CHARACTERISATION OF A NOVEL POTASSIUM CONDUCTANCE 
IN RAT CEREBELLAR GRANULE NEURONS.

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A thesis submitted for the degree of Doctor of Philosophy

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ABSTRACT.

Cultured cerebellar granule neurons (CGNs) possess transient and delayed rectifier type voltage-gated potassium (K⁺) conductances. An additional component of outward K⁺ current has been described in these cells which has been termed standing outward current (IKso). This current is outwardly rectifying, non-inactivating and is reversibly and concentration dependently inhibited by muscarine. The aim of this study was to characterise a number of biophysical and pharmacological properties of IKso in an attempt to identify the molecular correlate of the current and elucidate the mechanism of muscarinic modulation.

The effect of M₂ and M₃ muscarinic receptor antagonists on the muscarine concentration response curve was determined. The M₂ antagonist (methoctramine) had little effect on the control concentration response curve, while the same concentration of a M₃ antagonist (zamifenacin) produced a rightward shift identifying the M₃ receptor subtype as mediating the muscarine effect. (Additional concentrations of zamifenacin resulted in an estimated pA₂ value of 8.13). Inhibiting a classical downstream product of M₃ receptor activation (PLC), only slightly reduced the muscarinic inhibition of IKso, suggesting that M₃ receptors may act through a novel pathway to inhibit IKso in CGNs.

It has been proposed that the molecular correlate of IKso may be a member of the ether à go go (eag) family of K⁺ channels, since rat-eag (r-eag) channels when expressed in mammalian cells show similar properties to IKso. A feature of r-eag currents is a dramatic slowing of the activation kinetics on application of external Mg⁺⁺ in a concentration and voltage dependent manner. The activation kinetics of IKso were found to be unaffected by external Mg⁺⁺, arguing against eag being the molecular correlate of IKso.

IKso also shares certain functional properties with members of the two pore domain superfamily of K⁺ channels (K₇). A lack of voltage dependence of activation has been demonstrated for IKso, which is a diagnostic feature of K₇ channels. Pharmacologically IKso is inhibited by Ba²⁺, NMDG, external acidification, is weakly inhibited by quinine and quinidine, but is unaffected by arachidonic acid. These properties mean IKso bears closest resemblance to the K₇ clone TASK-1.

The properties of IKso are almost identical to those of TASK-1, and RT-PCR revealed mRNA for TASK-1 is expressed in CGNs. Additionally immunocytochemical experiments confirmed the presence of TASK-1 protein in both the membrane and cytoplasm of the cells. It seems likely therefore that IKso belongs to the K₇ superfamily of potassium channels.
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CHAPTER 1.

GENERAL INTRODUCTION.
1.1. Organisation of the cerebellum.

The brain is divided into three major anatomical regions, the fore, mid and hindbrain. These regions are further divided into distinct structures, with the hindbrain being composed of the Pons, Medulla and Cerebellum (see Figure 1.1).

![Figure 1.1. Schematic midsagittal section of the human brain, showing the position of the major brain structures.](image)

The cerebellum is a complex structure located below and towards the back of the brain, behind the brainstem. The word cerebellum come from the Latin for 'little brain', which is an apt description since it resembles the cerebrum in a number of ways. Like the cerebrum, the mammalian cerebellum is divided into hemispheres, whose surface is highly convoluted, giving the cerebellum a characteristic laminated appearance. Each fold of the cerebellum is known as a folium. The internal structure of the cerebellum also resembles that of the cerebrum, with an outer cortex (grey matter) surrounding the hemispheres, an inner white matter and a mass of deep centrally located neurons below the white matter known as the deep nuclei. Although the cerebellum may be 'little', constituting only 10 % of the total volume of the brain, it probably contains more neurons than are found in the whole of the rest of the brain (Herrup and Kuem erle, 1997).
The pioneering work of Ramón y Cajal at the beginning of the last century resulted in a detailed description of the anatomy of the cerebellum, which has hardly been modified by subsequent work. The cerebellar cortex contains five readily identifiable types of neuron: the granule cells, Purkinje cells, Golgi cells, stellate cells and basket cells. The architecture of the cortex is highly regular and relatively simple, with the pattern of intrinsic connections known in great detail. The repetition of the same basic circuit module results in the preservation of an identical cellular structure curving through every folium. The cortex of the cerebellum consists of three layers: the external molecular layer, the intermediate Purkinje cell layer and the internal granular layer. Figures 1.2 and 1.3 illustrate the general organisation of the cerebellar cortex.

Figure 1.2. The general organisation of the cerebellar cortex (a). Diagram of a single cerebellar folium that has been sectioned vertically, both in its longitudinal axis (right part of the diagram) and transversely (on the left). Diagram illustrates the five main types of neuron in the cerebellar cortex and the two kinds of afferents with their main interconnections. Purkinje cells (pink); inhibitory interneurons (black) including stellate, basket and Golgi cells; granule cells and parallel fibres (yellow); afferents (blue) both climbing and mossy fibres. Figure taken from Williams and Warwick, 1975.
The granular cell layer, as its name implies, is composed primarily of densely packed granule neurons. These cells are small glutamatergic neurons, which are by far the most numerous elements in the brain as a whole. They have an unmistakably characteristic shape; a globular cell body with three or four short, radiating dendrites which branch only at their ends, resulting in a claw like appearance (Palay and Chan-Palay, 1974). The axons of granule cells are known as parallel fibres. They originate from the cell body and ascend through the Purkinje cell layer to the molecular layer where the axon bifurcates like a T, giving rise to a pair of fibres running in opposite directions parallel to the longitudinal axis of the folium.

The Purkinje cell layer is composed of a single layer of the large cell bodies of Purkinje cells arranged side by side. These neurons are highly differentiated with a broad, flattened, fan-like dendritic tree extending into the molecular layer. The dendritic tree is strictly confined to the transverse plane of the folium meaning that
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The parallel fibres of the granule cells run at right angles to the Purkinje cell dendrites. The distal branchlets of the Purkinje cells are covered with spines, which the parallel fibres synapse on to as they pass through the Purkinje cell dendritic tree. A single parallel fibre contacts as many as 500 Purkinje cells, with a single Purkinje cell receiving input from approximately $10^5$ parallel fibres (Nolte, 1993). The sole output of the cerebellar cortex is via the Purkinje cell axons, which are GABAergic. These project primarily to the deep nuclei.

The remaining neurons, the stellate, basket and Golgi cells, are essentially inhibitory interneurons, which interconnect the various elements in the cerebellar cortex. The stellate and basket cells are located in the molecular layer, and as with Purkinje cells their dendritic tree is confined to the transverse plane of the folium. Stellate cells are smaller than basket cells and their dendrites span considerably smaller distances. However, both cell types possess dendritic spines, on to which the parallel fibres synapse. Both cell types synapse onto Purkinje cells although the basket cell axons predominately descend into the Purkinje cell layer to synapse with the cell body, while the stellate cell axons mainly terminate on Purkinje cell dendritic spines (Eccles et al, 1967, Williams and Warwick, 1975). Golgi cells are located in the granular layer of the cortex, with their cell bodies lying just below the Purkinje cell bodies. They have a series of large dendrites which enter the molecular layer and, unlike the other interneurons, the dendritic tree of a Golgi cell is not confined to one plane of the folium, but instead expands equally in all directions. The dendritic tree of a Golgi cell is about three times as wide as that of a Purkinje cell, and the dendritic trees of neighbouring Golgi cells do not overlap. The axon of Golgi cells also branches profusely and permeates the full thickness of the granular layer, terminating on the granule cell dendrites.

There are two major inputs to the cerebellar cortex, both of which are excitatory: climbing fibres and mossy fibres (Voogd and Glickstein, 1998). The climbing fibres are axons of neurons originating in the inferior olive nucleus of the medulla. They enter the cerebellum and establish two branches, one to the deep nuclei and one directly to the Purkinje cells. Each climbing fibre passes straight through the white matter and the granule layer to become attached to a single Purkinje cell. It then divides repeatedly and forms multiple synaptic contacts with the smooth areas of the
The Purkinje cells also receive indirect input from the second type of extracerebellar afferents, the mossy fibres. This input originates from a variety of sources and typically carries sensory and motor information. Mossy fibres synapse with granule cells, which in turn synapse with the Purkinje cells. The convergence of the mossy fibres and granule cells, along with the terminals of the Golgi cell axons, form a characteristic structure known as a glomerulus.

The cerebellum is a major associative centre for sensory input receiving information from cutaneous receptors, proprioceptors, the eyes, ears, brainstem reticular formation and cerebral cortex. Commonly it is associated with the motor system since normal functioning is necessary for smooth co-ordinated effector locomotor responses to occur. Further there is a growing consensus that the cerebellum contributes in some way to a significant number of higher cognitive functions (Middleton and Strick, 1998, Herrup and Kuemerle, 1997).

Granule neurons are well connected with the other types of neuron in the cerebellar cortex, and the activity of these interneurons will ultimately influence the output of the cerebellum via the Purkinje cells. Therefore understanding how the excitability of these cells can be modulated may prove to be physiologically important. It is these interneurons that provide the focus of this study.

1.2. Cell excitability.

All excitable cells possess a potential difference across their membrane, which is generated by the separation of charge due to the uneven distribution of ions across the membrane. If an intracellular microelectrode is inserted into a neuron it is found that the inside of the cell is negative to the outside by some tens of millivolts. This potential difference is essential for neuronal function as changes in the membrane potential underlies the signalling mechanism of the nervous system.
1.2.1. Historical perspective (Hille, 1992; Aidley, 1998).

It has long been appreciated by scientists that cells of the nervous system communicate through the propagation of electrical signals, known as action potentials. The idea that it was the movement of ions across the cell membrane that was responsible for generating these electrical signals (the membrane ionic theory of excitation) was proposed long before the technical advances were achieved that enabled direct measurement of ionic currents. Indeed, the first demonstration that ions play a pivotal role in the excitability of living cells came in the 1880's, when a series of pioneering experiments by Sidney Ringer demonstrated that for an isolated frog heart to continue beating the perfusing solution must contain Na\(^+\), K\(^+\) and Ca\(^{2+}\) in definite proportions.

The next major step towards understanding cell excitability came in 1902 with Julius Bernstein's 'membrane theory' of the resting membrane potential. It was already known that, if two different concentrations of a salt solution were separated by a selectively permeable membrane, a potential difference could be measured between these two solutions. For example, imagine a container divided into two compartments by a membrane that is selectively permeable to K\(^+\) ions. On either side of the membrane is a potassium salt solution of different concentrations. According to the laws of diffusion the ions of these salt solutions will want to mingle until the concentration on both sides is equal. Because of the presence of the selectively permeable membrane this is not possible and instead only K\(^+\) ions will diffuse across the membrane, down their chemical gradient from regions of high to low concentration. The movement of this positive charge causes a potential difference to be set up between the two compartments, which gets larger depending on the number of K\(^+\) ions that diffuse across. The chemical 'force' causing a net diffusion of K\(^+\) ions from one side of the membrane to the other is gradually countered by the growing electrical force tending to oppose the flow of K\(^+\) ions. Hence an equilibrium is reached where the chemical gradient just balances the electrical gradient and there is no net current flow. The potential difference at this point is known as the equilibrium potential.
In 1888 Walter Nernst, a physical chemist, derived an equation that could be used to predict the value of the equilibrium potential for any permeable ion, and it was Bernstein who was the first to apply the Nernst equation to the living cell.

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

**Equation 1.1. The Nernst equation.** $E_K$ is the value of the membrane potential at which $K^+$ is in equilibrium, $R$ is the gas constant, $T$ the temperature in Kelvin, $z$ is the valency of $K^+$, $F$ the Faraday constant and $[K^+]_o$ and $[K^+]_i$ the concentrations of $K^+$ inside and outside the cell.

Bernstein proposed that excitable cells were surrounded by a membrane that was selectively permeable to $K^+$ ions at rest, but during excitation its permeability to other ions increased via a process he termed 'membrane breakdown'. This membrane hypothesis predicted, using the Nernst equation, that the resting membrane potential of excitable cells was negative (see Table 1.1).

<table>
<thead>
<tr>
<th>Ion</th>
<th>Cytoplasm (mM)</th>
<th>Extracellular fluid (mM)</th>
<th>Nernst potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K^+$</td>
<td>400</td>
<td>20</td>
<td>-75</td>
</tr>
<tr>
<td>$Na^+$</td>
<td>50</td>
<td>440</td>
<td>+55</td>
</tr>
<tr>
<td>$Cl^-$</td>
<td>52</td>
<td>560</td>
<td>-60</td>
</tr>
<tr>
<td>$A^-$</td>
<td>385</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*Table 1.1. Distribution of the major ions across the membrane of the squid giant axon, including the Nernst potential of individual ions (taken from Hille, 1992).*

In the late 1930's, Kenneth Cole and Howard Curtis confirmed Bernstein's theory that permeability of the membrane increased during excitation. They demonstrated, using squid giant axons, that during the nerve action potential the resistance of the axon cell membrane was greatly reduced when compared to its resting value. This decrease in resistance was correctly interpreted as an increase in ionic permeability.
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The period that followed was an exciting time in the field of biophysics, with the development of reliable experimental methods that enabled direct measurement of the membrane potential both at rest and during conduction of an action potential. Alan Hodgkin and Andrew Huxley and Curtis and Cole in the late 1930's and early 1940's finally succeeded in measuring the full action potential of an axon using an intracellular pipette. They confirmed that the resting membrane potential was indeed negative, and showed that during the action potential the membrane potential became much more positive, overshooting zero by tens of millivolts.

These first measurements with intracellular microelectrodes showed that at physiological $[K^{+}]_o$, the resting membrane potential was more positive to that predicted by the Nernst equation for a $K^+$ selective membrane. This deviation was correctly interpreted to mean that the resting membrane in neurons is primarily $K^+$ selective, but also slightly permeable to some other ions.

Intracellular recordings also revealed the mechanism responsible for generating an action potential. According to Bernstein's theory the membrane would become permeable to many ions during an action potential, and so a rise in the membrane potential to approximately 0 mV was predicted. However the membrane reached a potential much more positive than this resulting in the idea that rather than becoming permeable to many ions during the action potential, the membrane becomes selectively permeable for $Na^+$. If this were the case then the electromotive force would be the $Na^+$ equilibrium potential, which is near +60 mV, explaining why the peak of the action potential overshoots 0 mV by such a large amount. The sodium theory was confirmed by a series of experiments using modified bathing solutions. The results showed that under conditions of reduced external $Na^+$ the action potential waveform rose less steeply, propagated less rapidly and reached less positive peak values.

Later Hodgkin and Huxley in the 1950's went on, using the newly developed voltage clamp technique, to propose that the current comprising the nerve action potential was composed of two major independent components occurring in parallel: an early transient phase of current carried by $Na^+$ ions and a delayed long lasting phase of current carried by $K^+$ ions. In each case the direction of current was determined by
the direction of the electrochemical potential of the ion concerned (Hodgkin et al., 1952). These conclusions were made on the basis of studies using Na⁺ replacement and electrochemistry. Thus the importance of ions to excitation was firmly established.

The plasma membrane of all cells is largely composed of a bilayer of lipid molecules with out-facing polar head groups and hydrophobic hydrocarbon tails. The hydrophobic nature of the cell membrane means that it is virtually impermeable to polar molecules, such as ions, making the membrane an effective barrier for separating the cell contents from the extracellular environment. So how do ions get across the cell membrane to generate ionic currents?

It was while studying K⁺ fluxes across giant nerve axons in 1955 that Hodgkin, on this occasion working with Richard Keynes, used the concept of a channel located in the axon membrane to explain the movement of potassium. A model in which ions are assumed to move single file through narrow channels or along chains of sites which stretch through the membrane (Hodgkin and Keynes, 1955), best fitted their flux ratio data. Therefore the existence of specialised pores, which are responsible for allowing the movement of ions across the cell membrane, was proposed approximately twenty years before Bert Sakmann and Erwin Neher in the late 1970's and early 1980's, developed and refined the revolutionary patch-clamp technique. This powerful technique allowed for the first time the tiny currents, which flow through individual ion channels when they are open, to be resolved and the properties of the channels and the accompanying currents to be studied.

1.3. Ion channels.

The concept of an actual membrane pore was first discussed in the 1840's (see Neher and Sakmann, 1995), more than a century before Hodgkin and Keynes incorporated their existence into a model to explain ion flux. It took until the 1970's for the idea to be universally accepted, with advances in electrophysiology and subsequently molecular biology, firmly establishing their existence.
Ion channels are integral membrane proteins which are anchored in the lipid bilayer forming an aqueous pore connecting the external medium to the internal cytoplasm, through which ions can diffuse down their electrochemical gradient. The conducting pathway of the ion channel can be composed of a single continuous polypeptide, as with Na\(^+\) and Ca\(^{2+}\) channels, or several peptide subunits as is the case for K\(^+\) channels (see section 1.3.3.1.3.). Ion channels are distinguished from other ion transporting membrane proteins (transporters and pumps) by their ability to move millions of ion per second. This movement of ions is much more rapid than can be achieved by any carrier system providing major evidence that ion channels are pores.

Water creates a polar environment due to the separation of charge within the molecule. Because ions are charged particles, in solution they attract water molecules and become surrounded by electrostatically bound water. It is extremely energetically unfavourable, and therefore improbable, for ions to leave this polar environment to enter the nonpolar environment of the lipid bilayer, demonstrating the need for a continuous aqueous environment across the membrane in the form of an ion channel.

Ion channels however are more than just pores across the membrane. It is clear from the description of action potential generation and membrane potential maintenance, that not all ion channels can be constantly in a conducting state and that ion channels must show a degree of selectivity.

The opening and closing of ion channels is a regulated process and it is commonly thought that there is some structure or property of the channel that is concerned with the transition between these two states. The word gate is used to describe this concept (Aidley and Stanfield, 1996). The transition of a channel from closed to open state is referred to as gating, and the gating event may be the binding of a chemical outside or inside the cell, such as neurotransmitters or cytoplasmic messenger molecules, a change in the membrane voltage or some kind of sensory stimuli such as membrane stretch. The former two cases can be used to classify channels into two major classes: the ligand-gated and voltage-gated channels.
When open channels exhibit the ability to discriminate between ions to varying extents. Each channel selects between possible permeant ions on the basis of ionic charge, allowing either cations or anions to permeate. Some cation-permeable channel types are relatively non-selective for the cations present in the extracellular fluid and are simply denoted non-selective cation channels. However the majority of ion channels are primarily permeable to a single type of ion and a channel is named after the ion for which it shows the greatest selectivity, e.g. Na\(^+\) channel, Cl\(^-\) channel, K\(^+\) channel.

Rather than attempt a review of all ion channels, which is beyond the scope of this thesis, the remainder of this report will focus on one class of ion channel, those that are selectively permeable to K\(^+\) ions. This will serve as a basis for the presentation and discussion of data presented in this thesis.

1.3.1. Potassium channels.

Potassium channels play an important role in controlling the excitability of cells. Different potassium channels are involved in establishing the resting potential, determining the duration of action potentials, modulation of transmitter release and in rhythmic firing patterns and delayed excitation (Hille, 1992). Open potassium channels will draw the membrane potential nearer to the potassium equilibrium potential, and therefore as a general rule increased potassium channel activity will tend to result in a decreased cell excitability, whereas decreased activity will increase excitability.

By physiological and pharmacological criteria a variety of potassium channels have been identified which vary in distribution among different cellular regions and cell types (Pongs et al, 1988). The many different potassium channels can be distinguished mainly by the way they gate. Some potassium channels open in response to membrane voltage changes, some respond to varying levels of intracellular Ca\(^{2+}\), in other potassium channels gating is tightly regulated by G proteins or the binding of extracellular ligands (Heginbotham et al, 1994), and some potassium channels do not seem to gate at all but are open constantly (Koester, 1991).
1.3.2. Potassium channel selectivity.

When potassium channels are open they are ten thousand times more permeable to $K^+$ ions than smaller $Na^+$ ions. How is this selectivity achieved? Hille argues that for an ion channel to be highly selective the pore must be narrow enough at some point to force the permeating ions into contact with part of the channel protein so that they can be 'sensed' by an interaction with the channel pore (Hille, 1992).

The idea that a narrow region of the channel pore conferred selectivity was first suggested by Bertil Hille in the early 1970's while working on the mechanism underlying $Na^+$ channel selectivity. He called the putative narrow region of the pore a 'selectivity filter'. The simplest way of thinking of the selectivity filter is to imagine a molecular sieve which purely differentiates between ion on the basis of size and shape, with only ions smaller than the dimensions of the filter passing through the channel. This explains why large ions cannot permeate a potassium channel, but not how $K^+$ ions are selected for over smaller ions. Therefore the selectivity filter model was adapted to include some kind of ion interaction with residues lining the pore.

From ion substitution experiments the narrow part of the potassium channel was estimated to be 3Å in diameter (Hille, 1973). The selectivity filter is so narrow that only a single fully dehydrated $K^+$ ion can pass through one at a time, which lends support for the single file movement of ions first postulated by Hodgkin and Keynes in 1955 (Choe and Robinson, 1998). If $K^+$ ions are to be stripped of their waters of hydration during permeation then an essential feature of the selectivity filter will have to be provision of atoms that will compensate for this loss, ensuring that ion permeation is energetically favourable. $K^+$ channels are thought to possess oxygen atoms within the selectivity filter which act as surrogate water molecules for the ion. To explain $K^+$ selectivity over $Na^+$ it was proposed that the oxygen atoms which bond to $K^+$ are fairly rigid in structure and that in order to permeate ions have to fit closely to this structure. A $K^+$ ion (radius 1.33Å) in a fixed pore of 3Å diameter, which is coordinated by oxygen atoms could have much the same energy as a $K^+$ ion in water. It follows that a structure smaller than a $K^+$ ion, such as a $Na^+$ ion (radius 0.9Å), will not fit into this rigid selectivity filter as well and form the same number of bonds as a
K\textsuperscript{+} ion. Therefore Na\textsuperscript{+} ions will have an energy higher than in water making it unlikely that Na\textsuperscript{+} will permeate the channel (Armstrong, 1998).

Up until recently, models concerning ion channel selectivity have been formed without any definite structural information. However the publication of the crystal structure of a bacterial potassium channel (KcsA; Doyle et al, 1998) has confirmed much of what has been proposed from functional studies on potassium channels. A narrowing of the pore at the extracellular mouth of the channel was observed, which corresponds to the selectivity filter. This region contains residues belonging to the potassium channel selectivity sequence (see section 1.3.3.2.1.), which have previously been demonstrated to be important in channel selectivity (e.g. Yool and Schwarz, 1991, Heginbotham et al, 1994). One surprising feature of the structure was that the side chains of amino acids contributing to the selectivity filter were in fact pointing away from the pore, meaning that the passage of K\textsuperscript{+} ions through the filter is in fact co-ordinated by the backbone carbonyls oxygens of the main chain atoms. These oxygens line the central pore axis to form a stack of oxygen rings separated by 3 - 4 Å (Choe and Robinson, 1998). The idea of a rigidity to the structure of the selectivity filter was also suggested by the crystal structure of KcsA. Positioned around the selectivity filter is a 'cuff' of aromatic amino acids which through hydrogen bonds with each other and surrounding residues produces a fixed structure. This organisation may allow the backbone carbonyl oxygens of the filter to be held in just the right position to only successfully co-ordinate ions of the same radius as K\textsuperscript{+}.

1.3.3. Potassium channel classification.

The diversity of potassium channel types is necessary to shape the electrical activity of cells in different tissues and the heterogeneity of potassium channel function is reflected in part by the large number of potassium channel genes (Xu et al, 1995). Over seventy potassium channels genes have been identified so far, however the total number of potassium channel proteins is even larger since many of these genes undergo RNA processing, such as alternative splicing resulting in multiple protein products from a single gene (Coetzee et al, 1999). Potassium channels therefore are the largest and most diverse class of ion channel.
Potassium channels can be classified into one of three superfamilies according to the secondary structure of their proteins - those that have six transmembrane domains (TMDs), those that have four TMDs, and those with only two TMDs (see Figure 1.4).

**Figure 1.4.** Schematic representation of the three groups of K⁺ channel principal proteins. They are classified into three groups in terms of their predicted membrane topology - those that have six transmembrane domains (TMDs), those with four TMDs and those with only two TMDs. Each group of proteins is divided into discrete families on the basis of sequence similarity. Each family can be further subdivided into several subfamilies, which often contain several closely related subfamily members. A functional classification places the voltage- and Ca²⁺-regulated K⁺ channels in the 6TMD group, the 'leak' channels in the 4TMD group and the inward rectifier K⁺ (Kir) channels in the 2TMD group. Figure taken from Coetzee et al, 1999.

### 1.3.3.1. Six transmembrane domain superfamily.

This superfamily is the oldest and most well studied superfamily. Functionally potassium channels belonging to this superfamily form voltage and/or Ca²⁺ activated potassium channels when expressed in heterologous expression systems. This group contains the voltage-gated potassium channel family, Kv (with eight subfamilies Kv1
- Kv6 and Kv8 - Kv9), as well as members of the KCNQ, eag, SK and slo families (Coetzee et al, 1999).

Electrophysiological studies have shown that voltage-gated and Ca\(^{2+}\) activated potassium currents can be recorded from CGNs (e.g. Cull-Candy et al, 1989, Fagni et al, 1991).

1.3.3.1.1. Kv family.

This family is the largest family of potassium channels within the 6TMD superfamily, and is also the most well described. Genes belonging to this family encode proteins that are responsible for the generation of delayed rectifier and transient A-type potassium currents.

1.3.3.1.2. Delayed rectifier current.

In their study of the action potential of squid giant axons Hodgkin and Huxley described a potassium current that activated with a delay in response to a step change in the membrane potential, and was responsible for the repolarisation of the action potential. This current was outwardly rectifying and rose along an S-shaped curve to a steady state amplitude that was sustained throughout membrane depolarisation. Following repolarisation of the membrane the current decayed in an exponential manner. This classic current became known as the 'delayed rectifier' and is frequently abbreviated to \(I_{\text{Kv}}\).

Many neuronal cells contain potassium currents which can be defined as delayed rectifiers (Rudy, 1988). It would appear that these currents may represent a composite of multiple \(I_{\text{Kv}}\) currents, based on their separation through pharmacological differences (Dubois, 1981). The activation thresholds of these currents lie above that of the action potential, typically between -50 to -10 mV, although in a few instances they have been detected at more negative potentials (Rudy, 1988). Studies using cloned Kv potassium channel subunits, whose currents also correspond to those of delayed rectifiers, illustrate similar activation thresholds together with an equal array of differential sensitivities to various pharmacological agents (Chandy and Gutman,
1995, Dolly and Parcej, 1996). Such differences may help to resolve the contributions of different potassium currents within the same cell. The main function of $I_{Kv}$ is in the rapid repolarisation phase of the action potential. Because of its delayed activation, the outward flow of potassium ions during an action potential coincides with the inactivation of the inward sodium current. Thus, $I_{Kv}$ ensures that the action potential is kept brief (Hille, 1992).

1.3.3.1.3. Transient A-type current.

A fast transient outward potassium current ($I_A$) was originally described in molluscan neurons by Connor and Stephens (1971) and is now known to exist in numerous excitable tissues (Rudy, 1988). Like the delayed rectifier the A current is activated by depolarising voltage steps rising along a sigmoidal curve. However A current can clearly be distinguished from $I_{Kv}$ because of its rapid inactivation after only a few tens of milliseconds.

$I_A$ activates in the subthreshold range for action potential generation typically at potentials more positive to -65 mV, although $I_A$ channels can only be activated when a cell is depolarised after a period of hyperpolarisation. Inactivation is steeply voltage dependent with a mid-point near -70 mV and almost complete inactivation at membrane potentials positive to -50 mV. The hyperpolarisation following an action potential, provided by $I_{Kv}$, is enough to remove inactivation causing the transient outward current to arise, temporarily keeping the cell membrane hyperpolarised. Only after inactivation of $I_A$ can the potential begin to depolarise again, eventually leading to another action potential. In this way $I_A$ helps maintain interspike intervals, and thus dictates the firing frequency of a neuron.

