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Developmental Control of Voltage-Gated Potassium Currents on Postnatal Rat Peripheral Neurons

by

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ABSTRACT

Voltage-gated potassium (K) channels are important in controlling a neuron's excitability. In this thesis I show that neonatal rat nodose and superior cervical ganglion (SCG) neurons express three voltage-gated K currents: a non-inactivating delayed rectifier type current (IK); a rapidly inactivating A-current (IAf), and; a slowly inactivating A-current (IAs). The channels that underlie IAf and IAs differ in their voltage-dependent, kinetic and pharmacological properties, but share the same single channel conductance, suggesting that rapidly and slowly inactivating A-channels are distinct subtypes of the same basic channel. My preliminary molecular biology experiments establish an approach for identifying the genes that encode for IAf, IAs and IK channels on SCG neurons. By studying the expression of IAf, IAs and IK on peripheral neurons during the first 2 postnatal weeks, I showed that there is no change in the expression of the 3 currents on nodose neurons, whereas the outward current on SCG neurons changes significantly from one dominated by IAs to one dominated by IAf. These results indicate that the pattern of developmental expression of similar types of K channels can differ for each neuron type. Next, I investigated various factors involved in the postnatal control of expression of K channels on SCG neurons. I demonstrated that neither preganglionic nor target factors influence postnatal changes in K currents. However, I observed that neonatal SCG neurons that develop in culture without other cell types lose their expression of IAf and IAs, suggesting that extrinsic factor(s) are involved in the regulation of the expression of these currents. I showed that this loss of A-currents is due, in part, to the absence of a soluble factor provided by nonneuronal cells. In addition, my analysis of the different patterns of expression of voltage-gated K currents suggests that peripheral neurons use intrinsic mechanisms to coordinate their expression of IAf, IAs and IK, so that changes in one K current are compensated for by reciprocal changes in one or more of the other K currents.

RÉSUMÉ

Les canaux potassiques (K) voltage-dépendants jouent un rôle important dans le contrôle de l'excitabilité du neurone. Dans cette thèse, je démontre que les neurones du ganglion nodose et du ganglion cervical supérieur (GCS) chez le rat nouveau-né expriment trois courants K voltage-dépendants: un courant qui ne s'inactive pas du type de la rectification retardée (IK); un courant A rapidement inactivé (IAr); un courant A lentement inactivé (IAl). Les canaux responsables du IAr et IAl diffèrent dans leur dépendance du voltage, leur cinétique et leur pharmacologie, mais partagent la même conductance unitaire. Cela suggère que les canaux A à inactivation rapide et lente représentent deux sous-types du même canal de base. Mes expériences préliminaires de biologie moléculaire établissent une approche pour l'identification des gènes qui codent les canaux IAr, IAl et IK des neurones du GCS. J'ai étudié l'expression du IAr, IAl et IK des neurones périphériques durant les deux premières semaines du développement post-natal. J'ai alors pu démontrer qu'il n'y a pas de changement dans l'expression de ces trois courants pour les neurones du ganglion nodose, tandis que le courant sortant des neurones du GCS passe, de façon significative, d'un courant dominé par IAl en un dominé par IAr. Ces résultats indiquent que lors du développement, les patrons d'expression de canaux K de types similaires sont particuliers à différents neurones. Par la suite, j'ai étudié les facteurs impliqués dans le contrôle de l'expression des canaux K des neurones du GCS post-natal. J'ai démontré qu'aucun facteur provenant des neurones préganglioniques ou des cibles n'influence les changements de courants K voltage-dépendants. En revanche, j'ai observé que les neurones du GCS de nouveaux-nés, se développant en culture sans autre type cellulaire, n'expriment plus IAr et IAl, suggérant ainsi qu'un ou des facteurs externes sont impliqués dans la régulation de l'expression de ces courants. J'ai démontré que cette perte de courant A est due, en partie, à l'absence d'un facteur soluble produit par les cellules non-neuronales. De plus, mon analyse des différents patrons d'expression de courants K voltage-dépendants suggère que les neurones périphériques utilisent des mécanismes intrinsèques pour coordonner leur expression de IAr, IAl et IK. En conséquence, les changements observés pour un courant K donné sont compensés par des changements réciproques d'un ou plusieurs autres courants K.

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To my friends, for making me laugh about all things funny and sad, and to my family, well, for everything.

PREFACE

In accordance with the Guidelines Concerning Thesis Preparation of the Faculty of Graduate Studies and Research of McGill University, the candidate has chosen the option of including as part of her thesis portions of the text of original papers submitted or suitable for submission to journals for publication. The text is as follows:

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CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

The experimental results presented in Chapters 3 and part of Chapter 4 have been published in a form similar, but not identical, to that which appears in this thesis (McFarlane and Cooper, 1991, 1992a). The denervation and axotomy experiments, and the results obtained with cultures of SCG neurons in the absence of other cell types in Chapter 5 have also been published (McFarlane and Cooper, 1992a). We are in the process of submitting the results presented in Chapter 5 on the effects of nonneuronal cells on K current expression (McFarlane and Cooper, 1992b).

I provide the first clear description of inactivating voltage-gated K currents on peripheral sensory neurons at the single channel and macroscopic level. My results suggest strongly that slowly and rapidly inactivating A-channels which share the same single channel conductance, are mediated by separate channels. I show for the first time that a slowly inactivating channel is also present on sympathetic neurons from the superior cervical ganglion. In fact, I demonstrate that the slowly inactivating current is the predominant voltage-gated outward K current on neonatal SCG neurons. I also characterized a rapidly inactivating current present on SCG neurons, as previously demonstrated by a number of labs.

I am the first person to study the in vivo postnatal developmental expression of voltage-gated K currents on rat peripheral sensory and sympathetic neurons. I show that for SCG neurons there is a switch from an outward current dominated by slowly inactivating currents, to one that is predominantly rapidly inactivating, over the first 2 postnatal weeks. I also demonstrate that preganglionic inputs and target influences are not responsible for the changes observed during the first 2 weeks postnatally. The experiments presented in Chapter 5 demonstrate that neonatal SCG neurons lose their expression of inactivating K currents when grown in the absence of other cell types. This loss can be prevented by a soluble factor from the nonneuronal cells of the ganglion. These studies demonstrate for the first time that nonneuronal cells can influence the expression of voltage-gated K currents on neurons. In addition, they indicate that the control of K current expression during development is not entirely intrinsic to the neurons, but can be influenced by epigenetic factors. These results also suggest caution in interpreting electrophysiological results obtained for neurons in culture, as the currents may not be the same as the currents expressed on neurons in vivo. The studies on nodose sensory neurons discussed in Chapter 4 and 5 are the first time that the postnatal control of K currents on these neurons has been examined. The studies on postnatal control of SCG sympathetic and nodose sensory neurons indicate that distinct classes of peripheral neurons show different postnatal control of their expression of K currents. Finally, the preliminary results discussed in Chapter 6 are the first to identify a K channel gene expressed in a mammalian sympathetic ganglion.

