.1 The dependence of the reversal potential on the pipette potassium concentration

The reversal potential of the currents was measured with different concentrations of potassium in the recording electrode. The potassium concentration was altered by replacement of sodium chloride with potassium chloride in solution A (table 1). The reversal potential in each solution was measured by stepping the membrane potential to a level at which the channel opened and then ramping the voltage (see section 2.6.3) in a negative direction, past the potential at which the channel current reversed.

A typical experimental record is shown in figure 4.16 where the voltage of a cell-attached patch has been ramped in this way. \([K^+]_p = 15\, \text{mM}\) and \([K^+]_b = 5\, \text{mM}\). The voltage was first pulsed by +90 mV, from a holding potential of -31 mV for 20 msec and then ramped at a rate of -2.5 mV/msec for 70 msec to end at -86 mV. The current illustrated in the figure reverses at -3 mV relative to the resting membrane potential of the cell.

Records such as that shown in figure 4.16 were averaged and the reversal potential of the channel was measured from the averaged current. Figure 4.17 shows averaged currents from two patches, one with \([K^+]_p = 30\, \text{mM}\) (A) and one with \([K^+]_p = 60\, \text{mM}\) (B) \([K^+]_b = 5\, \text{mM}\). The voltage was ramped from +59 mV at a rate of -2 mV/msec. The reversal potentials are marked by lines on the figure pointing to where the current crosses 0 pA. With \([K^+]_p = 60\, \text{mM}\) the current reverses at +8 mV while with \([K^+]_p = 30\, \text{mM}\) the current reverses at -4 mV relative to the resting membrane.
Figure 4.16

Unitary delayed outward current produced by ramping the membrane potential.

$[K^+]_p$ was 15 mM and $[K^+]_b$ 5 mM. The membrane potential was stepped from a holding potential of -31 mV by 90 mV for 20 msec and then ramped at a rate of -2.5 mV/msec to end at -86 mV relative to the membrane potential of the cell. The current illustrated reverses at -3 mV relative to the resting potential of the cell which is assumed to be approximately -27 mV (see section 3.2).
Unitary potassium currents

Figure 4.17

Averaged currents produced by ramping the membrane potential.

Currents were recorded with $[K^+]_b = 5 \text{ mM}$ and with $[K^+]_p = 30 \text{ mM}$ (A) and 60 mM (B). The membrane potential of the patches was held at -31 mV and stepped to +59 mV for 20 msec before being ramped at a rate of -2 mV/msec.

The current in B has a steeper slope than that in A and reverses at +8 mV while that in A reverses at -4 mV relative to the resting membrane potential of the cell.
potential of the cell. The dependence of reversal potential on the concentration of potassium in the pipette is shown in figure 4.18. The current could not be reversed with $[K^+]_p < 15$ mM because at these potassium ion concentrations the reversal potential becomes so negative that the channel closes before the reversal potential can be reached.

4.5.2 The slope conductance is dependent on the external potassium ion concentration

The slope conductance was measured from the slope of the ramped current where it passed through 0 pA. This is shown by the arrow in figure 4.16 and the lines labelled A and B in figure 4.17. Figure 4.19 shows current recordings from cell-attached patches ($[K^+]_b = 5$ mM) held at -31 mV and depolarized to +49 mV relative to the resting membrane potential but with different concentrations of potassium in the pipette; $[K^+]_p = 5$ mM in A, 15 mM in B and 30 mM in C. The currents passing through channel with a high external potassium ion concentration are larger than those through channels with a low external potassium pulsed to the same potential. The arrows show the closed level and states I and II as in figure 4.2. Arrow heads at the top of the figure show the extent of the depolarizing voltage pulse. The amplitudes of states I and II in the figure are approximately 0.8 and 1.2 pA (A), 1.0 and 1.4 pA (B) and 1.4 and 1.9 pA (C). Figure 4.17 shows that the slope conductance of the channel with $[K^+]_p = 30$ mM (A) is less than that when $[K^+]_p = 60$ mM (B). Figure 4.20 shows the values of slope conductance measured in this way plotted against external potassium ion concentration.
Unitary potassium currents

Figure 4.18

Reversal potential plotted against \([K^+]_p\).

The reversal potential was measured as the potential (relative to the resting membrane potential of approximately -27 mV) at which the averaged ramped current passed through 0 pA. Only one value is plotted with \([K^+]_p = 60\) mM and all other values represent the means of 3 results.

It can be seen that the reversal potential of the unitary currents is dependent on \([K^+]_p\). The dotted line represents the slope expected if the channel were purely selective for potassium.
mV
relative to $V_{rest}$

$mM$

-20  -10   0    10    20    40    80    160
Unitary potassium currents

Figure 4.19

Unitary delayed outward currents recorded with different concentrations of potassium in the electrode.

Currents were recorded from cell-attached patches held at -31 mV relative to the resting membrane potential of the cell ([K\(^{+}\)]\(_{b}\) = 120 mM) and depolarized by +80 mV. [K\(^{+}\)]\(_{p}\) = 5 mM in A, 15 mM in B and 30 mM in C.

The unitary current size increases with increasing [K\(^{+}\)]\(_{p}\). In A the amplitudes of states I and II are approximately 0.8 and 1.2 pA, in B they are 1.0 and 1.4 pA and in C 1.4 and 1.9 pA respectively.
Slope conductances were measured on cell-attached patches with the $[K^+]_\text{b} = 5$ mM. The measurements of slope conductance made using the voltage ramp cannot distinguish between state I and state II and so all measurements represent an average slope conductance for the two states. It can be seen that the conductance saturates with increasing $[K^+]_\text{p}$. This indicates that the independence principle (Hodgkin and Huxley, 1952a) is not obeyed at high $[K^+]$. This can be explained by analogy with the Michaelis-Menten model of enzyme kinetics (Hille, 1984) where the velocity of reaction reaches a saturating value at high substrate concentration because substrate molecules compete for active sites on the enzyme. Here, ions are competing for sites within the pore at high $[K^+]$ and there is therefore an upper limit on the ionic flux through the channel.
Figure 4.20

Slope conductance plotted against membrane potential.

Slope conductance is plotted against the membrane potential of the patch relative to the resting membrane potential of the cell ([K⁺]₀ = 5 mM). Slope conductance was calculated from the slope of the current recorded under a voltage ramp as it passed through 0 pA.

It can be seen that the conductance saturates with increasing [K⁺]₀.
4.6 DISCUSSION

In this section the properties of the unitary delayed rectifier potassium currents are discussed. The evidence for the presence of two open states of different conductance is summarized and the kinetics and possible state diagrams discussed; the open states are compared with multiple conductance states found in other unitary currents. The properties of the unitary currents are then compared with those of the delayed outward currents described in Chapter 3.

4.6.1 The evidence for the presence of two open states and possible state diagrams

Recordings of the unitary delayed rectifier potassium currents suggest the presence of two open states of different conductance (see figure 4.2). These two current levels could also be distinguished in amplitude histograms constructed from the recorded currents (figures 4.3 and 4.10) and were seen in all patches containing the delayed potassium channels. The patterns of openings to the two levels are described in section 4.4.1 and illustrated in figure 4.5. These suggest that it is unlikely that the two levels are due to two separate channels opening but are probably two open states of the same channel. This is also suggested by the finding that the openings to the two levels do not behave independently. Using the $\chi^2$ - test it was shown that if an opening to one of the conductance levels is seen during a voltage pulse then it is probable that an opening to the other conductance level will also be seen; that is, that the openings do not behave independently. This test showed that the probability that the openings behave independently, using this criterion of non-independence, was less than 0.1 %
(see table 4.1), making it likely that the two levels represent different states of the same channel. Further evidence for two open states is shown in figure 4.7 where two exponential distributions had to be used to produce an adequate fit to the combined-state open time histogram. Colquhoun and Hawkes (1981) showed that a single exponential fit to an open time histogram indicates one distinguishable mean open time and therefore one kinetically identifiable open state while two exponential distributions indicates the presence of at least two kinetically distinguishable open states. Voltage-dependence of the open states was demonstrated, the longer time constant increasing with increasing depolarization; this voltage dependence was paralleled by a change in the amplitude distributions (figures 4.10 and 4.11), the relative time spent by the channel in the higher conductance state increasing with increasing depolarization. Conversely, however, the closed time histograms show no voltage dependence, although double exponential distribution fits to these histograms suggest the presence of at least two closed states. Measurements of the latency to the first opening after a depolarizing voltage step show that the first latency distribution is adequately fitted with equation 4.3 (figure 4.15). If a channel passes through only one closed state on its way to opening following a depolarizing voltage step then the first latency distribution can be expected to be fitted with a single exponential distribution (Standen, Stanfield and Ward, (1985); Patlak and Horn (1982); Hille, (1984)); the peak in the histogram occurs at a time later than zero necessitating a fit with at least two exponential distributions suggesting that the channel passes through at least two closed states on its way to opening following a voltage pulse. The mean first latency does appear to show some voltage dependence. As the closed time histograms show no voltage dependence this suggests that there are three sequential closed states and that
the transition from the state farthest away from the open state (C₃) and the next closed state (C₂) is voltage dependent, the channel rarely entering state C₃ during a depolarizing voltage pulse. Figure 4.12 B shows a closed time histogram fitted with the sum of two exponential distributions. It can be seen that there are some long closed times which are not adequately fitted by the resultant distribution and are possibly closings to a state C₃. If so these closings should show voltage dependence. There are not enough of these, however, to justify fitting the histograms with the sum of three exponential distributions or to show voltage dependence or independence.

To construct possible state diagrams it is also necessary to consider the pathways which the channel may take between the open states and from these to and from the closed states. Table 4.1 shows the probability of seeing the possible transitions between C₁ and the two conductance states are given alongside the calculated probability of having missed an intermediate state given the time response of the recording system and the mean open times of the states (taken as the time constants of the exponentials fitted to the dwell time distributions of the various states). These results suggest that transitions between states C and I probably do occur (the probability of missing an intermediate state II is lower than that of seeing a transition between C and I) as do transitions between state I and state II. Transitions between states C and II, however, possibly do not occur since the probability of missing an intermediate state I is higher than that of seeing a transition between C and II. It has been mentioned that the time spent in II as a proportion of the total open time increases with increasing depolarization (figures 4.10 and 4.11). These observations lead to the following diagram as being the most simple state diagram to explain the results.
In addition to the states shown in this diagram there should be another state or states representing the slow inactivation observed and supported by runs analysis (table 4.2). I have no evidence to suggest from which of the states suggested above the channel may pass into the inactivated state, however, and so cannot hypothesize as to where this state might appear on a possible state diagram.

4.6.2 Comparison with other multiple-conductance-state channels

A description of other multiple-conductance-state channels was given in section 1.5 and it was suggested that enough information may be available about these types of channel to begin to make sub-classifications on the basis of the behaviour of the multiple conductance states. The channel described above has two open states, the conductance of the smaller being more than half that of the larger. The acetylcholine receptor channel of embryonic muscle (Trautmann, 1982; Takeda and Trautmann, 1984), and several potassium selective channels from skeletal muscle (Fox, 1985; Tomlins, Williams and Montgomery, 1984; Tomlins and Williams, 1986; Labarca and
Unitary potassium currents

Miller, 1981) show multiple conductance states of this type. On the basis of the suggested classification the channel described above can be closely compared with the three conductance state, voltage-activated potassium channel of frog sarcoplasmic reticulum described by Labarca and Miller (1981). This channel has two open states termed α and β. $P_{Na}/P_K$ is the same for both states but their voltage dependence differs. At negative voltages the closed state and state α dominate while at positive voltages states α and β dominate. Labarca and Miller also suggested a sequential scheme for the transition of the channel from the closed state to state α and then to state β using the same criteria as those described above; that is, that transitions between the closed state and state β were observed but with a frequency less than the probability of missing an intermediate state α because of the response time of the recording system. It is interesting but possibly not significant that both channels are voltage dependent potassium channels.