The isolation of $K_v$ channel clones has allowed enormous progress in understanding the structure and molecular mechanisms of function of these potassium channels. Hydrophobicity analysis of $K_v$ gene products predict proteins with six transmembrane domains (S1 - S6) and a hydrophobic region named H5 (also known as the pore or P domain) between S5 and S6, which dips into the plane of the membrane from the extracellular side and lines the pore (Figure 1.5; Yool and Schwartz, 1991). The S4 segment contributes a major part of the voltage sensor, having a repeating pattern of
positively charged amino acids every three residues; this is also seen in Na\(^+\) and Ca\(^{2+}\) channels (Robertson, 1997).

\[ \text{Figure 1.5. Proposed membrane topology of an } \alpha \text{ subunit belonging to the } K_v \text{ family.} \]

Comparison of \( K_v \) gene products to those encoding Na\(^+\) and Ca\(^{2+}\) channels revealed that the potassium channel proteins were much shorter. Na\(^+\) and Ca\(^{2+}\) channels are composed of a single protein with four homologous (but not identical) domains, and \( K_v \) proteins resemble one of these four internally homologous repeats. Therefore by analogy with Na\(^+\) and Ca\(^{2+}\) channels it was predicted that functional \( K_v \) channels were formed by the assembly of four proteins (Tempel \textit{et al}, 1987). This model has been experimentally supported through careful analysis of the results of toxin binding experiments on wild type and mutant channels (MacKinnon, 1991). Constructs containing four potassium channel domains linked in tandem can produce functional channels (Liman \textit{et al}, 1992) also supporting the theory of tetramer formation. The pore forming proteins that determine the channel infrastructure are known as \( \alpha \) subunits.

Functional \( K_v \) channels can be formed from the co-assembly of four identical \( \alpha \) subunits (homomultimers) from either the \( K_v \)1, \( K_v \)2 \( K_v \)3 or \( K_v \)4 subfamilies. Each \( K_v \) gene encodes \( \alpha \) subunits with varying amino acid compositions resulting in homomeric channels with different pharmacological and physiological properties. This contributes only in part to the enormous diversity of potassium channels \textit{in vivo} since different \( \alpha \) subunits can co-assemble to form heteromultimeric channels which have hybrid properties (Christie \textit{et al}, 1990, Isacoff \textit{et al}, 1990, Ruppersberg, 1990).
The only condition for this mixing is that α subunits must belong to the same subfamily. The presence of a specific interaction or tetramerisation domain (TI) in the N terminus of an α subunit appears to be chiefly responsible for subunit associations (Li et al, 1992, Shen et al, 1993).

Given the large repertoire of α subunits together with differential combinations in functional tetramers, elucidating exactly which α subunits underlie native voltage-gated potassium currents seems a daunting task. Indeed, the first examples of purified native channels suggests that these combinations may involve up to four different α subunits in a single channel (Shamotienko et al, 1997).

Several α subunits are known to be expressed in CGNs, including Kv1.1 (Veh et al, 1995), Kv1.5 (Drewe et al, 1992), Kv2.2 (Hwang et al, 1992), Kv3.1 (Shibata et al, 1999), Kv4.2 (Shibata et al, 1999, Serodio and Rudy, 1998) and Kv4.3 (Serodio and Rudy, 1998). Therefore delayed rectifier and A-type currents in CGNs could be the result of several theoretical α subunit combinations.

Correlating native currents to cloned channels is further complicated by the presence of auxiliary β subunits, which can associate with tetrameric channel complexes, changing their kinetic properties (Rettig et al, 1994). An additional source of diversity comes in the form of modulatory α subunits. These α subunits form the Kv5-6 and Kv8-9 subfamilies. They are highly similar to other Kv α subunits by primary sequence, yet they are unable to form homomeric conducting channels in heterologous expression systems (Drewe et al, 1992, Hugnot et al, 1996, Salinas et al, 1997, Stocker and Kerschensteiner, 1998). Inclusion of these α subunits into previously homomeric channels results in heteromeric channels with altered characteristics in comparison to homomeric channels (e.g. Kerschensteiner and Stocker, 1999).

1.3.3.1.4. Slo family.

Calcium activated K⁺ channels were also first described in molluscan neurons (Meech and Strumwaser, 1970) and have since been found in virtually every cell type. Action
potentials in neurons are followed by a hyperpolarisation which can last for up to several seconds. This hyperpolarisation has several phases that are mediated by the activation of different types of Ca\(^{2+}\) activated K\(^+\) currents. Patch clamp studies have revealed two families of K\(_{ca}\) channels, of small (SK\(_{ca}\)) and high (BK\(_{ca}\)) conductance. Activation of BK\(_{ca}\) channels contributes to action potential repolarisation, while SK\(_{ca}\) channels are thought to underlie the slow afterhyperpolarisation (sAHP). Therefore these types of potassium channels are fundamental regulators of neuronal excitability, participating in interspike interval and spike frequency adaptation (Vergara et al., 1998).

CGNs possess a K\(^+\) current which is dependent on intracellular Ca\(^{2+}\) levels, corresponding to a BK\(_{ca}\) conductance (Fagni et al., 1991).

The BK\(_{ca}\) channel is so called because it has a large single channel conductance. Single channel recordings using equal potassium concentrations on either side of the patch have revealed channel conductances of 250 pS (Vergara et al., 1998). Channels are activated by the concerted influences of membrane depolarisation and increased levels of intracellular Ca\(^{2+}\). At membrane potentials near to the resting membrane potential these channels require 1 - 10 \(\mu\)M Ca\(^{2+}\) for activation (Sah, 1996).

Functionally activation of BK\(_{ca}\) contributes to action potential repolarisation and fast AHPs. By hyperpolarising the membrane voltage in close proximity to Ca\(^{2+}\) channels BK\(_{ca}\) channels are thought of as feedback modulators of the activity of voltage-gated Ca\(^{2+}\) channels. In some neurons this may allow BK\(_{ca}\) channels to regulate the amount of neurotransmitter released from presynaptic terminals by modulating the duration of the presynaptic action potential. BK\(_{ca}\) channels are blocked by submillimolar concentrations of TEA, and some are sensitive to the scorpion venom extract charybdotoxin (Sah, 1996).

BK\(_{ca}\) channel proteins are encoded by the Slo gene, \(\alpha\) subunits are encoded by a single gene with splice variants generating functional diversity (Tseng-Crank et al., 1994). As with the K\(_{v}\) family there is evidence that functional channels result from the coassembly of four \(\alpha\) subunits (Shen et al., 1994). The primary protein sequences
share a high degree of homology with the S1-S6 sequences of the 6TMD Kv channels. However BKca channels have an additional transmembrane segment (S0) leading to an externally located N terminus. The S0 segment together with the N terminus determines β subunit modulation (Meera et al., 1997). The C terminus is made up of four hydrophobic segments, S7-S10, which are most probably cytosolic (Vergara et al., 1998). The Ca$^{2+}$ binding domain is within this region.

**Figure 1.6.** Proposed membrane topology of an α subunit from the Slo family.

### 1.3.3.2. Two transmembrane domain superfamily.

This superfamily is the second major group of potassium channels to be identified. Functionally potassium channels belonging to this superfamily are responsible for inwardly rectifying potassium currents. Inward rectification means that at any given driving force (voltage) the inward flow of potassium ions is greater than the outward flow for the opposite driving force (Nichols and Loptain, 1997). Inward rectification was originally termed anomalous rectification because it is opposite to the 'normal' outward rectification that is seen in delayed rectifier potassium channels. Hence the conductance of inwardly rectifying potassium channels increases under hyperpolarisation and decreases under depolarisation.

Currently there are seven subfamilies, (Kir1-7), most of which form potassium channels with various degrees of inward rectification when expressed in heterologous expression systems (Coetzee et al., 1999). In terms of their function inward rectifiers
can play an important role in maintaining the cell resting potential due to the slight outward conductance of potassium ions at membrane potentials more positive to the potassium equilibrium potential.

Structurally potassium channel α subunits responsible for generating inwardly rectifying potassium currents are characterised by having two TMDs connected by a H5/P domain. Like members of the six TMD superfamily functional inwardly rectifying potassium channels are the result of tetramerisation (Yang et al, 1995).

![Figure 1.7. Proposed membrane topology of an α subunit belonging to the 2TMD superfamily.](image)


### 1.3.3.2.1. Potassium channel signature sequence.

Although the six TMD and four TMD superfamilies differ functionally the channels underlying these currents share the unifying feature of potassium channel selectivity. Given that this is the case it is reasonable to look for conservation at the primary sequence level which would explain the common ion selectivity. Figure 1.8 shows a partial sequence alignment comparing K⁺ channels from each of the above superfamilies, including voltage-gated, Ca²⁺ activated and inward rectifier K⁺ channels. The sequences being compared are taken from the H5/P domain region. A complete alignment would reveal that outside of this region there is little sequence
similarity between some channels however within the P domain all channels are highly homologous over a stretch of eight amino acids. This homologous region is known as the signature sequence (TXXTXGYG) and is found in all $K^+$ channels so far cloned.

![P REGION](image)

**Figure 1.8.** Sequence alignment of the P-region from different cloned $K^+$ channels. Residues identical to those in the Shaker (Sh) channel are highlighted in light grey, with the most highly conserved residues highlighted in dark grey. Kv1.1, 2.1, 3.1 and 4.1 encode mammalian voltage-gated $K^+$ channels. mSlo is a Ca$^{2+}$ activated channel from mouse, and eag is a cyclic nucleotide-regulated channel. AKT1 and KAT1 are plant inward rectifying $K^+$ channels. ROMK1 and IRK1 are inwardly rectifying $K^+$ channels from rat outer medulla and mouse cardiac tissue. GIRK1 is a $G$ protein modulated $K^+$ channel. Figure adapted from Heginbotham *et al.*, 1994.

It was using the potassium channel signature sequence to search gene databases that the third potassium channel superfamily was discovered.

1.3.3.3. Four transmembrane domain superfamily.

This superfamily is the most recently identified group of potassium channels (Ketchum *et al.*, 1995). Functionally these channels are potassium selective channels that do not appear to gate in a manner observed with channels formed by $K_v$ or Kir $\alpha$ subunits. Generally currents respond to changes in external potassium in a manner described by the Goldman-Hodgkin-Katz equation and these channels are therefore referred to as 'leak' channels.
Currently eight genes have been identified whose protein products when expressed alone in heterologous expression systems results in the formation of functional channels. Recent studies show that these channels can be extensively modulated (e.g. by arachindonic acid or pH), and channels are assigned to subfamilies according to this modulation. The properties of these leak currents are discussed in more detail in Chapter 5.

In mammals proteins belonging to this superfamily have four TMDs and are unusual as they have two P domains in tandem (P1 and P2), as if they consisted of two Kir subunits joined together. This superfamily is therefore commonly known as the two-pore domain superfamily or the K\textsubscript{T} family (K = K\textsuperscript{+}, T = 2-pore). Functional channels are thought to be a result of dimerisation of subunits, thereby retaining the fourfold symmetry around the central pore (Coetzee \textit{et al.}, 1999).

1.4. Aims.

In addition to I\textsubscript{KV}, I\textsubscript{A}, and BKC\textsubscript{a} potassium conductances, CGNs in primary culture have been shown to possess an additional component of outward K\textsuperscript{+} current (Watkins and Mathie, 1996). This current is outwardly rectifying, non-inactivating and a distinguishing feature of this potassium conductance is its modulation by activation of acetylcholine muscarinic receptors. The molecular nature of this current is undetermined and therefore the aim of this study was to try and identify the molecular correlate of IK\textsubscript{so}. A further aim was to elucidate the intracellular mechanism coupling muscarinic receptor activation to inhibition of IK\textsubscript{so}. 

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{image}
\caption{Proposed membrane topology of an \(\alpha\) subunit belonging to the 4TMD superfamily.}
\end{figure}
Chapter 3 is concerned with identifying the $K^+$ currents in CGNs. The presence of $I_{Kv}$, $I_A$ and $I_{K_{so}}$ are demonstrated, focussing specifically on the basic characteristics of $I_{K_{so}}$. Experiments concerned with the cellular mechanism underlying muscarinic modulation are presented here. Chapter 4 compares the properties of $I_{K_{so}}$ to those of the **ether à go go (eag)** family of $K^+$ channels to establish whether $I_{K_{so}}$ is an **eag** current in CGNs. Chapter 5 attempts a broad pharmacological characterisation of $I_{K_{so}}$ and tests its voltage dependence of activation to determine whether $I_{K_{so}}$ belongs to the $K_T$ superfamily of $K^+$ channels, and Chapter 6 describes some molecular biology experiments which demonstrate the presence of a specific $K_T$ gene in CGNs.
CHAPTER 2.

METHODS AND MATERIALS.
METHODS.

The aim of this chapter is to provide a theoretical background to the experimental techniques utilised during this study, as well as providing specific details of how individual experiments were undertaken.

2.1. Introduction to electrophysiology.

The voltage clamp technique allows ion flow across a cell membrane to be measured as an electric current whilst the membrane voltage is held under experimental control. This method was originally developed by Cole (1949) and Hodgkin et al (1952), for use with the squid giant axon. Since then many variants of the technique have evolved and voltage clamp analysis has been extended to a wide range of tissues.

The most widely used method of recording macroscopic currents is the whole cell patch clamp (WCPC) technique (Hamill et al, 1981), which is an extension of the patch clamp technique developed by Neher and Sakmann (1976). This method uses a single electrode to both clamp the membrane potential and measure current responses.

A successful electrophysiological recording depends on the formation of a high resistance seal between the tip of the recording electrode and the membrane of the cell under study. The electrical resistance is typically in the region of 10 GΩ; such seals are known as 'gigaseals'. The physical and electrical isolation of the membrane patch greatly improves signal to noise resolution of current recordings by an order of magnitude since thermal movement of charges through a low resistance seal is a source of additional noise in the recording.

Once a gigaseal has been formed this is known as the cell attached configuration and is useful for single channel recordings. From the cell attached configuration it is possible to rupture the isolated section of cell membrane under the pipette tip, without destroying the gigaseal, by applying suction to the pipette interior. This allows low resistance access to the cell interior and produces electrical and chemical continuity between the pipette and the cell. Current flowing through all the open channels of the
whole cell membrane can now be recorded, hence this configuration is the whole cell configuration of the patch clamp technique (Hamill et al, 1981).

A consequence of whole cell recording is the equilibration of the cell cytoplasm with the pipette solution, removing any intracellular constituents which may be required for maintenance or modulation of membrane currents. This problem can be overcome using a variation of the WCPC technique, known as perforated patch.

2.1.1. Perforated patch whole cell recording.

The perforated patch configuration is obtained by introducing a pore forming substance, such as the antibiotic amphotericin B, into the pipette solution (Rae et al, 1991). This antibiotic incorporates into the patch of membrane under the tip of the electrode, forming membrane spanning channels which allow the movement across the membrane of monovalent ions or uncharged molecules which are no larger than approximately 0.8 nm. Since these channels are not permeant to molecules as large or larger than glucose, whole cell recording can be performed without disturbing the intracellular environment. As the number of channels perforating the cell membrane increases, the resistance of the isolated cell membrane patch is systematically reduced. This eventually allows electrical access to the interior of the cell, enabling manipulation of the cell voltage.

It was essential to use the perforated patch technique in this study since it has been shown that $I_{K_{so}}$ in cerebellar granule neurons (CGNs) displays rundown under conventional whole cell recording. The inclusion of amphotericin B in the internal solution allows the stable recording of $I_{K_{so}}$ for several tens of minutes without any decline in its magnitude (Watkins and Mathie, 1996).

2.2. Principles of the patch clamp recording technique.

The patch clamp technique allows accurate measurement of cellular currents, while simultaneously adjusting the pipette potential, therefore clamping the cell membrane at a required voltage.
To understand why the potential has to be adjusted, consider that a K⁺ channel in the membrane opens. Assuming the cell membrane potential is positive to the potassium equilibrium potential, K⁺ ions would flow out of the cell from high to low concentrations. The movement of K⁺ out of the cell and the corresponding net loss of positive charge would alter the membrane potential making it more negative with respect to the outside. If the pipette potential was not adjusted it would 'follow' the membrane potential. While such a technique is useful for measuring the membrane potential, it does not allow the measurement of currents produced at a constant voltage. To study currents at a constant potential, additional circuitry is required to counteract the changes in the membrane potential that normally accompany ionic flux. The basic requirement of the patch clamp technique is a resistance feedback circuit. This element of the patch clamp circuit is illustrated in Figure 2.1.

In very basic terms, when an operational amplifier in the headstage, A, is connected as shown in Figure 2.1, with a high megaohm resistor R_f (f = feedback), a current-to-voltage converter is obtained.

The pipette potential (V_p) is connected to the inverting input (-) of the amplifier and the desired potential, known as the command potential (V_cmd) is connected to the non-inverting input (+).

The properties of the operational amplifier and the circuit mean that the amplifier forces the potential at the inverting input to follow the potential at the non-inverting input (i.e. V_p = V_cmd).
Consider that $V_{cmd}$ is set to a potential more positive than the zero current potential of a cell, this will activate channels in the membrane resulting in current flow through the patch ($i_p$). When current flows across the cell membrane the membrane potential will change and the inputs to A will be different. To prevent this from happening the amplifier injects a current through $R_f$ ($i_0$) which will be equal but opposite to $i_p$, effectively cancelling it out, returning $V_p$ to $V_{cmd}$. In this way the currents flowing in response to a step change in $V_{cmd}$ can be measured while at the same time clamping $V_p$ at a constant value.

Associated with the patch clamp technique are potential sources of error. It is essential to appreciate the existence of such errors so that results can be correctly interpreted. The electronics of the patch clamp amplifier enables the measurement of some of these errors, and using additional circuitry some of these errors can be compensated for, enabling more accurate current recording.

2.2.1. Capacitance compensation.

The pipette and cell membrane act as capacitors since they are thin insulators between conducting solutions. In WCPC recordings the capacitance of the pipette ($C_{pip}$) and cell membrane ($C_{mem}$) must be charged in order to change the membrane potential. Charging of the pipette and membrane are often seen as capacitance spikes on the current traces during WCPC recordings, which if not compensated for can distort fast ionic currents.

Both $C_{pip}$ and $C_{mem}$ can be measured using the fast and slow compensation controls respectively. Once the compensation has been applied the current required to charge the capacitance is removed from the current trace and only steady state current remains.

2.2.2. Series resistance.

When cellular currents measured by the patch clamp amplifier are inverted and injected via the pipette to clamp the membrane potential at $V_{cmd}$, a resistance to flow of this current ($i_0$) exists, which is known as the series resistance ($R_s$).
Rs is the sum of $R_{\text{access}}$ and $R_{\text{external}}$. $R_{\text{access}}$ is composed of the pipette resistance plus any residual resistance of the ruptured/perforated patch, and $R_{\text{external}}$ is the resistance of the bath electrode and the electrolyte solution to the flow of the current from the pipette to the bath electrode. Typical Rs values following rupture of the patch are at least twice the original pipette resistance, so decreasing the resistance of the pipette tip by using wide pipettes is one way of reducing $R_s$. There is a trade off between having a pipette diameter small enough to allow frequent seal formation but large enough not to contribute to a large $R_s$.

Since $i_f$ has to pass across $R_s$ to clamp the cell, a voltage drop ($V_{\text{drop}}$) will occur, meaning the cell will not be clamped at the true $V_{\text{cmd}}$, but at a potential $V_{\text{cmd}} - V_{\text{drop}}$, where $V_{\text{drop}}$ is equal to $i_fR_s$. For example, consider a cell whose $R_s = 10 \, \text{M}\Omega$. When membrane potential is stepped to $V_{\text{cmd}} = -40 \, \text{mV}$, the current flowing at this potential is measured as 1 nA. In this case $V_{\text{drop}} = 10 \, \text{mV}$. So rather than being held at -40 mV the cell is actually being held at -50 mV, since $i_f$ is not large enough to clamp the membrane potential at $V_{\text{cmd}}$. Therefore for large currents and a large uncompensated $R_s$ the loss of voltage clamp that occurs can seriously limit the accuracy of voltage control in WCPC recordings. Therefore the effect of $R_s$ must be taken into consideration when interpreting the results as the currents recorded may not be an accurate estimate of the currents that would be activated by the 'true' $V_{\text{cmd}}$.

An additional consequence of large $R_s$ values is a slowing of the change in cell membrane potential in response to a step change in $V_{\text{cmd}}$. Following a step change in $V_{\text{cmd}}$, the membrane potential of the cell does not change instantaneously but with an exponential time course, whose time constant is given by $\tau = R_sC_{\text{mem}}$.

Electronic techniques exist for compensating the errors associated with $R_s$, but the compensation is never perfect. At best 80-90 % compensation is achieved resulting in acceptable levels of error in most cases. The series resistance was uncompensated in this study.
2.2.3. Space clamp.

When manipulating the membrane potential using patch clamp techniques, an investigator assumes that the whole of the cell membrane is maintained at the same potential as \( V_{cmd} \), i.e. the cell membrane is isopotential. This assumption however only holds for small spherical cells. In large cells or those with many arborisations the potential of the whole cell membrane in response to a command voltage will not be identical throughout the cell, but will decay exponentially with distance from the point of current injection.

In electronics the point in a cable where the voltage produced following an injection of current falls to half its original level is termed the 'electrical half-distance', or length constant. Therefore the membrane potential of the soma may be well controlled, while that of the axons or dendrites could differ substantially from \( V_{cmd} \) if the length of these processes greatly exceeds the electrical half distance. Space clamp problems therefore provide potential sources of error when making whole cell recordings since they cannot be compensated for.

The experiments described in this thesis were performed using primary cultures of CGNs. Characteristically these cells are small and spherical (<10 \( \mu \)m diameter) making them ideal for use in electrophysiological investigations since errors due to inadequate space clamp will be negligible.

2.2.4. Liquid junction potentials.

In patch clamp recording, the choice of experimental solutions is often dictated by the individual demands of the experiment. Usually this involves using pipette and bath solutions of different ionic concentrations. When these two solutions come into contact a potential difference will occur at their interface which is known as the junction potential \( (L_j) \), and is a result of the different solute concentrations and ionic mobilities. During patch clamp recording it is standard practice to zero the amplifier, and hence the junction potential, prior to seal formation. The junction potential is balanced by application of an equal but opposite potential provided by the amplifier.
Once the pipette is sealed against the membrane the junction potential is no longer present since there is now no interface between the pipette solution and the bath solution. The potential used to balance the junction potential however is still being applied by the amplifier, distorting the value of any $V_{cmd}$. This means any current measured is not produced by $V_{cmd}$ but by $V_{cmd} + L_j$. To accurately determine the potential being applied to the membrane the value of $L_j$ must be subtracted from $V_{cmd}$.

Liquid junction potentials can be measured experimentally or they can be calculated. Once the magnitude and polarity of the junction potential has been determined it can be corrected for at the time of the experiment, or at some point during analysis.

The method used to measure the liquid junction potential experimentally has been described by Neher (1992). Briefly, the recording bath and pipette are filled with internal solution, the amplifier switched to current clamp mode and the voltage output zeroed. The bath solution is then exchanged for the control external solution and the voltage output once stabilised is noted. Following this the bath solution is switched back to internal solution to check for reversibility. The value obtained for the liquid junction potential can be used for correction of the applied membrane potential. To accurately measure liquid junction potentials experimentally takes preparation since certain conditions need to be fulfilled (see Neher, 1992), and therefore it is often simpler to calculate the liquid junction potential using the generalised Henderson equation (see Barry and Lynch, 1991). This method was used to calculate liquid junction potentials generated between solutions used in the experiments described in this thesis. In most cases $L_j \leq 4$ mV, therefore results are presented without liquid junction potential compensation.

### 2.3. The Reverse Transcription – Polymerase Chain Reaction (RT-PCR).

Before a protein can be expressed in a cell the gene encoding that protein needs to be transcribed into mRNA and then this mRNA is translated into a sequence of amino acids that make up the protein. Therefore, if a cell is expressing a protein the mRNA encoding that protein will be present in the cytoplasm.
RT-PCR is a very sensitive way of detecting the presence of specific mRNA; it is an extension of the PCR method (Mullis, 1990). In RT-PCR, mRNA acting as the starting template is reverse transcribed into single stranded cDNA (RT step). Most mRNA species are present at low levels (5 - 15 molecules per cell), meaning that RT will in turn produce low levels of cDNA. This is amplified many times to give a detectable signal (PCR step).

The RT step of the process requires mRNA from the cells of interest to be isolated. In practice the total RNA of a cell can be obtained and used in the RT step since it is not necessary to obtain pure mRNA. It is necessary however to DNase treat the sample at this point to remove any genomic DNA which may cause contamination at a later stage. mRNA is then incubated with a reverse transcription enzyme (e.g. Moloney murine leukemia virus reverse transcriptase, MMLVRT), a reverse primer and the four deoxyribonucleoside triphosphates. The primers bind to complementary mRNA and this is transcribed into cDNA using the mRNA as a template. A choice of reverse primers can be used, random hexamer primers, oligo d(T) primers or sequence specific primers. The advantage of random hexamer and oligo d(T) primers is that they are not specific to any sequence, meaning the RT step will result in a mixture of cDNA species. This cDNA mix, produced from only one RT reaction, can subsequently be used in several PCR reactions to amplify cDNA of any target sequence, providing specific primers are used at this stage.

All mRNA species undergo a post translational modification known as polyadenylation, which is the addition of a length of adenosine bases to the 3' end of the mRNA. Oligo d(T) primers are a length of thymine bases which are complementary to this sequence and so will bind to all species of mRNA, producing a mixture of cDNA during the RT step. However because extension of an oligo d(T) primer always begins at the 3' end of mRNA, in practice, this end is preferentially reverse transcribed since reverse transcription often terminates prematurely. Therefore oligo d(T) primers are best used if the target sequence is relatively close to the polyadenylation site of mRNA.

Random hexamer primers, as the name suggests, are random six base sequences that will bind to mRNA with the complementary sequence. This sequence can occur at
any point along the length of the mRNA meaning that no one region of mRNA will be preferentially reverse transcribed, eliminating the problem associated with oligo
d(T) primers.

For the experiments presented in this study random hexamer primers were used. This produced cDNA of many mRNA species and the cDNA of interest, TASK-1, was then amplified using sequence specific primers in the PCR step.

Once cDNA has been made it is amplified by the three step process of PCR. PCR requires large amounts of the four deoxyribonucleoside triphosphates, a thermostable DNA polymerase and a pair of specific primers. Primers are oligonucleotides that are approximately twenty nucleotides long. These oligonucleotides are complementary to sequences that lie on opposite strands of the template DNA. Primers flank the segment of DNA that is to be amplified. The major product of PCR is the segment of double-stranded (ds) DNA whose termini are defined by the 5' termini of the primers, and whose length is defined by the distance between the primers. Primers are designed to have approximately the same guanine (G) and cytosine (C) content so that they anneal at the same temperature.

A diagrammatic illustration of the PCR method is shown in Figure 2.2. The first step of PCR is to denature the ds DNA by heating, to separate it into single strands (cycle 2, step 1, Figure 2.2b). The second step reduces the temperature allowing the primers to anneal to their complementary sequences on the DNA strands (cycle 2, step 2, Figure 2.2b). The third and final step is the extension of the annealed primers by the DNA polymerase to yield a complementary second strand of DNA (cycle 2, step 3, Figure 2.2b).

At the end of the RT step the product is single-stranded (ss) DNA, therefore the first cycle of the PCR reaction is atypical (Figure 2.2a). Although there is no ds DNA present at this point, a denaturing step is still included to ensure that all the ss DNA is linear, i.e. any hair pin loops or small regions of self annealing are removed by denaturing allowing the specific primers optimal access. The products of the first cycle are DNA molecules that are double stranded for some of their length, meaning that in all subsequent cycles the denaturing step also separates this into single strands.
Figure 2.2. Schematic representation of the polymerase chain reaction.
a). Cycle 1. Step 1. ss DNA molecule containing target sequence (black box) is denatured by heating, to linearise. Step 2. Temperature is lowered and primer (\( ^\ast \)) anneals to its complementary sequence on the cDNA. Step 3. Temperature is raised slightly and the primer is extended by the polymerase. Formation of new DNA (hatched boxes) occurs in the 5' to 3' direction.
b). Cycle 2. Product of cycle 1 is ds DNA. In cycle 2 the denaturing step separates the ds DNA molecule into two single strands of DNA. Both primers (\( ^\ast \) and \( \lambda \)) anneal to their specific complementary sequences and are extended by the polymerase. Extension in this cycle results in the formation of the first ss DNA of the required length. This three step process is repeated up to forty times.
c). Cycle 3. Specific ss DNA acts as a template resulting in the formation of the first ds DNA molecule of the required length (specific DNA products). From this point, with each cycle, the number of specific DNA products increases exponentially while the number of heterogenous DNA increases linearly.
The products of a successful first cycle of amplification are heterogeneously sized DNA molecules, whose lengths may exceed the distance between the binding sites of the two primers (cycle 1, Figure 2.2a). In the second cycle these molecules act as templates and generate ss DNAs of defined length (cycle 2, Figure 2.2b), which in turn act as templates themselves in the third cycle producing specific ds DNA products (cycle 3, Figure 2.2c). From this stage onwards there is an exponential growth in the amount of copies of specific ds DNA, which will continue until the amount of template exceeds that which the polymerase can extend in the time provided. In this way targeted DNA will form the dominant product of the reaction. It should be noted however that longer molecules will continue to be produced from the original template DNA during every cycle. These products will accumulate only at a linear rate and therefore will not contribute significantly to the final mass of the target sequences.