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1. INTRODUCTION

Chapter 1

Introduction

1.1 Introduction

The mammalian nervous system consists of an intricate arrangement of interconnections among approximately 100 billion nerve cells. The number and complexity of these connections is essential for the proper functioning of the system, however, it necessitates strict control over the associations made between pre and post synaptic neurons. Just as important is that the communication between connected neurons is appropriate.

Communication between nerve cells is both electrical and chemical. Signals are transferred from cell to cell at chemical synapses, but the signal generated by a neuron and transmitted along its axon is electrical in nature. The production and transmission of these electrical signals, called action potentials, is dependent on the presence of ionic channels in the surface membrane. Ionic channels are specialized proteins that span the membrane forming hydrophilic pores through which ions can pass.

A neuron has millions of ionic channels in its membrane. To produce an electrical signal there must be a mechanism to coordinate the operation of these ionic channels. As such, channels are designed to open in response to a biological stimulus; when the pores are open, ions flow, and because of their charged nature, change the membrane potential. This flow of ionic current mediates the various electrical changes in nerve cells including synaptic potentials and, in particular, the initiation and conduction of the action potential.

There are 3 main families of channels based on the type of stimulus that is required to gate, or open, the channel. Ligand-gated channels open when a ligand binds to the channel protein complex. The ligand-gated channels include the nicotinic acetylcholine, gamma aminobutyric acid

(GABA), glutamate and glycine receptor subfamilies. These channels are involved in fast communication at chemical synapses. The second family consists of channels that are gated by way of second messenger systems. In this family, a ligand binds to the membrane receptor and activates ion channels indirectly through activation of a G-protein; the activated G-protein engages a second messenger (which can involve increases in intracellular calcium concentration) that acts on the channel protein. Examples of this class include muscarinic potassium channels, and ion channels coupled to adrenergic, serotonergic, and peptidergic receptors (Rudy, 1988). These channels are also involved in synaptic transmission, however, the time course of second messenger mediated synaptic events is orders of magnitude slower than those mediated by ligand-gated channels. The third family consists of channels that are gated by changes in the voltage across the membrane of the cell, the voltage-gated channels. The channels in this family, which include sodium (Na), calcium (Ca) and potassium (K) channels, are primarily involved in the generation and transmission of action potentials.

Each channel family is made up of a large number of different members, and any given neuron will generally express a subset of members of each family. The particular repertoire of ionic channels a neuron expresses is important in determining how a neuron transmits information from other neurons that synapse on its cell body. For example, voltage-gated potassium (K) channels, which are part of the family of voltage-gated ion channels, have an important role in regulating a neuron's excitability. However, there are several subtypes of voltage-gated K channel, each with its own unique kinetic and voltage-dependent properties. As such, each subtype exerts a different effect on cellular excitability. Therefore, the particular set of voltage-gated K channels a neuron expresses, and the density of each member of the set, will be important in determining its electrical properties; such as the threshold to generate an action potential in response to an excitatory response, and the frequency that it fires action potentials with a maintained stimulus.

In view of the importance of ionic channels in communication between neurons, an important question of developmental neurobiology, that has received relatively little attention, is: what determines the appearance of different ionic channels on neurons? Many of the mechanisms that dictate which channel types a neuron expresses remain obscure.

In the following chapter, I discuss what is known about one particular class of ion channels, the voltage-gated K channels, and how the expression of these channels on neurons is regulated during development. First, I consider the functional, or electrophysiological, properties of voltage-gated K channels. Next, I discuss the structure of these channels as revealed by recent molecular biological studies. Then, I review what is known about the control of voltage-gated K currents during development, and finally the possibility that extrinsic factors are involved in this control.

1.2 Voltage-gated potassium currents

1.2.1 The ionic basis of neural signaling

Hodgkin and Huxley (1952) were the first to show that the action potential (AP) occurs by the flow of 2 different ionic currents, sodium and potassium. Their experiments using two electrode voltage clamp of the squid giant axon demonstrated that sodium (Na) ions are responsible for the upstroke of the AP, while potassium (K) ions are involved in the repolarization of the AP. This work not only revealed that ionic conductances are involved in neural signaling, but it established practical and theoretical approaches to studying the electrophysiological properties of ionic conductances, which 50 years later are still very much in use.

Initially such studies were restricted to invertebrate neurons, mainly for technical reasons because mammalian neurons were too small to impale with the two electrodes necessary for voltage clamp techniques. With the advent of the patch clamp technique by Neher and Sakmann (Neher et al., 1978; Hamill et al., 1981), almost forty years after Hodgkin and Huxley's experiments, two very important advances were made. First, their recordings with patch electrodes confirmed that ions flow through pores in the surface membrane: all-or-none currents were measured in a small patch of cell membrane under the electrode, consistent with the concept of a pore that opens and closes. Second, the use of a single electrode for passing and recording current made it possible to record from the much smaller mammalian neuron.

1.2.2 Properties of voltage-gated ion channels

For voltage-gated channels, ionic current flows through individual channels in response to a membrane voltage change. The summed activity of the current flowing through all channels that are open gives rise to the macroscopic, or whole-cell current. Which ions flow through a channel is dependent on a narrowing of a region of the channel pore, called the selectivity filter (Hille, 1984). A potassium channel preferentially conducts K ions over other available ions, and the ease by which it conducts K is called the single channel conductance. This property is defined by the pore region of the channel, therefore, if channels differ in their single channel conductance it implies that they are structurally different.

In general, voltage-gated channels can exist in 3 main states: open or activated; closed or deactivated; and inactivated. In the open state, which is triggered by changes in membrane potential, channels conduct ions. In the closed state, channels do not conduct, but are ready to open in response to a voltage change. Channels that inactivate, open in response to a depolarizing voltage step, but despite maintained depolarization the channels enter an inactivated state, which is different from the closed state mentioned above, where they do not conduct ions. To remove inactivation the membrane must be hyperpolarized.

Researchers have used electrophysiological kinetic data describing the movement between the closed and open states, and the open and inactivated states, to model ionic channel behaviour in the form of state diagrams representing transitions among different states (Armstrong and Bezanilla, 1977; Zagotta and Aldrich, 1990b). A full description of the contribution of this biophysical approach is outside the main focus of this thesis. The important point these models make, however, is that channels can exist in several closed, open and inactivated states (Hille, 1984); for simplicity I have referred to them as single states.

Hodgkin and Huxley (1952) hypothesized that the voltage-dependence of channel opening is due to charged particles associated with the channels. Movement of these particles, in response to voltage changes, causes conformational changes in the pore of the channel so that it conducts ions. The dependence of conformational change on the movement of these particles in response to a change in the electric field is what makes channels dependent on voltage. Armstrong and Bezanilla (1977) demonstrated the existence of such particles for voltage-dependent Na channels by measuring the currents associated with their movement through the membrane.