4.6.3 Comparison with the macroscopic delayed outward currents

Both the unitary currents and the macroscopic current are carried mainly by potassium ions and are voltage dependent. The time courses of the currents are also the same at equal potentials. Figure 4.13 shows summed single channel currents recorded at +18 mV and +38 mV alongside macroscopic currents recorded at +16 mV and +36 mV. This shows that the time courses of the two types of current are very similar and that the voltage dependence of the activation of the currents is the same. This is reflected in the activation time constants of the two currents, the similarity of which is shown in figures 4.13 and 4.14. This suggests that the single channel currents described above are the unitary current correlate of the macroscopic currents described

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in Chapter 3.

The similarity of the two time courses also supports the assumption made in Chapter 3 that the time course of the recorded macroscopic currents is the true time course of these currents and its slow activation kinetics compared with those of the delayed outward currents described by Moolenaar and Spector (1978) are not due to poor voltage-clamping of the single cells. If the latter was the case then the time course of the unitary currents would be expected to be faster than that of the macroscopic currents at the same potential; voltage-clamping of a small membrane patch is not likely to be subject to any significant time delays since there is no significant series resistance causing a voltage drop and because the area of membrane being voltage clamped is so small so that any time differences in voltage clamping different areas of the patch should be insignificant.

Both currents also show slow inactivation. This was demonstrated in the unitary currents by runs analysis which showed that it was likely that blank current trace records group together, indicating that the channel enters an inactivated state. A possible macroscopic current correlate of this behaviour is the run down of currents seen if the membrane potential of the single cell is subjected to depolarizing steps at a frequency of greater than once every 4 msec.

It therefore seems likely that the unitary potassium current described above is responsible for the macroscopic current described in Chapter 3 and that the behaviour of the channels is not altered when they are recorded in excised patches.

4.6.4 Comparison with other unitary potassium currents

The potassium channel described above has a conductance of approximately
11.1 pS (state I) and 16.4 pS (state II) with $[K^+]_i = 120$ mM and $[K^+]_o = 5$ mM. The current-voltage relation is linear between 0 and +120 mV at these potassium concentrations. The channel has at least two open states and at least two and possibly three closed states. The open time distribution is voltage dependent but the closed time distribution is not. The currents show slow inactivation but there is no evidence of fast inactivation. The activation time constant of the currents is 12.8 msec at +38 mV and the first latency is voltage-dependent. The mean open time of the channels is voltage dependent, the time constants of the exponential distributions fitted to the open time distributions are 0.53 and 7.61 msec at -2 mV and 0.85 and 17.35 msec at +38 mV.

The conductance of the delayed rectifier potassium current recorded by Conti and Neher (1980) from squid giant axon was 17.5 pS with $[K^+]_o = 460$ mM and $[K^+]_i = 1$ mM. The delayed rectifier of frog skeletal muscle has a unitary conductance of approximately 15 pS with $[K^+]_i = 120$ mM and $[K^+]_o = 2.5$ mM (Standen, Stanfield and Ward, 1985). In physiological solutions delayed rectifier channels from squid giant axon inserted into lipid bilayers have a conductance of 18 pS. The delayed rectifier channels of frog skeletal muscle appear to have one open state, three closed states and exhibit slow inactivation (demonstrated by runs analysis). The mean open time of these channels is voltage dependent being around 2 msec at -60 mV and 6 msec at +40 mV.

The conductance of the delayed outward potassium channels of mouse neuroblastoma is therefore similar to that of previously reported delayed rectifier channels. The channels are also similar in that their open times and first latencies are voltage-dependent and they both exhibit slow inactivation. The detailed kinetics are dissimilar however: the mean open times are different and the channels have different probable state diagrams.
Quandt (1988) has described delayed rectifier channels of mouse neuroblastoma N1E115 cells which had been differentiated in 2% DMSO for between 3 days and 2 weeks. These channels have a conductance of 35 pS in symmetrical 125 mM K⁺ solutions. In symmetrical 120 mM K⁺ the potassium channel described here has a slope conductance of approximately 34 pS. Quandt did not find two open states of different conductance in these channels but showed that they exhibit slow inactivation, the channel activity remaining constant for approximately 100 msec and then declining. The mean first latency of the channels is 32 msec at a membrane potential of 10 mV; the first latency of the outward potassium channels described in this chapter is 35.4 msec at a membrane potential of 0 mV.

The evidence presented suggests that the channels described above are delayed rectifier potassium channels and are the same as those described by Quandt (1988). The absence of multiple conductance states in the channels described by Quandt indicates that experimental conditions or differentiation time may determine the presence or absence of these multiple conductance states. However, the time resolution of the current traces shown by Quandt (1988) is much lower than that of the traces presented here and it may be that this caused transitions between different conductance states to be missed.
Chapter 5

BLOCK OF DELAYED RECTIFIER CHANNELS BY EXTERNALLY APPLIED TETRAETHYLAMMONIUM IONS

Tetraethylammonium ion (TEA\(^+\)), either applied externally or internally, blocks all of the known potassium channels. Externally applied TEA\(^+\) blocks the delayed rectifier of some preparations with a high affinity (for example in the node of Ranvier of \textit{Rana pipiens} (Hille, 1967) where the half-blocking concentration (\(K_a\)) is 0.4 mM but is ineffective in squid giant axon (Tasaki and Hagiwara, 1957; Armstrong and Binstock, 1965) at concentrations of up to 100 mM and at concentrations of up to 40 mM in \textit{Procambarus} (Shrager, Macey and Strickholm, 1969).

In some preparations externally applied TEA\(^+\) has been shown to block the delayed rectifier or delayed outward current more effectively than it does other potassium currents. For example, externally applied TEA\(^+\) blocks the delayed rectifier of frog muscle (Ildefonse, Regondaud and Rougier, 1969; Ildefonse and Rougier, 1971; Kao and Stanfield, 1970; Stanfield 1970, 1973, 1983) but the slowly activating potassium conductance is relatively unaffected (Stanfield, 1970; Almers and Palade, 1981) and in neurones of \textit{Helix pomatia} the delayed rectifier is blocked by 5 to 10 mM externally applied TEA\(^+\) while the fast transient outward current is blocked by 20 to 90 mM externally applied TEA\(^+\) (Neher and Lux, 1972). In neurones of \textit{Tritonia diomedia} the calcium-activated potassium current is only slightly sensitive to externally applied TEA\(^+\), being reduced by around 20% when [TEA\(^+\)] = 100 mM. The same concentration of TEA\(^+\) causes a 50% to 60% reduction in fast transient current
while the half-blocking concentration (K_d) for the delayed rectifier in the same preparation is 8 mM (Thompson, 1977). The delayed outward current was also found to be more sensitive to TEA⁺ than was the calcium-activated potassium current in neurones of the molluscs *Archidoris monteryensis* and *Anisodoris nobilis* (Aldrich, Getting and Thompson, 1979; Connor, 1978). The delayed outward current of *Helix aspersa*, however, has the same sensitivity to TEA⁺ around 10 mM as the calcium-activated potassium current (Meech and Standen, 1975), the first calcium-activated potassium current to be described. In neurones of *Aplysia* (Hermann and Gorman, 1981) and of *Helix pomatia* (Hermann and Hartung, 1981) the calcium-activated potassium current has been shown (at least when it is activated by calcium injection) to be more sensitive to TEA⁺ than is the delayed outward current.

Block of unitary ATP-regulated potassium channels by externally applied TEA⁺ has been demonstrated in frog skeletal muscle (Spruce, Standen and Stanfield, 1987a,b) as has block of the delayed rectifier in the same preparation (Spruce, Standen and Stanfield, 1987 c). Both of these studies demonstrate a fast block by TEA⁺ where the single-channel current amplitude is reduced without a detectable increase in the variance of the current noise of the open level occurring.

The results presented in this chapter demonstrate that the delayed rectifier of the neuroblastoma line N1E115 is blocked by low concentrations of TEA⁺ in a similar manner to that described by Spruce, Standen and Stanfield (1987 a,b, and c), the unitary current amplitude is decreased and individual blocking events cannot be resolved.
5.1 RESULTS

Single delayed rectifier channels were recorded from cell-attached patches on cells which had been grown for between 1 and 4 days in differentiation medium. The cells were bathed in solution A ([K⁺]₀ = 5 mM). The solution in the recording electrode was also solution A but with low concentrations of TEA⁺ (0.1 to 0.75 mM) achieved by substitution of TEA chloride for sodium chloride in this solution. The electrode solution also contained 3 μM TTX.

5.2 THE EFFECT OF TEA⁺ ON THE AMPLITUDE OF SINGLE CHANNEL CURRENTS

Figure 5.1 shows current traces recorded from cell-attached patches which were held at -30 mV relative to the resting membrane potential of the cell ([K]₀ = 5 mM) and stepped by +80 mV. The currents are thought to be unitary delayed rectifier potassium currents as described in the previous chapter because of their activation by depolarization, the currents activating with a delay after a depolarizing voltage pulse and having two open levels. The currents illustrated were recorded with no TEA⁺ (A), 0.1 mM TEA⁺ (B) and 0.25 mM TEA⁺ (C) in the electrode. It can be seen that increasing the concentration of TEA⁺ in the electrode solution decreases the amplitude of the unitary delayed rectifier currents. The amplitudes of the states I and II of the single channel currents illustrated are approximately 0.78 and 1.16 pA (A); 0.61 and 1.05 pA (B); and 0.58 and 0.80 pA (C) respectively.

Amplitude histograms were constructed from currents recorded with [TEA⁺]₀ = 0.1 mM using the method described in section 2.9.2. Currents recorded with
Unitary delayed rectifier currents recorded with different concentrations of TEA$^+$ present in the electrode.

Currents were recorded from cell-attached patches which were held at -30 mV relative to the resting potential of the cell ($[K^+]_b = 5$ mM). They were activated by a depolarizing voltage step of +80 mV the duration of which is shown by the arrowheads. The arrows represent the current levels of the baseline and the two open levels of the channel as in figure 4.1.

The concentration of TEA$^+$ in the electrode ($[TEA^+]_p$) was 0 mM in A, 0.1 mM in B and 0.25 mM in C. It can be seen that increasing $[TEA^+]_p$ decreases the amplitude of the currents. The amplitudes of states I and II of the currents illustrated are approximately 0.78 and 1.16 pA (A), 0.61 and 1.05 pA (B) and 0.58 and 0.80 pA (C).
higher concentrations of $\text{TEA}^+$ in the pipette are so small, however, that the amplitudes of baseline, state I and state II overlap greatly when amplitude histograms are constructed this method. That is, the drifts which occur in the baseline are large enough to obscure the single channel currents in an amplitude histogram which has been constructed in this way. The amplitudes of currents recorded with $[\text{TEA}^+]_p > 0.1 \text{ mM}$ were therefore measured by eye, using a horizontal cursor.

Figure 5.2 shows amplitude histograms constructed using the method described on in section 2.9.2 from current traces recorded with the control solution (solution A, $[\text{K}^+]_p = 5 \text{ mM}$) (A) and with solution A with $0.1 \text{ mM TEA}^+$ (B) in the electrode. The peaks corresponding to the baseline current level can be fitted with single Gaussian distributions (not shown) with mean = 0.05 pA (A) and 0.01 pA (B) and standard deviation = 0.14 (A) and 0.11 (B). The two peaks in each histogram which correspond to the open channel current levels have been fitted with the sum of two Gaussian distributions as described in section 2.9.2. In figure 5.2 A these two distributions (I and II) have mean = 0.62 (I) and 1.14 pA (II) and standard deviation = 0.18 (I) and 0.17 pA (II) while in 5.2 B they have area = mean = 0.31 (I) and 0.72 pA (II) and standard deviation = 0.18 (I) and 0.18 pA (II). The amplitude histogram shown in figure 5.3 was constructed from amplitudes measured by eye using a horizontal cursor. There are no values representing the baseline on the histogram since all amplitudes are measured as the difference between the baseline level just before the channel opening and the open channel level. The histogram shown was constructed from currents recorded when a cell-attached patch was stepped by +80 mV from -30 mV relative to the resting potential of the cell (The mean resting potential in these conditions ([K$^+$]o = 5 mM) is approximately -27 mV (see section 3.2).) with
Figure 5.2

Amplitude histograms constructed from currents recorded with and without TEA⁺ in the electrode.