The cycle of denaturation, annealing and extension is then repeated twenty to forty times, resulting in the synthesis of millions of copies of the targeted DNA fragment.

2.3.1. Optimisation of experimental conditions for RT-PCR.

RT-PCR is a very sensitive technique for producing large amounts of a specific DNA fragment. Each reaction is unique and optimal conditions vary depending on the primers and DNA polymerase used.

The temperature for each step in a PCR cycle can be optimised. The denaturing temperature can be raised slightly; the higher the temperature the more complete will be the denaturation of the DNA allowing primers optimal access for annealing. This is particularly important if target DNA is GC rich since a high GC content leads to stronger hybridisation between DNA strands and therefore requires a higher denaturing temperature. However raising the temperature too high will deactivate the polymerase enzyme, meaning no amplification will take place.

The annealing temperature can also be optimised for each set of primers. The ideal temperature is low enough to enable hybridisation between primer and template, but is high enough to prevent mismatched hybrids from forming. This temperature can
be estimated by determining the melting temperature ($T_m$) of the primer-template hybrid. The $T_m$ is the temperature at which the correctly base-paired hybrid dissociates; a temperature 1-2 °C below this should be low enough to allow the correct primer-template hybrid to form but too high for a hybrid with a single mismatch to be stable. The $T_m$ can be calculated from the formula:

$$T_m = (4 \times [G + C]) + (2 \times [A + T]) \degree C$$

$[G + C] =$ number of guanine (G) and cytosine (C) nucleotides in primer sequence.

$[A + T] =$ number of adenine (A) and thymine (T) nucleotides in primer sequence.

Higher GC content allows a higher annealing temperature resulting in a high stringency PCR reaction. The $T_m$ for all four primers for TASK-1 was calculated to be 62 °C, therefore an annealing temperature of 60 °C was used in the PCR.

Finally, the optimum temperature for extension of the primers is determined by the DNA polymerase used. In the experiments presented here Taq DNA polymerase or Pfx DNA polymerase were used, which have optimum temperatures of 72 and 68 °C respectively.

Although not altered here Mg$^{2+}$ concentration can also be varied to optimise the PCR conditions. DNA polymerase enzymes require free Mg$^{2+}$ ions for activity, however nucleotides chelate divalent cations so the Mg$^{2+}$ concentration in PCR reactions must be a few millimolar units higher than the nucleotide concentration. Mg$^{2+}$ and other salts also influence the hybridisation of primers to the template DNA. Some workers prefer to modify the Mg$^{2+}$ concentration, rather than the annealing temperature, to regulate primer specificity (Kocher and Wilson, 1991).
2.4. Preparation of neonatal rat cerebellar granule neurons.

For electrophysiological studies cultured cells are of particular value. Access to individual cells, which are readily identifiable, is uncomplicated by the presence of non-neuronal tissue and cell debris that needs to be removed to expose cells for study in slices. Environmental conditions are readily altered by exchanging the extracellular media bathing the cells, and the cells can be kept healthy for several days reducing the use of animals.

Successful culturing of cells in vitro requires the use of sterile techniques. All tissue culture protocols were carried out in a horizontal laminar air-flow bench (BASSAIRE, Southampton, U.K.), which was swabbed with 70% ethanol before and after use. All metalware used in tissue manipulation was treated with 70% ethanol and heat sterilised, and sterile plasticware and pipettes were always used. Similarly all tissue culture solutions were filter sterilised using a 0.22 μm filter unit.

Once isolated, CGNs were plated on to pre-prepared 13 mm diameter glass coverslips. The coverslips were sterilised with 100% ethanol before incubating in a solution of poly-L-lysine hydrobromide, prepared at a concentration of 15 mg l⁻¹ in sterile water, for at least 12 hours at room temperature. The coverslips were rinsed twice with sterile water and allowed to dry before use.

2.4.1. Tissue culture protocol.

CGNs were isolated from neonatal rats (Sprague-Dawley, 6-9 days old, of either sex) and cultured essentially as described by Wilkin et al (1976) with several modifications.

Animals were decapitated and the head ventral surface down, was firmly pinned to a Sylgard-lined petri dish. Using fine scissors the skin on the dorsal surface of the head was bisected along the mid-line, reflected away from the skull and secured with pins. The tip of the scissors was inserted into the skull at the point where the plates fuse and the skull was cut laterally to the left and right. The caudal plate was then bisected
down the midline, taking care not to damage the underlying brain tissue, and removal of this structure exposed the cerebellum.

Using a pair of curved forceps the cerebellum was removed as a whole and the tissue was placed in a 35 mm petri dish with a small volume of enriched phosphate buffered saline (PBS) solution which contained: 0.32 % MgSO\textsubscript{4}, 0.25 % glucose and 0.3 % bovine serum albumin (BSA).

The meningeal layers and connective tissue were dissected away as fully as possible and the cerebellum was then cross chopped (200 × 200 µm) using a McIlwain tissue chopper (Mickle Laboratory Engineering Company, U.K.). The cross chopped tissue was then transferred to a 50 ml Falcon tube where it was trypsinised for 20 mins at 37 °C in 10 ml of enriched PBS solution containing 0.025 % trypsin.

Proteolysis was terminated with the addition of 10 ml of enriched PBS solution containing 1.78 mM MgSO\textsubscript{4}, 8 µg ml\textsuperscript{-1} soyabean trypsin inhibitor (SBTI) and 10.24 Kunits ml\textsuperscript{-1} deoxyribose nucleotidase (DNase). The resulting suspension was agitated to prevent clumping and then centrifuged at 1200 rpm for 3 mins to pellet the cells (Dissociated cells tend to re-aggregate to form clumps as a result of chromatin release from damaged cells. DNase is therefore present to minimise this effect). The supernatant was discarded and the cells were resuspended and further dissociated by trituration through a fire polished Pasteur pipette in 5 ml of enriched PBS solution containing 3 mM MgSO\textsubscript{4}, 50 µg ml\textsuperscript{-1} SBTI and 64 Kunits ml\textsuperscript{-1} DNase, until tissue clumps disappeared. The resulting milky cell suspension was then transferred to a 15 ml Falcon tube and underlined with 2 ml of 4 % BSA in Earle's salt solution (g l\textsuperscript{-1}: 6.8 NaCl, 2.2 NaHCO\textsubscript{3}, 0.158 NaH\textsubscript{2}PO\textsubscript{4}, 0.4 KCl, 1 D-glucose, 0.01 phenol red).

Centrifugation at 1500 rpm for 5 mins removed cell debris, the supernatant was discarded and the pelleted cells were resuspended by gentle trituration through a sterile P1000 tip in 4-5 ml of minimum essential media (MEM: 2.5 % chick embryo extract, 39 mM D-glucose, 2 mM glutamine, 25 mM KCl, 50 IU penicillin and 50 µg ml\textsuperscript{-1} streptomycin) supplemented with 10 % foetal calf serum (FCS). The high concentration of potassium in the medium maintains the cells in a state of partial depolarisation. This increases calcium entry, resulting in a higher steady state
concentration of cell calcium, which has been proposed to be the mechanism of survival of granule cells in culture (Gallo et al, 1987).

80 µl aliquots of the cell suspension were allowed to settle on to pre-prepared coverslips in a four-well plate. The cells were then maintained overnight in a humidified atmosphere of 5 % CO₂ at 37 °C before coverslips were flooded with 500 µl of growth media (MEM + 10 % FCS). All electrophysiological experiments were carried out on cells that had been in culture for 6-14 days.

![Figure 2.3](image.png)

**Figure 2.3.** A phase contrast image of a typical field of CGNs. Individual neurons are surrounded by a bright halo. In electrophysiological experiments cells which were isolated and had a smooth, spherical appearance were used for recordings.

### 2.5. Electrophysiological recording.

All the data presented were recorded at room temperature (21-23 °C) using the perforated patch clamp technique. Patch clamp recordings were obtained using an Axopatch 1-D amplifier (Axon Instruments). Patch electrodes were mounted in a pipette holder connected to a pre-amplifier (CV - 4; gain ×1/100 headstage, Axon Instruments). The headstage was connected to a manual micromanipulator (Narishige). CGNs were visualised using an upright microscope with Nomarski
optics using a 40x water immersion objective (Zeiss). Electrostatic screening to prevent external electrical interference was achieved using a Faraday cage and by ensuring all equipment was connected to ground. To achieve mechanical stability and reduce vibration the microscope, headstage and micromanipulator were situated on an airtable (Technical Manufacturing Corporation).

Voltage protocols and data acquisition were controlled by a Gateway 2000 4DX-33 computer through a DigiData 1200 interface (Axon Instruments) using the pCLAMP 6.0.2 software package (Axon Instruments). Currents were low pass filtered at 5 kHz before sampling and capture on line.

Measurements of whole cell capacitance and series resistance gave mean values of 4.46 ± 0.14 pF and 20.75 ± 0.92 MΩ (n = 153) respectively.

The culture technique described results in a population of cells of which approximately 95 % have been shown to be CGNs (Thangnipon et al, 1983). Neurons were identified based on healthy morphological features such as their small size, spherical shape, smooth somatic appearance and firm adhesion to the underlying coverslip. Cells with this morphology do not show labelling with GFAP, which is a marker for glial cells (see Figure 6.8), additionally the small capacitance of the identified cells is diagnostic of them being granule neurons. Previous work confirms cells identified in this way are indeed neurons. Na current, although small, is present in these cells (e.g. Hirano et al, 1986, Cull-Candy et al, 1989) and excitability has been demonstrated in current clamp experiments where depolarising current pulse injections result in the firing of action potentials (Watkins and Mathie, 1996).

2.5.1. Electrode manufacture.

Pipette fabrication is critical for gigaseal formation and good WCPC recordings. Pipettes with smooth, clean tips of only a few microns in diameter are necessary. Patch pipettes used during this thesis for recording whole-cell currents and membrane potential were fabricated from thick walled borosilicate glass capillary tubing (o.d. 1.4-1.6 mm, i.d. 0.8-1.0 mm, with 2 mm fibre; Plowden and Thompson). All pipettes were pulled on the day of the experiment using a vertical two stage Narashige PC-10,
a LIST medical L/M-3P-A micropipette puller, or a Flaming/Brown P-87 (Sutter Instruments Co.) micropipette puller. Pipettes were firepolished to the desired shape and resistance using a Narashige MF-83 microforge. The resistance of filled pipettes was approximately 10 MΩ.

2.5.2. Composition of pipette solution.

The pipette solution used was a KCl based solution, the composition of which was (in mM): 125 KCl, 5 4-(2-hydroxyethyl)-1-piperazinethanesulphonic acid (HEPES), 5 MgCl₂, and 0.1 bis (2-aminophenoxy)-ethane-N, N', N' tetraacetate (BAPTA); titrated with KOH to pH 7.4. Solution was filtered using a 0.22 μm filter unit and warmed to room temperature prior to use. When not in use solution was kept refrigerated.

To enable perforated patch recordings to be made the pipette solution was supplemented with 320 μg ml⁻¹ of amphotericin B. A stock solution of amphotericin B was made up in DMSO to a concentration of 8 mg ml⁻¹. Once made the stock solution was then stored at -20 °C and if made up at the beginning of the week remained potent for the duration of that week.

To obtain amphotericin B supplemented pipette solution for use in recordings, 8 μl of the stock solution was added to 2 ml of filtered pipette solution, giving a final concentration of 320 μg ml⁻¹. This solution was stored in the dark at room temperature, as amphotericin B is light sensitive, and used within 4 hours. Once the amphotericin has been added to the pipette solution it cannot be filtered as the amphotericin B molecule will not pass through a 0.22 μm filter unit.

2.5.3. Composition of external solution.

The control external solution was composed of (in mM): 120 NaCl, 2.5 KCl, 2 MgCl₂, 0.5 CaCl₂, 10 HEPES and 5 D-glucose; titrated with NaOH to pH 7.4 for control solution, or to pH 6.4, 6.9, 7.9 as required. External solution was made up on alternate days and kept refrigerated when not in use.
In all experiments where the ionic composition of the external solution was altered, the solution was prepared by equimolar replacement of NaCl. In N-methyl-D-glucamine (NMDG) solutions all NaCl was replaced and the osmolarity of the solution altered to approximately 310 mosm with sucrose.

Once cells had been clamped they were continually perfused with control external solution. The perfusion system consisted of six 50 ml reservoirs from which solutions of differing composition flowed under gravity, into the bath. The choice of solution was controlled using a multiway tap. The flow of solution was maintained at approximately 4-5 ml min\(^{-1}\), with the fluid level kept constant by a suck off tube operated by a vacuum pump.

2.5.4. Experimental protocol.

The following method was routinely used to enable perforated patch whole cell recording from CGNs:

2.5.4.1. Seal formation

The formation of a seal was visualised by monitoring the resistance of the recording electrode.
- a glass electrode was backfilled with pipette solution supplemented with amphotericin B.
- a 5 mV voltage step was applied to the interior of the pipette, which when lowered in to the bath, produced a square current pulse allowing the resistance of the pipette to be calculated using Ohm's Law. A typical value for the resistance of the pipettes when filled with electrolyte in response to the 5 mV test pulse was 10 MΩ.
- pipette was advanced towards the cell and contact of the pipette with the cell membrane was characterised by a sudden increase in tip resistance, seen as a decrease in current amplitude of the test pulse.
- application of gentle suction to the interior of the pipette then drew a Ω shaped section of cell membrane into the lumen of the pipette and seal formation was
visualised as a dramatic increase in the tip resistance, such that virtually no current could pass between the pipette and bath electrode. This is because the distance between the cell membrane and the rim of the pipette had fallen to around 1 Å and the electrical resistance was typically in the region of 10 GΩ.

2.5.4.2. Whole cell access.

- the pipette potential was then set at -60 mV so that when access was achieved the membrane potential was clamped at a potential close to the resting membrane potential of the cell. At this point a small hyperpolarising voltage step produced capacitive transients, due to the patch pipette being charged up, and could be eliminated using the fast capacity compensation circuitry built into the patch clamp amplifier.

- amphotericin B became incorporated into the membrane and access began to occur, appearing usually within 2 - 4 mins, as development of larger wider capacitive transients due to the cell membrane being charged up. The time course of these transients are increasingly rapid as the patch becomes perforated. These transients could be eliminated using the slow capacity compensation circuitry. Commonly access was also visualised by applying a protocol for detecting K⁺ current. In this case the development of membrane current was a measure of access. As access to the cell improved currents could be seen to gradually increase in magnitude, until the access resistance reaches its final value. At this point experiments were performed providing the access resistance was not unusually high.

2.5.4.3. Voltage protocols.

The following voltage protocols were used in the experiments:

1). Records of IKs0 were obtained by holding the membrane potential at -20 mV and stepping to a potential of -60 mV for 800 ms before returning to the holding potential. The currents were sampled at 500 Hz and the inter-episode duration was 5 s.
2). To obtain a current-voltage relationship for $I_{K_{so}}$ the membrane potential was held at -30 mV and the potential was stepped to -90 mV for 1 s. The potential was then stepped to test potentials ranging from -80 to +10 mV in a series of 10 mV steps lasting 1 s. The membrane was then returned to the holding potential. The currents were sampled at 400 Hz and the inter-episode duration was 8 s.

3). An alternative protocol used to obtain a current-voltage relationship for $I_{K_{so}}$ was a ramp protocol. Cells were held at -20 mV and ramped slowly to -100 mV in 800 ms (10 ms mV$^{-1}$), before returning to the holding potential. The currents were sampled at 1 kHz and inter-episode duration was 5 s.

4). $I_A$ was evoked using a double pulse protocol. Cells were held at -30 mV and a 500 ms hyperpolarising pre-pulse to -120 mV was applied, before stepping to a constant test pulse of 0 mV for 400 ms. Cells were then returned to the holding potential. The currents were sampled at 500 Hz and inter-episode duration was 5 s.

5). The time course of removal of inactivation of $I_A$ was also investigated using a double pulse protocol. Cells were held at -50 mV, and a hyperpolarising pre-pulse to -120 mV of variable duration was applied, before stepping to a constant test pulse of 0 mV for 200 ms. Conditioning pre-pulses to -120 mV were applied for durations ranging from 1 to 121 ms, prolonged in 5 ms increments. The currents were sampled at 1 kHz and inter-episode duration was 5 s.

6). $I_{kv}$ was evoked by holding cells at -70 mV and stepping to -50 mV for 30 ms, to inactivate any residual $I_A$. Cells were then depolarised to a test potential of +10 mV for 150 ms before stepping back first to -50 mV for 30 ms and then to the holding potential. The currents were sampled at 2 kHz and the inter-episode duration was 6 s.

7). To obtain a current-voltage relationship for $I_{kv}$ protocol 6 was adapted, so that the test potential began at -50 mV and incremented by 10 mV each trial until a final test potential of +40 mV was reached. Again currents were sampled at 2 kHz and inter-episode duration was 6 s.
2.5.4.4. Parameter measurement and analysis.

Parameter measurements were carried out using CLAMPFIT 6.0.2 software (Axon Instruments). Details of how current parameters were determined are given at relevant points in the results chapters.

Once parameters had been measured the data obtained was manipulated using Excel (Microsoft) spreadsheets and graphically represented using Origin 5.0 (Microcal Inc.) software. Statistical comparison where necessary was achieved using a paired or unpaired Student's t-test, with a probability level of \( P < 0.05 \) to test for significance. Results are given as mean ± standard error of the mean (s.e.m.), with \( n \) being the number of experiments in which the currents were recorded.

2.6. RT-PCR experiments.

2.6.1. Preparation of CGNs for RT-PCR.

Both freshly dissociated cells and cells that had been in culture for several days were required for RT-PCR experiments. CGNs in both cases were obtained essentially as described previously.

For freshly dissociated cells, the cell pellet remaining after centrifugation through Earle's salts was used in the preparation of RNA. Cultured cells to be used for RT-PCR experiments were grown in a 75 cm\(^3\) flask rather than on coverslips. Fresh media was added to the flask every three days. Once cells had been cultured for the required length of time the media was decanted into a 50 ml Falcon tube and any remaining cells were obtained by incubation with 2 mls of 0.05 % trypsin solution for 2 min at 37 °C. Cells were dislodged by tapping the side of the flask and the resulting suspension was pooled with the media, which was then centrifuged at 1500 rpm for 5.5 min. Once the supernatant was removed the pelleted cells were used in the preparation of RNA.
2.6.2. Purification of RNA.

RNA was extracted from CGNs using a RNeasy mini prep kit (Qiagen, Crawley, UK) which uses the selective binding properties of a silica based gel membrane to isolate RNA. The cell pellets obtained as described above, were gently triturated with 350 μl of lysis buffer (supplied with the kit). This disrupts the cell membrane releasing cell contents, including RNA. The success of the trituration was judged by the viscosity of the solution, which increases greatly due to the presence of strands of genomic DNA. Lysis buffer contains highly denaturing guanidinium isothiocyanate (GITC) which immediately inactivates RNases to ensure isolation of intact RNA.

Once the cells were sufficiently disrupted the solution was homogenised by pipetting, and 350 μl of 70 % ethanol was added to the homogenised lysate and mixed well by pipetting. Ethanol is added to provide the correct conditions for binding of RNA to the silica gel membrane.

The sample is then added to the RNeasy mini prep spin column (Qiagen), which contains the silica gel membrane, and centrifuged for 30-60 s at 13000rpm. Centrifugation passes the sample through the membrane to which any RNA in the sample will bind. Flow-through can then be discarded. The silica gel membrane is then washed three times to remove any contaminants. The wash buffers are high salt solutions and therefore maintain the correct conditions to ensure RNA stays bound to the membrane during washing. Elution of the purified RNA is then achieved using 40 μl RNase free water.

2.6.3. DNase treatment.

To ensure there was no residual contamination with genomic DNA, which could give a false positive signal in the experiments, the sample underwent DNase treatment. Samples of RNA were DNase treated in a final volume of 60 μl, containing:

- 50 mM Tris-HCl
- 75 mM KCl
- 3 mM MgCl₂
- 10 mM dithiothreitol (DTT)
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- 40 units RNasin
- 5 units RQ DNase

RNasin was included to inhibit any RNase enzymes that could otherwise digest the RNA. This mix was incubated at 37 °C for 15-30 min and the reaction terminated either by incubation at 95 °C for 5 min, or addition of 5 μl stop solution (1.54 mM EGTA) in combination with incubation at 65 °C for 10 min. Samples were heated by placing tubes in a preheated thermal cycler block, with heated lid (Touchdown).

Following DNase treatment the DNase must be removed from the RNA, plus RNA needs to be isolated. This was achieved using the RNeasy mini prep kit (Qiagen). The sample volume was adjusted to 100 μl with RNase free water before adding 350 μl of lysis buffer and mixing thoroughly. 250 μl of 100 % ethanol was added to the sample, which was then centrifuged for 30-60 s at 13,000 rpm. The membrane was washed twice with wash buffer and the purified RNA eluted in 40 μl of RNase free water.

2.6.4. Reverse transcription (RT) of RNA to single stranded cDNA.

Samples of RNA were reversed transcribed in a final volume of 50 μl, containing:

- 50 mM Tris-HCl
- 75 mM KCl
- 3 mM MgCl₂
- 10 mM DTT
- 1.25 / 1.43 mM of each deoxyribonucleoside triphosphates (dNTP)*
- random hexamer primers
- 40 units RNasin
- 500 / 400 units Moloney murine leukaemia virus reverse transcriptase (MMLVRT)*

The mix was incubated at 37 °C for 1-2 hours.
* in later experiments (see Figure 6.3 onwards) a more concentrated solution of cDNA was required. In this case samples of RNA were reverse transcribed in a final volume of 35 µl containing the same concentration of reagents as above except for 1.43 mM dNTPs and 400 units of MMLVRT.

Samples were heated by placing in a preheated thermal cycler block, with heated lid (Touchdown) and the reaction was terminated by placing the tubes on ice.

Samples were diluted 1:1 with RNase free water before use in PCR experiments.

2.6.5. Primer design.

In order to screen the cDNA for TASK-1 expression, primers specific to this sequence need to be used in the PCR step. Primers were designed against the complete rat TASK-1 sequence as published on GenBank (GenBank accession number AF31384), and synthesised by GIBCO BRL.

The primers were designed to be 20 nucleotides long (20mers) to enable a reasonably high annealing temperature to be used during PCR. Additionally the GC content of all primers was approximately 50 % to ensure that all primers anneal at the same temperature.

The sequence of primers used to probe for TASK-1 were as follows (the figures in brackets refer to the base pair number of TASK-1):

F1 5' - CACCGTCATCACCACAATCG - 3' (367-386)
R1 5' - TGCTCTGCATCACGCTTCTC - 3' (863-882)
F2 5' - AGTACGTGGCCTTCAGCTTC - 3' (759-778)
R2 5' - TGCAGTACTGCAGCTTCTCG - 3' (1108-1127)

Actin is ubiquitously expressed in all cell types and the reverse transcription and amplification of its mRNA was employed as an internal control. The primer pairs used were provided by a colleague in the lab and had the following sequences:
ActinF 5’ - TTGTAACCAA CTGGGACGATATGG - 3’ (1554-1577)
ActinR 5’ - GATCTTGATCTTCATG GTGCTAGG - 3’ (2869-2846)

2.6.6. Amplification of cDNA by the polymerase chain reaction.

After completion of the RT step the resulting cDNA was amplified by PCR and screened for TASK-1 expression using specific primers. Amplification consists of a series of denaturing, annealing and extension steps, the temperatures of which vary depending on the polymerase enzyme and primer pair used.

PCR using Taq polymerase was performed in a final volume of 25 μl containing:

- 20 mM Tris-HCl
- 100 mM KCl
- 0.2 % Triton X-100
- 250 μM each dNTP
- 3 mM MgCl₂
- 2.5 / 5 units Taq polymerase*
- 0.8 / 1.6 μM TASK-1 or 0.8 μM actin primers*

* initial experiments with Taq polymerase used the lower concentrations of enzyme and TASK-1 primers (Figure 6.1 and 6.2). During the optimisation of PCR conditions it was found that increasing the concentration of these components produced a better signal (Figure 6.3 onwards).

Several different temperatures were tested (see Chapter 6) until the optimal cycling protocol was determined to be: 96 °C for 3 min (1 cycle); 96 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min (30 cycles); 72 °C for 5 min (1 cycle).

PCR using Pfx polymerase was performed in a final volume of 25 μl, containing:
5 µl 10x Pfx amplification buffer (supplied with enzyme)
- 250 mM each dNTP
- 3 mM MgCl₂
- 1.25 units Pfx polymerase
- 1.6 µM TASK-1 or 0.8 µM actin primers

Cycling protocol: 96 °C for 3 min (1 cycle); 96 °C for 30 s, 60 °C for 30 s, 68 °C for 1 min (30 cycles); 68 °C for 5 min (1 cycle).

In all cases tubes were placed in a preheated thermal cycler block, with heated lid (Techne, progene or Touchdown) which was programmed with the required protocol. Once the protocol was complete the tubes were cooled to 4 °C or frozen at -20 °C until required.

2.6.7. Preparation of agarose gel.

PCR products were run on a 2 % agarose gel. Depending on the size of the gel to be cast either 2 g or 0.8 g of agarose was melted in 100 or 40 ml respectively of TAE electrophoresis buffer (40 mM tris-acetate, 1 mM EDTA), until a clear transparent solution was achieved. The agarose solution was then cooled to approximately 60 °C, while being kept in constant motion, before pouring into an electrophoresis tank and allowed to set. Once this had occurred the gel was covered to a depth of approximately 5 mm with TAE.

2.6.8. Preparation of samples for electrophoresis.

8 µl of gel loading buffer (0.25 % bromophenol blue, 40 % sucrose, 0.1 M EDTA, 0.5 % sodium lauryl sulphate (SDS)) was added to each 25 µl sample. Depending on the size of the gel used either 20 or 13 µl of sample was loaded into individual wells of the gel. 6 µl of DNA size markers (100 bp ladder, 1µg ml⁻¹) were also used. The gel was run at 110 V for 45 min.
2.6.9. Visualisation of the DNA bands.

The gel was gently shaken in a 1 μg ml⁻¹ solution of ethidium bromide for approximately 15 min. The bands were then visualised with an ultra violet light source. A Polaroid photograph was taken of the illuminated gel to give a permanent record of the results.

MATERIALS.

Cell culture: All enzymes and chemicals used during the isolation of CGNs were purchased from Sigma Chemicals Ltd. (UK), except for Earles salt's solution, chick embryo extract, glutamine, FCS and penicillin/streptomycin which were obtained from GIBCO BRL, Life Technologies, (UK).

Electrophysiology: Salts used in the preparation of internal and external solutions were purchased from BDH, except for NaCl which was purchased from Sigma. Glucose, HEPES, BAPTA and DMSO were all obtained from Sigma. Pharmacological agents were purchased from Sigma, except for methoctramine and U73122 (1-[6-(((17β)-3-Methoxyestra-1, 3, 5(10)-trien-17-yl)amino)hexyl]-1H-pyrrole-2, 5-dione) which were purchased from Research Biochemicals International (RBI), and zamifenacin which was a kind gift from Pfizer.

Molecular biology: All components of the RT-PCR experiments were obtained from Promega (UK), except for Pfx polymerase and TASK-1 primers which were obtained from GIBCO BRL. Agarose, gel loading buffer and ethidium bromide were purchased from Sigma, and 100 bp ladder was purchased from Amersham Pharmacia Biotech.

All drugs were made up as stock solutions in distilled water and stored at -20 °C (except for quinidine and quinine which were dissolved in ethanol and amphotericin which was dissolved in DMSO). Drugs were made up to required concentrations on the day of experiments using control external solution.
CHAPTER 3.

CHARACTERISATION OF POTASSIUM CURRENTS IN CEREBELLAR GRANULE NEURONS.
3.1. INTRODUCTION.

Cerebellar granule neurons (CGNs) comprise the largest population of identifiable neurons in the vertebrate brain (Hockberger et al, 1987), by some estimates 80 - 85 % of all human neurons are CGNs (Lange, 1975). These cells are excitatory interneurons relaying mossy fibre input, one of the two major inputs, to the Purkinje cells, the sole output of the cerebellum. Purkinje cells can receive up to 200 000 synaptic inputs, the majority of which come from CGNs which synapse exclusively upon the dendritic spines of Purkinje cells. CGNs also receive inhibitory inputs themselves directly from Golgi cells. Since the activity of these interneurons will ultimately influence the output of the cerebellum, via the Purkinje cells, the modulation of the excitability of these cells may prove to be physiologically important.

Until the development of the patch clamp technique (Hamill et al, 1981) little was known about the membrane properties of CGNs since their small size precluded them from conventional microelectrode techniques (Jalonen et al, 1990). The electrical properties of CGNs have since been investigated in different cerebellar preparations and characterisation of voltage and neurotransmitter activated ionic currents have been reported (e.g. Cull-Candy et al, 1988, 1989).