For channel inactivation, the current hypothesis is that after the channel opens, the inside of the pore gets plugged with a charged portion of the channel protein (Armstrong and Bezanilla, 1977; Bezanilla et al., 1991); this is a rapid event occurring in msecs. To remove inactivation, equated with the unplugging of the channel, the cell must be hyperpolarized. Recent elegant experiments by Zagotta and co-workers (1990) provide strong support for this hypothesis. A slower type of inactivation also occurs, which involves entry into a second inactivated state whose stability is controlled by a different portion of the K channel protein (Iverson and Rudy, 1990; Hoshi et al., 1991).

Hodgkin and Huxley (1952) showed that the processes of activation (i.e. the process whereby a channel opens), deactivation (i.e. the process whereby a channel closes), and inactivation are dependent on voltage and time. However, the voltage and time dependencies are different for each type of channel, and therefore can be used in the classification of channel subtypes. Channels can also be defined in terms of the pharmacological agents that act on them: drugs or toxins will specifically block certain channels and not others, and as such, the repertoire of agents that act on a given channel can help in its classification.

1.2.3 Voltage-gated K currents

Channels that conduct K ions are found in all eukaryotic cells, and vary widely in their voltage-dependence and kinetic behaviour. Research has clearly demonstrated the role of one class of K channels, the voltage-gated K channels, in regulating neuronal excitability (Thompson and Aldrich, 1980; Adams and Galvan, 1986). Currents which flow through voltage-gated K channels can either pass into or out of the cell. Inward currents, making up the class of inward rectifiers, are observed in cardiac and skeletal muscle, eggs and many vertebrate and invertebrate neurons (Rudy, 1988). These K currents activate upon membrane hyperpolarization, and because the membrane potential is below the Nernst equilibrium potential for potassium (Ek), the direction of positive current flow is into the cell. This class of current was not studied in this thesis, and as such will not be discussed further.

1.2.4 Voltage-gated outward K currents

Outward K currents are activated by depolarized potentials that bring the membrane potential above Ek. As such, the opening of these K channels results in outward, hyperpolarizing currents, which bring the membrane away from the threshold potential for AP generation. As a result, outward K currents decrease cell excitability. Outward K currents are traditionally classified on the basis of their inactivation kinetics as either transient A-currents, or delayed rectifiers (Thompson and Aldrich, 1980; Rudy, 1988).

1.2.4.1 Delayed Rectifiers:

Hodgkin and Huxley (1952) using two electrode voltage clamp were the first to measure and characterize a K current. They showed that in the squid giant axon a voltage-gated K conductance is activated with a delay upon depolarization, and unlike the Na current does not inactivate over the duration of the voltage step of 10 ms. This current was termed a delayed rectifier current (IK) because the onset of current activation was delayed as compared to the activation of the Na current, and because the current rectified in the outward direction (i.e. passed current better in the outward, than inward direction). In modeling the ionic basis of the AP, Hodgkin and Huxley were able to show that activation of the K conductance, coupled with inactivation of the Na conductance, is responsible for repolarizing the action potential.

It is the properties of this current in squid giant axon that are used to describe the class of K currents known as delayed rectifiers. Non transient K currents are often classified as delayed rectifiers by default, and, as a result, the family of delayed rectifiers consists of non transient currents whose properties can differ considerably.

1.2.4.2 Transient A-currents:

The other main class of voltage-gated K currents include the fast activating, transient currents, first observed in onichidium by Hagiwara and co-workers (1961). This current, referred to as the A-current, was subsequently characterized in molluscan neurons (Connor and Stevens, 1971a; Neher, 1971). A-currents activate rapidly (5-10ms) in response to a depolarizing voltage step, and then inactivate rapidly with time constants of tens of msecs, despite maintained depolarization. To remove inactivation, the membrane must be hyperpolarized. As such, A-currents are analogous to Na currents, first characterized by Hodgkin and Huxley (1952), however, the rate constants of these processes for Na channels are an order of magnitude faster than for A-channels.

Transient currents are found in a large number of nonneuronal and neuronal cell types from both vertebrate and invertebrate species (reviewed by Rogawski, 1985; Rudy, 1988; Llinas, 1988). The fact that transient currents with similar properties are found in very evolutionary distant species suggests that the A-channel protein has been conserved during evolution. The recent cloning of a large number of K channel genes from a number of distant species shows that this conservation of K channels holds true down to the molecular level (Wei et al., 1990; Salkoff et al., 1992).

A-channels are found on presynaptic nerve terminals (Shimahara, 1983; Bourque, 1990; Thorn et al., 1991), and postsynaptically on cell bodies (Connor and Stevens, 1971a; Belluzzi et al., 1985). Shimahara (1983) has shown that A-currents in nerve terminal membranes of Aplysia neurons shorten the duration of the AP that invades the terminals, thereby decreasing the amount of Ca that enters the nerve terminal through voltage-dependent Ca channels. Since transmitter release is dependent on increases in intraterminal Ca concentration, A-channels are important in regulating the amount of neurotransmitter released at synapses.

Postsynaptically, the role of A-currents is more varied. For example, in spontaneously active neurons, depolarizations activate the A-current which slows down the rate of return of the membrane to AP threshold, thereby prolonging the interval between APs; the hyperpolarization following an AP would be sufficient to remove inactivation of the A-channels so they could continue to operate (Connor and Stevens, 1971b). In this way, the A-current regulates the firing frequency of spontaneously active neurons, or those firing in response to a tonic depolarization (Gustaffson et al., 1982; Segal et al., 1984). A-currents on heart cells play a similar role (Giles and Van Ginneken, 1985). Other roles for A-currents in postsynaptic membranes, as defined in sympathetic neurons, include repolarizing the AP (Belluzzi et al., 1985), and counteracting excitatory synaptic potentials (Cassell and McLachlan, 1986).

However, for many neurons the A-current is not activated in response to depolarization, as the majority of A-channels are inactivated at the resting potential. Neurons often rest at a potential where small changes in membrane potential result in large changes in the proportion of channels that are inactivated. As such, in neurons where A-currents are inactivated at rest, small hyperpolarizations could mean that A-currents would become available to play similar roles as mentioned above. For example, hyperpolarization of guinea pig nucleus tractus solitarius neurons that express A-currents, causes a decrease in the duration of AP bursts and delays the onset of burst activity (Dekin et al., 1987); presumably, because the outward, hyperpolarizing A-current is now available for activation upon depolarization of the cell membrane. In addition, in hippocampal neurons gamma aminobutyric acid (GABA) shifts the voltage-dependence of inactivation of an A-channel to more depolarized potentials. This allows the A-channel to be activated from potentials at which it was previously inactivated and therefore unresponsive (Gage, 1992). Thus, A-currents which under normal resting conditions may not be available to the neuron, can become available upon modulation of the channel protein, or the cell membrane potential.