The histogram in A was constructed from currents recorded with the control solution (solution A, [K⁺]₀ = 5 mM) in the electrode while that shown in B was constructed from currents recorded with solution A with 0.1 mM TEA⁺ in the electrode.

The smooth curves represent the sum of two Gaussian distributions (I and II) fitted to the data using the least squares fitting method (Marquardt, 1963). In A these have area = 263.51 (I) and 177.71 (II), mean = 0.62 (I) and 1.14 pA (II) and standard deviation = 0.18 (I) and 0.17 pA (II) while in B they have area = 148.84 (I) and 138.33 (II), mean = 0.31 (I) and 0.72 pA (II) and standard deviation = 0.18 (I) and 0.18 pA (II).
Amplitude histogram constructed from amplitudes measured by eye.

The histogram was constructed from currents measured at +50 mV with [TEA⁺]₀ = 0.25 mM. Amplitudes of openings to the two current levels were measured by eye using a horizontal cursor. There are no values representing the baseline since all amplitudes were measured as the difference between the baseline level just before the channel opening and the open channel level. This minimises the effect of baseline drift on the measured amplitudes. The means and standard deviations of the currents measured were 0.30 +/- 0.08 pA (state I) and 0.58 +/- 0.02 (state II).
[TEA\(^+\)]_p = 0.25 mM. The means and standard deviations of the currents measured were 0.30 +/- 0.08 pA (state I) and 0.58 +/- 0.02 (state II).

The blocking action of TEA\(^+\) in decreasing the amplitude of the single channel currents does not appear, from inspection of traces such as those in figure 5.1, to be accompanied by an increase in the noise level of the current when the channel is open. In order to discover whether TEA\(^+\) produces an increase in open-channel noise as well as a decrease in single channel current amplitude, the open-channel noise was measured for currents recorded with solution A and with solution A with [TEA\(^+\)] = 0.1 mM present in the pipette. The open-channel noise was measured by subtracting the baseline current variance (given by the variance of the Gaussian distribution fitted to the baseline current amplitude histogram) from the open-channel current variance (given by the variance of the Gaussian distributions fitted to the open channel current amplitude histograms) to give the variance of the open channel current.

At +50 mV relative to the resting potential of the cells ([K\(^+\)]_b = 5 mM) the resultant variance for the two open states is 0.062 +/- 0.02 pA\(^2\) and 0.12 +/- 0.003 pA\(^2\) (4 patches) with no TEA\(^+\) present in the electrode solution and 0.042 +/- 0.01 pA\(^2\) and 0.037 +/- 0.02 pA\(^2\) (4 patches) with 0.1 mM TEA\(^+\) present. This agrees with the inference from the appearance of the current traces (such as figure 5.1) that TEA\(^+\) does not produce a significant increase in open channel current noise.

The results have therefore been treated as though the decrease in amplitude in the presence of TEA\(^+\) is not accompanied by an increase in open channel current noise. This suggests that the interruptions in current caused by the channel blocking action of TEA\(^+\) are faster than the response time of the recording system. These interruptions in current are therefore filtered out by the recording system so that
instead of seeing the block as many brief closings interrupting openings to the control amplitude the block is manifested as a reduction in channel amplitude. The reduction in current, as a proportion of the control amplitude, will therefore be equal to the proportion of time during an open period which the channels spends in the blocked state (Coronado and Miller, 1979; Spruce, Standen and Stanfield, 1987c; Spruce, 1987).

If this block is treated in a similar way to that used by Spruce, Standen and Stanfield, 1987c for the TEA⁺ block of delayed rectifier in frog skeletal muscle (which has only one open state but also shows a decreased single channel amplitude in the presence of TEA⁺ and no change in open time or open-channel noise) and the state diagram suggested in section 4.6.1 is assumed, then the effect of TEA⁺ concentration can be considered in terms of two schemes:
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In each case the dissociation constant $K_d = k_y/k_b$. For either scheme the measured open time is the time the channel spends in the states O and OB (for a very fast block) and the fractional current amplitude (amplitude in the presence of TEA$^+$ / control amplitude) will be the fraction of this time that the channel spends in state O. This is given by

$$\frac{k_b}{(k_b + k_b[TEA^+])}$$

so, for each state

5.1 fractional current = $1 / (1 + ([TEA^+] / K_d))$

5.2.1 Concentration dependence of the block

The unitary delayed rectifier currents illustrated in figure 5.1 suggest that TEA$^+$ decreases the amplitude of the single channel current and that this decrease is dependent on the concentration of TEA$^+$ present in the electrode. The amplitudes of states I and II were measured as described in section 5.2 from currents recorded from cell-attached patches stepped by +80 mV from a holding potential of -30 mV relative to the resting potential of the cell ([K]$_b$ = 5 mM; [K]$_p$ = 5 mM). The mean amplitudes of states I and II recorded in these conditions were 0.76 +/- 0.15 pA and 1.09 +/- 0.18 pA (4 patches) when recorded with no TEA$^+$ in the electrode whereas with [TEA$^+$]$_p$ = 0.1 mM the amplitudes were decreased to 0.68 +/- 0.02 pA and 0.79 +/- 0.02 pA (4 patches) and with [TEA$^+$]$_p$ = 0.75 mM they were 0.28 +/- 0.02 pA and 0.36 +/- 0.04 pA (4 patches).
Block by TEA⁺

Figure 5.4

Fractional current plotted against [TEA⁺]ₚ.

The fractional current was calculated by finding the mean open levels from 4 patches at each concentration of TEA⁺ and dividing this by the mean open amplitudes measured from between 4 and 7 patches stepped to the same potential (+50 mV) with no TEA⁺ in the pipette. A mean Kₐ was calculated for each state by using equation 5.1 (fractional current = 1/1+([TEA⁺]ₚ/Kₐ) to find Kₐ for each mean fractional current and averaging the resultant values. This gave values of 0.27 +/- 0.03 mM for state I and 0.4 +/- 0.04 mM for state II. These values were then used to construct curves (solid lines) which predict the fractional currents at different [TEA⁺]ₚ. The dashed lines assume 2:1 binding are calculated from fractional current = 1/1+([TEA⁺]ₚ/Kₐ) and do not fit the data.
In figure 5.4 the fractional current for each state (current measured in the presence of TEA$^+$ / current measured at the same potential in the absence of TEA$^+$) is plotted against the log of [TEA$^+$]. The solid lines are drawn according to equation 5.1 and so assume that one TEA$^+$ molecule binds to one receptor. The dissociation constants ($K_d$) were calculated by finding the mean fractional current of each state for 4 patches at each concentration of TEA$^+$ and using equation 5.1 to find $K_d$ from this. The resultant $K_d$s were averaged to give a mean $K_d$ for each state of 0.27 +/- 0.03 mM (state I) and 0.40 +/- 0.04 mM (state II). It can be seen that the 1:1 binding curves (calculated from equation 5.1) fit the data well. The broken lines assume 2:1 binding (two molecules of TEA$^+$ binding to one receptor producing an interruption in current) and do not fit the data. These were calculated from the equation

\[ 5.2 \quad \text{fractional current} = \frac{1}{1+([\text{TEA}]/K_d)^2} \]

This finding that one TEA$^+$ molecule binds to one receptor on the channel is consistent with other findings for TEA$^+$ block of potassium channels (for example Spruce, Standen and Stanfield, 1987c).
5.3 THE EFFECT OF TEA⁺ ON THE CHANNEL KINETICS

5.3.1 Open times

Single channel current recordings from cell-attached patches on cells bathed in solution A ([K⁺]₀ = 5 mM) with solution A +/- TEA⁺ as the electrode solution were used to form combined-state open-time histograms as described in section 4.4.4. Patches were held at -30 mV and stepped to +50 mV relative to the resting potential of the cell. Figure 5.5 A shows an open time histogram constructed from currents recorded without TEA⁺ in the electrode and 5.5 B shows a similar histogram constructed from currents recorded with [TEA⁺] = 0.1 mM. The histograms have been fitted with the sum of two exponential distributions (smooth curves). The exponentials shown in A have time constants of 0.40 (τ₁) and 6.23 msec (τ₂) while those shown in B have time constants of τ₁ = 0.64 and τ₂ = 4.98 msec. The means of τ₁ and τ₂ for histograms constructed from 4 patches stepped to +50 mV in the absence of TEA⁺ with the control solution in the electrode were τ₁ = 0.94 +/- 0.55 and τ₂ = 6.55 +/- 0.92 whereas the means from 4 patches stepped to +50 mV with 0.1 mM TEA⁺ present in the pipette were τ₁ = 0.81 +/- 0.21 and τ₂ = 6.50 +/- 2.02. This suggests that TEA⁺ has no effect on the open time of the channel.

I was not able to record enough data in higher concentrations of TEA⁺ to enable open-time histograms to be constructed from enough values to allow exponential fits to be performed with any accuracy; with the small currents present in these concentrations of TEA⁺ the baseline noise must be smaller to allow exact measurements of when channels open and close. It can be seen from figure 5.2 that even in 0.1 mM TEA⁺ the open-channel current levels in the amplitude histogram are
The histogram in A was constructed from open times measured from a cell-attached patch whose membrane potential was stepped from -30 to +50 mV relative to the resting potential of the cell ([K\textsuperscript{+}]_b = 5 mM). The electrode contained solution A ([K\textsuperscript{+}]_p = 5 mM) with no TEA\textsuperscript{+} in A and solution A with [TEA\textsuperscript{+}]_p = 0.1 mM in B.

The histograms have been fitted with the sum of two exponential distributions (1 and 2; smooth curves) which have time constants in the absence of TEA\textsuperscript{+} (A) of 0.40 (τ\textsubscript{1}) and 6.23 msec (τ\textsubscript{2}) while those in B ([TEA\textsuperscript{+}]_p = 0.1 mM) have time constants of 0.64 (τ\textsubscript{1}) and 4.98 msec (τ\textsubscript{2}).
overlapping considerably with the baseline level. Mean open times were calculated, however, from channels recorded with 0.25 mM TEA$^+$ in the electrode. If the channel was only able to close when TEA$^+$ was not bound to its receptor then the open times of the channel, and therefore the mean open time, would be expected to be increased by the presence of TEA$^+$. When the membrane potential of cell-attached patches was stepped from -30 mV to +50 mV relative to the resting membrane potential of the cell ($[K^+]_b$) with $[TEA^+]=0.25$ mM the mean open time was 4.29 +/- 0.34 msec (5 patches) which compares with a mean open time of 5.0 +/- 0.99 msec (4 patches) when $[TEA^+]_p=0.1$ mM and 6.89 +/- 1.1 msec (4 patches) with no TEA$^+$ present in the electrode. The blocking action of TEA$^+$ does not therefore appear to be accompanied by changes in the channel kinetics. As no change in open-channel kinetics is seen it is concluded that the blocked channel is able to move between states in the same way as the unblocked channel.

5.3.2 Activation kinetics

Figure 5.6 shows summed single channel currents recorded from cell-attached patches whose membrane potential was held at -30 mv and stepped to +50 mV relative to the resting potential of the cell ($[K^+]_b=5$ mM). The currents summed to produce A were recorded with no TEA$^+$ in the electrode and those which produced B with 0.1 mM TEA$^+$. Currents have been fitted with equation 3.7 as in chapters 3 and 4. The mean time constant of activation for channels recorded in these conditions was 9.37 +/- 2.08 (4 patches) for currents recorded in the presence of 0.1 mM TEA$^+$ and 6.0 +/- 1.7 (4 patches) for those recorded in the absence of TEA$^+$. If these results are tested using the t-test there is probability of greater than 0.95 that
these results come from the same statistical population. It was therefore concluded that there is no evidence for an effect of TEA$^+$ on the activation time course of the currents.