CGNs have been shown to possess voltage activated inward currents (Hirano et al, 1986, Hockberger et al, 1987, Cull-Candy et al, 1989, Jalonen et al, 1990). Cull-Candy et al (1989) demonstrated that inward current could be evoked over a wide range of potentials (-50 to +80 mV) when stepping from a pre pulse potential of -100 mV. The peak inward current initially increased with depolarisation, reaching maximum amplitude at approximately -20 mV, then declining towards zero as the voltage became more positive. These currents were abolished in Na\(^+\) free solution and in the presence of 100 nM TTX, identifying the current as a sodium current. Other studies confirm the presence of Na\(^+\) current in CGNs. However Hirano et al (1986) observed an additional late component of inward current that was unaffected by removal of Na\(^+\) ions. Manipulation of the extracellular solution revealed that the late component of inward current was carried by Ca\(^{2+}\) ions.
Subsequently using appropriately designed external solutions several groups examined the \( \text{Ca}^{2+} \) current in more detail. The current activates around -25 mV, reaches peak amplitude at +10 mV and reverses around +80 mV. Application of the calcium channel agonist Bay K 8644 enhanced the current amplitude, while the calcium channel antagonist nimodipine inhibited the current by 25 \% (Marchetti et al 1991, Amico et al 1995). This indicates that L-type \( \text{Ca}^{2+} \) channels carry a component of the whole cell calcium current in CGNs. Treatment with \( \omega \)-conotoxin GVIA (cgtx) and \( \omega \)-agatoxin IVA (agatx), putative specific blockers of N- and P-type \( \text{Ca}^{2+} \) currents respectively, produced an inhibition of the whole cell \( \text{Ca}^{2+} \) current by 17 and 47 \%. The actions of nimodipine, cgtx and agatx appeared to be additive suggesting the presence of a mixed population of \( \text{Ca}^{2+} \) channels in CGNs (Amico et al, 1995). However the effects of toxins do not appear to be completely selective in CGNs since application of agatx, in the presence of cgtx and a L-type antagonist, reduced the whole cell current by only 4.5 \% (Pearson et al, 1995). Therefore the relative contribution of each \( \text{Ca}^{2+} \) channel type to the whole cell \( \text{Ca}^{2+} \) current is undetermined, and a large component of the whole cell \( \text{Ca}^{2+} \) current remains unclassified.

The failure of some authors to detect a calcium conductance in CGNs has been attributed to incomplete block of outward currents which mask the small calcium current (Marchetti et al, 1991), or wash out of the current in whole cell recording (Hockberger et al, 1987, Cull-Candy et al, 1989). Alternatively it is possible that \( \text{Ca}^{2+} \) current is only present at certain stages of development, for example Marchetti et al (1991) reported that the average \( \text{Ca}^{2+} \) current increased in amplitude with days in vitro.

In addition to evoking inward currents depolarising voltage protocols evoke outward currents in CGNs. These outward currents are abolished when CGNs are voltage clamped with internal Cs\(^+\) solution (Hirano et al, 1986), indicating that the outward current recorded with internal K\(^+\) solution is carried by voltage dependent K\(^+\) channels. The whole cell K\(^+\) current in CGNs has been well characterised. A depolarising voltage step to +30 mV from a holding potential of -120 mV evokes an outward current, which clearly decays in two phases. The current activates rapidly and reaches an early peak, then declines over tens of milliseconds to leave a late
current which decays more slowly, with a half time of the order of seconds (Cull-Candy et al, 1989). These transient and sustained components of whole cell K⁺ current corresponds to A type (Iₐ) and delayed rectifier (Iₖᵥ) conductances (Hockberger et al, 1987, Carignani et al, 1991, Watkins and Mathie, 1994).

It is possible to study these currents in isolation using appropriate voltage protocols. For example cells are held at either -80 or -50 mV and stepped in 10 mV increments to more positive values. Currents evoked from cells held at -80 mV contained both Iₐ and Iₖᵥ components. In contrast currents evoked from cells held at -50 mV consisted solely of Iₖᵥ. Currents obtained from the -50 mV holding potential protocol can then be subtracted from currents obtained using the -80 mV holding potential protocol to reveal Iₐ in isolation (Carignani et al, 1991). A hyperpolarising pre-pulse is required to evoke Iₐ to relieve the channel from steady state inactivation. Inactivation of Iₐ in CGNs is virtually complete at -60 mV and half maximal at approximately -75 mV (Cull-Candy et al, 1989, Bardoni and Belluzzi, 1993).

Pharmacological separation of Iₐ and Iₖᵥ are less easily achieved. 4-AP and TEA have previously been described as relatively specific antagonists of Iₐ and Iₖᵥ respectively in a number of preparations (Hille, 1992). In CGNs conflicting reports of the effects of 4-AP and TEA exist. Cull-Candy et al (1989) reported that 2 mM 4-AP substantially reduced Iₐ, but a clear reduction of Iₖᵥ was also observed. Carignani et al (1991) saw similar results of 4-AP on Iₐ with almost complete inhibition at high concentrations (EC₅₀ = 0.7 mM), however no effect of 4-AP on Iₖᵥ was observed. Conversely Watkins and Mathie (1994) found 1 mM 4-AP reduced Iₐ by only 13 % while Iₖᵥ was reduced by approximately 32 %.

TEA appears to be more selective in CGNs than 4-AP with Carignani et al observing a 30 % reduction of Iₖᵥ with 3 mM TEA and Watkins and Mathie observing a 59 % reduction with 5 mM TEA. No significant reduction of Iₐ was measured in either case. Bardoni and Belluzzi (1993) found that 20 mM TEA completely abolished Iₖᵥ and used this treatment to provide virtually pure Iₐ recordings. In contrast Cull-Candy et al did not observe such a potent or specific effect of 20 mM TEA on Iₖᵥ. TEA treatment predominately reduced Iₖᵥ but a small measurable effect on Iₐ was also apparent.
The sustained outward current can be further separated into a $\text{Ca}^{2+}$ dependent and a $\text{Ca}^{2+}$ independent component by preventing $\text{Ca}^{2+}$ inflow with $\text{Ca}^{2+}$ channel blockers (Bardoni and Belluzzi, 1993). This result is in accordance with the earlier description of a maxi-K ($\text{BK}_{\text{Ca}}$) conductance in CGNs (Fagni et al, 1991).

In addition to the sustained and transient type potassium conductances CGNs in primary culture have been shown to possess an additional component of outward potassium current (Watkins and Mathie, 1996). This component can be observed as a maintained 'standing outward' current ($\text{IK}_{\text{so}}$) at -30 mV, which does not exhibit any apparent inactivation. The I/V relation for $\text{IK}_{\text{so}}$ is outwardly rectifying with little inward current seen with strong hyperpolarisation, the current appears to activate at potentials just slightly positive to the $\text{K}^+$ equilibrium. Increasing the external $\text{K}^+$ concentration from 2.5 to 22.5 mM shifts the reversal potential from approximately -89 mV to a more positive value, in a manner consistent with the current being carried by $\text{K}^+$ ions.

$\text{IK}_{\text{so}}$ develops over several days in culture. Hardly any detectable current is present the first couple of days in vitro, gradually increasing with time in culture. As the level of $\text{IK}_{\text{so}}$ increases with time the resting membrane potential of the cells becomes more hyperpolarised, stabilising at around -90 mV after seven days in culture. Interestingly $\text{IK}_{\text{so}}$ can only be recorded using the perforated patch clamp technique, since under conventional whole cell recording the current runs down within minutes.

One important feature of $\text{IK}_{\text{so}}$ is its inhibition by activation of muscarinic acetylcholine receptors. Bath application of muscarine reduces the current in a dose dependent and fully reversible manner. Under current clamp conditions application of 10 $\mu$M muscarine resulted in a shift of the zero current potential in the depolarising direction, and inhibition of $\text{IK}_{\text{so}}$ also resulted in an increased input resistance of the cells. This data implies that muscarine acts as a depolarising influence and will enhance the excitability of CGNs through modulation of $\text{IK}_{\text{so}}$.

Muscarinic receptors are members of the superfamily of GTP-binding protein (G protein) coupled receptors. These receptors transduce their signals by coupling with
heterotrimeric G proteins, which initiate a variety of intracellular processes such as modulation of second messenger levels and ion channel activity. Five subtypes of muscarinic receptor have been identified by molecular cloning (M₁ to M₅; Felder, 1995), and these can be grouped according to the second messenger pathway to which they couple. M₁, M₃ and M₅ receptors are generally accepted to couple to increased phosphoinositide hydrolysis, resulting in increased levels of intracellular Ca²⁺. M₂ and M₄ receptors decrease adenylyl cyclase activity, resulting in decreased levels of cAMP. CGNs have been shown to possess both M₂ and M₃ subtypes of muscarinic receptor (Fukamauchi et al, 1991), although the receptor mediating the inhibition of IKₙ₀ has yet to be determined.

The aim of this chapter was to describe the variety of K⁺ currents in CGNs, focussing specifically on IKₙ₀. To elucidate the subtype of muscarinic receptor involved in the inhibition of IKₙ₀, the selective muscarinic receptor antagonists methoctramine (M₂) and zamifenacin (M₃) were used. In addition, an attempt was made at identifying the second messenger pathway coupling receptor to channel.
3.2. RESULTS.

3.2.1. Voltage activated outward currents.

3.2.1.1. Voltage activated outward currents, $I_{KV}$.

The delayed rectifier component of outward current, $I_{KV}$, can be studied in relative isolation using an appropriate voltage protocol. Figure 3.1a shows representative data traces generated using a voltage protocol designed to obtain a current-voltage relation for $I_{KV}$. Cells were held at a potential of -70 mV, pre-pulsed to -50 mV for 30 ms, before stepping to test potentials ranging from -50 to +40 mV. The current traces demonstrate the distinct profile of $I_{KV}$, namely a sustained current at depolarised potentials, which shows little or no inactivation over the duration of the step.

The amplitude of $I_{KV}$ was measured as a mean over 15 ms, 134 ms following the step to the test potential. This value was then plotted against the appropriate voltage to give an I/V relation for $I_{KV}$ in CGNs, Figure 3.1b. A small outward current is seen at potentials more positive than -60 mV, and at potentials more positive to -20 mV the amplitude of $I_{KV}$ increases in a non-linear fashion. This agrees with an activation threshold of -20 mV for $I_{KV}$ in these cells (Watkins and Mathie, 1994, Yeung et al, 1999). The mean amplitude of $I_{KV}$ at +40 mV is $472.8 \pm 29.3$ pA (n = 35).

The data traces in Figure 3.1c illustrate the effect of 10 mM TEA on $I_{KV}$. In the presence of 10 mM TEA the current amplitude was reduced. For the example shown the amplitude of $I_{KV}$ recorded at +10 mV was decreased from 416.3 pA in control to 240.8 pA in the presence of 10 mM TEA. This effect was observed in a further 5 cells producing a mean inhibition of $44.9 \pm 5.5 \%$.

3.2.1.2. Voltage activated outward currents, $I_A$.

In order to obtain records of the transient component of outward current $I_A$, a protocol which includes a hyperpolarising pre-pulse was utilised. Figure 3.2a shows representative data traces obtained when cells were stepped from a holding potential
of -30 mV to a pre-pulse potential of -120 mV, before stepping to a test potential of 0 mV.

$I_A$ can clearly be seen as a large transient peak. Bath application of 10 mM 4-AP decreases the amplitude of the peak until this component is completely abolished. The current resulting when currents measured at point • are subtracted from those measured at point ■ was taken as a measure of $I_A$ and plotted as a function of time in Figure 3.2b. The time course demonstrates the complete and reversible inhibition of $I_A$ by 10 mM 4-AP. Application of 10 mM 4-AP slightly increases the steady state current at 0 mV, which subsequently in subtraction analysis results in values for the amplitude of $I_A$ that fall below zero.

3.2.1.3. Removal of inactivation of $I_A$.

The time course for removal of inactivation was investigated using a double pulse protocol. Cells were held at -50 mV, and a hyperpolarising pre-pulse of variable duration applied before a constant test pulse to 0 mV. Conditioning pre-pulses to -120 mV were prolonged in 5 ms increments. Figure 3.3a shows that peak transient current at 0 mV increased as the pre-pulse to -120 mV was lengthened. This is also demonstrated in Figure 3.3b by plotting peak current against pre-pulse duration. The curve is exponential in form and, in keeping with Cull-Candy et al (1989), was fitted with the expression:

$$I/I_{\text{max}} = 1 - \exp(-t/\tau)$$

where, $I$ is the current evoked by a test depolarisation to 0 mV after a pre-pulse of duration $t$ at -120 mV; $I_{\text{max}}$ is the maximum current evoked at 0 mV; and $\tau$ is the time constant for removal of inactivation. A mean value for $\tau$ of 22.7 ± 2.5 ms ($n = 4$) at -120 mV was obtained. Cull-Candy et al (1989) reported a mean value for $\tau$ of 35 ± 2 ms at -100 mV.
3.2.2. Properties of Standing Outward current, IK_{so}.

Figure 3.4a demonstrates the presence of a non-inactivating standing outward current IK_{so}, at a holding potential of -20 mV, in a cell that had been in culture for 14 days. Typically cells were held at -20 mV and stepped to -60 mV for 800 ms before returning to the holding potential. The amplitude of IK_{so} at -20 mV was measured as a mean over 176 ms directly preceding the hyperpolarising step to -60 mV. The mean amplitude of IK_{so} at -20 mV in cells that had been in culture for 8 days was $295.2 \pm 16.3$ pA, n = 65.

At -20 mV any current measured will predominantly be composed of IK_{so} since IKV activates at potentials just positive to this value. Additionally any IKV or IA present will have inactivated during the 5 s period between protocols.

Bath application of 10 μM muscarine produces inhibition of IK_{so} which can be seen in Figure 3.4a as a reduction in the amplitude of IK_{so} at both potentials. The current profile is not altered in the presence of muscarine, the current is simply scaled down. The amplitude of IK_{so} during application of 10 μM muscarine was measured and plotted against time, Figure 3.4b, demonstrating the fully reversible nature of the inhibition by muscarine. In the example shown IK_{so} was inhibited by 69.5 %, with 10 μM muscarine producing a mean inhibition of $67.1 \pm 1.4$ % (n = 74).

Muscarine inhibits IK_{so} in a concentration dependent manner. The concentration-response relationship for inhibition of IK_{so} is illustrated in Figure 3.4c. Concentrations of 0.1, 0.3, 1 and 30 μM inhibited IK_{so} by $21.5 \pm 2.8$ % (n = 16), $37.7 \pm 6.4$ % (n = 6), $47.8 \pm 2.2$ % (n = 16) and $62.9 \pm 1.9$ % (n = 5) respectively. The data were fitted with a conventional logistic function, with the expression:

$$y = \frac{(A_1 - A_2)}{(1+(x/x_0)^p)} + 2$$

where, A1 is the minimum inhibition attainable (constrained to zero), A2 is the maximum inhibition attainable (unconstrained), x0 is the EC_{50} value and p is the slope factor.
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This gave an EC$_{50}$ value for muscarine of 0.25 μM. The EC$_{50}$ value agrees well with the EC$_{50}$ of 0.17 μM reported by Watkins and Mathie (1996).

Figure 3.5 demonstrates the presence of a standing outward current in primary cultures of mouse CGNs. The current profile is identical to that recorded in rat CGNs and the current is inhibited by bath application of 10 μM muscarine with a mean inhibition of 66.3 ± 2.1% (n = 4). The mean amplitude of IK$_{so}$ in mouse CGNs that had been in culture for 7 days was 126.2 ± 17.7 pA, n = 4.

3.2.3. Muscarinic receptor subtype involved in the modulation of IK$_{so}$.

CGNs have been shown to express M$_2$ and M$_3$ subtypes of muscarinic receptor (Fukamauchi et al, 1991). It is generally accepted that M$_2$ receptors are coupled via a PTX sensitive pathway to the inhibition of adenylyl cyclase, whilst M$_3$ receptors are linked to increased phosphoinositide hydrolysis via a PTX insensitive pathway. Previous experiments (Watkins and Mathie, 1996) demonstrated that pre-treating CGNs with PTX had no significant effect on the level of muscarinic inhibition of IK$_{so}$ by 10 μM muscarine, suggesting the involvement of M$_3$ receptors in muscarinic modulation. To confirm if this was the case the selective muscarinic antagonists methoctramine (M$_2$) and zamifenacin (M$_3$) were used.

Figure 3.6 demonstrates that muscarinic inhibition of IK$_{so}$ is unaffected by 100 nM methoctramine. Current traces in Figure 3.6a were generated using the standard protocol and were obtained in control conditions, in the presence of 1 μM muscarine and in the presence of 1 μM muscarine plus 100 nM methoctramine. In the example shown 1 μM muscarine produces 44.9 % inhibition of IK$_{so}$ in the presence of the M$_2$ antagonist, with a mean % inhibition of 48.8 ± 3.7 % (n = 7). This value is not significantly different from the 47.8 ± 2.2 % inhibition produced by 1 μM muscarine in the absence of antagonist (n = 16; P = 0.81, unpaired t-test).

Figure 3.6b shows the concentration-response curves for the inhibition of IK$_{so}$ by muscarine alone, and in the presence of 100 nM methoctramine. The EC$_{50}$ values are 0.25 and 0.44 μM respectively. The two curves overlie one another with no
significant difference in the amount of inhibition seen at any muscarine concentration. This result confirms that M$_2$ receptor activation does not lead to modulation of IK$_{so}$.

Figure 3.7 demonstrates that the muscarinic inhibition of IK$_{so}$ is reduced in the presence of 100 nM zamifenacin. Current traces in Figure 3.7a were generated using the standard protocol and were obtained in control conditions, in the presence of 1 μM muscarine and in the presence of 1 μM muscarine plus 100 nM zamifenacin. In the example shown 1 μM muscarine has no effect on the amplitude of IK$_{so}$ when the M$_3$ antagonist is present, the same result was seen in a further 2 cells.

Figure 3.7b shows the concentration response curves for the inhibition of IK$_{so}$ by muscarine alone and in the presence of 100 nM zamifenacin. The concentration-response curve is shifted to the right in the presence of antagonist resulting in an EC$_{50}$ value of 11.81 μM. This result confirms that M$_3$ receptor activation is responsible for modulation of IK$_{so}$.

The effect of 10 and 30 nM zamifenacin on the muscarine concentration-response curve were also tested. When the maximum response attainable was constrained to 75.64 and 100 % the EC$_{50}$ values for 10 nM zamifenacin were 0.74 and 3.22 μM respectively. 30 nM zamifenacin resulted in an EC$_{50}$ value of 0.46 μM.

Figure 3.7c is a Schild plot of the data. A straight line was fitted to the data, while fixing the slope at 1, using a linear regression model, which resulted in a pA$_2$ value of 8.13.

3.2.4. Mechanism of muscarinic modulation.

The results presented so far suggest that activation of M$_3$ receptors is responsible for inhibition of IK$_{so}$. It is generally accepted that M$_3$ receptors are linked to increased phosphoinositide hydrolysis via activation of PLC. If activation of this second messenger pathway is responsible for the inhibition of IK$_{so}$ then application of a PLC inhibitor, such as U73122, would be expected to diminish the inhibition of IK$_{so}$ by muscarine.
The effect of U73122 on the level of muscarinic inhibition of IK_{so} is shown in Figure 3.8a. 10 μM muscarine was bath applied and produced a characteristic reduction of the current. Muscarine was washed off and IK_{so} recovered to control levels. The cell was then incubated in 10 μM U73122 for no less than 4 min and the muscarine challenge repeated in the continuing presence of the PLC inhibitor. In five cells application of 10 μM muscarine in control conditions produced a mean inhibition of 75.4 ± 3.5 %. Following incubation in 10 μM U73122, 10 μM muscarine produced a mean inhibition of 62.8 ± 3.6 % in the same five cells. The second application of muscarine produced a significantly reduced response (P = 0.003; paired t-test).

To determine whether the decreased response was a direct result of U73122 treatment, or due to some kind of desensitisation of the response, a further eight cells were subjected to repetitive doses of muscarine in the absence of U73122. Figure 3.8b shows a representative result. The first application of muscarine produced a mean inhibition of 62.6 ± 4.3 %, whereas the second application produced a 60.4 ± 4.3 % inhibition. Statistical comparison showed that there was no significant difference between the two responses (P = 0.56; paired t-test), meaning that the decrease in the response seen with U73122 seems to be a direct result of PLC inhibition.

From this result it can be concluded that activation of the PLC second messenger pathway may have a small role to play in the modulation of IK_{so}. However, even when this pathway is blocked by U73122 IK_{so} is still substantially inhibited by muscarine suggesting that an alternative mechanism predominates in the modulation of the current.
Figure 3.1. Properties of I_{Kv} in CGNs.

a). \(I_{Kv}\) can be seen as a sustained current, which shows no inactivation during a depolarising voltage step. Cells held at \(-70\) mV and pre-pulsed to \(-50\) mV, to inactivate the \(I_A\) component of outward current, before stepping to test potentials ranging from \(-50\) to \(+40\) mV.

b). I/V relation of \(I_{Kv}\) in CGNs. The amplitude of \(I_{Kv}\) was measured as a mean over 15 ms, 134 ms following the step to the test potential.

c). Effect of 10 mM TEA on the amplitude of \(I_{Kv}\) evoked by a test potential to \(+10\) mV.
Figure 3.2. Properties of $I_A$ in CGNs.

a). $I_A$ can be seen as a large transient peak in control conditions. Representative data traces obtained by applying a voltage step to -120 mV from a holding potential of -30 mV before stepping to a test potential of 0 mV. Application of 10 mM 4-AP completely abolishes this component of potassium current.

b). Illustration of the time course of reversible inhibition of $I_A$ on bath application of 10 mM 4-AP. The amplitude of $I_A$ was obtained by subtracting currents measured at point • from those measured at point ■.
Figure 3.3. Time course of removal of inactivation of the transient outward current, $I_{h}$.

a). The cell was clamped at $-50$ mV and a hyperpolarising pre-pulse to a constant potential of $-120$ mV applied for durations varying between 1 and 121 ms in 5 ms increments. This was followed in each case by a test depolarisation to 0 mV in order to elicit transient outward currents. The size of the peak current increased with longer duration pre-pulses.

b). Normalised peak transient outward current plotted against pre-pulse duration. A logarithmic fit to this curve gives the time constant of removal of inactivation as 22.7 ms at $-120$ mV.
Figure 3.4. Effect of muscarine on $I_{K_{SO}}$

a). $I_{K_{SO}}$ is seen as a non-inactivating current at $-20 \text{ mV}$ and is instantaneously reduced in amplitude when cells are stepped to $-60 \text{ mV}$ for 800 ms, once every 6 s. The current is inhibited by 10 $\mu$M muscarine.

b). Illustration of the time course of inhibition of $I_{K_{SO}}$ on bath application of 10 mM muscarine. The amplitude of $I_{K_{SO}}$ was measured at $-20 \text{ mV}$ as a mean over 176 ms directly preceding the step to $-60 \text{ mV}$, and plotted as a function of time. Inhibition by 10 $\mu$M muscarine is fully reversible.

c). Concentration-response curve for muscarine. Data points were fitted using a conventional logistic function, giving a calculated $EC_{50}$ value of 0.25 $\mu$M.
Figure 3.5. Presence of $I_{K_{so}}$ in mouse CGNs.

a). Using the standard protocol detailed in Figure 3.4, $I_{K_{so}}$ can be detected in mouse CGNs. Similarly the amplitude of $I_{K_{so}}$ is reversibly reduced in the presence of 10 μM muscarine.

b). Time course of inhibition of $I_{K_{so}}$ in mouse CGNs on bath application of 10 μM muscarine.
Figure 3.6. Effect of a M2 antagonist on the muscarinic inhibition of IKso

a). Inhibition of IKso by 1 μM muscarine in the absence and presence of 100 nM methoctramine. Representative data traces obtained using the standard protocol.

b). Concentration-response curve for muscarine alone and in the presence of 100 nM methoctramine. Each data point represents the mean ± s.e.mean for number of experiments indicated. Data points were fitted using conventional logistic function, giving a calculated EC50 value of 0.25 μM in control and 0.44 μM in the presence of the M2 antagonist, with a slope factor of 0.84 and a maximum inhibition of 71.47%.
Figure 3.7. Effect of a M3 antagonist on the muscarinic inhibition of IKso.

a). Inhibition of IKso by 1 μM muscarine in the absence and presence of 100 nM zamifenacin. Representative data traces obtained using the standard protocol.

b). Concentration-response curve for muscarine alone and in the presence of 100 nM zamifenacin. Each data point represents the mean ± s.e.mean for number of experiments indicated. Data points were fitted using conventional logistic function, giving a calculated EC50 value of 0.25 μM in control and 11.81 μM in the presence of the M3 antagonist, with a slope factor of 1.41 and a maximum inhibition of 77.33%.

c). EC50 values in the presence of 10 and 30 nM zamifenacin were also obtained (data not shown) and used to produce a Schild plot. A linear regression model with a slope fixed at 1 was used to fit a straight line to the data. The point at which the line crosses the x-axis gives a pA2 value of 8.13.
Figure 3.8. Effect of a PLC inhibitor (U73122) on the muscarinic inhibition of $I_{K_{so}}$

a). Time course plot showing the effect of 10 $\mu$M muscarine on the amplitude of $I_{K_{so}}$ both before and following incubation with 10 $\mu$M U73122. Each point is a measure of the mean amplitude of $I_{K_{so}}$ at -20 mV, taken over 176ms.

b). Time course plot showing the effect of repetitive doses of muscarine on the amplitude of $I_{K_{so}}$. Data points obtained as in (a). This experiment acts as a control to determine whether a second application of muscarine produces a similar degree of inhibition as the first.
3.3. DISCUSSION.

The results presented in this chapter demonstrate that CGNs have a transient ($I_A$) and sustained ($I_{Kv}$) voltage dependent component of whole cell $K^+$ current, whose characteristics agree well with published data. Additionally the presence of a maintained outward current known as $I_{Ks0}$ has been confirmed in these cells. Inhibition of this current by muscarinic receptor activation has been demonstrated and experiments with selective muscarinic antagonists suggest that the activation of $M_3$ receptors is responsible for the modulation of $I_{Ks0}$. The mechanism coupling receptor to channel remains to be determined. Preliminary work indicates that although activation of PLC may play a small role in the inhibition of $I_{Ks0}$, it is not the dominant mechanism.

3.3.1. Muscarine sensitive currents.

$I_{Ks0}$ is not unique in its muscarine sensitivity, a variety of $K^+$ channels in mammalian neurons share the feature of muscarinic modulation. Generally $I_A$ and $I_{Kv}$ currents are unaffected by muscarine, however there are some exceptions. For example in striatal neurons $I_A$ is enhanced on application of carbachol (Atkins et al, 1990), whereas $I_{Kv}$ in ventromedial hypothalamic neurons is decreased (ffrench-Mullen et al, 1994). In hippocampal CA1 neurons $I_A$ is inhibited (Nakajima et al, 1986) while $I_{Kv}$ is reduced by muscarinic activation (Zhang et al, 1992). The $Ca^{2+}$ activated $K^+$ current, $I_{AHP}$, in hippocampal pyramidal cells is also reduced by muscarinic stimulation (e.g. Lancaster and Adams, 1986, Madison et al, 1987).

The most well characterised neuronal muscarine sensitive current is the M current ($I_{KM}$; Brown, 1988). $I_{KM}$ is a voltage dependent current, which is activated near the resting membrane potential in a variety of neurons. The current has slow activation and deactivation kinetics and does not inactivate even with prolonged depolarisation. The defining feature of $I_{KM}$ is its inhibition by muscarine, and the excitatory action of ACh on certain neurons has been attributed to its inhibitory action on $I_{KM}$ (e.g. Halliwell and Adams, 1982).
Additionally $K^+$ selective leak conductances in a variety of cells have also been reported to be reduced by muscarinic receptor activation (e.g. Madison et al, 1987, Benson et al, 1988, Pitler and Alger, 1990, Womble and Moises, 1992, Guerineau et al, 1994).

3.3.2. Modulation of $IK_{so}$ and possible mechanisms of inhibition.

CGNs have been shown to possess the $M_2$ and $M_3$ subtypes of muscarinic receptor, but not $M_1$ or $M_4$ (Fukamauchi et al, 1991). These receptors are functionally distinct, and belong to different subgroups of mAChRs which are defined by the $G$ protein and second messenger pathway to which they couple.

To date cDNAs that encode 21 distinct mammalian $G$ protein $\alpha$ subunits have been cloned; these can be divided into four major subfamilies according to amino acid sequence similarity ($G_s$, $G_i$, $G_q$ and $G_{12}$). In addition at least five distinct $\beta$ subunits and twelve $\gamma$ subunits have been identified (Clapham, 1996). This allows for a vast number of theoretical combinations and therefore signal transduction options. However some selectivity between $\beta$ and $\gamma$ subunits exists, meaning not every theoretical combination is possible (Clapham and Neer, 1993).