1.2.5 Problems with the separation of K currents

One of the main problems in classifying or characterizing voltage-gated K currents in a given neuron is to separate the different K currents that make up the macroscopic current. A number of methods are currently used to separate K currents based on differences in their pharmacological, voltage-dependent, kinetic or single channel properties. However, in many neurons, separation of K currents can be problematic because the above mentioned properties are not sufficiently different.

Traditionally, K currents are separated from one another with the use of pharmacological agents, or by taking advantage of differences in the voltage-dependence of inactivation of the K currents. Thompson (1977) showed that A-currents and delayed rectifier (IK) currents in molluscan neurons can be distinguished on the basis of their differential sensitivity to two pharmacological agents, 4-aminopyridine (4-AP), and tetraethylammonium (TEA). Thompson showed that when applied to the external surface of the neuron, 4-AP specifically blocked the A-current, and TEA specifically blocked IK. Researchers have used this criterion to classify currents that are 4-AP sensitive as A-currents (Segal and Barker, 1984; Giles and Van Ginneken, 1985; Greene et al., 1990; Numann et al., 1987), and those that are TEA sensitive as delayed rectifiers (Numann et al., 1987; Cobbett et al., 1989; Greene et al., 1990).

TEA and 4-AP are useful in neurons where they block a specific K current. In these neurons, the agents can be used to isolate K currents for further characterization, or to study the effects of blockade of a specific K current on the electrical behaviour of the cell. However, it is important to realize that in many neurons it is not possible to use either TEA or 4-AP to separate the different K current components (Cobbett et al., 1989; Ficker and Heinemann, 1992; McFarlane and Cooper, 1991, see Chapter 3); the K currents do not differ sufficiently in their sensitivity to these agents. In addition, in some cells, the pharmacological effects of 4-AP and TEA are not as described for molluscan neurons. For example, in guinea pig trigeminal neurons a rapidly inactivating current is blocked by TEA, but not by 4-AP (Spigelman and Puil, 1989).

The other classical means to separate different K currents present in the same cell is to take advantage of differences in their voltage-dependence for inactivation, and thereby use subtraction protocols; such methods were established for molluscan neurons (Connor and Stevens, 1971a). In these neurons, a transient A-current can be maximally activated when depolarizing steps are made from a membrane potential of -90mV, but the current is completely inactivated at a membrane potential of -40mV. However, the non-transient current shows no inactivation over this range of potentials. This difference in voltage-dependence of inactivation of these 2 currents is used in their isolation: depolarizing steps from a membrane potential of -90mV will activate both transient and non-transient currents, whereas depolarizing steps from a membrane potential of -40mV will only activate the non-transient current. Therefore, subtraction of the non-transient currents evoked from a membrane potential of -40mV, from the total current evoked from a membrane potential of -90mV, will isolate the transient current. This subtraction procedure only works however, if there is no overlap in the range of voltages over which the different K channels exhibit inactivation. In many neurons, this is not the case, and therefore this procedure does not adequately separate the currents.

Probably the best method to distinguish between different K currents is to record the behaviour of the channels that underlie them. However, only a few studies on mammalian cells have related the single channels to the macroscopic K currents (Marty and Neher, 1985; Hoshi and Aldrich, 1988a,b; Solc and Aldrich, 1988; Lynch and Barry, 1991; McFarlane and Cooper, 1991, see chapter 3). Combined single channel and macroscopic analysis of K currents helps eliminate two main problems: (1) That physically different channels can underlie macroscopic currents with similar voltage-dependent and kinetic properties. For example, studies on pheochromocytoma cells (Hoshi and Aldrich, 1988a), adrenal chromaffin cells (Marty and Neher, 1985), and amphibian spinal neurons (Harris et al., 1988) showed that several separate conductance channels are responsible for the non-transient macroscopic K currents expressed by the cells. These results indicate that without the corresponding single channel data it is possible to inappropriately group several conductances together as a single homogenous macroscopic current. (2) It is possible to mistakenly classify two currents as being different on the basis of macroscopic properties, while the single channels underlying the currents are fairly similar. For example, Cooper and Shrier (1989) showed that in cultured rat sensory neurons (nodose ganglion) similar 22pS conductance channels underlie both a rapidly (τ=10-30ms) and a more slowly inactivating macroscopic current (τ=100-300ms and 1-3s).

The problems in separating and identifying the different K currents present in neurons has resulted in confusion in classifying K currents (see review by Rudy, 1988). In particular, there are problems with the classification of the non-transient K currents. Recently, several investigators demonstrated that "non-inactivating" delayed rectifier K currents can actually be divided further into two broad categories. Those that slowly inactivate over hundreds of msecs (at least one order of magnitude more slowly than classical A-currents), and those that do not inactivate, except perhaps over very long time scales (at least tens of seconds); the latter currents are referred to as non-inactivating currents in this thesis. In many cells, including frog skeletal muscle (Adrian et al., 1970), rat hippocampal neurons (Segal and Barker, 1984), melanotrophs of the rat pituitary (Kehl, 1989) and the neurons of the rat supraoptic nucleus (Cobbett et al., 1989), the current called the delayed rectifier actually inactivates slowly. Even the prototypical delayed rectifier of the squid giant axon shows slow inactivation (Chabala, 1984).

Unfortunately, on the basis that they inactivate, slowly inactivating currents have also been classified as A-currents. For example, currents very similar to the slowly inactivating delayed rectifiers described above are found in guinea pig hippocampal neurons (Gustaffson et al., 1982), guinea pig DRG neurons (Kasai et al., 1986), molluscan neurons (Serrano and Getting, 1989) and rat hypothalamic neurons (Greene et al, 1990), but are labeled as slowly inactivating A-currents. Matters are further complicated by the fact that non-inactivating and slowly inactivating currents behave very similarly over short 100 ms depolarizing voltage steps often used in electrophysiological experiments. Therefore, if both currents are present in a cell, they are often mistakenly considered as a single macroscopic current.

Classification of slowly inactivating currents on the basis of pharmacological blockade by either 4-AP, or TEA, is not helpful in clarifying the situation. For example, while TEA blocks slowly inactivating currents in some neurons (Lukasiewicz and Werblin, 1988; Kehl, 1989; Cobbett et al., 1989), in other neurons slowly inactivating currents are even more sensitive to 4-AP than the classical A-currents (Stansfeld et al., 1986; Storm, 1988; Spigelman and Puil, 1989). Two other pharmacological agents, mast cell degranulating peptide (MCDP) from bee venom, and dendrotoxin (DTX) from snake venom, have been used to classify some slowly inactivating K currents (Rudy, 1988; Castle et al., 1989; Rehm and Tempel, 1991); they are thought to bind to the same brain membrane protein. For example, DTX blocks a slowly inactivating K current in adult nodose neurons (Stansfeld et al., 1986), and the slowly inactivating K currents expressed in Xenopus oocytes injected with mRNA for a rat brain K channel clone (Stuhmer et al., 1989). However, not all slowly inactivating mammalian K channels are sensitive to DTX (Rehm and

Tempel, 1991; Stuhmer et al., 1989).