### 5.4 Voltage Dependence of the Block

Figure 5.7 shows the logarithm of the dissociation constant ($K_d$) for each state plotted against membrane potential ($V_m$). This shows that the dissociation constant is only slightly dependent on voltage, the block being relieved somewhat at more depolarized potentials. The dashed lines have a slope which gives an e-fold change in $K_d$ for a change in membrane potential of 153 mV. The data from state I (●) fits a straight line well although the data from state II (♦) fits less well. This voltage-dependence may be explained if TEA$^+$ binds at a site which is within the membrane field (Woodhull, 1973); as TEA$^+$ is positively charged it will enter the membrane field from the extracellular side of the patch less easily when the intracellular side is made more positive relative to the extracellular side. The $K_d$ at any voltage is given by

$$K_d = K_d(0) \exp(zdVF/RT)$$

where $K_d(0)$ is the dissociation constant at zero membrane potential, $d$ is the fraction of the membrane voltage experienced at the blocking site and $z$ is the valency of the blocking ion. This equation predicts an e-fold change in $K_d$ for a 153 mV change in membrane potential if $d = 0.16$. 

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Figure 5.6

Summed single channel currents fitted with equation 3.7.

Currents were recorded when the membrane potential of a cell-attached patch was stepped from -30 to +50 mV relative to the resting membrane potential of the cell ([K\(^+\)]_p = 5 mM). [TEA\(^+\)]_p = 0 mM in A and 0.1 mM in B. The resultant current traces which contained channel openings were summed together and were fitted with equation 3.7 which assumes \( g_K = g_{K_0}(V-E_K)n^2 \). The activation time constant, \( \tau_a \), was estimated from the fit to be 15.84 msec in A and 17.4 msec in B.
The mean $K_d$ for each state was found from between 6 and 8 patches at each potential. The values for state I (●) fit a straight line well but those from state II (♦) fit much less well. The line of best fit drawn through the points for state I has a slope which gives an e-fold change in $K_d$ for 153 mV change in voltage.
5.5 DISCUSSION

It has been shown above that TEA$^+$ reduces the amplitude of unitary, delayed rectifier potassium currents of mouse neuroblastoma N1E115 cells without increasing the noise of the open channel current. The simplest kinetic schemes which may be used to represent the results, which suggest that the proportion of open time which the channel spends blocked is directly proportional to the decrease in current amplitude produced by the block, are given in section 3.2 and are repeated below for clarity:
These schemes also assume that the block is produced by the binding of one TEA\(^+\) molecule to one TEA\(^+\) receptor in accordance with the results presented in section 5.2.1. For either of the two proposed schemes, the \(K_d\) for a channel state is equal to \(k_d/k_b\) for that state, yielding equation 5.2

\[
\text{fractional current} = \frac{1}{1+(\text{[TEA}^+\text{]}/K_d)}.
\]

The block of unitary delayed rectifier potassium channels of frog skeletal muscle is also produced by 1:1 binding of TEA\(^+\) molecules to TEA\(^+\) receptors. At -3 mV, however, the dissociation constant for these channels is 5.8 mM (Spruce, Standen and Stanfield, 1987c). The dissociation constants for the channel described here are 0.27 mM (state I) and 0.4 mM (state II) at +50 mV which indicate that this channel has a higher affinity for TEA\(^+\). This is comparable with other neuronal delayed rectifier currents. For example, the delayed rectifier of node of Ranvier of *Rana pipiens* has a \(K_d\) for TEA\(^+\) of 0.4 mM (Hille, 1967).

TEA\(^+\) does not appear to have any affect on the kinetics of the channel. It was shown in section 5.3.1 that the blocking action of TEA\(^+\) does not appear to be accompanied by changes in the open time of the channel and in section 5.3.2 that the activation kinetics are probably not affected by TEA\(^+\).

5.5.1 Voltage dependence of the block

A weak voltage dependence of the TEA\(^+\) block of the channel was demonstrated in section 5.4, \(K_d\) changing e-fold for a change in membrane potential of 153 mV. Such a voltage dependence is explained if it is assumed that the TEA\(^+\) molecules block the potassium current when they are bound to a site which is within the membrane field (Woodhull, 1973) so that the positively charged molecule enters...
from the extracellular side more easily when the intracellular side is more negative with respect to the extracellular side. The results can be explained if TEA\(^+\) binds to a site which is 0.16 of the way through the membrane. Studies of the TEA\(^+\) block of squid delayed rectifier (French and Shoukimas, 1981; Clay, 1985), calcium-activated potassium channels (Blatz and Magleby, 1984; Yellen, 1984) and delayed rectifier of frog skeletal muscle (Spruce, Standen and Stanfield, 1987c) all show TEA\(^+\) binding at sites somewhere between 0.15 and 0.26 of the way through the membrane.

**5.5.2 Comparison with the macroscopic current**

The dissociation constant of TEA\(^+\) calculated for the macroscopic delayed rectifier currents described in Chapter 3 was 0.73 +/- 0.22 mM at +26 mV (section 3.3.4). The dissociation constants for the unitary currents are 0.27 +/- 0.03 mM (state I) and 0.4 +/- 0.04 mM (state II) at +50 mV (section 5.2.1). The most likely explanation for this difference (which cannot be explained by the difference in membrane potentials because \(K_d\) increases with increasing membrane potential (figure 5.7)) is that the measurement of block of unitary currents is likely to be more accurate than that of the macroscopic currents where the measured current may be passing through other channels as well as the delayed rectifier channels and where the concentration of TEA\(^+\) at the membrane surface may be slightly different from that of the extracellular solution.

These results support the conclusions made in Chapter 4 that the unitary currents are passing through delayed rectifier potassium channels. They also indicate that this channel may have the properties expected in a neuronal delayed rectifier
channel since the TEA$^+$ dissociation constants are close to values previously found for neuronal delayed rectifier currents.
UNITARY CALCIUM-ACTIVATED CURRENTS

6.1 INTRODUCTION

6.1.1 Unitary non-selective cation currents

Colquhoun, Neher, Reuter and Stevens (1981) reported a unitary, calcium-activated, non-selective cation current in cultured rat cardiac cells. They postulated that this may be the unitary current correlate of the oscillatory inward currents which occur in cardiac Purkinje fibres after depolarizing voltage steps during exposure to ouabain or to high extracellular calcium concentrations (Lederer and Tsien, 1976; Kass, Tsien and Weingart, 1978; Vassalle and Mugelli, 1981). A similar channel was reported in the mouse neuroblastoma N1E115 by Yellen (1982) and a calcium-activated non-selective cation channel which is probably that reported by Yellen is described here. Similar channels have been described in the membrane of non-excitable cells (Maruyama and Petersen, 1982; Bevan, Gray and Ritchie, 1984) and in a rat insulinoma cell line (Sturgess, Hales and Ashford, 1986).

6.1.2 Calcium-activated potassium currents

Whittam (1968) found that the potassium permeability of red blood cells increased when the intracellular concentration of free calcium ions was raised. Subsequently this property of potassium permeability was also discovered in *Aplysia* neurones (Meech and Strumwasser, 1970; Meech, 1972) and in cat spinal...
motoneurones (Krnjevic and Lisiewicz, 1972). In *Aplysia* neurones it was found that calcium influx caused a post-tetanic hyperpolarization (Meech 1974b) and that injection of EGTA prolonged the action potential (Meech, 1974c) and Meech and Standen (1974) and Meech (1974a) found a calcium-sensitive potassium conductance in neurones of *Helix aspersa*. Meech and Standen (1975) concluded that depolarization of *Helix* neurones activates two types of potassium channel, one which is voltage dependent and one whose activation is dependent on the influx of calcium.

Unitary calcium-activated potassium currents were first reported by Marty (1981) in bovine chromaffin cells and by Pallotta, Magleby and Barrett (1981) in cultured rat muscle. Since then unitary calcium-activated potassium currents with large conductances (typically greater than 200 pS in symmetrical 140 mM KCl) have been recorded in the surface membranes of a diverse range of cells.

Moolenaar and Spector (1979a,b) recorded a slow outward current with a rise time of several seconds when differentiated cells of the mouse neuroblastoma clone N1E115 were depolarized to potentials more positive than -20 mV in high [Ca$^{2+}$] solutions. They found that the current was carried by potassium, was insensitive to TEA$^+$ and was blocked by Ca$^{2+}$ antagonists. In his report of single Ca$^{2+}$-activated non-selective cation channels in neuroblastoma N1E115 (1982) Yellen also reported that he had observed Ca$^{2+}$-activated K$^+$ - selective unitary currents in excised inside-out and outside-out patches. Yellen (1982) reported that these currents "have about 10 times the conductance of the Ca$^{2+}$-activated nonselective channels" which have a conductance of 22 +/- 1.5 pS at 24°C with 125 mM NaCl outside and 125 mM KCl inside.
Calcium-activated currents

In this chapter I will describe a unitary, large conductance non-selective cation current which is probably that reported by Yellen (1982) and a unitary, large conductance potassium current recorded from the membrane of differentiated cells of the mouse neuroblastoma clone N1E115. The activation of both of these currents is dependent on the presence of calcium at the intracellular surface of the membrane patch.
6.2 RESULTS

Large conductance unitary currents were sometimes observed in cell-attached patches. These currents were investigated using the ripped-off patch recording mode (see section 2.5.2) in which the intracellular face of the membrane patch can be exposed to different solutions using the method illustrated in figure 2.3. It was found that there were two types of unitary current which could be activated by the presence of calcium in the intracellular solution. By measuring the reversal potentials of these currents it was found that one was mainly carried by potassium ions. The other appeared to flow through a channel with equal selectivity for sodium and potassium ions. This channel is probably the calcium-activated non-selective cation channel reported by Yellen (1982). Results from calcium-activated non-selective cation channels will be described first, followed by those from experiments on calcium-activated potassium channels.
6.3 UNITARY NON-SELECTIVE CATION CURRENTS

Excised membrane patches (see section 2.5.2) were formed from cells which were placed in solution A with zero calcium after being differentiated in medium containing DMSO for between one and four days. The intracellular face of these patches was exposed to different solutions by moving the patch electrode between flow pipes as described in section 2.5.2. The flow solution used to bathe the intracellular face of a membrane patch was either solution C (table 1) ([K⁺] = 5 mM; [Cl⁻] = 9.4 mM; [SO₄²⁻] = 72.5 mM; [Na⁺] = 147 mM; [Mg²⁺] = 2.2 mM) or solution F (table 1) ([K⁺] = 152.3 mM; [Cl⁻] = 150 mM; [Mg²⁺] = 2.2 mM). The concentrations of sodium and potassium in these solutions were varied by substitution of one for the other and the concentration of calcium was changed by using different CaEGTA buffers as described in section 2.2.3. The pipette solution was solution A with [K⁺] = 5, 60 or 150 mM.

6.3.1 The ionic nature of the currents

The ionic nature of the currents was investigated by measuring the membrane potential at which the currents reversed in different ionic conditions. The reversal potential of the currents was found by either applying a depolarizing voltage step to the membrane patch and then ramping the membrane potential of the patch by applying an increasingly hyperpolarizing voltage as described in section 2.6.3, or by measuring the unitary current elicited by holding the membrane potential at different voltages. Figure 6.1 shows the current recorded when such a voltage ramp was applied to a membrane patch with solution A in the pipette ([K⁺]ₚ = 150 mM, [Na⁺]ₚ = 0 mM,
Calcium-activated currents

Figure 6.1

Unitary current recorded by ramping the membrane potential.