It is generally accepted that $M_2$ receptors couple to their effector via the $G_i$ class of $G$ proteins. Activation of this class of $G$ proteins characteristically leads to attenuation of adenylyl cyclase activity, thereby reducing intracellular levels of cAMP (Felder, 1995). $\alpha$ subunits of the $G_i$ subfamily possess a specific cysteine residue near the carboxyl terminus which can be ADP ribosylated by pertussis toxin (PTX). This modification results in a $G$ protein that is no longer accessible to receptor mediated activation (ffrench-Mullen et al, 1994, Felder, 1995). $M_3$ receptors couple to their effector via a different class of $G$ proteins, $G_q$. Activation of this class of $G$ proteins results in increased phosphoinositide hydrolysis, generating IP$_3$ which subsequently mobilises intracellular calcium. The $G_q$ class of $G$ proteins are not modified by PTX, therefore the effect of PTX on a muscarinic response can be used in the preliminary identification of the subtype of receptor involved.
In the case of muscarinic inhibition of $I_{K_{so}}$, pre-treatment of CGNs with 120 mg ml$^{-1}$ of PTX for 17-20 hours produced no significant difference either in the magnitude of $I_{K_{sc}}$ at $-30$ mV or its inhibition by 10 $\mu$M muscarine, suggesting the involvement of M$_3$ receptors (Watkins and Mathie, 1996). To confirm whether the M$_3$ receptor mediates the muscarinic effect, experiments with the selective muscarinic antagonists methoctramine (M$_2$) and zamifenacin (M$_3$) were performed. From published pA$_2$ values for these antagonists it is possible to estimate the effect of each compound on the muscarine concentration response curve, assuming either M$_2$ or M$_3$ receptors are involved. For example methoctramine has a pA$_2$ value of 7.9 for M$_2$ receptors and 6.2 for M$_3$ receptors (Hulme et al, 1990). If the endogenous receptors underlying muscarinic inhibition are M$_2$ then 100 nM methoctramine would be expected to produce about a 20 fold rightward shift of the concentration response curve, while if M$_3$ receptors are involved 100 nM methoctramine would result in only an approximate 2 fold shift of the concentration response curve. The exact effect of zamifenacin is harder to predict since this compound has published pA$_2$ values of either 9.2 or 7.9 for endogenous M$_3$ receptors, depending on their tissue localisation, and a pA$_2$ value of 7.14 for M$_2$ receptors (Wallis, 1995). Thus, if M$_3$ receptors underlie muscarinic inhibition 100 nM zamifenacin would be expected to produce a rightward shift of the concentration response curve by at least 20 fold, compared to around a 2 fold shift if M$_2$ receptors are important. Experiments presented in this chapter show that 100 nM methoctramine has virtually no effect on the control muscarine concentration response curve, while 100 nM zamifenacin shifts it to the right by about 50 fold. These results strongly suggest that the muscarinic inhibition of $I_{K_{so}}$ is mediated via the M$_3$ receptor subtype.

Additional experiments using 10 and 30 nM zamifenacin resulted in a pA$_2$ value of 8.13 being calculated for zamifenacin in CGNs. However the large scatter of the results with these two concentrations (which would be predicted to cause relatively small shifts in the muscarine EC$_{50}$) make it difficult to be confident of an absolute pA$_2$ value for zamifenacin based on the Schild analysis shown in Figure 3.7c.

Confirmation of an M$_3$ linked increase in phosphoinositide metabolism in CGNs has been provided by several studies (e.g. Xu and Chuang, 1987, Whitham et al, 1991,
Del Rio et al, 1994). To establish whether activation of this signalling pathway has a role in the inhibition of $I_{K_{so}}$ CGNs were treated with the phospholipase C inhibitor U73122 (Jin et al, 1994). This compound disrupts the signalling pathway in its initial stages and in CGNs the increase in intracellular $Ca^{2+}$ observed on application of 10 $\mu$M muscarine is abolished following pre-incubation in 10 $\mu$M U73122 (Boyd and Mathie, 2000). Results of experiments performed in this chapter show that the muscarinic inhibition of $I_{K_{so}}$ is slightly attenuated following incubation in 10 $\mu$M U73122. This suggests that a PLC dependent pathway may have a small contribution in the inhibition of $I_{K_{so}}$, but it is by no means the dominant pathway since a significant inhibition of $I_{K_{so}}$ remains following incubation with the PLC inhibitor. This suggests that $M_3$ receptors are acting predominantly via a PLC independent mechanism to produce muscarinic modulation of $I_{K_{so}}$.

CGNs possess at least 10 receptor populations which have been shown to regulate PLC activity (Xu and Chuang, 1987, Billon-Carter and Chuang, 1989, Lin et al, 1990, Del Rio et al, 1994). These include those for serotonin (5-HT) and noradrenaline (NA). Application of both these neurotransmitters had no clear effect upon the magnitude of $I_{K_{so}}$ in CGNs (Watkins and Mathie, 1996), providing additional evidence for modulation of $I_{K_{so}}$ through a PLC independent pathway.

The effects of downstream products of $G_{q/11}$ activation on $I_{K_{so}}$ have not been fully tested here, but experiments with U73122 indicated that an increase in intracellular $Ca^{2+}$ is not the signal for muscarinic inhibition of $I_{K_{so}}$. However $I_{K_{so}}$ does not appear to be completely $Ca^{2+}$ independent. Removal of $Ca^{2+}$ significantly increases the magnitude of the current at $-30$ mV, which is reversed upon perfusion with external solution containing 0.5 mM $Ca^{2+}$. Additionally the level of muscarinic inhibition of $I_{K_{so}}$ is attenuated when $Ca^{2+}$ is removed from the extracellular solution. It is suggested that $I_{K_{so}}$ is under some kind of tonic modulation by $Ca^{2+}$ (Watkins and Mathie, 1996).

The mechanism underlying muscarinic $I_{K_{so}}$ inhibition could prove to be as elusive as that underlying the inhibition of $I_{K_{M}}$ in sympathetic neurons. Although the signalling mechanism has been examined extensively for this current no pathway has been
conclusively identified (Marrion, 1997a). In rat sympathetic neurons the microinjection of specific G protein antibodies demonstrated that muscarinic inhibition of $I_{Km}$ occurs through $G_{aq/11}$ (Caulfield et al, 1994, Haley et al, 1998), but like $I_{Ks0}$ this inhibition does not involve the activation of PLC. Muscarinic modulation of $I_{Km}$ is still evident after incubation with U73122, when IP$_3$ receptors are blocked with heparin and when intracellular Ca$^{2+}$ is buffered (Cruzblanca et al, 1998, Del Río et al, 1999). In contrast other groups have reported a disruption of muscarinic suppression of $I_{Km}$ when intracellular Ca$^{2+}$ is manipulated (Beech et al, 1991), as well as a direct action of Ca$^{2+}$ on $I_{Km}$ (Selyanko and Brown, 1996). Present thinking proposes that rather than Ca$^{2+}$ ions acting as a signal for $I_{Km}$ inhibition themselves, instead they play a permissive role. This role may be the result of some Ca$^{2+}$ sensitive step in the signalling pathway, or because a particular functional state of M channels exists that is sensitive to basal levels of intracellular Ca$^{2+}$ (Cruzblanca et al, 1998). Interestingly $I_{Km}$ in rat sympathetic neurons can also be inhibited by bradykinin through $G_{aq/11}$. Inhibition in this case seems to be through a PLC dependent mechanism as treatments which disrupt this pathway result in the loss of $I_{Km}$ suppression by bradykinin (Cruzblanca et al, 1998). The question of how two receptors acting through $G_q$ in the same cell couple to quite different signalling mechanisms remains to be answered.

Although $M_3$ receptors couple predominantly to PTX insensitive G proteins ($G_{q/11}$) to activate PLC, interactions with other classes of G protein and signalling effectors can readily be observed in a variety of cell types. For example when the $M_3$ receptor is transfected into either CHO or HEK cells, evidence exists which shows the receptor can couple with both PTX sensitive as well as PTX insensitive G proteins (Offermans et al, 1994, Burford et al, 1995). Additionally $M_3$ receptors in rat parotid gland, where a homogenous $M_3$ population is claimed, couple to both Ca$^{2+}$ mobilisation and a decrease in cAMP formation (Dai et al, 1991).

It has already been established that PTX has no effect on muscarinic modulation of $I_{Ks0}$ (Watkins and Mathie, 1996), so the possibility that $M_3$ receptors act via a $G_i$ transduction pathway is not being suggested here (although the results of the PTX experiments do rule out the possibility of direct coupling of $G_{ai}$ subunits to $I_{Ks0}$).
Instead it is intended to demonstrate that activation of one receptor subtype can generate diverse signals.

M₃ receptors have also been shown to couple to PLA₂ and PLD, as well as tyrosine kinase and a novel class of voltage insensitive Ca²⁺ channels (Felder, 1995). Therefore there are several possible mechanisms still to be investigated before the pathway responsible for the inhibition of IK_{s0} is elucidated. It may be that a novel mechanism in CGNs is responsible for IK_{s0} inhibition, similar to that recently demonstrated for a delayed rectifier K⁺ channel. This study identifies the small GTP-binding protein, RhoA, as a necessary component in the M₁ mediated modulation of Kv1.2 channels (Cachero et al, 1998).

Alternatively members of the mitogen activated protein kinase (MAPK) family may be involved in the modulation of IK_{s0}. Rosenblum et al (2000) recently demonstrated that activation of muscarinic receptors in hippocampal slices, primary cortical neurons and transfected COS-7 cells couple to activation of ERK1/II (a member of the MAPK family) via a Ca²⁺ independent mechanism which involves the βγ subunit of G proteins. While activated ERK1/II can translocate to the nucleus and have longterm effects on gene expression, an immediate effect of ERK1/II activation on LTP in the hippocampus was also demonstrated. Thus suggesting that ERK1/II may have immediate cytosolic targets. It is possible therefore that specific ion channels are a potential downstream target of the Ca²⁺ independent MAPK signalling cascade, resulting in the modulation of their activity in a variety of cell types.

The experiments investigating muscarinic modulation of IK_{s0} are still in the initial stages and have certainly not exhausted all the downstream products of Gq/11 activation. In fact, even a direct action of Gq/11 subunits on IK_{s0} cannot be ruled out with the present evidence. Therefore, before a novel signalling pathway is proposed to be responsible for the inhibition of IK_{s0}, obvious additional experiments are required.
CHAPTER 4.

COMPARISON OF *ETHER Á GO GO*, M CURRENT AND $I_{K_{so}}$. 
4.1. INTRODUCTION.

The isolation and molecular characterisation of behavioural mutants in *Drosophila* that alter membrane excitability has proven to be a powerful method of identifying genes encoding ion channel subunits. The existence of flies which displayed an ether-sensitive, leg-shaking phenotype led to the discovery of a novel family of potassium channels known as the *ether à go-go (eag)* family.

The *eag* gene was cloned and sequence analysis of cDNA revealed that the encoded protein shares sequence similarities with the *Shaker (Sh)* family of potassium channels (Warmke *et al*, 1991). Most notably there are seven hydrophobic segments (six transmembrane segments plus a pore domain). Within this hydrophobic core region *eag* shows about 15% identity with the voltage activated potassium channels in the extended *Sh* family. The degree of amino acid identity is especially striking in S4 (voltage sensor) and P (pore) domains. However the *eag* polypeptide also shares similarities with cyclic nucleotide gated cation channels, since it possesses a cyclic nucleotide binding domain and shares numerous amino acid identities within the hydrophobic core (Guy *et al*, 1991). Therefore it was concluded that *eag* encodes a novel type of voltage gated potassium channel, which is the prototype of an entire new family of related potassium channel polypeptides that parallels the *Sh* family (Ganetzky *et al*, 1999).

Expression of *eag* channels in *Xenopus* oocytes or mammalian cell lines produces non-inactivating, voltage dependent, outwardly rectifying currents, with a threshold of activation of about −40 mV. A characteristic feature of *eag* currents is that activation becomes slower and more sigmoidal with increasingly negative prepulses or holding potentials (Ludwig *et al*, 1994, Robertson *et al*, 1996). An additional novel feature of *eag* currents is their sensitivity to Mg$^{2+}$. Increasing the extracellular concentration of Mg$^{2+}$ causes a dramatic slowing of the activation kinetics in a concentration and voltage dependent manner (Terlau *et al*, 1996). Expressed channels are relatively insensitive to the classical potassium channel blockers TEA and 4-AP, but do display sensitivity to muscarine, with activation of muscarinic receptors producing a block of *eag* current.
To date the physiologically relevant mammalian counterpart of eag channels is unresolved. However the properties of eag currents have led to the suggestion that they may be the molecular correlate of the potassium current known as the M current (Stansfeld et al, 1997).

M current was first described in frog sympathetic ganglion cells and was found to be inhibited by acetylcholine acting via muscarinic receptors (hence M current; Brown & Adams 1980). The M current is a voltage and time dependent potassium current which has a low threshold for activation (around -65 mV). On depolarisation M current activates with slow kinetics (in the order of tens to hundreds of milliseconds) and does not inactivate during the period of the depolarising step. On stepping back to a more hyperpolarised level the current also inactivates slowly. Single M channels are of relatively low conductance and they are difficult to identify pharmacologically since they are insensitive to any of the classical potassium channel blockers except barium ions. Recently however, two compounds have emerged that seem to show selectivity for M current, linopirdine and XE991 (Aiken et al, 1995, Lamas et al, 1997, Wang et al, 1998)

Since its discovery analogous currents have been described in a variety of other neuronal and non-neuronal cells such as rat and rabbit superior cervical sympathetic ganglia, cultured spinal cord neurons, toad stomach smooth muscle cells, hippocampal pyramidal cells and NG108-15 cells (Brown, 1988). Also the range of neurotransmitters which inhibit M current has been extended from muscarinic agonists to include bradykinin, substance P, LHRH and angiotensin. In addition activation of purinergic, β-adrenergic, opioid and glutamate metabotropic receptors have been shown to suppress M current in certain cell types (Marrion, 1997a). Because the precise kinetic and pharmacological properties of the current vary somewhat in different cell types the name ‘M-like’ is often applied to this current family (Selyanko et al, 1999).

Although much has been published on the M current, and its kinetics and pharmacology in many cell types has been well characterised, until recently the molecular nature of the current has remained elusive.
Chapter 4 - *Eag*, M current and $\text{IK}_{\text{so}}$. 

Stansfeld *et al* (1997) have suggested that *eag* may be the molecular correlate of M current, based on a number of functional similarities. These included an absence of inactivation, inhibition by muscarinic receptor activation and insensitivity to the classical potassium channel blockers TEA and 4-AP, but not Ba$^{2+}$. The strongest evidence presented was the similarity between the kinetic profiles of *eag* current and M current. Stansfeld *et al* (1997) compared *drosophila-eag* (d-eag) currents expressed in CHO cells with M currents in rat superior cervical ganglia, and demonstrated that the kinetic profiles were indeed very alike. However if one compares rat-*eag* (r-eag) channels with the same M current then there are obvious differences in kinetics. The deactivation kinetics of r-*eag* are so different that a comparison with M current no longer seems convincing. Closer inspection reveals additional differences in permeation, conductance, pharmacology and expression patterns, which when taken together suggest that the molecular identity of M current does not lie with *eag* (Marrion, 1997b).

Instead it has been proposed that $\text{IK}_{\text{so}}$ could be the native current correlate to r-*eag* (Marrion, 1997b). They share the fundamental characteristics of outward rectification, non-inactivation and sensitivity to muscarine, plus the deactivation kinetics of r-*eag* are more similar to $\text{IK}_{\text{so}}$ than M current.

The aim of this chapter was to come closer to identifying the molecular nature of $\text{IK}_{\text{so}}$. The sensitivity of $\text{IK}_{\text{so}}$ to the selective M current blocker linopirdine was tested to determine whether $\text{IK}_{\text{so}}$ could be an M-like current in CGNs. Also the effect of Mg$^{2+}$ on the activation of $\text{IK}_{\text{so}}$ was studied. A unique feature of r-*eag* currents is that the activation kinetics strongly depend on the concentration of external Mg$^{2+}$, with the removal of Mg$^{2+}$ speeding up activation (Terlau *et al*, 1996). If this regulatory action of Mg$^{2+}$ is unique to *eag* channels then it would be expected that $\text{IK}_{\text{so}}$ would share this sensitivity to Mg$^{2+}$ if it does indeed belong to the *eag* family of potassium channels.
4.2. RESULTS.

4.2.1. Effect of the specific M current blocker, linopirdine, on the amplitude of IK\textsubscript{so}.

The data traces in Figure 4.1a illustrate the effect of 100 µM linopirdine on IK\textsubscript{so}. In the presence of 100 µM linopirdine the current amplitude was reduced. For the example shown the amplitude of IK\textsubscript{so} recorded at -20 mV was decreased from 146 pA in control to 113 pA in the presence of 100 µM linopirdine. The effect of linopirdine was completely reversed upon washing, as is shown in Figure 4.1b.

Figure 4.1c shows the mean percentage inhibition of IK\textsubscript{so} recorded at -20 mV, caused by 10 and 100 µM linopirdine. At 10 µM, linopirdine had very little effect on the amplitude of IK\textsubscript{so}, producing a mean inhibition of 0.4 ± 5.2 % (n = 4). Increasing the concentration to 100 µM resulted in a mean inhibition of 27.4 ± 4.3 % (n = 5).

4.2.2. Activation kinetics of IK\textsubscript{so}.

The activation of IK\textsubscript{so} was investigated to allow a comparison of the kinetics with those of r-eag currents. Figure 4.2 shows a current-voltage relationship designed to look at the activation of IK\textsubscript{so}. The cell is held at a potential of -30 mV where a hyperpolarising step to -90 mV for 1 s produces rapid deactivation of IK\textsubscript{so}. Currents were then activated by depolarising from -90 mV to test potentials ranging from -70 to +10 mV in 10 mV steps.

Figure 4.2 demonstrates the difficulty in resolving the activation of IK\textsubscript{so} in cerebellar granule neurons, (CGNs), due to the contamination by other currents. Even at quite hyperpolarised potentials the activation is masked by the transient A current (I\textsubscript{a}).

4.2.2.1. Isolation of IK\textsubscript{so} activation using subtraction analysis.

IK\textsubscript{so} is inhibited by muscarine and therefore it is possible to isolate the muscarine sensitive component by subtracting the currents recorded in the presence of 10 µM
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muscarine from control currents. *I*<sub>A</sub> should be eliminated by subtraction, meaning the activation of the difference current can be quantified. To quantify the activation of *IK*<sub>so</sub> sigmoidal curves are fitted to the activating section of the current traces and the time taken to reach the half maximal value is obtained and expressed as a *t*<sub>50</sub> value. Figure 4.3 shows example data for which subtraction analysis has been performed. For subtraction analysis to work in this instance *I*<sub>A</sub> must not vary between protocols. In many experiments this was not the case and subtracted data resulted in unusable traces. It was possible on occasion to quantify the subtracted data using sigmoidal curves, but the *t*<sub>50</sub> values obtained were extremely inconsistent. For example the *t*<sub>50</sub> values calculated when stepping to a test potential of -40 mV ranged from 1.9 to 10 ms. An alternative method for isolating the activation of *IK*<sub>so</sub> was therefore employed.

### 4.2.2.2. Isolation of *IK*<sub>so</sub> activation using a pharmacological blocker of *I*<sub>A</sub>.

4-aminopyridine (4-AP) has previously been described as a relatively specific blocker of transient outward current in a number of preparations, and is routinely used in the pharmacological separation of potassium currents (Cull-Candy *et al*, 1989). Earlier experiments demonstrated that 10 mM 4-AP is a potent blocker of *I*<sub>A</sub> in CGNs (see Figure 3.2), thereby providing a more reliable means of isolating the activation of *IK*<sub>so</sub>.

*I*<sub>A</sub> is activated when a cell is depolarised after a period of hyperpolarisation. To evoke *I*<sub>A</sub> in CGNs, cells were stepped from a holding potential of -30 mV to a hyperpolarising potential of -120 mV, before stepping to a test potential of 0 mV. *I*<sub>A</sub> can clearly be seen in Figure 4.4a as a large transient peak, with bath application of 10 mM 4-AP gradually reducing peak amplitude. *I*<sub>A</sub> is measured as the difference between the points ○ and ■ in Figure 4.4a, and this is plotted as a function of time in Figure 4.4b.

Figure 4.4b demonstrates that *I*<sub>A</sub> is completely abolished by 10 mM 4-AP in a fully reversible manner. It should be noted that the level of *I*<sub>A</sub> seems to fall below zero, this is due to the steady state current at point ○ in Figure 4.4a increasing slightly on
application of 4-AP. Additionally Figure 4.4b shows the lack of effect of 4-AP on the amplitude of IKso, which is measured as a mean over 90 ms at -30 mV.

The current voltage protocol detailed in Figure 4.2 was performed in the presence and absence of 10 mM 4-AP. For clarity, only the trace on stepping to a test potential of -40 mV is shown in Figure 4.4c and 4.4d. Again the inhibition of IA can clearly be seen in the presence of 4-AP.

Once it had been established that 4-AP could reliably remove IA contamination, all subsequent experiments regarding the activation of IKso were performed in the presence of 4-AP.

4.2.3. Effect of external Mg^{2+} on the activation of IKso.

Figure 4.5 shows the effect of varying the external Mg^{2+} concentration to 0 and 10 mM on the activation of IKso. Current traces were generated using the current voltage protocol detailed in Figure 4.2. On first examination it seems that there is no effect of external Mg^{2+} on the activation of IKso. This observation was quantified by analysing the traces as previously described. An example of the curve fitting procedure is shown in Figure 4.6.

tso values were obtained for test potentials up to -20 mV in all Mg^{2+} concentrations. Quantifying the activation of IKso was only performed for potentials more negative than -20 mV as potentials more positive to this begins to bring in contamination by the delayed rectifier component of outward potassium current, even in the presence of 10 mM 4-AP.

Figure 4.7 demonstrates that the tso values range from about 0.3 to 2.5 ms for all potentials and Mg^{2+} concentrations studied. Significance tests showed that the tso values obtained in 0 and 10 mM Mg^{2+} did not differ significantly from control values obtained in 2 mM Mg^{2+}. For example in 0 mM Mg^{2+} the mean tso value was 1.79 ± 0.21 ms (n = 8) at -40 mV which did not differ significantly from control values of 1.84 ± 0.24 ms (n = 10; P = 0.88, unpaired t-test). The mean tso value in 10 mM Mg^{2+}
was 1.65 ± 0.18 ms (n = 6), which again did not significantly differ from control values (P = 0.59, unpaired t-test). This lack of effect indicates that external Mg$^{2+}$ does not regulate the activation of IK$_{so}$, which is in contrast to eag currents.
FIGURE 4.1. The effect of linopirdine on $I_{K_{so}}$.

a). Representative data traces were generated by applying a voltage step to -60 mV from a holding potential of -20 mV, before stepping back to the holding potential, once every 6 s. The protocol was performed under control conditions and in the presence of 100 pM linopirdine.

b). Illustration of the inhibition of $I_{K_{so}}$ on bath application of 100 pM linopirdine (inhibition can also be seen in (a) as a reduction in the amount of current present at the holding potential). The amplitude of $I_{K_{so}}$ was measured as a mean over 176 ms directly preceding the hyperpolarising step to -60 mV, and plotted as a function of time.

c). Graph of the mean percentage inhibition of the control current at -20 mV produced by 10 (n = 4) and 100 pM linopirdine (n = 5).
FIGURE 4.2. Contamination of current-voltage relation by $I_A$. Current-voltage relationship is produced by stepping from a holding potential of $-30 \text{ mV}$ to test potentials of 1 s duration ranging from $-70$ to $+10 \text{ mV}$, following a 1 s prepulse to $-90 \text{ mV}$. The presence of $I_A$ can clearly be seen at all potentials above $-70 \text{ mV}$. 
FIGURE 4.3. Isolation of IK<sub>so</sub> using subtraction analysis.
a). Current-voltage relationship, obtained in control conditions, using protocol detailed in Figure 4.2. Only traces for test potentials in the range -70 mV to -20 mV are shown.
b). As (a) but recorded in the presence of 10 μM muscarine.
c). Muscarine sensitive current obtained by subtraction of current recorded in the presence of 10 μM muscarine from control currents.
FIGURE 4.4. Effect of 4-AP on $I_A$ and $I_{K_{so}}$ in CGNs.
a). Data traces were generated by applying a voltage step to $-120$ mV from a holding potential of $-30$ mV, before stepping to a test potential of $0$ mV, once every $5$ s. $10$ mM 4-AP was bath applied, producing inhibition of the transient peak.
b). Illustration of the inhibition of $I_A$ on bath application of $10$ mM 4-AP. Current measured at point • in (a) was subtracted from the current present at point ■, to give a measure of $I_A$. This is plotted as a function of time, demonstrating the complete and reversible inhibition of $I_A$ by $10$ mM 4-AP.
The lack of effect of $10$ mM 4-AP on $I_{K_{so}}$ is also illustrated. The amplitude of $I_{K_{so}}$ is measured at $-30$ mV as a mean over $90$ ms directly preceding the hyperpolarising step to $-120$ mV.
c). Single data trace evoked on stepping to a test potential of $-40$ mV, using the protocol detailed in Figure 4.2, in control solution.
d). As in (c) but in the presence of $10$ mM 4-AP.
FIGURE 4.5. Effect of external Mg$^{2+}$ on the activation of IK<sub>sa</sub>. Data traces obtained in 2 mM (a), 0 mM (b) and 10 mM (c) Mg$^{2+}$. Currents were generated in the presence of 10 mM 4-AP using the protocol detailed in Figure 4.2.
FIGURE 4.6. Fitting of sigmoidal curves to current traces.
The activating section of current traces on stepping from a prepulse potential of -90 mV to a test potential of -40 mV are shown on an expanded time scale, for all [Mg$^{2+}$].
Sigmoidal curves are fitted to the current traces using the built in function of Origin 5.0. Time taken to reach 50% activation is expressed as a $t_{50}$ value for each example shown.
FIGURE 4.7. $t_{50}$ values for activation of $\text{IK}_{\text{so}}$ in varying external $\text{Mg}^{2+}$ concentrations. Plot of the mean $t_{50}$ value for each test potential at all $[\text{Mg}^{2+}]$ studied. Standard error is shown as error bars. This graph demonstrates that at all potentials tested it was found that the $t_{50}$ values obtained in 0 and 10 mM $\text{Mg}^{2+}$ did not differ significantly from control values obtained in 2 mM $\text{Mg}^{2+}$. (■, n = 8, ●, n = 10 ▲, n = 6).
4.3. DISCUSSION.

The results of experiments described in this chapter have shown that $IK_{so}$ is not an $M$-like or related current. The activation of $IK_{so}$ is much faster than $M$ current and $IK_{so}$ showed little sensitivity to linopirdine at concentrations which would produce about a 50% block of native $M$ currents. Experiments showed that the activation kinetics of $IK_{so}$ were unaffected by changes in external $Mg^{2+}$, indicating that $IK_{so}$ is not a member of the $eag$ family of potassium channels.

4.3.1. Ether à go go and $M$ current.

$Eag$ was originally suggested to be the molecular correlate of $M$ current on the basis of a comparison between $d$-$eag$ currents expressed in CHO cells and $M$ current in rat superior cervical ganglia. The data presented was visually compelling and the kinetic profile between $d$-$eag$ and $M$ current was very similar. The problem with this comparison however is the species difference. Stansfeld et al (1997) are not comparing like with like. $D$-$eag$ currents are not representative of mammalian $eag$. Although $d$-$eag$ and $r$-$eag$ share 67% amino acid identity the corresponding potassium channels exhibit remarkable differences when expressed in $Xenopus$ oocytes (Ludwig et al, 1994), most notably in their ion selectivity. $R$-$eag$ is highly selective for potassium, whereas $d$-$eag$ is less selective, exhibiting an unusually high permeability ratio for $Cs^+$ ions. The slowing of activation kinetics with increasingly negative prepulses is a characteristic feature of $eag$ currents, however this phenomenon is much more pronounced in $r$-$eag$ than $d$-$eag$ (Ludwig et al, 1994). Additionally $r$-$eag$ currents have quite different, faster deactivation kinetics than $d$-$eag$, and $d$-$eag$ currents exhibit partial inactivation whereas mammalian $eag$ are sustained for the duration of the activating voltage command (Robertson et al, 1996). Therefore, as suggested by Mathie & Watkins (1997), it would be more prudent to compare $M$ current in rat superior cervical ganglia to the $eag$ clone of the same species. This comparison however is not as visually compelling as that with $d$-$eag$ and it no longer seems convincing that $r$-$eag$ is the molecular correlate of $M$ current.