Results from single channel studies also suggest that caution should be taken when classifying slowly inactivating currents. A single class of channels, based on conductance, in rat nodose neurons (Cooper and Shrier, 1989), rabbit atrial cells (Clark et al., 1988) and guinea pig DRG neurons (Kasai et al., 1986) gives rise to both a slowly inactivating current, and the classical A-current. Without the benefit of the single channel data, these researchers might have classified the rapidly and slowly inactivating currents as being quite different. Studies such as these indicate that without corresponding single channel data, it becomes very difficult to properly classify outward K currents as delayed rectifiers, or A-currents, simply on the basis of observed macroscopic properties.

In summary, electrophysiological studies have identified voltage-gated K currents that are broadly classified on the basis of their inactivation properties; some currents inactivate rapidly with time constants of 10-30ms, others inactivate more slowly over hundreds of msecs, whereas a third group of currents shows little or no inactivation. A single neuron can express voltage-gated K currents from all three groups. The voltage-dependent and pharmacological properties of these three types of K current can overlap. Therefore, when a single neuron expresses several K currents it is often difficult to properly separate them for further characterization. It is also becoming increasingly clear from electrophysiological experiments that the present system of classifying voltage-gated K currents as delayed rectifiers or A-currents is inadequate, given that there can be considerable overlap in the properties of different types of K current. In fact, it has been suggested that only with the molecular characterization of K channels will a classification system become possible (Rudy, 1988).

1.3 Molecular biology of K channels

The electrophysiological experiments demonstrate that there are several different K channels, with varying properties and functions. However, the recent cloning of a large number of K channel genes indicates that the diversity of K channels is much greater than suggested from electrophysiological experiments. These results underline the fact that a knowledge of the molecular biology of K channels is almost essential for understanding the K channels responsible for the electrophysiological properties of neurons. Molecular biology has led to at least partial answers for the following questions: How is the diversity of K channels produced? Are K channels with different physical properties encoded for by completely different genes, by separate but homologous genes, or by the same gene with alternative splicing to produce different K channel transcripts? Which domains of the protein are important for the different functional characteristics of the channel, and what changes in these regions are required to produce K channels with different properties?

In the short time since the first K channel gene was cloned (Papazian et al., 1987; Kamb et al., 1987; Pongs et al., 1988) researchers have determined many of the molecular mechanisms involved in producing a large number of different K channels (see reviews by Pongs, 1989; Salkoff et al., 1992; Jan and Jan, 1990). More recently, experiments have concentrated on elucidating the functional domains of the protein that are important for gating, voltage-dependent and kinetic properties, pharmacology, and ion selectivity.

1.3.1 Cloning of the Shaker locus

The approach taken towards cloning the first K channel was different from that taken to clone the Na channel (Noda et al., 1984) and the nicotinic acetylcholine receptor (Noda et al., 1983). A classical approach was used to clone both these channels: A specific irreversible ligand for the channel was used to isolate the channel protein from a highly enriched source, the electric organ of Torpedo Californica. The amino terminal of the isolated protein was sequenced, and based on this sequence, an oligonucleotide probe was made which was used to screen a complimentary DNA (cDNA) library from T.Californica to isolate the gene(s) for the channel. This approach was not feasible for K channels for two main reasons: (1) there is no rich source of K channels (K channels make up a very small amount of total cell protein); (2) as yet, there is no specific irreversible ligand for K channels. Therefore, the cloning approach taken by 3 different labs (Papazian et al., 1987; Kamb et al., 1987; Pongs et al., 1988) was to take advantage of a particular mutant in Drosophila, the Shaker mutant, which was known to be deficient in a rapidly inactivating A-current (Salkoff and Wyman, 1981b). Since the precise chromosomal location of the mutation was known, the strategy was to clone the genomic DNA corresponding to the Shaker locus, using a method called chromosomal walking.

This approach proved to be successful: The Shaker locus, when sequenced, was found to consist of a large transcription unit (>110kB) containing several exons and introns (Tempel et al., 1987; Kamb et al., 1987; Pongs et al., 1988). The Shaker locus codes for a protein of approximately 70kD. From hydropathy analysis it is thought that the protein consists of 6 (S1-S6) alpha helical membrane spanning domains (see Fig. 1.1). The amino and carboxyl termini are thought to be cytoplasmic; this idea is consistent with several extracellular N-linked glycosylation sites, and intracellular cAMP-dependent phosphorylation sites. To confirm that the DNA cloned from the Shaker locus codes for a K channel protein, messenger RNA (mRNA) was transcribed in vitro, and injected into Xenopus oocytes. Injected oocytes, recorded with two electrode voltage clamp, displayed rapidly inactivating, K selective currents (Timpe et al., 1988 a,b; Iverson et al., 1988) similar to K currents expressed in native Drosophila muscle (Salkoff and Wyman, 1981a, 1983). The oocyte expression studies confirmed that the Shaker locus encodes for a K channel protein.

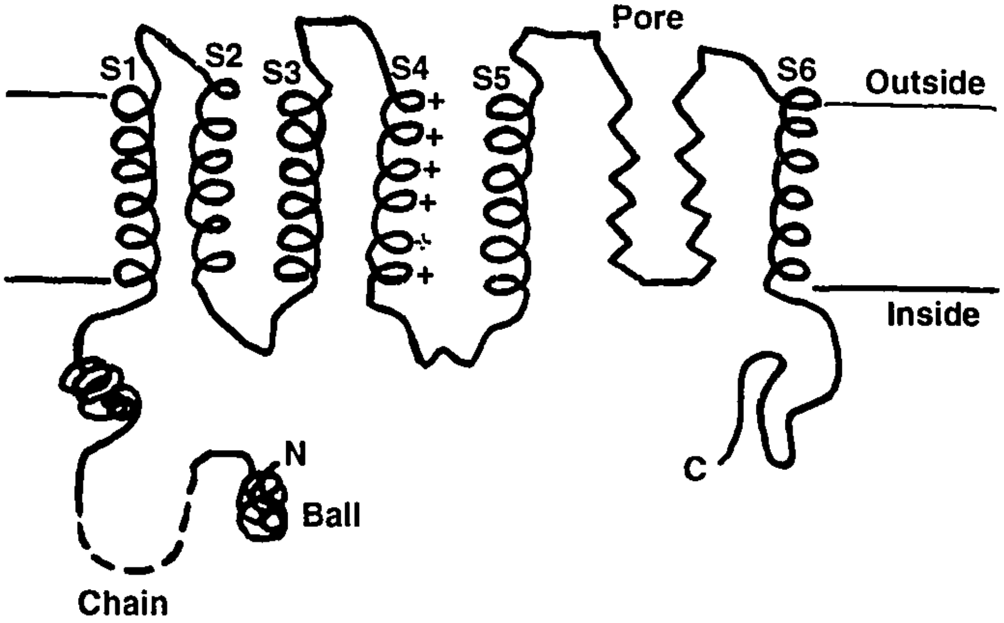

Figure 1.1 Functional domains of voltage-gated K channels. A widely accepted transmembrane folding model for voltage-gated K channels which arose as a result of mutational analysis. (modified from Hartmann et al., 1991)

1.3.2 Molecular mechanisms to produce channel diversity

In Drosophila, several different mechanisms exist to produce a large number of different K channels.