The current was recorded with $[K^+]_p = 150 \text{ mM}$, $[Na^+]_p = 0 \text{ mM}$ and $[Cl^-]_p = 154.4 \text{ mM}$ and with $[K^+]_{\text{low}} = 5 \text{ mM}$, $[Na^+]_{\text{low}} = 147 \text{ mM}$ and $[Cl^-]_{\text{low}} = 9.4 \text{ mM}$.

The current shown reverses at $+10 \text{ mV}$ (marked by the arrow).
Calcium-activated currents

and $[\text{Cl}]_p = 154.4$ mM) and solution C ($[\text{K}]_{\text{flow}} = 5$ mM, $[\text{Na}]_{\text{flow}} = 147$ mM and $[\text{Cl}]_{\text{flow}} = 9.4$ mM) as the flow solution. The membrane potential of the patch was held at 0 mV, stepped by +70 mV for 20 msec and then ramped by -2 mV/msec for 70 msec. The current shown reverses at +10 mV. Figure 6.2 shows the current-voltage relation obtained from a membrane patch in the same ionic conditions but whose membrane potential was held at different voltages, each for a period of approximately 30 seconds. The current amplitudes were measured by fitting Gaussian curves to amplitude histograms as described in section 2.9.2). The unitary currents in figure 6.2 reverse at approximately -2 mV.

It was found that the reversal potential of the currents did not depend on the concentration gradients of potassium, sodium or chloride. When the pipette potassium concentration, $[\text{K}]_p$, was 150 mM and the concentration of potassium in the flow solution, $[\text{K}]_{\text{flow}}$, was 5 mM, the reversal potential of the unitary currents was found to be $+3.3 \pm 2.7$ mV (8 patches). In these conditions, the equilibrium potential for potassium ions, predicted by the Nernst equation, is +86 mV. The concentrations of chloride in the pipette and flow solutions give a predicted chloride equilibrium potential of +70 mV ($[\text{Cl}]_{\text{flow}} = 9.4$ mM and $[\text{Cl}]_p = 154.4$ mM) and sodium currents are not expected to reverse ($[\text{Na}] = 0$ mM, $[\text{Na}]_{\text{flow}} = 147$ mM). These results suggest that the channels through which these currents are passing do not select between cations but pass sodium and potassium with equal selectivity. The total monovalent cation concentration in these conditions was 152 mM at either face of the membrane patch, giving an expected reversal potential for non-selective cation currents of 0 mV. The actual reversal potential of approximately +3 mV is very close to the theoretical.
Calcium-activated currents

Figure 6.2

Current-voltage relation for one membrane patch.

Amplitude histograms were constructed from currents recorded from a membrane patch whose potential was held at different voltages for periods of approximately 30 seconds. The electrode solution was solution A ([K⁺]₀ = 150 mM, [Na⁺]₀ = 0 mM and [Cl⁻]₀ = 154.4 mM) and the flow solution was solution C ([Na⁺]₀ = 147 mM and [Cl⁻]₀ = 9.4 and [K⁺]₀ = 5 mM).

The current-voltage relation is linear with the currents reversing at approximately -2 mV and the slope conductance is approximately 43 pS.
The reversal potential would also be close to 0 mV if the current was carried by chloride and sulphate ions with equal selectivity but this is unlikely since no sulphate-permeable channels have yet been reported.

The unitary slope conductance of these channels was measured by measuring the slope of the current elicited by the ramped voltage as the current passed through 0 pA (see the arrow in figure 6.1), and by measuring the slope of current-voltage relations such as the one shown in figure 6.2. The unitary slope conductance in these conditions, with the total monovalent cation concentration equal to 152 mM on either side of the membrane, was 25.3 +/- 2.7 pS (8 patches). This compares with a value of 22 pS given by Yellen (1982) for a total monovalent cation concentration of 150 mM on either side.

6.3.2 Calcium dependence

Membrane patches were excised from cells which were bathed in solution A containing no calcium and 1 mM EGTA. The unitary non-selective currents were not seen until the pipette was moved into the mouth of a flow pipe through which was flowing a calcium-containing solution. When patches were moved from a solution containing 10 μM calcium to one containing no calcium and 1 mM EGTA the channel activity decreased and ceased within several seconds. The effect of calcium on the probability of being open was investigated extensively in one patch which contained a single channel. The patch was moved from a flow solution containing 5 mM K⁺ and 9.4 mM Cl⁻ with [Ca²⁺] = 5 μM to one containing the same concentrations of potassium, sodium and chloride (solution C, table 1) but with no calcium.
Calcium-activated currents

Figure 6.3

Currents recorded before and after moving a membrane patch into calcium-containing solution.

Currents were recorded from a membrane patch which was excised into solution A with no calcium and 1 mM EGTA (A). The electrode solution was solution A. The patch was then moved into the mouth of a flow pipe containing solution C with $[Ca^{2+}]_{flow} = 5 \, \mu M$ $[K^+]_{flow} = 152 \, mM$ and $[Na^+]_{flow} = 0 \, mM$ (B). The patch was held at -20 mV.

No unitary non-selective cation currents were recorded until the membrane patch was moved into the mouth of the flow pipe.
The current-voltage relation measured from this patch is shown in figure 6.2 where the current reverses at approximately -2 mV. The pipette was moved from the solution containing 5 µM calcium to one containing no calcium three times. In each case the channel activity ceased after the patch had been bathed in the zero calcium solution for several seconds. The membrane potential of the patch was held at -20 mV throughout. The current became too noisy to measure the probability of the channel being open when the pipette was moved into the zero calcium solution after having been moved from the 5 µM calcium solution to the zero calcium solution and back into the 5 µM calcium solution. Figure 6.3 A shows traces recorded from this patch with the membrane potential held at -20 mV with the intracellular face of the patch bathed in solution A with no calcium and 1 mM EGTA and figure 6.3 B shows traces recorded while the intracellular face of the patch was bathed in a solution with \([Ca^{2+}]_{\text{low}} = 5 \mu\text{M}\). The probability of being open was calculated for this channel for consecutive periods of 2 seconds while the patch was introduced into the 5µM calcium solution and moved from this into the zero calcium solution and back again. These results are plotted in figure 6.4. It can be seen that the probability of being open \(P_{\text{open}}\) increased to approximately 0.34 while the patch was bathed in 5µM calcium, decreased to zero in the zero calcium solution and increased to approximately 0.16 when the intracellular face of the membrane patch was reintroduced into the 5 µM calcium solution. It can also be seen that these changes did not occur immediately when the solutions bathing the intracellular face of the patch were changed. Possible reasons for this are discussed later in this chapter.

Figure 6.5 shows open time histograms constructed from currents recorded
Calcium-activated currents

Figure 6.4

Probability of being open calculated from continuously recorded currents.

The probability of being open was calculated, using equation 2.3 given in section 2.9.5, from unitary currents recorded in the presence and absence of calcium. The probability of being open was calculated for consecutive periods of 2 seconds. Solution A was the electrode solution and the flow solution was solution C (table 1).

It can be seen that the probability of being open decreases when the intracellular face of the membrane is moved into the calcium-free solution but increases again when the patch is reintroduced into the solution containing 5μM calcium.
Calcium-activated currents

from the same membrane patch at a holding potential of -20 mV while the intracellular surface of the membrane patch was bathed in 5 μM calcium (A) and with the intracellular surface bathed in a zero calcium solution. (The flow solutions were solution C with \([K^+] = 5\) mM and the pipette solution was solution A with \([K^+] = 150\) mM.) The histograms have been fitted with single exponential distributions. These fit the data well, indicating that the channel probably has only one open state (Colquhoun and Hawkes, 1981). The distributions have time constants of 0.928 msec in A and of 0.917 msec in B. This suggests that calcium does not affect the distribution of channel open times. However, if the delay in the effect of changing the solution bathing the membrane patch (apparent from figure 6.4) was due to a difference between the time at which the flow solution changes and the time at which the calcium concentration at the membrane surface changes then both figure 6.5 A and 6.5 B would probably show the open time distribution for a channel in the presence of 5 μM calcium. This possibility is shown to be unlikely in section 6.4.2.

6.3.3 Voltage dependence

The voltage-dependence of the probability of being open and of the open time distribution of the channel were investigated. Figure 6.6 shows the probability of being open measured from a membrane patch, the intracellular face of which was bathed in solution C with \([K^+] = 60\) mM and \([Ca^{2+}] = 5\)μM (solution A was the electrode solution, \([K^+]_p = 5\) mM). Currents were identified as non-selective cation currents by their reversal potentials. \(P_{\text{open}}\) is plotted against voltage and increases from 0.038 at -40 mV to 0.233 at +50 mV.
Calcium-activated currents

Figure 6.5

Open time histograms constructed from data recorded in the presence and absence of calcium.

Open times were calculated for data recorded from a membrane patch held at a membrane potential of -20 mV. The electrode solution was solution A ([K⁺]p = 150 mM, [Na⁺]p = 0 mM) and the intracellular face of the patch was bathed in solution C ([K⁺]flow = 5 mM, [Na⁺]flow = 147 mM). In A solution C contained 5μM calcium and contained no calcium in B. The histograms have been fitted with single exponential distributions with time constants of 0.928 msec (A) and 0.917 msec (B).

The results indicate that the channel has only one kinetically identifiable open state and suggest that the open time distribution does not depend on calcium.
Calcium-activated currents

$Ca^+$

Figure 6.6

$P_{\text{open}}$ plotted against membrane potential

$P_{\text{open}}$ was calculated from currents recorded at different voltages from a membrane patch whose intracellular surface was bathed in solution C with $[K^+]_{\text{low}} = 60$ mM.

It can be seen that the probability of being open is voltage dependent, being approximately constant between -40 and -10 mV but increasing with increasing membrane potential at potentials of +10 mV and higher. $P_{\text{open}}$ increases from 0.038 at -40 mV to 0.233 at +50 mV.
Calcium-activated currents

This voltage dependence is reflected in the voltage-dependence of the mean open time. Open time histograms measured from currents recorded from the same membrane patch at -40 mV (A), +10 mV (B) and +50 mV (C) are shown in figure 6.7. These have been fitted with single exponential distributions with time constants of 0.86 msec (A), 2.11 msec (B) and 3.65 msec (C). It can be seen that the histograms are fitted well with a single exponential distribution again indicating that the channel has a single open state (Colquhoun and Hawkes, 1981). The mean open time of the channel is equal to the time constant of the fitted exponentials and the figure suggests that this shows voltage-dependence. The mean open time is plotted against voltage in figure 6.8. It can be seen that the voltage dependence of the probability of being open closely follows that of the mean open time voltage dependence. The channel has a short mean open time and low probability of being open at hyperpolarized potentials. At membrane potentials which are more depolarized than -10 or -20 mV the open time and probability of being open increase with increasing membrane potential.

Closed time histograms were calculated from the currents which were used to generate the open time histograms. Examples of such histograms are shown in figure 6.9 A from data recorded at +40 mV and in figure 6.9 B from data recorded at -40 mV. These have been fitted with the sum of two exponential distributions indicating the presence of at least two closed states (Colquhoun and Hawkes, 1981). The time constants of these exponential distributions are, at -40 mV, 0.54 msec ($\tau_1$) and 3.9 msec ($\tau_2$) and are 0.7 msec ($\tau_1$) and 5 msec ($\tau_2$) at +40 mV. This suggests that the closed time distributions are not very dependent on voltage. This is also suggested by figure 6.10 where the time constants of the two exponentials used to fit the closed time distribution are plotted against membrane potential.
Open time histograms calculated from currents recorded at different membrane potentials.

Open time histograms were constructed from the currents whose $P_{\text{open}}$ was shown in figure 6.6, recorded at -40 mV (A), +10 mV (B) and +50 mV (C). These have been fitted with single exponential distributions with time constants of 0.86 msec (A), 2.11 msec (B) and 3.65 msec (C).

The results indicate that the channel has a single open state and that the mean open time of this state (equal to the time constant of the exponential distribution fitted to the histogram) is voltage-dependent. There is possibly a second component but this could not be fitted.
Calcium-activated currents

Figure 6.8

Mean open time plotted against membrane potential.