Quantitative comparison between $r$-$eag$ and $M$ current reveals several differences. Most obviously the kinetics of the two currents differ considerably, with the
deactivation kinetics of r-eag being much faster. The permeability properties of r-eag and M current do not match. Mammalian M channels can conduct Cs\(^+\) ions while r-eag cannot, indicating that the pore of r-eag is different from that of the mammalian M channel (Marrion, 1997b). Both currents are inhibited by activation of muscarinic receptors and in the case of r-eag it is the resulting increase in intracellular calcium that is responsible (Stansfeld et al, 1996). The pathway that induces block of M currents however is still under dispute. The pharmacology of M current and r-eag also differ, r-eag currents are insensitive to linopirdine and XE991, selective blockers of M current which are increasingly being used as diagnostic tools in the identification of M-like currents (Wang et al, 1998).

A unique feature of r-eag channels is that their activation kinetics strongly depend on the concentration of external Mg\(^{2+}\), with the removal of Mg\(^{2+}\) speeding up activation. This property is not shared with M current. In general M current is relatively insensitive to external Mg\(^{2+}\) with neither the activation or deactivation kinetics being materially altered on removing Mg\(^{2+}\) (Adams et al, 1982). It has been reported that the amplitude of the M current in NG108-15 cells is reduced by Mg\(^{2+}\) (IC\(_{50}\) = 16.3 mM), but this seems to be a block of the steady state current and is without effect on the activation kinetics (Robbins et al, 1992).

One final difference between the two is that the expression pattern for eag mRNA does not overlap with the occurrence of M current. For example mRNA for r-eag is almost absent in hippocampal pyramidal cells which possess M current, but is abundant in CGNs which do not possess M current (Marrion, 1997b). Taken together this evidence suggests that the molecular identity of M current does not lie with eag channels.

In fact it is now known that the molecular correlate of the M current is the KCNQ family of potassium channels. Wang et al (1998) have shown that co-expression of KCNQ2 and 3 subunits produces heteromulitmers which form a channel with essentially identical biophysical and pharmacological sensitivities to the native M current in rat sympathetic neurons. The pattern of KCNQ2/3 gene expression is consistent with these genes encoding the native M current.
It is not yet known whether all M-like channels are composed of these two subunits, or homologs thereof. Since M-like currents in different cell types are not identical in kinetics and pharmacology it is possible that members of other potassium channel gene families might contribute to the generation of M-like currents. For example it has previously been suggested that the eag locus produces subunits that, in addition to forming homomeric channels with novel properties, also contribute to potassium channel diversity by combining with peptides from different potassium channel genes (Zhong & Wu, 1991, 1993).

Voltage clamp analysis of Drosophila larval smooth muscle revealed that eag mutations affected all identified potassium currents in this tissue, including those specifically eliminated by mutations in the Sh genes. Therefore it is possible that the channels generating M-like currents in different cell types may be heterogenous in molecular composition. Indeed Selyanko et al (1999) have attributed the M-like current in NG108-15 cells to a combination of two different types of potassium channel: the mouse ether à go-go related gene (merg1a) and KCNQ2/3.

So, although it seems the expression of homomeric eag subunits does not underlie M current the involvement of eag subunits in the generation of M-like currents should not be ruled out completely. Instead, perhaps it would be better to conclude that eag subunits could contribute to M-like currents in specific cell types.

4.3.2. M current and IK\textsubscript{so}

IK\textsubscript{so} shares the fundamental characteristics of outward rectification, non-inactivation and sensitivity to muscarine with both M-like currents and expressed eag currents. Therefore IK\textsubscript{so} could be considered an M-like current in CGNs.

Although IK\textsubscript{so} shows some resemblance to M current a more rigorous comparison reveals several differences between the two currents, in much the same way as has been shown for eag. The major difference between IK\textsubscript{so} and M current is their kinetics. M current is described as a slowly activating/deactivating current, whereas the kinetics of IK\textsubscript{so} are almost instantaneous.
The kinetics of M current have been studied in detail and both the activation and deactivation show voltage sensitivity. Pennefather (1986) explored the activation of M current at potentials up to +40 mV and found that the time constant of activation accelerated with increasing potentials, but only to a minimum of 16 ms. Results in this chapter show that the $t_{50}$ (time taken for half maximal activation) values for $I_{K\alpha}$ range from 0.3 to 2.5 ms over a range of test potentials indicating that the activation of $I_{K\alpha}$ is not only faster than M current, but additionally doesn’t show any voltage sensitivity.

The time constant of deactivation for M current in contrast accelerates with decreasing potentials, up to a minimum of 11 ms at -100 mV (Robbins et al., 1992, Owen et al., 1990). Previous studies with $I_{K\alpha}$ have shown that the deactivation kinetics are much faster than those reported for M currents. The deactivation time constant of $I_{K\alpha}$ on stepping from -30 to -80 mV is 0.5 ms, compared to 122 ms for M current in NG108-15 cells (Watkins & Mathie, 1996, Robbins et al., 1992).

The pharmacological profile of M current and $I_{K\alpha}$ initially appear similar, with both being insensitive to the classical potassium channel blockers TEA and 4-AP, but showing some sensitivity to Ba$^{2+}$. These compounds are broad spectrum potassium channel blockers and cannot be used alone to confirm the identity of a potassium current. However, as previously mentioned, selective blockers of M-like currents are now available. By using one such compound, linopirdine, experiments in this chapter have shown that $I_{K\alpha}$ lacks the sensitivity to linopirdine seen with M-like currents. Studies have shown that for M current in rat superior cervical ganglion cells and CA1 hippocampal pyramidal cells the IC$_{50}$ for linopirdine is 3.4 and 8.5 μM respectively (Aiken et al., 1995, Lamas et al., 1997). A concentration of 10 μM, which for M-like currents would be expected to produce more than a 50% block, barely had any effect on $I_{K\alpha}$ (0.4 ± 5.2 % inhibition). Increasing the concentration to 100 μM inhibited $I_{K\alpha}$ by 27.4 ± 4.3 %. These results therefore indicate that M current and $I_{K\alpha}$ are not related currents. In retrospect this is not surprising since mRNA for only KCNQ2, but not KCNQ3 is expressed to any significant level in CGNs (Wang et al, 1998).
Chapter 4 – *Eag, M current and IK*<sub>so</sub>.*

**4.3.3. Ether à go go and IK*<sub>so</sub>**

There is sufficient evidence to conclude that the molecular correlate of M current is not *eag*, at least not in homomeric form. However Marrion (1997b) suggested that IK<sub>so</sub> may be the native current correlate to r-*eag*. They share all the fundamental characteristics and the deactivation kinetics of r-*eag* are more similar to IK<sub>so</sub> than M current. Furthermore r-*eag* mRNA is highly expressed in CGNs.

If IK<sub>so</sub> does belong to the *eag* family of potassium channels then it would be expected that IK<sub>so</sub> would share the unique property of Mg<sup>2+</sup> sensitivity with r-*eag* currents.

Terlau *et al* (1996) showed that the presence of Mg<sup>2+</sup> in the external solution significantly slowed the activation of r-*eag* currents. For example in the absence of external Mg<sup>2+</sup> the time constant of activation on stepping to 0 mV is approximately 8 ms compared to about 250 ms in the presence of 2 mM Mg<sup>2+</sup>.

Experiments in this chapter determined the t<sub>50</sub> values for IK<sub>so</sub> activation over a range of potentials and Mg<sup>2+</sup> concentrations. These results show that the activation of IK<sub>so</sub> is unaffected by changes in external Mg<sup>2+</sup>, with the t<sub>50</sub> values obtained in 0 and 10 mM Mg<sup>2+</sup> not differing significantly from those obtained in control conditions of 2 mM Mg<sup>2+</sup>. Since IK<sub>so</sub> lacks the diagnostic feature of r-*eag* currents it therefore seems unlikely that it is the native current correlate of r-*eag*.

It should be appreciated that the t<sub>50</sub> values cannot be used as a true measure of the time constant of activation for IK<sub>so</sub>. Sampling rate limitations mean that insufficient data points were obtained during the voltage step to allow optimal fitting of a sigmoidal curve. Although the t<sub>50</sub> values cannot be used as a direct measure of current activation, the values do show that extracellular Mg<sup>2+</sup> has no effect on the activation of IK<sub>so</sub> comparable to that seen for r-*eag* currents. If the activation of IK<sub>so</sub> were to be slowed in the presence of extracellular Mg<sup>2+</sup> in the same way as has been demonstrated for r-*eag*, then this slowing would be revealed by the protocol even when the sampling rate limitations are taken into consideration.
Nevertheless, as was the case with M current, it can only be concluded that a homomeric association of eag subunits does not underlie IK_{so}. There is more than one eag gene present in the mammalian genome (Ludwig et al, 1997) and it may be that a heteromeric combination of subunits from the eag family is a more reasonable suggestion for the molecular basis of IK_{so}. Terlau et al (1996) suggested that the interaction between Mg^{2+} and r-eag may involve a unique site at the r-eag channel protein which may not be present in other potassium channels. It may be that this site is only present in a homomeric arrangement of subunits.

Alternatively it could be the association of r-eag subunits with an additional protein that is responsible for IK_{so} in CGNs. Hoshi et al (1997, 1998) injected cerebellar poly (A)^+ RNA into Xenopus oocytes and recorded currents that were nearly identical to IK_{so}. Using suppression cloning the expression of this current was blocked which allowed the isolation of a clone encoding an important regulatory polypeptide. This protein was designated KCR1. When KCR1 and r-eag were co-expressed in COS cells the voltage dependence of activation of r-eag currents shifted 10 mV in the hyperpolarising direction. The presence of KCR1 also resulted in r-eag currents whose activation kinetics were insensitive to changes in external Mg^{2+}, producing currents which behave similarly to IK_{so}.

It should be noted that while the protocol used to identify IK_{so} was the best that could be devised, it is not perfect. The current measured may be additionally contaminated by other components, which may include I_{KV} and possibly other, as yet unidentified components of the ‘leak’ current.

Therefore there are a several suggestions for the possible molecular identity of IK_{so}. Further molecular and electrophysiological data are needed before the true nature of IK_{so} is revealed.
CHAPTER 5.

POTENTIAL MOLECULAR CHARACTERISATION OF $\text{IK}_{so}$. 
5.1. INTRODUCTION.

Molecular cloning techniques have led to the isolation and functional expression of numerous genes that encode pore-forming α subunits of potassium channels. Once a gene encoding a particular α subunit has been identified, the amino acid sequence of the resulting protein can be determined, and the structure of the protein is then predicted by hydrophobicity analysis. Determination of the structure of α subunits in this way has meant that potassium channels can be classified according to the structure of their subunits. Until recently there were thought to be two structural superfamilies: one containing subunits with one pore-forming P domain and six transmembrane domains (6TMD; Figure 5.1a), and one containing subunits with one P domain and two transmembrane domains (2TMD; Figure 5.1b).

The first potassium channel gene to be isolated, and the founding member of the 6TMD superfamily, was Shaker. This gene was identified as the cause of a motion disorder in Drosophila (Papazain et al, 1987). Subsequently additional members of this superfamily have been identified on the basis of their sequence similarity to Shaker. These include the Shab, Shaw and Shal genes. All these genes encode subunits which generate voltage-gated, outwardly-rectifying potassium currents, corresponding to rapidly inactivating A type and delayed rectifier type channels (Butler et al, 1989, Wei et al, 1990). Mammalian counterparts of these genes are the Kv1, Kv2, Kv3 and Kv4 subfamilies.

The problem with homology screening is that the method is biased towards isolation of genes that share substantial similarity to the first gene isolated, in this case Shaker. Other K⁺ channel genes only distantly related to Shaker will not be identified by this approach. Slo and eag are examples of such genes. These genes also encode subunits belonging to the 6TMD superfamily but are missed by homology screening since their overall similarity to Shaker is very low. Instead they were identified by molecular analysis of other Drosophila behavioural mutants.
Figure 5.1. Putative topological organisation of individual $K^+$ channel $\alpha$ subunits from the three structural superfamilies.

a). 6TMD subunit – voltage gated and calcium gated $K^+$ currents.


c). 2P domain 8 TMD subunit – yeast leak $K^+$ currents.

More recently genes for $K^+$ channels have been isolated by expression cloning, a method which does not select for homology but identifies genes whose products show ion channel function (Goldstein et al, 1998). This method led to the discovery of the second structural superfamily of $K^+$ channels, containing subunits with 2TMD (Ho et al, 1993). Members of this family form inwardly rectifying $K^+$ channels.

Although these two superfamilies generate different potassium currents they do have some similarities. That is they both require four subunits to form a functional channel and each subunit contains one pore-forming P domain.

Within the P domain there is a stretch of eight amino acids which has been found to be highly conserved between potassium channels (see section 1.3.3.2.1.). This region is responsible for the common ion selectivity seen in essentially all potassium channels, and is referred to as the signature sequence (Heginbotham et al, 1994). This unique sequence has been identified in all $K^+$ channels so far cloned, regardless of their structure.

Unexpectedly, within the last four years a third major superfamily of potassium channels has emerged, known as the 2-pore domain family of potassium channels ($K_T; K = K^+, T = 2$-pore; Ketchum et al, 1995). Using the P domain from several potassium channels, gene databases were searched to identify channel like motifs. Along with many known potassium channel genes an additional gene was identified in yeast which was unusual as it contained two signature sequences. Subsequent hydropathy analysis of the gene product predicted a subunit which had two P domains and 8TMD (Figure 5.1c). In fact the subunit structure appears like a union of a 6TMD subunit of voltage-gated $K^+$ channels and a 2TMD subunit of inward rectifier channels (Salkoff and Jegla, 1995), however there is no region comparable to the S4 voltage sensor of voltage-gated potassium channels.

Expression studies using Xenopus oocytes showed that injection with cRNA for this yeast channel gene gave rise to an outwardly rectifying, non-inactivating potassium current, whose activation is not voltage dependent. Therefore this channel was named TOK1 (Tandem of P domains in an Outwardly rectifying $K^+$ channel; Ketchum et al, 1995).
The novel way in which TOK1 was identified has subsequently led to the discovery of several $K_T$ channels in other species, including mammals, *Drosophila*, nematode worms and plants (e.g. Lesage *et al.*, 1996a, Goldstein *et al.*, 1996, Salkoff and Jegla, 1995, Czempinski *et al.*, 1997). The cloned channels from all these organisms have 4TMD, a bit like a union of two subunits from the 2TMD superfamily (Figure 5.1d). Again there is no region corresponding to the S4 domain of voltage-gated $K^+$ channels. The 8TMD structure of TOK1 is therefore atypical of the $K_T$ channel superfamily so far.

The functional properties of these newly cloned channels have been examined by expression in Xenopus oocytes. As predicted for $K^+$ channels lacking a voltage-sensing region all these channels, just like TOK1, are voltage insensitive. In fact the lack of voltage dependent activation is a diagnostic feature of the $K_T$ channel superfamily.

In mammals six functional members of the $K_T$ superfamily with 4TMD have been identified to date, TWIK-1; TWIK-2; TREK-1; TRAAK; TASK-1 and TASK-2. Despite their similar topology these clones share low sequence similarity and generate extraordinarily diverse channels in terms of both their distribution and functional properties.

TWIK-1 (Tandem of P domains in a Weak Inwardly rectifying $K^+$ channel; Lesage *et al.*, 1996a) was the founding member of mammalian $K_T$ channels. As its name suggests expression of TWIK-1 in oocytes produced a channel with weak inward rectification properties. The current is non-inactivating, with time and voltage independent kinetics. The I/V relation is almost linear over a wide range of potentials (-120 to 0 mV), saturating for stronger membrane depolarisations. The rectification at positive membrane potentials is due to voltage dependent block by $Mg^{2+}$ (Lesage *et al.*, 1996a). Pharmacologically TWIK-1 is insensitive to the classical potassium channel blockers TEA and 4-AP, but is blocked by $Ba^{2+}$, as well as quinine and quinidine. Activity is also inhibited by intracellular acidification, and is stimulated by activation of protein kinase C. Northern blot analysis shows TWIK-1 has a wide tissue distribution, being found most abundantly in heart and brain.
Recently another $K_T$ channel has been cloned which shares significant sequence similarity to TWIK-1. This channel also produces weak inwardly rectifying currents and has been named TWIK-2. Electrophysiological properties and mRNA expression patterns of TWIK-2 are similar to those of TWIK-1, however TWIK-2 channels are pharmacologically distinct from TWIK-1 channels since they are not inhibited by quinidine or quinine and are relatively insensitive to block by $Ba^{2+}$ (Chavez et al, 1999).

TREK-1 (TWIK RElated $K^+$ channel; Fink et al, 1996) was the first outward rectifier of this family to be described. This clone generates non-inactivating, outward currents which are potentiated by intracellular acidification, application of arachidonic acid and membrane stretch (Fink et al, 1996, Patel et al, 1998, Maingret et al, 1999a). Activation of both protein kinase A and C inhibits this current.

TASK-1, TASK-2 and TRAAK currents behave as open rectifiers, with no rectification other than that predicted from the constant field assumptions for an open channel (Maingret et al, 1999a, Duprat et al, 1997, Leonoudakis et al, 1998, Fink et al, 1998, Reyes et al, 1998). Despite this common property, TASK-1, TASK-2 and TRAAK exhibit very different modulations of their activity. TASK-1 and 2 are regulated by external pH variations (hence TWIK related Acid Sensitive $K^+$ channel), with a small drop of the external pH near to the physiological range producing an inhibition of the current (Duprat et al, 1997, Leonoudakis et al, 1998, Reyes et al, 1998). TASK-1 and TASK-2 can be distinguished from one another since the activation kinetics of TASK-2 currents are relatively slow when compared to TASK-1. They also have distinct individual expression patterns. TASK-1 has widespread distribution, being abundant in the brain, whereas TASK-2 is mainly expressed in the kidney and is essentially absent in the brain.

TRAAK is stimulated by arachidonic acid (TWIK Related Arachidonic Acid stimulated $K^+$ channel; Fink et al, 1998), as well as other unsaturated fatty acids, and is also activated by membrane stretch (Maingret et al, 1999b). These properties are shared with TREK-1 currents, however TRAAK can be distinguished from TREK-1 on the basis of their differing pharmacology and localisation. TRAAK is insensitive
to intracellular acidification and cAMP. It is expressed exclusively in neuronal tissues including brain, spinal cord and retina, whereas TREK-1 is widely distributed. Despite their different functional properties, all these \(K_T\) channels express quasist�aneous and non-inactivating currents (although the activation kinetics of TASK-2 are relatively slow), which do not display voltage dependent activation thresholds. This means these channels can be open at all potentials and any apparent rectification is due to the uneven distribution of potassium ions across the membrane or some kind of extrinsic rectification. Therefore these channels are thought of as background or leak channels, and are believed to be involved in the generation and modulation of the resting membrane potential in a variety of cell types.

Based on some of the properties described \(K_T\) channels are presently classified into three functional subfamilies (Reyes et al, 1998, Salinas et al, 1999). Clones which display a sensitivity to small changes in extracellular pH, such as TASK-1 and TASK-2 make up one subfamily. Clones which are mechanosensitive and are enhanced by arachidonic acid, such as TREK-1 and TRAAK are grouped into a second subfamily, and the final subfamily includes clones which generate currents with inwardly rectifying I/V relations, such as TWIK-1 and TWIK-2.

To date no functional correlate of \(K_T\) channels has been identified in mammalian neurons. However two of these clones, TREK-1 and TASK-1 are highly expressed in the cerebellum, and their currents are instantaneously activating, outwardly rectifying and non-inactivating (Fink et al, 1996, Leonoudakis et al, 1998), just like \(I_{Ks0}\). Therefore it is possible that \(I_{Ks0}\) is the first functional correlate of this novel family to be identified in native neurons.

The aim of this chapter was to determine whether \(I_{Ks0}\) shares the diagnostic feature of \(K_T\) channels, which is a lack of voltage dependent activation. Additionally further pharmacological characterisation of \(I_{Ks0}\) is required to allow a more direct comparison of the current with cloned members of the \(K_T\) superfamily.
Chapter 5 – Potential molecular characterisation of $\text{IK}_{\text{s}}$

5.2. RESULTS.

5.2.1. Effect of increasing extracellular potassium concentration on the I/V relation of $\text{IK}_{\text{s}}$.

To ensure that $\text{IK}_{\text{s}}$ is studied in isolation the sensitivity of the current to muscarine has again been exploited. Because this current is sensitive to muscarine it is possible to isolate the muscarine sensitive component by subtraction and, using a ramp protocol, I/V relations for the difference current can be obtained under varying external potassium concentrations.

Previously it has been shown that following step voltage changes $\text{IK}_{\text{s}}$ reaches steady state with a time constant of 0.5 ms (Watkins and Mathie, 1996), therefore a ramp protocol was designed to be sufficiently slow (10 ms mV$^{-1}$) to allow $\text{IK}_{\text{s}}$ to reach steady state at each potential.

Panel a in Figure 5.2 shows the time course for a series of experiments. The amplitude of $\text{IK}_{\text{s}}$, recorded at -20 mV, was measured as a mean over 176 ms, once every 5 seconds and plotted as a function of time (closed squares). The numbered points represent the time at which the ramp protocol was performed, where cells were held at -20 mV and ramped down to -100 mV in 800 ms, before returning to the holding potential.

Panel b contains traces obtained when the ramp protocol was performed in control conditions (2.5 mM [K$^+$]$_o$), and in the presence of 10 µM muscarine. Panel c contains traces obtained when the ramp protocol was performed in 25 mM [K$^+$]$_o$ alone and in 25 mM [K$^+$]$_o$ in the presence of 10 µM muscarine. Subtracting the traces in panel b from one another and plotting the amount of difference current against voltage gives a conventional I/V relation for $\text{IK}_{\text{s}}$ in control conditions, see Figure 5.3a (black trace). Similarly subtracting the traces in panel c from one another provides a measure of the amount of $\text{IK}_{\text{s}}$ present in 25 mM [K$^+$]$_o$, and plotting this against voltage gives an I/V relation for $\text{IK}_{\text{s}}$ in conditions of 25 mM external potassium, see Figure 5.3a (red trace).
Figure 5.3a shows the effect of raising the external potassium concentration from 2.5 mM to 25 mM on the I/V relation of $I_{K_{so}}$. In control conditions the I/V relation for $I_{K_{so}}$ is outwardly rectifying, with very little inward current being seen at hyperpolarising potentials. The reversal potential in this example is $-89$ mV. Raising the external potassium concentration to 25 mM produces a shift of the reversal potential in the depolarising direction to $-40$ mV. This effect was observed in a further three cells. On average the reversal potential was $-90 \pm 4$ mV ($n = 4$), which is in good agreement with the predicted equilibrium potential for potassium in control conditions ($-98$ mV). In 25 mM $[K^+]_o$ the reversal potential was shifted to $-39 \pm 1$ mV ($n = 4$), this is virtually identical to the predicted potassium equilibrium potential ($-40$ mV), indicating that $I_{K_{so}}$ is a potassium selective current. Additionally raising the external potassium concentration demonstrates that $I_{K_{so}}$ can be measured at all potentials, with the inward component of the current being much larger in these conditions. Since $I_{K_{so}}$ is active at all potentials it has no threshold for activation and therefore shares the diagnostic property of $K_T$ channels. Raising the external potassium concentration further to 100 mM shifts the reversal potential even more positive and the I/V relation can clearly be seen to linearise. This open rectifying behaviour is exactly that predicted by the Goldman-Hodgkin-Katz equation for a leak channel, and has been reported for certain $K_T$ channels.

5.2.2. Pharmacological characterisation of $I_{K_{so}}$.

Presently no specific pharmacological tools are available to aid identification of specific $K_T$ currents. Instead the effect of a whole range of compounds is determined in an attempt to build up a unique pharmacological profile for a particular channel. Pharmacological characterisation of $I_{K_{so}}$ was undertaken to allow a more direct comparison of $I_{K_{so}}$ with cloned members of the $K_T$ family.

Figure 5.4 shows the effect of $Ba^{2+}$ on the amplitude of $I_{K_{so}}$. Panel a contains example traces obtained under control conditions and in the presence of various concentrations of $Ba^{2+}$. $Ba^{2+}$ can be seen to produce a concentration dependent inhibition of $I_{K_{so}}$, 0.3, 1 and 3 mM $Ba^{2+}$ produced $17.1 \pm 1.6$ % ($n = 11$), $55.7 \pm 1.5$ (n = 10) and $91.9 \pm 0.8$ % ($n = 6$) inhibition respectively.
In panel b the amplitude of $I_{K_{so}}$ at -20 mV in control conditions and in the presence of $Ba^{2+}$ is plotted as a function of time. This plot demonstrates that the inhibition produced by $Ba^{2+}$ can be fully reversed on washing. In panel c the percentage inhibition produced by 0.1 to 10 mM $Ba^{2+}$ is plotted as a function of concentration. When the concentration effect data is fitted with a conventional logistic function the curve indicates that the concentration of $Ba^{2+}$ required to produce the half maximal inhibitory effect, ($EC_{50}$), is 0.83 mM, with a slope factor of 1.74. A similar value for the $EC_{50}$ was obtained if the slope was fixed at 1, however the fit was not as good as when the slope factor was unconstrained.

Figures 5.5 and 5.6 show the effect of 100 µM quinidine and 100 µM quinine on the amplitude of $I_{K_{so}}$. In both cases panel a contains example traces obtained in control conditions and in the presence of the test compound. The time course for the experiments is plotted in panels b. On average 100 µM quinidine and quinine produce a reversible inhibition of $41.7 \pm 3.3 \%$ (n = 8) and $47.7 \pm 4.7 \%$ (n = 6) respectively.

Figure 5.7a and b illustrates the effect of $Na^{+}$ substitution on $I_{K_{so}}$. Panel a contains example traces obtained under control conditions and when external $Na^{+}$ was replaced with NMDG. Panel b shows the time course of the experiment. NMDG produces a reversible inhibition of $54.6 \pm 4.1 \%$ (n = 7). In contrast the effect of NMDG on the $K_{DR}$ current in these cells is minimal, as shown in panel c.

5.2.3. Effect of arachidonic acid on the amplitude of $I_{K_{so}}$.

Although there is no specific pharmacological tool available to identify a specific $K_T$ channel, the effect arachidonic acid (AA) has on $K_T$ channels can be used to classify them into a particular subfamily. Both TREK-1 and TRAAK belong to the same subfamily because they are substantially potentiated by application of AA. To come closer to identifying the molecular nature of $I_{K_{so}}$ the effect of AA on the current amplitude was tested.
Figure 5.8 shows a typical response of \( \text{IK}_{50} \) to 10 \( \mu \text{M} \) AA. AA has little effect on \( \text{IK}_{50} \); rather than enhance the current AA caused a transient inhibition of \( 10.6 \pm 2.2 \% \) (\( n = 9 \)), which was not maintained (steady-state inhibition \( 0.2 \pm 1.8 \% \), \( n = 9 \)). This result means that \( \text{IK}_{50} \) does not belong to the same subfamily as TREK-1 and TRAAK, and argues against the possibility that \( \text{IK}_{50} \) is due to the expression of TREK-1.

**5.2.4. Effect of changing external pH on the amplitude of \( \text{IK}_{50} \).**

The modulation of certain \( K_T \) channels by changes in extracellular pH, such as TASK-1 and TASK-2, has been reported. As with AA, sensitivity of \( K_T \) currents to external pH changes can be used to classify them into a specific subfamily. To come closer to identifying the molecular nature of \( \text{IK}_{50} \) the effect of altering external pH was investigated.

The data traces in Figure 5.9 illustrate the effect of changing extracellular pH on \( \text{IK}_{50} \). The external pH was either reduced by 0.5 or 1.0 pH unit from pH 7.4 to pH 6.9 and 6.4, or increased by 0.5 pH unit to pH 7.9. Panels a, b and c in Figure 5.9 demonstrate that extracellular acidification results in an inhibition of \( \text{IK}_{50} \). A typical example of the effect pH 6.4 has on \( \text{IK}_{50} \) is shown in panel a. Current traces generated in pH 6.4 are reduced in amplitude compared to control (pH 7.4). For the example shown, the amplitude of \( \text{IK}_{50} \) recorded at -20 mV was decreased from 107.5 pA in control to 37.5 pA during exposure to pH 6.4. This inhibition is completely reversed on washing as is shown by Figure 5.9b.

pH 6.9 also produced an inhibition of \( \text{IK}_{50} \), although to a lesser extent. In the example shown in Figure 5.9c, the amplitude of \( \text{IK}_{50} \) recorded at -20 mV was decreased from 384.5 pA in control to 259.5 pA during exposure to pH 6.9. Again this inhibition was completely reversed on washing.

In contrast to the effect of a decrease in the extracellular pH, an increase in pH resulted in a slight enhancement of \( \text{IK}_{50} \). In the example shown in Figure 5.9d the amplitude of \( \text{IK}_{50} \) recorded at -20 mV was increased from 302.7 pA in control to
338.6 pA on exposure to pH 7.9. Like the effects of a decrease in the external pH the effects caused by an increase in external pH are fully reversible upon washing.