1.3.2.1 Alternative splicing:

The primary Shaker gene product is alternatively spliced to produce as many as 24 different mRNA variants (Pongs et al., 1988; Schwarz et al., 1988; Kamb et al., 1988). The primary Shaker gene product is spliced so that mRNAs consist of alternate combinations of exons. The Shaker mRNA variants encode for proteins with common membrane spanning regions (the core region), but different combinations of carboxy and amino termini. Each mRNA variant induces A-currents when injected in oocytes, however the kinetic properties of the currents differ, suggesting that splice variants form subtypes of A-channels (Timpe et al., 1988a,b; Iverson et al., 1988; Iverson and Rudy, 1990). These results indicate that alternative splicing of a primary K channel gene product is one mechanism by which the diversity of K channels is achieved.

1.3.2.2 Separate but homologous genes:

Sole and co-workers (1987) postulated that additional Shaker-type K channel genes are present in the Drosophila genome on the basis of their experiments that showed that rapidly inactivating K currents were expressed by mutant Drosophila whose Shaker locus was deleted (Sole et al., 1987). On this assumption, Butler and co-workers (1989) used low stringency screening of a Drosophila cDNA library with a Shaker cDNA probe to clone three Shaker-like, but separate, K channel genes they called Shaw, Shab and Shal. The proteins encoded for by these three genes share a similar organization with that encoded for by Shaker; however, the K channels encoded for by the 4 different genes vary in their kinetic, and voltage-dependent properties (Wei et al., 1990). Shaker, Shal, Shaw and Shab define 4 separate K channel subfamilies. All vertebrate K channel genes isolated subsequently fall into one of these subclasses. A recent report has proposed that 2 other subfamilies exist (Drewe et al., 1992), although the mRNAs for these putative subfamilies have not been expressed in oocytes, and as such it is not clear whether they encode for K channel proteins.

Within the membrane spanning region (S1-S6) of the channel protein, there is 50-80% conservation at the amino acid level between each member of a subfamily. However, in comparing the same region between members of the different subfamilies there is only 40% conservation at the amino acid level. Presumably different gene subfamilies arose as a result of gene duplication and subsequent divergence of the genes during evolution. Thus, K channel diversity can also result from several different, but related genes, along with alternate splicing of the primary transcript.

1.3.2.3 Heteromultimeric channels:

K channels are thought to be multimers made up of more than one subunit. Such reasoning comes from what is known about the genes encoding voltage-gated Na and Ca channels: Na and Ca channels are large polypeptides consisting of 4 homologous domains in a tandem repeat (Catterall, 1988). The 70kD protein coded for by the Shaker locus appears to correspond to one of these homologous domains (compare Fig. 1.2A and 1.2 B,C). As such, the hypothesis is that K channels are actually multimers: A functional K channel would be made up of several (possibly 4) Shaker products, in analogy with the Na and Ca channels.

Injection of a single Shaker mRNA is sufficient to produce a functional K channel in Xenopus oocytes, suggesting that if K channels are multimers there is no requirement for more than one subunit type. However, heteromultimeric channels consisting of more than one type of subunit may also exist. The approach taken by several labs to test the possibility of heteromultimeric channels was to co-inject K channel mRNA species encoding for channels with different properties into Xenopus oocytes. If K currents are expressed which show a strictly additive effect of the properties of the K currents expressed when mRNA species are injected separately, then, only homomultimeric channels are formed and expressed in the oocytes. Alternatively, if the K currents