Mean open times were given by the time constants of single exponential distributions used to fit open time histograms such as those shown in figure 6.7. The open time histograms were constructed from the currents which were used to generate figures 6.6 and 6.7.

It can be seen that the mean open time of the channel is voltage-dependent, being constant between -40 mV and -20 mV and increasing with increasing membrane potential at potentials of -10 mV and above. This voltage-dependence is similar to that of $P_{\text{open}}$ shown in figure 6.6.
Calcium-activated currents

Figure 6.9

Closed time histograms.

Closed time histograms were constructed from currents recorded from a membrane patch, the intracellular surface of which was exposed to solution C with $[K^+]_{\text{in}} = 60 \, \text{mM}$ and $[Ca^{2+}]_{\text{in}} = 5 \, \mu\text{M}$. The histograms could not be fitted satisfactorily with a single exponential distribution but were fitted well with the sum of two exponential distributions, indicating that the channel probably has two closed states. A histogram constructed from currents recorded with the membrane potential held at $-40 \, \text{mV}$ (A) is shown alongside one constructed from currents recorded with the membrane potential held at $+50 \, \text{mV}$ (B). The histograms have been fitted with the sum of two exponential distributions with time constants of $0.54 \, \text{msec} (\tau_1)$ and $3.9 \, \text{msec} (\tau_2)$ (A) and of $0.7 \, \text{msec} (\tau_1)$ and $5.0 \, \text{msec} (\tau_2)$ (B).

The results suggest that the closed time distribution shows little or no voltage dependence.
Calcium-activated currents

Figure 6.10

Time constants of the exponential distributions fitted to the closed time histograms at different membrane potentials.

Closed time histograms were generated from currents recorded from membrane patches whose intracellular faces were bathed in solution C with \([K^+]_{\text{flow}} = 60\) mM and \([Ca^{2+}]_{\text{flow}} = 5\) µM. Solution A was the electrode solution. The time constants of the exponential distributions fitted to these histograms are plotted against membrane potential.

The results suggest that neither of the exponential distributions fitted to the closed time histograms is voltage dependent.
The figure shows two sets of data points. The x-axis represents mV (millivolts) ranging from -40 to 50. The y-axis represents time in milliseconds (msec) ranging from 0 to 5.

- The solid dots represent data points for \( \tau_1 \) (lower set).
- The diamond-shaped dots represent data points for \( \tau_2 \) (upper set).

The data suggests a positive correlation between mV and both \( \tau_1 \) and \( \tau_2 \) as mV increases.
6.4 UNITARY CALCIUM-ACTIVATED POTASSIUM CURRENTS

A second type of unitary current which opened when the intracellular face of an excised patch was exposed to a calcium-containing solution was identified. The channels through which these currents passed had a greater conductance than the non-selective cation channels and were mainly selective for potassium. They are possibly the unitary current correlate of the calcium-activated potassium current recorded by Moolenaar and Spector (1978) and are probably the currents mentioned by Yellen (1982) and described by Quandt (1988).

6.4.1 The ionic nature of the currents.

The ionic nature of the currents was again identified by measuring their reversal potential, either by the voltage ramping procedure described in section 2.6.3 or from the current-voltage relation of unitary currents measured by finding the amplitude of currents recorded at different holding potentials using the method described in section 2.9.2. Figure 6.11 A shows the current-voltage relation from currents recorded with solution F with $[K^+] = 30$ mM as the flow solution and with solution A with $[K^+] = 150$ mM in the pipette. It can be seen that the current reverses at approximately $+52$ mV which is reasonably close to the theoretical reversal potential for potassium ions of $+45$ mV in these conditions. The slope conductance of the channel was approximately 60 pS. When the patch was moved into a flow solution containing solution F with $[K^+] = 150$ mM the current reversed at approximately $+8$ mV; the theoretical reversal potential for potassium ions in these conditions is 0 mV.
Calcium-activated currents

Figure 6.11 A

Current-voltage relation for a membrane patch where $[K^+]_p = 150$ mM and $[K^+]_{flow} = 30$ mM.

The current-voltage relation was formed from current amplitudes measured by the method described in section 2.9.2. The electrode solution was solution A (table 1) with $[K^+]_p = 150$ mM and the flow solution was solution F (table 1) with $[K^+]_{flow} = 30$ mM.

The theoretical reversal potential for potassium ions in these conditions, calculated using the Nernst equation, is $+45$ mV. The currents reverse at approximately $+52$ mV and have a slope conductance of approximately 60 pS.
Figure 6.11 B

Current-voltage relation for the same membrane patch with $[K^+]_p = 150$ mM and $[K^+]_{flow} = 150$ mM.

The membrane patch whose current-voltage relation is shown in figure 6.11 A was moved into a flow pipe containing solution F with $[K^+]_{flow} = 150$ mM. (The electrode solution was solution A with $[K^+]_p = 150$ mM.)

The theoretical reversal potential for potassium ions in these conditions, calculated using the Nernst equation, is 0mV. The figure show that the currents reversed at approximately +8 mV and the slope conductance was approximately 146 pS. This suggests that the currents are carried mainly by potassium ions.
Calcium-activated currents

The slope conductance in these conditions was approximately 146 pS. This is shown in figure 6.11 B which shows the current voltage relation for currents recorded from a patch in these conditions. This suggests that the currents are carried mainly by potassium ions.

Figure 6.12 shows a unitary current obtained from the same patch when the membrane potential was ramped from +50 mV by -1.5 mV/msec. The mean reversal potential of currents recorded in this way from three patches in these conditions ([K⁺]₀ and [K⁺]₀ = 150 mM) was +6.3 mV +/- 3.8 mV and the mean slope conductance (the slope of the ramped current where it passes through 0 mV, shown by the arrow in figure 6.12) was 152 +/- 6 pS.

6.4.2 Calcium dependence

In all cases the patches were excised into solution A with no calcium and currents were not seen until the pipette was moved into a flow pipe containing a solution in which [Ca²⁺] = 5 μM. The effect of moving a patch held at -20 mV from a flow pipe containing solution F with [K⁺] = 150 mM ([K⁺]₀ = 150 mM also) and [Ca²⁺] = 5 μM to one containing the same solution but with [Ca²⁺] = 0 μM is shown in figure 6.13. This shows currents recorded from the membrane patch before (A) and after (B) it was moved; currents were not recorded whilst the patch was being moved (this takes approximately 30 seconds). It can be seen that the unitary currents ceased when the patch was moved into the solution containing no calcium. This cessation of unitary currents in zero calcium flow solutions occurred in all currents where the membrane patch was moved from a solution containing calcium to one containing no calcium.
Calcium-activated currents

Figure 6.12

Unitary current produced by ramping the membrane potential.

\[ [K^+]_p \text{ and } [K^+]_{\text{flow}} \text{ were both 150 mM. The membrane potential was ramped from a holding potential of } +50 \text{ mV by } -1.5 \text{ mV/msec. The current shown has a reversal potential of approximately } -8 \text{ mV, which is reasonably close to the theoretical equilibrium for potassium ions of } 0 \text{ mV, and has a slope conductance of approximately 150 pS.} \]
Calcium-activated currents

Figure 6.13

Currents recorded in the presence and absence of calcium.

Currents were recorded from a membrane patch with the membrane potential held at -20 mV. The electrode solution contains solution A with [K\(^+\)]_p = 150 mM. The patch was moved from a flow pipe containing solution F with [K\(^+\)]_{flow} = 150 mM and [Ca\(^{2+}\)]_{flow} = 5 \mu M (A) to one containing solution F with [Ca\(^{2+}\)]_{flow} = 0 \mu M (B).

It can be seen that channel activity was high in the calcium-containing solution but no activity was observed while the patch was in the calcium-free solution (the time between the end of recording A and the beginning of recording B was approximately 30 seconds).
6.4.3 Voltage dependence

The voltage dependence of $P_{\text{open}}$ was investigated for three patches with $[K^+]_p = 5 \text{ mM}$ and $[K^+]_{\text{flow}} = 150 \text{ mM}$ using equation 2.3 (section 2.9.5). The mean $P_{\text{open}}$ of the three patches is plotted against membrane potential in figure 6.14. It can be seen that $P_{\text{open}}$ is voltage dependent, increasing with increasing depolarization.

Open and closed time histograms were constructed for data recorded from one of these patches which appeared to contain only a single channel (openings to multiples of the unitary current amplitude were not seen). The electrode contained solution A with $[K^+]_p = 5 \text{ mM}$ and the flow solution was solution F with $[K^+]_{\text{flow}} = 150 \text{ mM}$. Figure 6.15 shows open time histograms for channel activity recorded at 0 mV (A) and +60 mV (B). The histograms could not be satisfactorily fitted with single exponential distributions but have been fitted with the sum of two exponential distributions, indicating the presence of at least two open states. The time constants of the two exponentials in A are 0.42 and 12.207 msec and in B are 0.696 and 20.37. This suggests that the mean open times of the two states may be voltage dependent, the mean open time of each state increasing with increasing depolarization. Closed time histograms constructed from the same data are shown in figure 6.16 A (0 mV) and B (+60 mV). These also could not be fitted with single exponential distributions but have been fitted with the sum of two exponential distributions, indicating the presence of at least two closed states. The time constants of these exponentials are 0.614 and 9.906 msec (A) and 0.361 and 3.343 msec (B). This suggests that the time spent in the closed states is also voltage dependent, decreasing with increasing depolarization. A voltage dependence was also observed in the presence of several long closed times at more negative voltages.
Calcium-activated currents

Figure 6.14

Voltage-dependence of $P_{\text{open}}$.

Currents were recorded from three patches with solution A ($[K^+]_p = 5$ mM) in the electrode and solution F ($[K^+]_{\text{flow}} = 150$ mM) in the flow pipe. $P_{\text{open}}$ was calculated using equation 2.3 (section 2.9.5) and the mean for the three patches is plotted against membrane potential.

It can be seen that $P_{\text{open}}$ is voltage dependent, increasing with increasing depolarization.
Open time histograms.

Open time histograms were constructed from currents recorded from a patch which probably contained a single channel (no openings to multiples of the unitary current amplitude were seen). Solution A ([K+]p = 5mM) was in the electrode and solution F ([K+]flow) was in the flow pipe. Currents were recorded at membrane potentials of 0 mV (A) and +60 mV (B). The histograms could not be fitted with single exponential distributions but have been fitted with the sum of two exponential distributions, indicating that the channel has at least two open states (Colquhoun and Hawkes, 1981).

The results suggest that the open time distribution of the channel is voltage dependent.
Closed time histograms.

Closed time histograms were constructed from the data used to construct the open time histograms in figure 6.15. These histograms could not be satisfactorily fitted with single exponential distributions but have been fitted with the sum of two exponential distributions, indicating that the channel has at least two closed states. The histogram shown in A was constructed from currents recorded at a membrane potential of 0 mV and has been fitted with exponential distributions with time constants of 0.614 msec and 9.906 msec; that shown in B was constructed from currents recorded at a membrane potential of +60 mV and has been fitted with exponential distributions with time constants of 0.361 and 3.343 msec.

The results suggest that the closed times of the channel are voltage-dependent.
Closed time histograms were constructed from the data used to construct the histograms shown in figure 6.16 but the first 5 msec of the histograms were discarded. The bins used for the histograms were 5 msec long, compared with 1 msec long in figure 6.16, so that very long closed times which were excluded from the histograms in figure 6.16 were included. It can be seen that there are several long closed times at 0 mV (A) but fewer at +60 mV (B). The histogram in B has been fitted with the sum of two exponential distributions with time constants of 6.96 msec and 24.74 msec. The histogram in A does not have enough data for a successful exponential fit.