Figure 5.9e shows the mean percentage inhibition of IKso recorded at -20 mV caused by acidification and alkalisation. pH 6.4 produced a mean inhibition of 77 ± 3 % (n = 10), pH 6.9 produced a mean inhibition of 31 ± 4 % (n = 4), and pH 7.9 produced a mean enhancement of 7 ± 3 % (n = 5).

These results suggests that IKso belongs to a specific subfamily of Kt channels, the TASK subfamily.

5.2.5. Voltage dependency of the pH effect.

The traces in Figure 5.10a were generated using a ramp protocol. Cells were held at -20 mV and ramped down to -110 mV in 100 ms. The protocol was performed in control conditions and during exposure to pH 6.4. The resulting traces demonstrate that decreasing the extracellular pH causes a reduction in the current amplitude at all potentials. A similar result was observed in a further four cells.

In Figure 5.10b the mean percentage inhibition of the control current produced on exposure to pH 6.4 is plotted against voltage. The graph shows that the percentage inhibition remains relatively constant with changing voltage, indicating that the pH effect is not voltage dependent.

5.2.6. Effect of extracellular acidification on the resting membrane potential and excitability of CGNs.

IKso is open at all potentials including the resting membrane potential. In the light of previous results extracellular acidification would be predicted to act as a depolarising influence on CGNs.

The effect of external acidification on the resting membrane potential of a neuron was studied under current clamp. In the example in Figure 5.11 the zero current potential
of the cell is -78.8 mV. Application of external solution of pH 6.4 produces a depolarisation with the zero current potential shifting in this case by approximately 16 mV to -63 mV. This result was reproduced in a further four cells. On average the resting membrane potential of CGNs was -78.1 ± 3.7 mV, with exposure to pH 6.4 producing a depolarisation of 19.2 ± 1.3 mV to -58.9 ± 3.3 mV (n = 5).

The input resistance of CGNs close to the resting membrane potential was calculated in both control conditions and during exposure to pH 6.4. Values were assessed from the slope of the currents evoked by the ramp protocol, as described in Figure 5.2, at potentials between -70 and -90 mV. In control conditions the input resistance was 477 ± 56 MΩ increasing to 1517 ± 367 MΩ (n = 5) at pH 6.4. This indicates that the excitability of CGNs is increased in acidic conditions.
Figure 5.2. Experimental protocol to obtain current-voltage relations for $I_{K_{so}}$

a). Representative time course for the series of experiments. The amplitude of $I_{K_{so}}$ at a holding potential of $-20$ mV was measured as a mean over 176 ms once every 6 s and plotted as a function of time. The numbered points represent the time at which a ramp protocol was performed.

b). Cells were held at $-20$ mV and hyperpolarised to $-60$ mV by means of a ramp waveform at $10$ mV ms$^{-1}$, over 800 ms, once every 6 s in the presence and absence of $10$ µM muscarine.

c). Cells were treated as described in (b), except in raised external $[K^+]$ (25 mM).
Figure 5.3. I/V relations of $I_{K_{so}}$ in varying external $[K^+]$.

a). The muscarine sensitive current during ramps is obtained by subtraction and is plotted against voltage to give an I/V relation for $I_{K_{so}}$ in control conditions and 25 mM external $[K^+]$.

b). I/V relations for $I_{K_{so}}$ in control conditions and in 100 mM external $[K^+]$. Currents were obtained as described in (a).
Figure 5.4. Effect of Ba$^{2+}$ on IK$_{so}$.

a). Representative data traces were generated using a standard protocol. Cells were stepped to -60 mV from a holding potential of -20 mV, before stepping back to the holding potential, once every 6 s. The protocol was performed under control conditions and in the presence of varying Ba$^{2+}$ concentrations.

b). Time course of the experiment in (a), illustrating the reversible inhibition of IK$_{so}$ on bath application of 0.3, 1 and 3 mM Ba$^{2+}$. Amplitude of IK$_{so}$ was measured as a mean over 176 ms directly preceding the step to -60 mV and plotted as a function of time.

c). Concentration-response curve for the percentage inhibition of IK$_{so}$ by Ba$^{2+}$. Each data point represents the mean ± s.e.mean for number of experiments indicated.
Figure 5.5. Effect of quinidine on IK$_{so}$.
a). Representative data traces obtained using the standard protocol, in control conditions and in the presence of 100 μM quinidine.
b). Time course of the experiment in (a), illustrating the reversible inhibition of IK$_{so}$ produced on bath application of 100 μM quinidine. Amplitude of IK$_{so}$ measured at the holding potential of -20 mV.
Figure 5.6. Effect of quinine on $\text{IK}_{\text{so}}$.

a). Representative data traces obtained using the standard protocol, in control conditions and in the presence of 100 μM quinine.

b). Time course of the experiment in (a), illustrating the inhibition of $\text{IK}_{\text{so}}$ produced on bath application of 100 μM quinine. Amplitude of $\text{IK}_{\text{so}}$ measured at the holding potential of −20 mV.
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Figure 5.7. Effect of NMDG on IK$_{so}$ and IK$_{KV}$.

a). Representative data traces obtained using the standard protocol, in control conditions and when external Na$^+$ was replaced by NMDG.

b). Time course of the experiment in (a), illustrating the reversible inhibition of IK$_{so}$ produced on exposure to NMDG. Amplitude of IK$_{so}$ measured at the holding potential of –20 mV.

c). To determine whether NMDG inhibition was specific to IK$_{so}$ the effect of Na$^+$ substitution on IK$_{KV}$ was tested. IK$_{KV}$ was evoked by holding cells at –70 mV, pre-pulsing to –50 mV, then depolarising to a test potential of +10 mV before stepping back first to –50 mV and then to the holding potential. Na$^+$ substitution had very little effect on IK$_{KV}$.
Figure 5.8. Effect of arachidonic acid (AA) on $I_{K_{so}}$.

a). Representative data traces obtained using the standard protocol, in control conditions and in the presence of 10 μM AA. 10 μM AA initially produces a small transient inhibition of $I_{K_{so}}$ (transient) which is not maintained for the duration of the experiment (sustained).

b). Time course of the experiment in (a), illustrating the transient inhibition of $I_{K_{so}}$ on bath application of 10 μM AA. Amplitude of $I_{K_{so}}$ measured at the holding potential of −20 mV.
Figure 5.9. Effect of changing external pH on the amplitude of $I_{K_{so}}$.

a). Representative traces obtained using the standard protocol, in control conditions and pH 6.4, demonstrating the inhibition of $I_{K_{so}}$ on external acidification.

b). Time course of the experiment in (a), illustrating the reversible inhibition of $I_{K_{so}}$ on bath application of external solution at pH 6.4. Amplitude of $I_{K_{so}}$ measured at the holding potential of $-20$ mV.

c). As in (b), but using external solution at pH 6.9.

d). As in (b), but using external solution at pH 7.9. A slight enhancement of $I_{K_{so}}$ can be seen to occur in these conditions.

e). Graph of the mean percentage inhibition of the control current at $-20$ mV, produced by pH 6.4 ($n = 10$), 6.9 ($n = 4$) and 7.9 ($n = 5$).
Figure 5.10. Voltage dependence of the pH effect.

a). Cells were held at -20 mV and hyperpolarised to -110 mV by means of a ramp waveform at 1.1 ms mV⁻¹, over 100 ms, once every 5 s in control conditions and pH 6.4. IK₉₀ is inhibited by pH 6.4 at all potentials tested.

b). The mean percentage inhibition of the control current produced on exposure to pH 6.4 was calculated over a range of voltages. Plotting this value against voltage illustrates that percentage inhibition remains relatively constant with changing voltage.
Figure 5.11. Effect of changing external pH on the membrane potential of CGNs. Example of current clamp data showing the effect of extracellular acidification on the resting potential of a cell.
5.3. DISCUSSION.

The results of experiments presented in this chapter provide compelling evidence for the molecular nature of $I_{K_{so}}$ being a member of the TASK family of $K_T$ channels. $I_{K_{so}}$ was found to behave as an open rectifier, and so shares the diagnostic feature of $K_T$ channels which is a lack of voltage dependent activation. Additional experiments to determine the pharmacology of $I_{K_{so}}$ revealed that the current was relatively unaffected by arachidonic acid (AA), but was exquisitely sensitive to changes in external pH in the physiological range. These results show that $I_{K_{so}}$ shares the properties of the TASK subfamily of $K_T$ channels, indicating that $I_{K_{so}}$ could potentially be the first $K_T$ channel to be identified in mammalian neurons.

5.3.1. Potential molecular characterisation of $I_{K_{so}}$.

The possibility that $I_{K_{so}}$ could be the first functional correlate of $K_T$ channels arose initially through comparison of the current with cloned members of this superfamily. Two mammalian clones, TREK-1 and TASK-1, show striking similarities to $I_{K_{so}}$. Their mRNA is highly expressed in the granule cell layer of the cerebellum and their currents are instantaneously activating, outwardly-rectifying and non-inactivating. A diagnostic feature of $K_T$ channels is a lack of voltage dependence of activation. If $I_{K_{so}}$ is to be considered a member of this superfamily then it was essential that the activation characteristics of the current were investigated, to determine whether $I_{K_{so}}$ shows true voltage sensitivity.

5.3.2. Voltage dependence of $I_{K_{so}}$.

Recordings of $I_{K_{so}}$ in CGNs reveals that, in physiological conditions, the channels underlying $I_{K_{so}}$ pass much more outward current upon depolarisation than inward current upon hyperpolarisation. For this reason $I_{K_{so}}$ can be classed as an outwardly rectifying potassium channel.

Classical voltage-gated potassium channels ($K_v$) belonging to the 6TMD superfamily also have outwardly rectifying $I/V$ relations in physiological conditions. These
currents activate at a fixed threshold potential and therefore within the protein there must be a region responsible for detecting this activation threshold.

The first voltage-gated channel to be cloned was a Na\(^+\) channel from the eel *Eletrophorus electricus* (Noda *et al*, 1984). One of the striking features of the primary structure was the nature of the S4 domains. Each S4 domain contains stretches where every third residue is either an arginine or a lysine, both of which are positively charged. It was postulated that this charged S4 segment functions as the voltage sensor. Credence was given to this theory since the positively charged region was found to be highly conserved amongst all voltage-gated channels, including potassium channels (Tempel *et al*, 1987, Bezanilla *et al*, 1994). Mutagenesis studies with *Shaker* have subsequently confirmed that this region is important in detecting the membrane voltage since point mutations of the positive charges alter the voltage dependence of activation (Papazain *et al*, 1991, Logothetis *et al*, 1992).

The threshold potential for individual Kv channels is determined by the properties of their particular voltage sensor, however no current will be observed until this potential is reached regardless of the experimental conditions. This behaviour is distinct from that of IK\(_{so}\). Experiments in this chapter, designed to isolate IK\(_{so}\), show that the current can be measured at all potentials tested, with inward current observed at potentials negative to the potassium equilibrium potential. This indicates that IK\(_{so}\) does not have a fixed threshold for activation. If external potassium is raised sufficiently, so that the potassium concentration across the membrane is almost symmetrical, then the I/V relation of IK\(_{so}\) linearises. This is exactly the behaviour predicted by the Goldman-Hodgkin-Katz equation for an open channel, meaning that the rectification seen in physiological conditions is solely due to the uneven potassium distribution across the membrane rather than some kind of intrinsic voltage sensitivity of the channel. In the light of this result IK\(_{so}\) should be more correctly referred to as an open rectifier rather than an outwardly rectifying current.

This lack of a defined activation threshold for IK\(_{so}\) means that it cannot be considered a member of the 6TMD superfamily, and therefore it can be concluded with certainty that IK\(_{so}\) is not a member of the *eag* potassium channel family (see Chapter 4). Instead the molecular nature of IK\(_{so}\) could lie with the K\(_T\) channel superfamily, since
a lack of voltage sensitivity is a diagnostic feature of currents belonging to this superfamily. Analysis of the primary sequence of \( K_T \) channels reveals that there is no region comparable to the S4 voltage sensor, which is in keeping with \( K^+ \) channels lacking any activation threshold voltage.

### 5.3.3. \( IK_{so} \) and TREK-1.

The lack of voltage sensitivity of \( IK_{so} \) provides compelling evidence for it being a member of the \( K_T \) channel superfamily. As already mentioned \( IK_{so} \) shares a number of similar properties to TREK-1 currents, and it is possible that \( IK_{so} \) could be due to expression of TREK-1 in CGNs. Although TREK-1 is an outwardly rectifying current in physiological conditions it cannot be classed as an open rectifier. Increasing external potassium concentration does not linearise the I/V relation; instead TREK-1 currents retain a certain degree of rectification. This unusual behaviour has led to TREK-1 being referred to as an 'unconventional' outward rectifier (Fink et al, 1996), and the only other channel to share this property is the 8TMD yeast \( K_T \) channel TOK1 (Ketchum et al, 1995, Lesage et al, 1996b). The rectification of the yeast channel has been shown to persist in the absence of external divalent ions and so cannot be due to voltage dependent Mg\(^{2+}\) block as is seen for inwardly rectifying potassium channels. Instead the tendency for TOK1 to preferentially pass outward currents has been attributed to a mechanism intrinsic to the protein itself (Lesage et al, 1996b). A model has been proposed where an endogenous blocking particle such as an external peptide domain of the channel enters the permeation pathway upon hyperpolarisation, producing the outwardly rectifying behaviour. Preliminary results with TREK-1 also seem to indicate that its rectification is due to an intrinsic mechanism, i.e. to structural elements contained in the protein sequence of the channel (Fink et al, 1996).

TREK-1 currents belong to a subfamily of \( K_T \) channels that are characterised by their mechanosensitivity and concentration dependent stimulation by AA (Patel et al, 1998, Maingret et al, 1999a). Addition of 10 \( \mu \)M AA to the external medium strongly potentiates (530 ± 78%) the whole cell TREK-1 current in transiently transfected COS cells (Patel et al, 1998). If \( IK_{so} \) is to be attributed to expression of
TREK-1 it is predicted that the current will be significantly enhanced on application of AA. Results from experiments detailed in this chapter showed that AA had very little effect on the amplitude of $I_{K_{so}}$, producing an initial transient inhibition of the current which was not maintained. Taken together these results argue against the idea that TREK-1 underlies $I_{K_{so}}$ in CGNs.

5.3.4. $I_{K_{so}}$ and TASK-1.

Another potential candidate in the search for the molecular correlate of $I_{K_{so}}$ is TASK-1. TASK-1 currents behave as open rectifiers and therefore show a high degree of similarity to $I_{K_{so}}$.

TASK-1 belongs to a subfamily of $K_T$ channels whose members can be distinguished from other family members by virtue of their sensitivity to small changes in the external pH, close to physiological values. If $I_{K_{so}}$ is to be considered the functional correlate of TASK-1 in CGNs then it was essential that pH sensitivity of the current was demonstrated.

$I_{K_{so}}$ was recorded under conditions of varying pH and data obtained showed that the amplitude of the current was reduced on acidification and enhanced in alkaline conditions, in much the same way as has been reported for TASK-1 (Duprat et al, 1997, Leonoudakis et al, 1998). This effect was voltage independent and, concurrent with inhibition of potassium channels, acidification produced an increased input resistance and was shown to act as a depolarising influence on CGNs under current clamp recording.

Effects of changes in external pH have been reported to have a significant effect on many kinds of ion channel (Hille, 1992). The mechanism of pH dependent control of ion channel function is not completely understood at the molecular level, however several different mechanisms have been proposed. It is thought that protonation of a single residue is sufficient to produce $H^+$ block of channels, whether protonation occurs on a residue within the channel pore itself or on an external residue seems to depend upon the channel under investigation.
Recent work with L-type Ca$^{2+}$ channels has implicated residues within the pore as being important for H$^+$ block. The P region glutamate in repeat I was mutated to a glutamine, which is equivalent to permanent protonation of the residue. This mutation mimicked the effect of protonation on wild type conductance and also prevented external H$^+$ from binding to its native site (Chen et al., 1996). H$^+$ block of cyclic nucleotide gated channels occurs in a similar manner, with a reduction in conductance also attributed to protonation of P region glutamates (Root and Mackinnon, 1994). In the case of the inward rectifier potassium channel, IRK3, it has been demonstrated by mutagenesis studies that protonation of an external cysteine residue outside of the permeation pathway reduces channel conductance by an allosteric mechanism (Coulter et al., 1995). Protonation of this residue stabilises a channel conformation that allows a different titratable group to influence ion permeation.

Presently no molecular mechanism for proton block of K$\text{}_T$ channels has been proposed. One possibility for TASK-1 is that protonation of a histidine residue at position 98, just after the GYG sequence in P1, is important in conferring pH sensitivity to the channel. This hypothesis comes from an extension of experiments with K$\nu$ 2.1 channels, where a neutral residue located within the P region, at position 369, was mutated to a histidine residue, resulting in a mutant channel that was blocked by protons much more effectively than the wild type channel. The histidine substitution also disrupted the site for external blockade by TEA, indicating the site appears to be near the external mouth of the pore in a surface position. This is consistent with external protons blocking the channel in a voltage independent manner (De Biasi et al., 1993).

At present there are no specific blockers of K$\text{}_T$ channels to aid in the correlation of cloned and native currents. As an alternative, a comprehensive pharmacological profile of IK$_{so}$ was obtained to allow a more direct comparison of the current with TASK-1. The findings are summarised in Table 5.1.
Chapter 5 – Potential molecular characterisation of IK\textsubscript{so}.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>rTASK-1</th>
<th>IK\textsubscript{so}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zn\textsuperscript{2+}</td>
<td>100 µM: 39 ± 9</td>
<td>10 µM: 11 ± 5</td>
</tr>
<tr>
<td>quinidine</td>
<td>100 µM: 29 ± 4</td>
<td>100 µM: 41.7 ± 3.3</td>
</tr>
<tr>
<td>TEA</td>
<td>100 mM: 33 ± 5</td>
<td>5 mM: no significant inhibition</td>
</tr>
<tr>
<td>Ba\textsuperscript{2+}</td>
<td>100 µM: 19 ± 1</td>
<td>100 µM: 5.6 ± 1.5</td>
</tr>
<tr>
<td>NMDG substitution</td>
<td>28 ± 2</td>
<td>54.6 ± 4.1</td>
</tr>
<tr>
<td>4-AP</td>
<td>5 mM: &lt; 15</td>
<td>5 mM: no significant inhibition</td>
</tr>
<tr>
<td>pH\textsubscript{o}</td>
<td>pH 6.4: ≈ 100</td>
<td>pH 6.4: 77.3 ± 2.6</td>
</tr>
<tr>
<td>Enhanced by AA</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>

N.B. all values are % inhibition.

Table 5.1. Pharmacology of IK\textsubscript{so} and rTASK-1 expressed in Xenopus oocytes. (rTASK-1 data taken from Leonoudakis \textit{et al}, 1998, IK\textsubscript{so} data taken from Watkins and Mathie, 1996).

The response of IK\textsubscript{so} to NMDG adds to the evidence for the current not being due to TREK-1 expression. Complete Na\textsuperscript{+} substitution by NMDG produces almost complete inhibition of TREK-1 currents (Fink \textit{et al}, 1996). By comparison this treatment results in only 55 % inhibition of IK\textsubscript{so}.

Studies on cloned K\textsubscript{T} channels have shown that some of these channels can be significantly enhanced by volatile anaesthetics. Four volatile agents (isofluorane, halothane, diethyl ether and chloroform) were tested on mTREK-1, mTRAAK and hTASK-1 expressed in COS cells (Patel \textit{et al}, 1999). All four anaesthetics activated mTREK-1 channels but had no effect on mTRAAK channels. hTASK-1 channels on the other hand, were activated by isofluorane and halothane, unaffected by chloroform and inhibited by diethyl ether. The result reported for hTASK-1 with isofluorane is in conflict with that reported by Leonoudakis \textit{et al} (1998) who found no significant effect of isofluorane on rTASK-1 currents. Although not tested here, it is possible that by adding to the pharmacological profile of IK\textsubscript{so} the response of the current to treatment with these agents may be useful in the correlation of cloned and native currents.
The pharmacological profile of \( \text{IK}_{\text{so}} \) and rTASK-1 are similar but not identical. It is possible that the different cell types may explain the discrepancy between the results. Alternatively \( \text{IK}_{\text{so}} \) could be composed of subunits encoded by a splice variant of rTASK-1, or an as yet unidentified gene. Work with \( \text{C. elegans} \) has shown that of more than eighty potassium channel genes known in this organism, over fifty of them belong to the \( \text{K}_\text{T} \) channel superfamily (Bargmann, 1998). It is likely therefore that the few \( \text{K}_\text{T} \) channels identified so far in mammalian neurons represents only the initial members of a large extended family. For example preliminary reports claim to have identified several additional \( \text{K}_\text{T} \) channel genes including TREK-2 and TASK-3 (Kim \textit{et al}, 2000a, b, Vega-Saenz de Miera \textit{et al}, 2000, Bang \textit{et al}, 2000).

Another possibility is that \( \text{IK}_{\text{so}} \) is the result of a heteromeric association of subunits. The 6TMD and 2TMD superfamilies need four subunits to form a functional channel (MacKinnon, 1991, Yang \textit{et al}, 1995), suggesting the requirement of four P domains in the formation of the potassium selective ion conducting channel pore. By extension, if both P domains in the \( \text{K}_\text{T} \) channels are functional then it is predicted that functional \( \text{K}_\text{T} \) channels will be composed of two subunits. To date this has only been demonstrated for TWIK-1 subunits (Lesage \textit{et al}, 1996c), which form homodimers by covalent association via a disulphide bridge. This assembly involves a 34 amino acid domain that is localised to the extracellular loop between M1 and P1. It is a cysteine residue at position 69 that is implicated in the formation of the covalent bond. This particular residue is conserved in TREK-1 (Duprat \textit{et al}, 1997) but not TASK-1 (Leonoudakis \textit{et al}, 1998), suggesting that its presence is not an absolute requirement for assembly of \( \text{K}_\text{T} \) subunits.

As well as forming homomultimers 6TMD and 2TMD channels can form heteromultimers (Isacoff \textit{et al}, 1990, Ruppersberg \textit{et al}, 1990, Sheng \textit{et al}, 1993, Wang \textit{et al}, 1993, Krapivinsky \textit{et al}, 1995), providing the \( \alpha \) subunits comprising the channel belong to the same subfamily (Covarrubias \textit{et al}, 1991). There is a domain in the N terminal region of each subunit that is conserved between members of the same subfamily. This allows heteromultimeric assembly within a subfamily but prevents co-assembly between subfamilies. There is no reason to suspect that this is not also the case for \( \text{K}_\text{T} \) channels, and subunits could form heterodimers with new channel
properties. Perhaps dimerisation of TASK-1 with a non-identical subunit could reproduce more exactly the pharmacological profile of $\text{IK}_{\text{so}}$.

### 5.3.5. Neuromodulation of TASK-1.

All the evidence presented so far suggests that TASK-1 currents underlie $\text{IK}_{\text{so}}$ in CGNs. Nevertheless one of the most potent and characteristic responses reported for $\text{IK}_{\text{so}}$ is inhibition by muscarine. The question of whether TASK-1 currents can also be modulated by activation of neurotransmitter receptors needs to be addressed before correlation of $\text{IK}_{\text{so}}$ and TASK-1 can be established.

$\text{IK}_{\text{so}}$ has been shown to be inhibited by activation of M3 receptors, which are generally accepted to increase phosphoinositide hydrolysis via activation of the $G_{q/11}$ class of G proteins (see Chapter 3). Treatments which disrupt this second messenger pathway do not prevent modulation of $\text{IK}_{\text{so}}$ by muscarine, and inhibition of $\text{IK}_{\text{so}}$ seems to occur through some other, as yet unidentified, mechanism. Published results from both hTASK-1 and rTASK-1 show that the current is largely unaffected by treatments that activate or inhibit signalling pathways commonly associated with the $G_{q/11}$ class of G proteins (Duprat et al, 1997, Leonoudakis et al, 1998), such as intracellular IP$_3$ injection, intracellular calcium buffering and treatment with phorbol esters. However in the light of the results with $\text{IK}_{\text{so}}$ it cannot be concluded that TASK-1 is not subject to neurotransmitter modulation. In fact, Talley et al (1999, 2000) recently demonstrated that direct activation of a $G_{q/11}$ coupled receptor resulted in inhibition of TASK-1. Application of Thyrotropin Releasing Hormone (TRH) significantly reduced the acid sensitive currents in HEK 293 cells transfected with TASK-1 and the TRH receptor, whereas it had essentially no effect on potassium currents in HEK 293 cells transfected with TRH receptors alone.

Additionally TASK-1 currents expressed in Xenopus oocytes have also been found to be subject to neuromodulation. Oocytes possess endogenous muscarinic receptors which are thought to be, primarily, of the M3 subtype (Kusano et al, 1982, Davidson et al, 1991). Activation of these receptors by 100 $\mu$M carbachol produced a 52 ± 6 % ($n = 4$) inhibition of TASK-1 currents which was fully reversible and associated with
a decrease in membrane conductance (Millar et al., 2000). Thus this data indicates that TASK-1 can indeed be inhibited by activation of cell surface receptors, strengthening the evidence for the correlation of $I_{K_{so}}$ with TASK-1.

Therefore taking all the biophysical and pharmacological data together provides strong evidence to suggest that TASK-1 underlies $I_{K_{so}}$ in CGNs. This makes $I_{K_{so}}$ the first functional correlate of $K_T$ channels to be identified in native neurons.
**CHAPTER 6.**

**DETECTION OF TASK-1 mRNA IN CGNs.**
6.1. INTRODUCTION.

The previous chapters have been concerned with the electrophysiological and pharmacological properties of \( I_{K_{so}} \). The results of these chapters provide compelling evidence to suggest that \( I_{K_{so}} \) may be a member of the \( K_r \) channel family. Of all the members of this family to be cloned so far TASK-1 is most similar to \( I_{K_{so}} \).

\( I_{K_{so}} \) has many of the properties predicted for this background channel, in particular, its lack of a defined voltage-threshold for activation and its exquisite sensitivity to changes in pH of the extracellular solution. In addition northern blot analysis of rat, mouse and human mRNA has revealed the presence of TASK-1 transcript in the brain (Duprat et al, 1997, Leonoudakis et al, 1998). More specifically TASK-1 mRNA has been detected in the granule cell layer of the cerebellum using \emph{in situ} hybridisation (Duprat et al, 1997).

Therefore the aim of this chapter was to determine if TASK-1 mRNA could be detected in cultured CGNs, in order to provide further evidence for the correlation of \( I_{K_{so}} \) with TASK-1. Analysis of CGN mRNA was carried out using the technique of reverse transcription-polymerase chain reaction (RT-PCR). A description of this technique is provided in Chapter 2, along with details of reaction mixtures for individual experiments.
6.2. RESULTS.

6.2.1. Detection of TASK-1 mRNA in freshly dissociated CGNs.

The initial aim of the experiments was to determine whether TASK-1 mRNA could be detected in freshly dissociated CGNs from neonatal rats, aged 6-9 days. Total RNA was extracted from CGN cultures and the RT step carried out using random hexamer primers, producing a mixture of cDNA products. This mix was divided into aliquots, which were then used in different PCR reactions with specific primer pairs.

Figure 6.1 shows the results of a PCR using two sets of specific primers for TASK-1 (F1:R1 and F2:R2; see methods) and a set of primers for actin, which act as a positive control. The reaction was carried out in the presence of Taq polymerase using the following cycling protocol: 3 min initial denaturation at 95 °C, followed by 30 cycles of 95 °C, 30 s; 60 °C, 30 s; 72 °C, 1 min, and 5 min final elongation at 72 °C.

In lane 5, a sample of the reaction mix, which contained primers for actin, was run. The DNA product of this reaction was expected to be 818 base pairs long. Using marker lanes the presence of a band of the predicted length can be seen. Actin is a ubiquitously expressed protein and therefore should always give a positive result. The detection of a band for actin confirms that all the necessary components for amplification were present, Taq polymerase was stable over the range of cycling temperatures used and also that the RNA extraction and RT steps had worked.

In lane 1 a sample of the reaction mix, which contained primer pair F1:R1, was run. The DNA product of this reaction is expected to be 516 base pairs long.

In lane 3 a sample of the reaction mix, which contained primer pair F2:R2, was run. The DNA product of this reaction is expected to be 369 base pairs long. Only lane 3 shows any detectable signal. The band is faint but it is of the predicted size and therefore shows that mRNA for TASK-1 is present in CGNs. The lack of signal in lane 1 may be because the primer pair F1:R1 is inefficient, or the conditions of PCR may not be optimal for this set of primers.