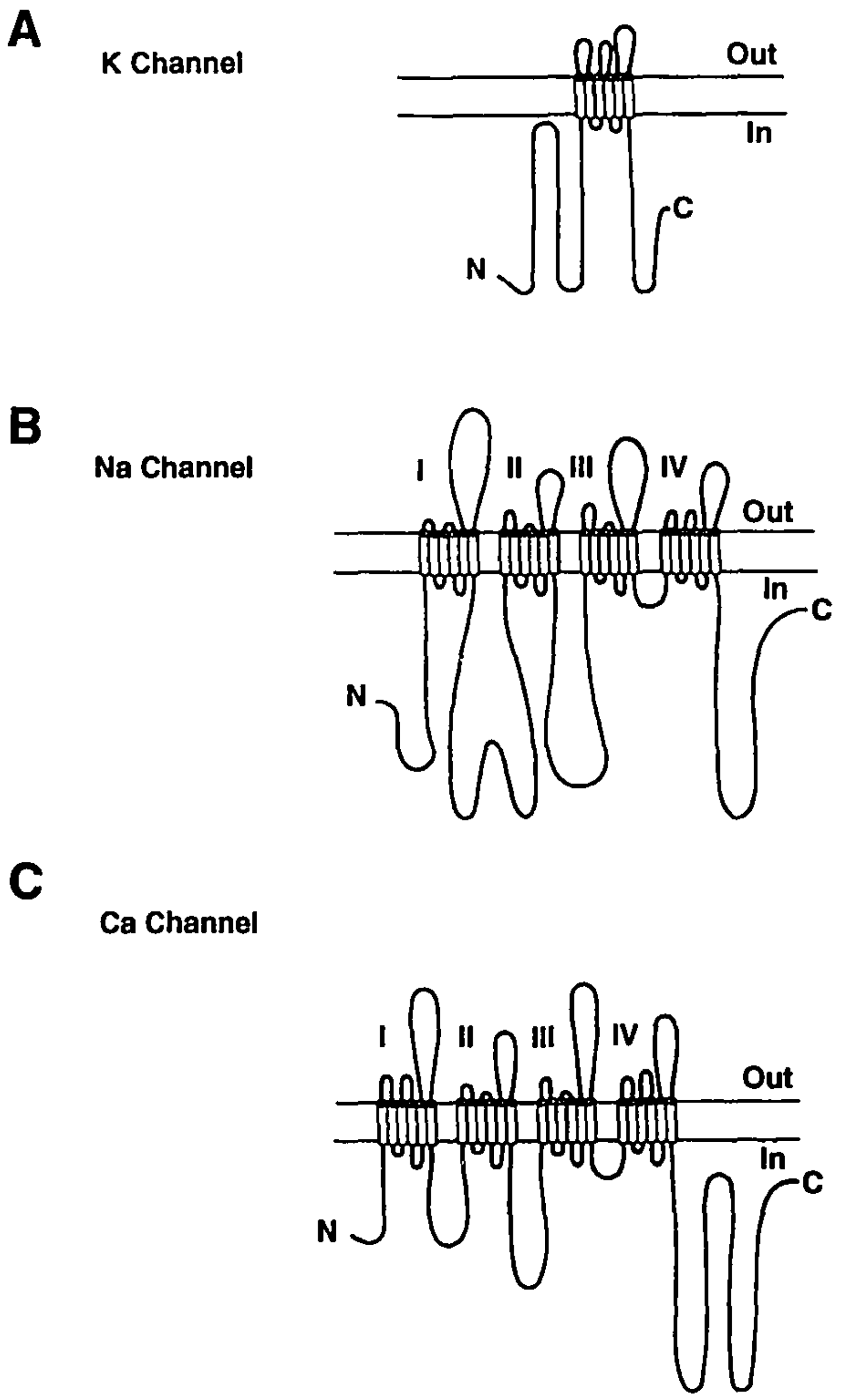

Fig 1.2 Proposed transmembrane arrangements of the principal subunits of Na, Ca and K channels. A: The K channel protein corresponds to one of the 4 homologous domains of 6 membrane spanning segments proposed for the Na and Ca channel protein subunits (B and C). (Modified from fig. 3 of Catterall, 1988).

of oocytes injected with 2 different K channel mRNA species have properties that can not be explained by simple addition of the properties of the K currents when mRNA species are expressed separately, it suggests that heteromultimeric channels are being assembled. Results from such experiments support the idea that, at least in oocytes, K channels can be made from more than one type of subunit (Isacoff et al., 1990; Ruppersberg et al., 1990; MacKinnon, 1991; Christie et al., 1990).

If 4 subunits make up a functional K channel protein, each of the 4 subunits can conceivably differ, and there are a large number of different subunits, the potential exists for a large number of different K channels. However, recent experiments using the same reasoning as the co-injection studies described above, show that each of the different Drosophila K channel subfamilies can only form hybrid channels with members of the same subfamily (Covarrubias et al., 1991). This sets certain limits to the amount of subunit mixing, and as a result, to the number of different K channels. Drosophila neurons express independent K channel systems (Salkoff and Wyman, 1981a,b; Solc et al., 1987) as shown by the fact that mutant flies exist whose neurons have selectively lost one of the K channel systems. Restricting mixing between K channel subunits of different subfamilies is one mechanism to generate these distinct K channel systems.

1.3.3 Mammalian K channels

K channel diversity is achieved somewhat differently in the mammalian nervous system. Regions of Shaker, Shab, Shal, and Shaw cDNAs were used under low stringency conditions as probes to clone a number of homologous K channel cDNAs from mouse brain (Tempel et al., 1988; Chandy et al., 1990; Pak et al., 1991a,b) and rat brain (Baumann et al., 1988; Christie et al., 1989; Frech et al., 1989; Stuhmer et al., 1989; Swanson et al., 1990). In contrast to Drosophila, which has only one gene member of each subfamily, at least 4 Shaw genes, 2 Shal genes, 1 Shab gene, and as many as 12 Shaker genes are present in the mammalian genome (see review by Salkoff et al., 1992). Except for one recent report (Luneau et al., 1991), there is little evidence for different mRNA species produced by alternative splicing. In fact, cloning and sequencing the genomic DNA for 3 mammalian brain K channel clones has shown that the genes do not contain introns (Chandy et al., 1990). Apparently, in the mammalian nervous system gene duplication and divergence, and possibly subunit mixing, are the major mechanisms for producing K channel diversity.

Keeping in mind the earlier discussion about the electrophysiological classification of K channels, it is important to note that until recently (Pak et al., 1991b; Schroter et al., 1991), low stringency screens with Shaker probes isolated only cDNAs encoding slowly inactivating K channels, although Shaker encodes for a rapidly inactivating K channel. This finding is surprising in that it suggests that slowly inactivating channels are very similar at the genetic level to rapidly inactivating channels.

1.3.4 Structure-function relationships determined from mutational analysis

Much is known about the different functional domains of the K channel protein from mutational analysis, which involves mutating specific amino acid residues of the cDNA encoding for a K channel protein, and expressing the mutated mRNAs in Xenopus oocytes (For a recent review of the subject see Miller, 1991). The effects of these mutations on different functional properties of the K channel help in determining which regions of the gene are responsible for the channel pore, and properties such as voltage-dependence, kinetics, and pharmacology. However, it is important to remember that changing one amino acid residue may be sufficient to alter the conformation of the channel, and therefore the data from such studies must be interpreted cautiously.

1.3.4.1 Channel pore:

Several pieces of evidence support the idea that the conductive pore of the channel consists of the linker between the 5th and 6th membrane spanning domains (see Fig. 1.1). First, single amino acid mutations in this region affects the blockade by toxins such as charybdotoxin (MacKinnon and Miller, 1989; MacKinnon et al., 1990), and drugs such as TEA (MacKinnon and Yellen, 1990; Yool and Schwarz, 1991), which are known to interact near or in the pore. Second, Hartmann and co-workers (1991) showed that transplanting a 24 amino acid segment of this region from one K channel clone, NGK2, which has a single channel conductance of 22pS, to the same region in a second K channel clone, DRK1, which has a single channel conductance of 8pS, resulted in hybrid DRK1 channels with the single channel conductance of an NGK2 channel. Therefore, the hypothesis is that this region between the 5th and 6th membrane spanning domains forms the pore. However, given the relatively short length of this segment it is unlikely for physical reasons that the region spans the membrane twice in an α-helical conformation. Rather, it is believed that this linker region crosses the membrane twice, in a fully extended ß-pleat form. Further studies have pinpointed certain amino acids within this pore region, specific for ligand binding (MacKinnon and Yellen, 1990), and ion selectivity (Yool and Schwarz, 1991).