The results suggest that there may be three closed states, one of which has a long mean dwell time.
This is illustrated in figure 6.17 where closed time histograms were constructed from the same data but the first 5 msec of the histograms were discarded. Bins were 5 msec long (compared with 1 msec long in figure 6.16); this meant that very long closed times which were excluded from the other histograms (figure 6.16) were included in these histograms. It can be seen that there were several long closed times when the patch was held at 0 mV while fewer and shorter long closed times were seen when the patch was held at +60 mV (this has been fitted with the sum of two exponential distributions with time constants of 6.96 and 24.74 msec; attempts were made to fit the histogram shown in figure 6.16 A with exponential distributions but these failed because of the lack of data).

Figures 6.15, 6.16 and 6.17 therefore indicate that the voltage dependence observed in the probability of being open is explained by voltage dependence of both open times and closed times. Figures 6.16 and 6.17 considered together also suggest the presence of three closed states and that the channel may exhibit bursting.

6.4.4 Bursting

The presence of long closed times, and a qualitative assessment based on the appearance of the unitary current recordings, suggested that these channels may exhibit bursting (Magleby and Pallotta, 1983; Colquhoun and Sakmann, 1985). A preliminary study was made which substantiates this. The critical time chosen to define a burst was 10 msec and was chosen by inspection of the current traces and the closed time histograms. This time is not critical since the mean length of gaps between bursts was much longer than that of the closings within bursts but for further study of the bursting properties of the channels it would be necessary to minimise the proportion of short
intervals which were misclassified as long intervals using the method of Colquhoun and Sakmann (1985).

Currents were recorded from membrane patches with solution A ($[K^+]_p = 5$ mM) in the electrode and solution F ($[K^+]_{flow} = 150$ mM, $[Ca^{2+}]_{flow} = 5$ μM) in the flow pipe. The results of the analysis are shown in table 6.1. These suggest that the interburst interval is voltage dependent. This suggests the presence of a third closed state, the time spent in which is voltage-dependent.
Table 6.1

<table>
<thead>
<tr>
<th>$V_m$ (mV)</th>
<th>$P_{open}$</th>
<th>Mean openings/burst</th>
<th>Mean burst duration (msec)</th>
<th>Mean interburst interval (msec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+30</td>
<td>0.785</td>
<td>12.95</td>
<td>317.53</td>
<td>59.12</td>
</tr>
<tr>
<td>+20</td>
<td>0.540</td>
<td>14.79</td>
<td>289.50</td>
<td>243.60</td>
</tr>
<tr>
<td>+10</td>
<td>0.526</td>
<td>13.00</td>
<td>271.93</td>
<td>230.56</td>
</tr>
<tr>
<td>0</td>
<td>0.159</td>
<td>11.77</td>
<td>278.28</td>
<td>1219.39</td>
</tr>
</tbody>
</table>
6.5 DISCUSSION

The results presented in this chapter described the voltage and calcium dependence of a non-selective cation channel and of a channel which passes a current carried mainly by potassium ions. The properties of these channels are summarized here and are compared with those of other similar channels and, in the case of the potassium channel, with a possible macroscopic current correlate in the same preparation.

6.5.1 Non-selective cation channel

This channel was shown to be non-selective by the insensitivity of its reversal potential to the sodium or potassium concentration gradients. Changing the concentrations of chloride and sulphate ions also had no effect on the channel’s reversal potential, indicating that the channel is not a chloride channel. These results leave the possibility that the channel is either a non-selective cation channel or that the channel passes sulphate ions and is non-selective for anions. Since the likelihood of the latter is very low (no sulphate-permeable channels have been reported) it is likely that the channel is a non-selective cation channel. The conductance of these channels was approximately 25 pS in solutions giving symmetrical cation concentrations of 152 mM. Yellen (1982) reported similar channels recorded from neuroblastoma N1E115; these had a conductance of 22 +/- 1.5 pS at 24°C with 125 mM NaCl at the extracellular face of the patch and 125 mM KCl at the intracellular face of the patch.
6.5.1.1 Voltage-dependence

Analysis of the open and closed times of the channel showed that the open time histograms were best fitted by a single exponential distribution (figure 6.5), indicating the presence of only one kinetically distinguishable open state, while the closed time histograms were best fitted by the sum of two exponential distributions indicating the presence of at least two closed states. It was shown that the open times are voltage-dependent, the open time increasing discontinuously with increasing depolarization (see figure 6.8), increasing with increasing depolarization at membrane potentials of -10 mV and above and being relatively low at more negative potentials. The closed times, however, showed no voltage dependence. These results differ from those of Yellen (1982) who reports that "Gating of the channels is not strongly voltage dependent over a range from -120 to +80 mV." He does not, however, give any data on the channel's probability of being open at different voltages nor on the open or closed time distributions so it is difficult to compare his results with those given here. Yellen used calcium concentrations of between 1 and 50 μM and does not say in which concentration he recorded data suggesting that the channel does not show voltage-dependence. It is possible, therefore, that the position of the discontinuity seen in the probability of being open and the mean open time of the channel, and their dependence on membrane potential, are dependent on the concentration of calcium at the intracellular surface and that in the concentrations of calcium used by Yellen the probability of being open was relatively flat over a large voltage range. Colquhoun, Neher, Reuter and Stevens (1981) also report only a weak voltage-dependence in a non-selective cation channel present in cultured cardiac cells.
6.5.1.2 Calcium-dependence

The calcium-dependence of $P_{\text{open}}$ is shown in figure 6.4. It can be seen that the probability of being open decreases slowly to zero when the solution bathing the intracellular face of the membrane patch is changed from one containing 5 μM calcium to one containing no calcium. Yellen (1982) reported that if calcium is removed from the intracellular membrane surface of a patch containing these channels, the channel openings become less frequent and eventually (sometimes after 1 or 2 minutes) cease but that when calcium is restored to the intracellular membrane surface the channels begin to open almost immediately. It can be seen from figure 6.4 that although some channel openings did occur soon after the channel was returned to a 5μM calcium solution, it took the same amount of time for channel activity to increase appreciably after restoration of calcium to the intracellular face of the membrane patch as it did for activity to cease after calcium was removed from the intracellular face. These delays may be due to a dependence of the channel kinetics on the concentration of calcium present coupled with a gradual exchange of calcium-free with calcium-containing solution at the surface of the membrane patch. The delay in cessation of the activity was seen in all non-selective channels observed in the presence and absence of calcium. This was not seen, however, in the calcium-activated potassium channels whose activity ceased as soon as calcium was removed from the intracellular membrane surface and returned soon after calcium was restored to the intracellular surface. Since the same techniques were used in both sets of experiments it seems unlikely that the delay in change of activity of the non-selective cation channel is due to factors such as the geometry of the patch making the solution change at the intracellular surface slower than that in the surrounding solution, suggesting that the
delay may be a property of the channel itself. It is difficult, however, to imagine what
the kinetic changes might be which could cause this delay.

6.5.1.3 Possible function

The non-selective channels reported by Colquhoun et al (1981) in cultured
rat cardiac cells show similar properties to the oscillatory inward currents that occur in
cardiac Purkinje fibres after depolarizing voltage steps during exposure to ouabain or
high extracellular calcium (Lederer and Tsien, 1976; Kass, Tsien and Weingart, 1978;
and Vassalle and Mugelli, 1982) in that they are activated by calcium ions, are not
strongly directly voltage-dependent and have a reversal potential which suggests poor
selectivity between sodium and potassium ions. Kass et al (1978) speculated that these
currents might be due to non-selective leak channels which would provide the
background current for the pacemaking activity of the cells. The currents reported here,
however, are more strongly voltage-dependent than those in cultured rat cardiac cells
and sympathetic neurones show no pacemaker activity.

6.5.2 The calcium-activated potassium channel

This channel was shown to be selective for potassium ions by the
dependence of its reversal potential and slope conductance on the potassium gradient
across the membrane. The channel has a slope conductance of approximately 146 pS at
a temperature of 19°C. The calcium-activated potassium channel of cultured rat muscle
(Barrett, Magleby and Pallotta, 1982) has a conductance of 100 pS at 1°C and 300 pS
at 37°C in symmetrical 144 mM K⁺ and that of cultured rat sympathetic neurones has a
conductance of 200 pS in symmetrical 150 mM K⁺ with [K⁺]ᵢᵣ and [K⁺]ᵢᵣₒᵦ = 150 mM.
The calcium-activated potassium current described above is probably that recorded by Quandt (1988) which had a conductance of 140 pS at 22°C in symmetrical 150 mM K⁺. Its properties are summarized here and compared with those of other calcium-activated potassium currents described.

6.5.2.1 Voltage dependence

It was shown above that both the open and closed time histograms for this channel were best fitted by the sum of two exponential distributions, indicating that the channel has at least two open and two closed states. The time constants of the exponential distributions fitted to the open and closed time histograms were voltage-dependent, the channel spending less time closed at more depolarized potentials. Sections 6.4.3 and 6.4.4 suggest the presence of a third closed state which confers bursting properties on the channel and the time spent in which is voltage dependent. These voltage dependencies lead to a steep voltage-dependence of P_{open} (see figure 6.14).

Moolenaar and Spector (1979b) described a slowly activating potassium current the size of which depended on both membrane potential and the calcium current of the cell. The currents described here are probably the unitary current correlate of this macroscopic current. Smart (1987) described a calcium-activated potassium channel in rat sympathetic neurones. The open time distribution of this channel was fitted by one exponential at depolarized potentials and the closed time distribution by three exponentials. Depolarization caused the long mean open time to increase while the short mean open time and the closed times were unaffected. The kinetics of this channel are therefore dissimilar to those of the calcium-activated
potassium channel of mouse neuroblastoma which is also of sympathetic origin. This shows more similarity to the calcium-activated potassium channel of cultured rat muscle (Barrett, Magleby and Pallotta, 1982; Magleby and Pallotta, 1983a,b) which has two open states and three closed states; the open times and the closed times are voltage-dependent. In addition the channel exhibits bursting with two classes of burst: short and long. The calcium-activated potassium channel of mouse neuroblastoma recorded by Quandt (1988) has a voltage-dependent $P_{\text{open}}$.

6.5.2.2 Calcium-dependence

The channel activity in an excised patch never began until the intracellular face of the patch was bathed in a calcium-containing solution ($[\text{Ca}^{2+}]_{\text{low}} = 5 \, \mu\text{M}$) and always ceased immediately if calcium was removed from the intracellular surface. The activity of the calcium-activated potassium channel of cultured rat sympathetic neurones (Smart, 1987) was virtually zero when $[\text{Ca}^{2+}]_i = 0.01 \, \mu\text{M}$. The mean dwell time in the two open states of the calcium-activated potassium channel of cultured rat muscle (Barrett, Magleby and Pallotta, 1982) were 0.34 and 2.2 msec with $[\text{Ca}^{2+}]_i = 0.1 \, \mu\text{M}$ and 0.47 and 6.9 msec with $[\text{Ca}^{2+}]_i = 0.5 \, \mu\text{M}$. The number of openings per short burst was relatively independent of calcium concentration while the number of openings per long burst increased with increasing $[\text{Ca}^{2+}]_i$. This increase was almost entirely due to an increase in the time spent in the long open state. The calcium-dependence of the kinetics of the channel described here should be further investigated with different calcium concentrations. The voltage for the half-maximal activation of the channel recorded by Quandt (1988) becomes more negative with increasing calcium concentration.
6.5.2.3 Possible function

The calcium-activated potassium channel is likely to be involved in the regulation of repetitive activity and the spacing of action potentials as a calcium influx accompanying an action potential will activate the channels, causing a hyperpolarizing current which, because of its calcium and voltage dependence, will resist further depolarization until the calcium concentration within the cell has dropped.
Chapter 7

DISCUSSION

The purpose of this discussion is to summarize the conclusions which have been drawn above about the ionic currents in neuroblastoma cells. The extent to which these findings are consistent with work on similar ionic currents, and on the same preparation, is discussed and the significance of the presence of these currents in these cells is considered. The extent to which the above comparisons support the assumption that neuroblastoma cells are a valid model system for electrophysiological studies on mammalian neuronal currents is discussed.