Lanes 2, 4 and 6 are negative controls. In this case negative controls are PCR reaction mix which contain some of the isolated CGN mRNA that did not go through
the RT step. No bands are seen for the negative controls, indicating that PCR products are due to amplification of cDNA from RT of mRNA and not from contaminating genomic DNA. If bands were present then this would indicate contamination at some point in the procedure. In subsequent experiments H₂O negative controls show that no contamination has occurred.
Figure 6.1. Results of RT-PCR using primers for TASK-1 and actin.
PCR products run on a 2% agarose gel, stained with ethidium bromide (1 μg ml⁻¹).
PCR was carried out in the presence of Taq polymerase, using the following cycling protocol: 3 min initial denaturation at 95 °C, followed by 30 cycles of 95 °C, 30 s; 60 °C, 30 s; 72 °C, 1 min, and 5 min final elongation at 72 °C.
Lanes 1 and 3 contain products of a PCR using TASK-1 primer pairs F1:R1 and F2:R2 respectively, and lane 5 is a positive control using a primer pair for actin.
Lanes 2, 4 and 6 are negative controls, and lanes designated M are 100 bp size markers.
6.2.2. Optimisation of RT-PCR conditions.

Having determined that the primer pair F2:R2 was able to detect TASK-1 mRNA in CGNs, additional experiments were undertaken to determine the optimal conditions for PCR. The cycling temperatures were adjusted in an attempt to maximise the PCR product.

Figure 6.2 shows the results of PCR carried out in the presence of Taq polymerase using the following cycling protocols: (a) 3 min initial denaturation at 97 °C, followed by 30 cycles of 97 °C, 30 s; 50 °C, 30s; 72 °C, 1 min, and 5 min final elongation at 72 °C. (b) 3 min initial denaturation at 98 °C, followed by 30 cycles of 98 °C, 20 s; 60 °C, 20 s; 72 °C, 1 min, and 5 min final elongation.

Lanes 1-6 are as detailed in Figure 6.1. Products obtained using cycling protocol (a). A clear band can be seen in lane 5 for actin, meaning Taq polymerase is stable at 97 °C. Almost no signal can be detected in lanes 1 and 3 for TASK-1, suggesting this set of conditions is not optimal for either primer pair.

In lanes 7-10 PCR was carried out using cycling protocol (b), lanes 7 and 8 used the TASK-1 primer pair that had previously given a detectable signal (F2:R2), and lanes 9 and 10 used the actin set of primers. The lack of a band in lane 9 (positive control) suggests that a denaturing temperature of 98 °C is too high, rendering Taq polymerase inactive.

Figure 6.3 shows the results of a PCR performed in the presence of Taq polymerase, using the following cycling protocol: 3 min initial denaturation at 96 °C, followed by 30 cycles of 96 °C, 30 s; 60 °C, 30 s; 72 °C, 1 min, and 5 min final elongation at 72 °C. Primer pair F2:R2 was used, plus the actin set of primers. In this case and in all subsequent experiments a more concentrated cDNA solution was used and the concentration of Taq polymerase and primers were increased (see Chapter 2 for details). A clear band can be seen for actin, in lane 3, and a fainter band is present in lane 1 for TASK-1. This combination of temperatures gives the best signal for TASK-1.
Figure 6.2  Optimisation of RT-PCR conditions for TASK-1 primers: Alteration of denaturing and annealing temperatures.

PCR products run on a 2 % agarose gel, stained with ethidium bromide (1 µg ml⁻¹).

In lanes 1-6 PCR was carried out in the presence of Taq polymerase, using the following cycling protocol: 3 min initial denaturation at 97 °C, followed by 30 cycles of 97 °C, 30 s; 50 °C, 30 s; 72 °C, 1 min, and 5 min final elongation at 72 °C.

Lanes 1 and 3 contain products of a PCR using TASK-1 primer pairs F1:R1 and F2:R2 respectively. Lane 5 is a positive control using a primer pair for actin.

In lanes 7-10 PCR was again carried out in the presence of Taq polymerase, this time using the following cycling protocol: 3 min initial denaturation at 98 °C, followed by 30 cycles of 98 °C, 20 s; 60 °C, 20 s; 72 °C, 1 min, and 5 min final elongation at 72 °C.

Lane 7 contains products of a PCR using TASK-1 primer pair F2:R2, and lane 9 is a positive control using a primer pair for actin.

Lanes 2, 4, 6, 8 and 10 are negative controls, and lanes designated M are 100 bp size markers.
Figure 6.3. Optimisation of RT-PCR conditions for one pair of TASK-1 primers: Alteration of denaturing temperature. PCR products run on a 2% agarose gel, stained with ethidium bromide (1 μg ml⁻¹).

PCR was carried out in the presence of Taq polymerase using the following cycling protocol: 3 min initial denaturation at 96 °C, followed by 30 cycles of 96 °C, 30 s; 60 °C, 30 s; 72 °C, 1 min, and 5 min final elongation at 72 °C.

Lane 1 contains products of a PCR using TASK-1 primer pair F2:R2. Lane 3 is a positive control using a primer pair for actin. Lanes 2 and 4 are negative controls, and lanes designated M are 100 bp size markers.
6.2.3. Use of Pfx DNA polymerase to detect TASK-1 mRNA in CGNs.

Other polymerase enzymes are available for PCR and one such enzyme is Pfx DNA polymerase (Gibco BRL). This enzyme is supplied with an enhancer solution, which is designed to improve the amplification of guanine (G) and cytosine (C) rich templates. TASK-1 has high GC content and so PCR performed in the presence of this enzyme would be expected to result in the increased amplification of TASK-1, producing a stronger signal.

Figure 6.4 shows the results of PCR using Pfx polymerase with primer pair F2:R2 and the following cycling protocol: 3 min initial denaturation at 96 °C, followed by 30 cycles of 96 °C, 30 s; 60 °C, 30s; 68 °C, 1 min, and 5 min final elongation at 68 °C.

A band for TASK-1 can be detected in lane 1 in the absence of enhancer buffer. Lanes 3 and 5 contain 1X and 2X enhancer buffer respectively and the bands grow fainter as the concentration of buffer is increased, the opposite of what is predicted. No band can be seen in lane 7 for actin, and the presence of smears in the negative control lanes (2, 4 and 6), casts some doubt over the reliability of the results. Therefore the experiment was repeated. This time both TASK-1 primer pairs were used with Pfx polymerase, in the absence of enhancer buffer. Figure 6.5 shows a clear band in lane 6 for actin confirming PCR has been successful, and the lack of smears in the negative control lanes rules out any contamination. No band is present in lane 1 using primer pair F1:R1, but a band for TASK-1 is present in lane 3 using primer pair F2:R2.

So, although Pfx polymerase can be used to show the presence of TASK-1 mRNA, it gave no improvements over Taq polymerase. Therefore all subsequent experiments were carried out using Taq polymerase.
Figure 6.4. Results of RT-PCR using primers for TASK-1 and actin in the presence of *Pfx* DNA polymerase.

PCR products run on a 2% agarose gel, stained with ethidium bromide (1 μg ml⁻¹).

PCR was carried out in the presence of *Pfx* polymerase, using the following cycling protocol: 3 min initial denaturation at 96 °C, followed by 30 cycles of 96 °C, 30 s; 60 °C, 30 s; 68 °C, 1 min, and 5 min final elongation at 68 °C.

Lanes 1, 3 and 5 contain products of a PCR using TASK-1 primer pair F2:R2 in conjunction with varying concentrations of enhancer buffer (0, 1X and 2X respectively). Lane 7 is a positive control using a primer pair for actin.

Lanes 2, 4, 6 and 8 are negative controls, and lanes designated M are 100 bp size markers.
Figure 6.5. Results of RT-PCR to confirm Pfx polymerase can detect presence of TASK-1 mRNA in CGNs. PCR products run on a 2% agarose gel, stained with ethidium bromide (1 µg ml⁻¹).
Conditions of PCR as in Figure 6.4, with no enhancer buffer included in any reaction mix.
Lanes 1 and 3 contain products of a PCR using TASK-1 primer pairs F1:R1 and F2:R2 respectively. Lane 6 is a positive control using a primer pair for actin.
Lanes 2, 4 and 7 are negative controls (lane 5 is empty), and lanes designated M are 100 bp size markers.
6.2.4. Detection of TASK-1 mRNA in freshly dissociated and ten day old CGNs.

Figure 6.6 shows the results of RT-PCR using optimal conditions as determined by the above experiments. (PCR performed in the presence of Taq polymerase using cycling protocol: 3 min initial denaturation at 96 °C, followed by 30 cycles of 96 °C, 30 s; 60 °C, 30 s; 72 °C, 1 min, and 5 min final elongation at 72 °C).

In lanes 1 and 2 PCR was carried out using primer pair F1:R1, in lanes 3 and 4 PCR was carried out using primer pair F2:R2 and in lanes 5 and 6 PCR was carried out using an additional combination of primers; F1:R2. The DNA product using this primer pair is expected to be 760 base pairs long. As in all previous experiments mRNA has been isolated from freshly dissociated cells.

A band for actin can be seen in lane 7, and clear bands for TASK-1 can be seen using all combinations of primers.

Watkins and Mathie (1996) reported that the amplitude of IKso develops with time in culture, consequently the experiment was repeated using cells that had been in culture for ten days.

Figure 6.7 shows the results. Again bands for TASK-1 are present with all primer combinations, but in this case the bands are much brighter. To determine whether this corresponds to higher TASK-1 expression would require quantitative RT-PCR.

At this stage the PCR products obtained with all primer combinations were sequenced to ensure the primers used resulted in the specific amplification of TASK-1.
Figure 6.6. Results of RT-PCR using mRNA isolated from freshly dissociated cells.

PCR products run on a 2% agarose gel, stained with ethidium bromide (1 μg ml⁻¹).

PCR was carried out in the presence of Taq polymerase using the following cycling protocol: 3 min initial denaturation at 96 °C, followed by 30 cycles of 96 °C, 30s; 60 °C, 30s; 72 °C, 1 min, and 5 min final elongation at 72 °C. Lanes 1, 3 and 5 contain products of a PCR using TASK-1 primer pairs F1:R1, F2:R2 and F1:R2 respectively. Lane 7 is a positive control using a primer pair for actin. Lanes 2, 4, 6 and 8 are negative controls, and lanes designated M are 100 bp size markers.
Figure 6.7. Results of RT-PCR using mRNA isolated from cells that have been in culture for 10 days. PCR products run on a 2 % agarose gel, stained with ethidium bromide (1 μg ml⁻¹). Conditions of PCR and order of bands as in Figure 6.6.
6.2.5. Immunocytochemical studies to detect TASK-1 protein expression in CGNs.

The presence of mRNA for TASK-1 has been confirmed in CGNs, however this does not necessarily equate with functional expression of the channel. To determine if TASK-1 protein is expressed in the membrane, immunocytochemical studies using antibodies against TASK-1 need to be performed.

A commercial antibody is available and has been used here to demonstrate the presence of TASK-1 protein in seven day old CGN cultures. Anti TASK-1 is a polyclonal antibody raised in rabbit against a highly purified peptide (TASK 252-269), corresponding to residues 252-269 of human TASK-1. This is a specific epitope for TASK-1 and is highly conserved in mouse and rat TASK-1. (Staining was abolished by preabsorption of the antibody with 10 µg/ml of the peptide confirming specificity of the antibody).

CGNs that had been in culture for seven days were incubated with anti TASK-1 (dilution 1:100) and antibodies against glial fibrillary acid protein (GFAP; clones 4A11, 1B4, 2E1; PharMingen; dilution 1:100). GFAP detects the presence of glial cells and acts as a background control.

Figure 6.8 shows labelling for TASK-1 antibody (green) and GFAP (red) in seven day old CGN cultures in the same field of view. TASK-1 protein appears to be expressed in both the cytoplasm and surface membrane of CGNs. There is some overlap between anti TASK-1 and GFAP labelling, indicating that TASK-1 protein is also expressed by glial cells in culture. This result was obtained using anti TASK-1 from two different sources, Alomone Labs and Chemicon.

1 day old CGN cultures were also treated in an identical manner with negligible labelling for TASK-1 being detected (results not shown). This result is consistent with the previous lack of any measurable IK_{so} in such cells (Watkins and Mathie, 1996).
All antibody work was undertaken by members of Dr Robert Fyffe's laboratory, Department of Anatomy, Wright State University, Ohio. Results are reproduced with his kind permission.
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Figure 6.8. Expression of TASK-1 protein in CGNs. TASK-1 antibody (green) and GFAP (red) labelling in seven day old CGN cultures in the same field of view. TASK-1 protein appears to be expressed in both the cytoplasm and surface membrane of CGNs. Overlapping staining between anti TASK-1 and GFAP indicates TASK-1 is also expressed by glial cells in culture.
6.3. DISCUSSION.

The results of the experiments in this chapter show that TASK-1 mRNA is expressed in cultured cells. In addition the level of TASK-1 mRNA appears to be greater in cells that have been longer in culture. This is in agreement with the observation that the amplitude of $I_{K_{so}}$ increases with time in culture. The expression of TASK-1 protein was confirmed with immunocytochemical studies, showing that protein could be detected in both the cytoplasm and membrane of CGNs. These results, taken together with electrophysiological and pharmacological data presented in previous chapters, provide compelling evidence for the correlation of $I_{K_{so}}$ with TASK-1.

6.3.1. Verifying target sequence amplification.

RT-PCR of mRNA from CGNs, using three combinations of specific primers, resulted in only DNA bands of the predicted length being detected. No non-specific bands were ever detected, even when the annealing temperature was as low as 50 °C. Such specificity infers that PCR is specifically amplifying TASK-1 sequence.

Although the products were of the size expected it is possible that their sequences were incorrect. In the RT step of the process mRNA was transcribed into cDNA for use in the PCR step. Since random hexamer primers were used in the RT step cDNA of many proteins will have been made. It is conceivable that during PCR the specific primers for TASK-1 bound to non target sequences and amplified a region of DNA the same size as the expected product, but with a completely different sequence.

Primer pairs are designed to give a single amplification product. Primers are twenty nucleotides long (20mer) and the frequency of a 20mer sequence is once every $4^{20}$, which is a figure greater than the length of the human genome. This means a 20mer primer would be expected to have just one hybridisation site in total species DNA. If conditions are not optimal during PCR then the primers can bind non-specifically producing amplification of a non target sequence of DNA.
The only absolute way of ensuring it is the TASK-1 sequence that has been amplified is to sequence the PCR product and compare this to the published TASK-1 sequence. The PCR products obtained with all primer combinations were therefore sequenced and were found to be identical to the appropriate region of the published TASK-1 sequence (Page, personal communication), thereby confirming the expression of TASK-1 mRNA in CGNs.

6.3.2. mRNA isolation from a heterogeneous cell population.

All the RT-PCR experiments were conducted using mRNA isolated from cells that had been prepared in the same way as for electrophysiological studies. It is very difficult to achieve a pure population of cells and although the proportion of CGNs will be high using this culture method, other cell types will be present. This is not a problem for electrophysiological investigations since individual CGNs can be selected on the basis of their morphology. However since total mRNA was isolated from a heterogeneous population of cells it is possible that the TASK-1 mRNA detected by RT-PCR could have come from cell types other than CGNs.

To show conclusively that TASK-1 mRNA is expressed in CGNs the mRNA from individual neurones should be isolated and used in RT-PCR. This process is known as single cell RT-PCR.

The method of single cell RT-PCR was first described by Rappolee et al (1988a,b). The technique was used in their case to analyse the regulation of growth factor transcripts and proteolytic enzymes. The application of single cell RT-PCR in combination with electrophysiological recordings was soon realised and has been used in a wide variety of studies. For example the subunit composition of glutamate receptors has been determined in a number of different cell types (Lambolez et al, 1992, Mackler and Eberwine, 1993, Jonas et al, 1994, Bochet et al, 1994) and the range of potassium channel subunits in stomatogastric ganglia of the lobster has been elucidated (Baro et al, 1996).

The basic method of single cell RT-PCR involves performing a whole cell patch clamp recording on a cell in culture, to characterise its biophysical and
pharmacological properties. During this recording the cell interior is dialysed with internal solution and after sufficient time has elapsed the electrode solution contains RNA. The remaining cell contents are then harvested into the patch pipette using suction. The tip of the pipette is broken into the reaction tube and the contents expelled. RT-PCR is then carried out in the normal manner (Sucher and Deitcher, 1995). To obtain sufficient DNA for visualisation on a gel two rounds of PCR are generally required. The second round uses a small sample of the first round reaction product as a starting template.

Due to time constraints single cell RT-PCR was not attempted during this study.

6.3.3. Development of TASK-1 mRNA in a time dependent manner.

Watkins and Mathie (1996) reported that the amplitude of $I_{K_{so}}$ increased with time in culture. Neurones that had been in culture for thirteen days possessed approximately 350 pA of $I_{K_{so}}$ at a membrane potential of -30 mV, compared with almost no detectable level of $I_{K_{so}}$ in neurones that had been only two days in culture. If TASK-1 underlies $I_{K_{so}}$ then it is possible that the levels of TASK-1 mRNA will increase in a similar manner, with older neurones showing a higher expression level of TASK-1 mRNA.

RT-PCR was carried out using freshly dissociated cells and cells that had been in culture for ten days. Isolation of mRNA was identical in both cases, as were the contents of the reaction mix and the cycling protocols used. Both experiments demonstrated the presence of mRNA for TASK-1 in CGNs, however the intensity of the bands obtained from older cells was much greater.

Assuming that the amount of product synthesised is proportional to the amount of template cDNA present at the start of the reaction, it is possible to conclude that bands with greater intensity indicates the presence of greater amounts of starting template cDNA. Comparing the intensity of the bands in this way gives an estimate of the relative amount of product from two PCRs (Brown, 1995). Using this criteria the results presented here seem to suggest that older cells have higher expression of
TASK-1 mRNA than freshly dissociated cells, since they produce bands of a greater intensity.
Assuming that mRNA expression correlates with functional channel expression this result is in accordance with TASK-1 underlying $I_{K_{s0}}$.

Using the results of RT-PCR in this way to quantitate levels of mRNA expression is not completely reliable. The main shortcoming is principally due to the exponential nature of PCR, whereby small variations in amplification efficiency dramatically affect the yield of amplification product (Foley et al., 1993). Even the minimal deviations that occur between adjacent wells of a thermocycler can lead to significant differences in the extent of amplification between otherwise identical reactions (Gilliland et al., 1990). This means that two separate reactions, which contain exactly the same amount of starting template cDNA, can give bands with vastly different intensities. Additionally the RNA extraction and RT step can vary between samples, meaning that two different cell samples with the same level of TASK-1 mRNA expression could also produce bands of differing intensities.

To quantitate the product of RT-PCR exactly substantial effort is required to design appropriate controls (Souazé et al., 1996, Zimmermann and Mannhalter, 1996, Reyes-Engel et al., 1996) and other techniques may sometimes be equally effective and easier to implement (Foley et al., 1993).

Rather than design a new protocol for exact quantification of PCR, antibody labelling was undertaken to determine conclusively whether TASK-1 protein is expressed in the same time dependent manner as the ability to record $I_{K_{s0}}$.

6.3.4. Immunocytochemical studies of TASK-1 protein.

The RT-PCR experiments showed that TASK-1 mRNA was present in cell cultures, however the presence of mRNA transcripts does not necessarily equate with the assembly of functional channels. To confirm that the expression of TASK-1 mRNA leads to expression of protein it was necessary to combine RT-PCR results with those of antibody staining experiments.
Dual staining of cell cultures with anti-TASK antibody and GFAP revealed that strong staining is seen for TASK-1 protein in both the membrane and cytoplasm of ten day old CGNs. Cells which had been in culture for only one day were also tested and no detectable TASK-1 labelling was seen (Fyffe, personal communication). This result is consistent with the observation that both TASK-1 mRNA and $I_{K_{so}}$ amplitude increases with time in culture. It should be noted that some TASK-1 labelling also was observed in glial cells. It is possible therefore that a component of the mRNA detected by RT-PCR could have originated from this source.

The results of RT-PCR in combination with immunocytochemistry show conclusively that TASK-1 is expressed in CGNs.
CONSOLIDATION.
This study has further characterised the properties of IK_{so}, an outwardly rectifying, non-inactivating potassium current in CGNs, which is reversibly and concentration dependently inhibited by muscarine. The results of the experiments presented here have led to the identification of the M_3 muscarinic receptor subtype as being responsible for mediating IK_{so} inhibition. Although the mechanism underlying this inhibition remains undetermined it can be concluded from experiments with specific blockers that modulation is predominantly via a PLC independent pathway. Most interestingly this study has also led to the potential molecular characterisation of IK_{so}.

Original studies with IK_{so} categorised the current as voltage dependent because of its outwardly rectifying I/V relation. A well studied voltage dependent, muscarine sensitive potassium current is the M current, however IK_{so} is not considered an M-like current in CGNs since its activation and deactivation kinetics are much faster and IK_{so} was found to be insensitive to the specific M current blocker, linopirdine. Cloned members of the ether á go go (eag) family of potassium channels share certain properties with IK_{so}, including muscarinic modulation. Characteristically the activation kinetics of eag are slowed in the presence of extracellular Mg^{2+} (Terlau et al., 1996), a feature IK_{so} was found not to share. It was concluded therefore that the molecular correlate of IK_{so} does not lie with the eag family of potassium channels.

Major evidence that IK_{so} is not an M-like or eag current lies with its voltage dependence of activation. In physiological conditions the I/V relation of IK_{so} is outwardly rectifying with an apparent activation threshold of around -90 mV. When the external K^+ concentration was increased to 25 mM the I/V relation was shifted rightward, with the reversal potential of the current closely following E_K. An inward current was revealed at potentials more negative to E_K meaning that IK_{so} could be measured at all potentials. Raising external K^+ even further (100 mM) shifted the I/V relation further to the right and linearised the I/V relation. This behaviour of IK_{so} is very different from that of the classical outward rectifier, voltage-gated K^+ channels belonging to the 6TMD superfamily. These channels are activated upon depolarisation and open from a fixed threshold potential determined by the properties of a voltage sensor. Currents are only recorded at potentials positive to this threshold potential, which is always positive to E_K.
The rectifying behaviour of IK_{so} can be approximated by the Goldman-Hodgkin-Katz equation, which predicts a curvature of the I/V relation under asymmetric K^{+} conditions. This result indicates that IK_{so} lacks intrinsic voltage sensitivity and consequently the molecular correlate of the current is unlikely to be a member of the 6TMD superfamily. Instead the lack of a defined threshold for activation means that IK_{so} shares the diagnostic feature of the recently cloned K_{r} superfamily of potassium channels. Furthermore the open rectifier behaviour of IK_{so}, its regulation by muscarinic receptor activation and its exquisite sensitivity to changes in the pH of the extracellular solution strongly suggest that IK_{so} is the functional correlate of the K_{r} channel TASK-1. Earlier in situ hybridisation studies of Duprat et al. (1997) showed that TASK-1 mRNA was present in the granule cell layer of the cerebellum. This observation has been extended here using RT-PCR and a selective TASK-1 antibody to show that TASK-1 is expressed in CGNs themselves and that the protein is found in the plasma membrane of these cells. To date, members of the K_{r} superfamily have been identified by searching gene databases for the K^{+} channel signature sequence, this study provides evidence for the existence of functional K_{r} channels in native neurons.

**Putative physiological role of K_{r} channels.**

The expression of cloned K_{r} channels in *Xenopus* oocytes or mammalian cell lines results in cells with a membrane potential near to the potassium equilibrium potential (E_{K}). This property, along with the almost time independent gating of K_{r} currents, is evidence to suggest this class of potassium channel has a physiological role as a background K^{+} conductance and is responsible for setting the resting membrane potential of many cell types (Lesage and Lazdunski, 1999). In common with their role of setting a cell’s resting membrane potential, genes belonging to the K_{r} family have been identified in the genome of many organisms, with channels cloned from *C. elegans*, *Drosophila*, yeast, plants and mammals. The sequencing of the entire genome of *C. elegans* revealed approximately 50 genes belonging to the K_{r} superfamily, and by extension it is expected many more will soon be identified in other species. Work with *C. elegans* has shown that the expression of some of these channels can be extremely specific, with most K_{r} genes expressed in only a small number of cells. In one instance a single interneuron is the major site of expression.
Other genes have expression limited to motorneurons, two pairs of interneurons, small subsets of sensory cells, muscle or epidermis. The distinct expression patterns of $K_T$ channels led to the proposal that individual channels may be intended to customise the electrical properties of small groups of cells (Salkoff et al, 1999).

The identification of this class of channel, the determination of their localisation and role in normal physiology may provide new targets for the development of therapeutic agents and uncover clues to the molecular basis for disorders that result from ion channel dysfunction (Goldstein et al, 1998). So far $K_T$ channels have been implicated as a possible target for volatile anaesthetics with some, but not all, members of this family being activated by volatile agents. Exactly how important this action is in the production and maintenance of the anaesthetised state remains unclear (Franks and Lieb, 1999). Additionally riluzole, a neuroprotective drug with anticonvulsant and anti-ischaemic properties, was found to increase the activity of the $K_T$ channel TRAAK by a factor of four, leading to the proposal that part of the drugs beneficial effects may be due to the stimulation of this class of channel (Fink et al, 1998).

**Physiological relevance of $I_{K_{so}}$.**

The presence of $I_{K_{so}}$ in CGNs determines the resting membrane potential of these cells as predicted for a channel belonging to the $K_T$ superfamily. $I_{K_{so}}$ develops over several days in culture (Watkins and Mathie, 1996), and concomitant with increased levels of the current CGNs develop a more hyperpolarised zero current potential. Additionally when CGNs were studied under current clamp conditions application of either muscarine or external solution at pH 6.4 produced a depolarisation. The high input resistance of these cells means that inhibition of $I_{K_{so}}$ will have a profound effect on the resting membrane potential, resulting in an increased excitability of granule neurons.

Granule neurons relay mossy fibre inputs via parallel fibres to Purkinje cells and other neurons in the cerebellar cortex. Mossy fibres are capable of firing at very high frequencies ($> 100$ Hz) and one function of granule neurons is thought to be a filtering of the mossy fibre input before distributing it to the Purkinje cells (Gabbiani
et al, 1994). If precise information is encoded in the firing frequency of CGNs then understanding how the excitability of granule neurons can be modulated may be of physiological relevance.

Although the presence of muscarinic receptors in CGNs is well established, for the action of muscarine on $I_{K_{so}}$ to have physiological relevance a cholinergic input to the cerebellum must be demonstrated. Immunohistochemical studies using specific antibodies for choline acetyltransferase have labelled a number of regions in the cerebellum including a subpopulation of mossy fibres and glomerular rosettes in the granule cell layer (Boegman et al, 1988, Ojima et al, 1989). From this distribution the existence of a cholinergic mossy fibre input to the cerebellar cortex has been proposed (Boegman et al, 1988). Alternatively glutamate has been found to have an inhibitory effect on $I_{K_{so}}$, although to a lesser degree than muscarine (Watkins and Mathie, unpublished observations), and it may be that glutamatergic mossy fibre activity is responsible for the physiological modulation of $I_{K_{so}}$.

The sensitivity of $I_{K_{so}}$ to small changes in pH is of particular physiological importance since extracellular acidification will, like muscarine, both depolarise CGNs and increase their excitability after block of $I_{K_{so}}$. Neuronal activity gives rise to changes in extracellular pH (reviewed in Chesler, 1990, Chesler and Kaila, 1992). pH shifts can be observed in pathophysiological situations such as epileptic seizures and in ischaemia where large acidifications are known to accompany increases in extracellular glutamate levels. Physiological conditions such as electrical stimulation of Schaffer collateral fibres in hippocampal slices and parallel fibre stimulation in the cerebellum also result in pH shifts (Duprat et al, 1997). These variations in pH have been characterised using extracellular pH sensitive microelectrodes and a lowering of pH by around 0.6 of a unit has been reported, with the possibility that this decrease may be larger in the vicinity of the synaptic cleft. Furthermore transient acid shifts occur during the release of neurotransmitters from storage vesicles which are themselves highly acidic (Anderson and Orci, 1988, Miesenböck et al, 1998). This property is consistent with the putative localisation of TASK-1 channels to the synapse. The C terminus of TASK-1 channels contains a sequence (S/TXV) which, in the case of $K_v1$ and Kir2 subfamilies, is sufficient to promote the association of
channels with synaptic proteins such as PSD95 or SAP90 (Lesage and Lazdunski, 1999).

In conclusion the biophysical and pharmacological properties of $I_{Ks0}$ are strikingly similar to those of TASK-1. These data make a persuasive case for the identification of $I_{Ks0}$ as an endogenous TASK-1 channel. The demonstration that a mammalian $K_T$ channel is functional in native cells highlights the channel's likely importance in controlling cell excitability and also suggests a mechanism for how agents that modify its activity, such as muscarinic receptor agonists and hydrogen ions, can profoundly alter both the neuron's resting potential and its excitability.
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