7.1 Delayed rectifier potassium currents

The results presented in Chapter 3 show that macroscopic delayed potassium currents may be recorded from mouse neuroblastoma cells using the whole-cell recording technique and those presented in Chapter 4 reported the characteristics of the probable unitary current correlate of these macroscopic currents. The unitary currents were shown probably to be responsible for the macroscopic currents by the voltage-dependence of their activation kinetics. When unitary currents recorded during many voltage sweeps are summed, the time course of the resultant current is the same as that of the macroscopic currents for each membrane potential measured. Both types of current also show slow inactivation. The macroscopic and the unitary currents both differ, however, from the macroscopic delayed outward potassium currents recorded in the same cell line using conventional two-microelectrode voltage clamp (Moolenaar
and Spector, 1978). These currents were recorded from cells which had been differentiated in DMSO-containing medium for between 1 and 3 weeks while the currents described here were recorded from cells which had been differentiated in the same way for less than three days. The currents recorded by Moolenaar and Spector (1978) show similar voltage-dependency (the currents recorded in Chapter 3 show half-maximal activation at a membrane potential of approximately -8 mV while those recorded by Moolenaar and Spector (1978) show half-maximal activation at a membrane potential of -9 mV). The activation of the currents described in Chapters 3 and 4 is, however, much slower than that described by Moolenaar and Spector (1978) (see section 3.5). It was concluded (section 3.5) that the most likely explanation for this is the presence of a fast transient outward current in the cells recorded from by Moolenaar and Spector (1978). This current might be present in the current recordings of Moolenaar and Spector but absent in those described here for two reasons:

1. Moolenaar and Spector recorded currents from cells whose membrane potential was held at -85 mV while the currents described here were recorded from cells which were held at -54 mV. At the latter membrane potential fast transient currents would almost certainly be completely inactivated (Connor, 1978; Cooper and Shrier, 1985; Taylor, 1987) but may not be at the former.

2. Cells which have been differentiated for a shorter period of time may not yet have developed fast transient currents. Differences in the development times of different neuronal currents have been shown in *Drosophila melanogaster* (Salkoff, 1983; Salkoff and Wyman, 1983; Salkoff, 1985). Kimhi et al (1976) found that the rates of rise and fall of the action potential of cells grown in 1 and 2% DMSO decreased after 2 days in DMSO-containing medium but had increased by the eighth
day in such medium. Although these differences are probably due to differences in channel density and cannot therefore explain the discrepancies discussed here, the increase in rate of fall of the action potential may have possibly been partly due to changes in the types of channel which were present in the membrane and not just their density.

While conducting the studies on unitary delayed rectifier currents, quickly activating outward currents which were probably carried by potassium were seen very occasionally. These may have been fast transient currents.

7.1.1 Multiple conductance states

The channels described in Chapter 4 appear to have the same characteristics as those described by Quandt (1988) except that the channels described here have been shown probably to have two open states of different conductance (see section 4.3 and 4.4) while Quandt did not report multiple conductance states. Quandt did, however, record on long time scales and the time resolution of the traces which he shows is much less than that of the traces shown in here (see figures 4.2, 4.19 and 5.1). This may have caused the transitions between open states to have been missed.

Multiple conductance states have been seen in a variety of channels (see section 1.5). A potassium-selective, voltage-dependent channel which has similar properties to the channel described here has been described in frog skeletal muscle by Labarca and Miller (1981). Both channels have two open states of different conductance, the larger conductance being equal to less than twice the smaller; and both channels appear to be constrained to opening to the smaller state before opening to the larger. The question remains as to why the channels have multiple-conductance
states. Multiple conductance states have been reported in channels whose activity is modulated by excitatory amino acids (Jahr and Stevens, 1986; Hamill, Bormann and Sakmann, 1983; Cull-Candy and Usowicz (1987). In each case the states to which the channel opens are determined by the particular amino acid which is applied to the membrane surface. This allows the membrane potential to be manipulated in a complex manner by different amino acids having different effects on the same channel. The possible purpose of multiple conductance states in voltage dependent channels is less easily explained. It has been shown (4.4.5) that the relative time which the channel spends in the two states is probably voltage-dependent, the channel opening to the larger conductance state more frequently at more depolarized potentials. This will cause the current flowing through the channel to be more steeply voltage dependent than it would be if the channel only ever opened to the smaller state and possibly allows the macroscopic current to be controlled by membrane potential in a more complex manner than for a single state channel. The possibility remains, however, that the presence of multiple conductance states in this channel is abnormal. The cells are a rapidly dividing tumour cell line and the currents which they possess may have deviated from the physiological currents in the cells from which the tumour was derived.

Sodium (Numa et al, 1984) and potassium (Papazian et al, 1987; Schwarz et al, 1988) channels have been sequenced and shown to possess subunits. The sequencing of channels which have multiple conductance states may, in the future, suggest the molecular processes which allow the channels to exhibit these multiple states. The presence of subunits, which may confer the sigmoidicity of channels' activation kinetics, may be significant in conferring multiple conductance states; or the
molecular changes to the protein which cause its conductance to change may be very small. For example, if a positively charged part of the molecule moved away from the part of the protein which forms the pore through the membrane this movement might be voltage-dependent and might increase the likelihood of positively charged ions entering the channel, increasing its conductance. Matsuda (1988) found that magnesium block of inward rectifier potassium channels of guinea-pig heart cells reveals two open states of different conductance. It may be that the multiple conductance states of the delayed rectifier described above are also caused by a similar block. This seems unlikely, however, since it has been shown (section 4.4.3) that the channel probably opens to the smaller conductance state before opening to the larger and then moves between the two; this makes it unlikely that the smaller state is due to partial block of the larger. It is also unlikely that the multiple conductance states seen in channels regulated by excitatory amino acids could be caused by a similar block.

7.2 Calcium-activated, non-selective cation channels

Non-selective cation currents which are probably the same currents reported by Yellen (1982) were described in Chapter 6. It was shown that these show voltage as well as calcium dependence whereas those described by Yellen show no voltage dependence and a similar channel in cultured cardiac cells (Colquhoun et al, 1981) shows only weak voltage dependence. If the concentration of calcium causes a shift in the voltage activation of the channel as described in calcium-activated potassium channels (Quandt, 1988), this difference in voltage-dependence of the channels may be due to differences in the calcium concentrations used (see section 6.5.1.1). Non-selective cation channels have been implicated in pacemaker activity but it is
difficult to see what their function might be in a neuronal cell. After periods of electrical activity and influx of calcium the channel might open but the value of this to a neurone is unclear. If it were found that the presence of voltage- as well as calcium-dependence is a general property of neuronal non-selective cation channels whereas cardiac non-selective cation channels show only weak voltage dependence, this might indicate a difference in function of the two types of channel.

7.3 Calcium-activated potassium channels

The calcium-activated potassium channel described in Chapter 6 has at least two open states and two closed states. Preliminary studies indicate that the channel also appears to exhibit bursting. The probability of being open is steeply voltage dependent as with other calcium-activated potassium channels (Barrett, Magleby and Pallotta, 1982; Magleby and Pallotta, 1983a,b; Smart, 1987). Calcium-activated potassium currents have been found to cause hyperpolarization after action potentials in neurones (Meech and Strumwasser, 1970; Meech, 1972; Krnjevic and Lisiewicz, 1972; Meech, 1974a,b; Meech and Standen, 1975) and in neuroblastoma (Moolenaar and Spector, 1979a,b). This current is therefore a typical neuronal current. It will regulate repetitive firing in neurones since it will activate during an action potential (because of the depolarization and influx of calcium) and will prevent further depolarization until the calcium concentration inside the cell drops. The after-hyperpolarization caused by the current (Moolenaar and Spector, 1979a,b) will place an upper limit on the firing rate of the cells.
Discussion

7.4 Neuroblastoma cells as a model system for electrophysiological studies of mammalian neuronal currents

Sodium and delayed rectifier potassium currents have been described above. The activation kinetics of these currents are slower than those of mature neuronal cells, although the kinetics of the currents recorded in neuroblastoma cells differentiated for longer periods of time (Moolenaar and Spector, 1978) are closer to those expected in neuronal cells. The delayed rectifier potassium channel is blocked by low concentrations of TEA\(^+\). This behaviour is similar to that of other neuronal delayed rectifier currents (for example, Hille, 1967; Adams, Smith and Thompson, 1980; Stanfield, 1983). The cells possess calcium-activated potassium currents which behave in a similar way to other neuronal calcium-activated potassium currents but possess a calcium-activated non-selective cation current which is not a characteristic neuronal current.

Neuroblastoma cells may be induced to differentiate by culture in DMSO-containing medium. This causes the cells to differentiate morphologically, growing processes, causes an enhancement in the electrical excitability of the cells (Nelson et al, 1969; Kimhi et al, 1976; Chalazonitis and Greene, 1974) and causes the cells to respond to acetylcholine application.

The above evidence indicates that neuroblastoma cells possess morphological and electrical characteristics of neuronal cells. The kinetics of activation of the potassium currents described in Chapters 3 and 4 may indicate a difference between these cells and normal neuronal cells or may be due to the small degree of differentiation in the neuroblastoma cells used; and the presence of non-selective cation currents in the cells may either indicate that the cells possess
currents which are atypical of neurones, or that non-selective cation currents have physiological functions other than their probable role in pacemaker activity.

The presence of multiple conductance states in the delayed rectifier potassium channels may be abnormal. The voltage-dependence of their kinetics, however, indicates that they may have a function in allowing the membrane currents to be manipulated in a more complicated manner than could be achieved with a single conductance state channel. All potassium channels are involved in stabilising the membrane potential of a cell since the equilibrium potential of potassium ions is very negative, in physiological conditions, compared with the threshold potential for excitability. There is a diversity of potassium channels, it being possible for there to be several types of potassium channel in the same membrane each having a different role. There is therefore necessity for the membrane potential to be stabilised in different conditions and in a more complex manner than the depolarization caused by a single type of sodium channel. The presence of multiple conductance states may add to this complexity.
Appendix I

Ramp generator
Appendix II

Karnowski’s fixative

Formalin 38% Formaldehyde 4 ml
Glutaraldehyde 25% 8 ml
Cacodylate buffer pH 7.3 18 ml (0.2 M)

made up to 50 ml with distilled water
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(Numa et al.) Noda, M., Shimizu, S., Tanabe, T., Takai, T., Kayamo, T., Ikeda,


Patch-clamp studies of macroscopic and single-channel currents in mouse neuroblastoma cells
by Rosalind Anita Jane Smith, MA.

Abstract

Cells from the clonal cell line N1E115, a mouse tumour of sympathetic origin, differentiate both morphologically and electrophysiologically when cultured in medium containing dimethylsulphoxide. This technique was used to obtain cells for electrophysiological recording.

Macroscopic currents were measured using the whole-cell patch-clamp technique and two currents, one thought to be carried mainly by sodium ions and one mainly by potassium ions, were identified. The kinetics of the potassium current, which are voltage dependent, suggested that this was a delayed rectifier.

A single-channel current which was selective for potassium ions was recorded using the cell-attached patch-clamp recording technique. The channel was thought to be the unitary current correlate of the macroscopic potassium current on the basis of its activation kinetics. The channel was shown to have two open states of different conductance, one having approximately two-thirds the conductance of the other. The single-channel kinetics were measured and it was shown that the open time distribution of the channel, the latency to its first opening after a voltage step and the relative times spent in each of the two open states are voltage dependent. The amplitude of these currents was reduced by application of tetraethylammonium ions to the extracellular face of the membrane patch and the concentration- and voltage-dependence of this block were investigated.

Two further unitary currents were identified using the excised patch-clamp recording technique: a calcium-activated non-selective cation current and a calcium-activated potassium current. The conductance and voltage-dependence of both currents were investigated.