1.3.4.2 Voltage-dependence:

As described earlier, the movement of charge on the channel protein must be coupled to conformational changes leading to channel opening. The fourth membrane spanning region (S4) has positively charged amino acids every third residue, a motif that is seen only for voltage-gated Na, Ca and K channels (Catteral, 1988). Conceivably, this positively charged region (see Fig 1.1) acts as the voltage sensor, and the outwardly directed movement of S4, in response to changes in the membrane electrical field, is required for channel opening. This idea is supported by the fact that mutations in this region have effects on voltage-dependence (Papazian et al., 1991; Lopez et al., 1991). The S5 region may also be involved in determining the voltage-dependence of a K channel. A genetic mutation localized to the 5th membrane spanning region (Gautam and Tanouye, 1990) has effects on the voltage-dependent gating of a Drosophila Shaker channel (ShA1) (Salkoff and Wyman, 1981b; Zagotta and Aldrich, 1990a).

While this hypothesis is attractive, other evidence suggests that additional regions may be involved. Mutations in other regions of the protein sometimes have effects on the voltage-dependence of the channel (Papazian et al., 1991). In addition, Shaker proteins in Drosophila photoreceptors, and those in muscle, differ by 40-50 mV in their voltage-dependencies for activation and inactivation, but share identical S4 and S5 regions (Hardie et al., 1991).

1.3.4.3 Inactivation:

Because a single K channel mRNA expressed in oocytes can inactivate and recover from inactivation with multiple exponential components (Christie et al., 1989; Hoshi et al., 1991), it is postulated that there are different types of inactivation of K channels that occur by distinct molecular mechanisms (Iverson and Rudy, 1990; Hoshi et al., 1991). *N-type* inactivation occurs as rapidly as a few msecs, and is thought to involve the intracellular amino terminal region of the protein. Armstrong and Bezanilla (1977) hypothesized that inactivation involves the plugging of the open channel pore by a cytoplasmic inactivation "ball", which is part of the channel protein. For the "ball" to be able to swing into the pore it is connected to the rest of the channel protein by a "chain" of amino acid residues. Recently, in a very elegant set of experiments, Hoshi, Aldrich and Zagotta provided strong support for this "ball and chain" mechanism for channel inactivation (Hoshi et al., 1990; Zagotta et al., 1990). They showed that deletions of amino acids in the amino terminus of a Shaker cDNA clone (ShB) encoding for a rapidly inactivating K channel eliminated the ability of the channel to inactivate. Channel inactivation was reconstituted by adding a 20 amino acid residue peptide, identical to the deleted sequence, to the internal face of the membrane. Deletion of this 20 amino acid peptide, Armstrong and Bezanilla's ball, eliminates inactivation, whereas providing the "ball" in free form supplies the inactivation plug, and therefore restores inactivation (see Fig 1.1). It is important to note that localization of fast type inactivation to the amino terminal may only be true for Shaker channels. For instance, deletion of the amino terminal of the mouse Shal (mShal) K channel protein does not radically alter its inactivation properties (Pak et al., 1991b).

C-type inactivation occurs more slowly and can be seen as a slow decay in the current, and/or a slow recovery from inactivation (Iverson and Rudy, 1990; Hoshi et al., 1991). This inactivation is independent of voltage, and coupled to, but not dependent on, N-type inactivation (Hoshi et al., 1991). Hoshi and co-workers have looked at the effects of alterations in the carboxy terminal region of Shaker channels, and show that differences in C-type inactivation can be localized to a single amino acid (position 463) in the 5th membrane spanning domain.

In summary, while molecular biological techniques provide a powerful tool for studying K channels, few studies have attempted to identify which genes code for the K channels recorded from specific neurons. For example, a large number of different K channel clones have been isolated from Drosophila. However, to date none of the neuronal K channels mRNAs expressed in oocytes are identical in their electrophysiological properties to the native channels. As such, it is not known which K channel genes are responsible for any of the native Drosophila K channels. The same situation is true for K channel genes isolated from the mammalian nervous system. Most researchers have identified neuronal K channel cDNAs from entire brain cDNA libraries. However, in the nervous system there are so many cell types, expressing different K currents, that isolating genes from whole brain provides no information about what genes are expressed in which cells.

Since, for the most part, the identities of the K channel genes expressed in specific neurons are unknown, many of the basic questions pertaining to the expression of K channels in the nervous system remain unanswered. Questions such as, which K channel genes are actually expressed in given cells, whether subunits assemble as homomultimers or heteromultimers, and whether additional subunits encoded for by a completely separate family of genes are required to produce certain K channels (Rudy et al., 1988; Zhong and Wu, 1991).

Recently, investigators have started to identify the transcripts expressed in known cell types. For example, transfection of an Aplysia Shaker channel, AK01A, into identified Aplysia neurons produces transient K channels whose properties are identical to those in the native system (Kaang et al., 1992). A different study, using the polymerase chain reaction, has identified 4 K channel transcripts, distinct from Shaker, which are responsible for the rapidly inactivating A-current of Drosophila photoreceptors (Hardie et al., 1991).

Identifying the K channel genes expressed in specific neurons also leads the way to addressing questions concerning the regulation of K channel expression at the molecular level. Questions such as, how do neurons know which K currents to express, and when to express them? What is involved in the maintenance of K current levels? How do neurons selectively change their expression of certain ionic currents? How is it that neurons within the same population express different sets of ionic currents? Are there set genetic programs, or are extrinsic factors involved in these choices? If so, what is their nature, and can these factors exert their effect throughout the life span of the neurons? These are important questions, as the ionic currents expressed by a neuron will determine its electrical behaviour.

1.4 Developmental expression of K currents

Despite the huge advances in K channel molecular biology, most of what is currently known about the control of K channel expression has come from electrophysiological experiments. For signaling between neurons to continue to be appropriate in face of changes in the number or strength of presynaptic inputs, it would be advantageous for the electrophysiological properties of neurons to retain a certain amount of plasticity. Examples of such plasticity exist. For instance, associative conditioning decreases the phototactic response of the nudibranch mollusc Hermissenda. This change in phototactic response is a result of decreases in the A-current and Ca-activated K conductance of photoreceptors (Alkon, 1984; Nelson et al., 1990). In addition, Woody and co-workers (1991) showed decreases in the A-current of cat cortical neurons during Pavlovian conditioning.

However, it is during development that most of the changes in K channel expression occur. Developmental studies show that the ionic currents expressed by mature neurons are often quite different from those expressed by the neurons at earlier stages, suggesting that the currents a neuron expresses, and their levels, are determined during development. Therefore, studying ionic channel expression during development will hopefully lead to a better understanding of the mechanisms underlying the control of ionic current expression, some of which may persist into adult life and provide a mechanism for plasticity.

Little is known about the control mechanisms involved in ionic current expression. Developmental studies have concentrated on examining the sequential appearance of ionic currents, and subsequent changes in the "immature" ionic profile of the neurons to its "mature" form. Investigators have mainly directed their studies to discovering whether there is any order to the appearance of ionic currents, and if so, are there general rules that can be applied to developmental changes in their expression (reviewed by Spitzer, 1985).

1.4.1 Development of the action potential mechanism

Inward Na and outward K currents form the basis of the AP and cellular excitability (Hodgkin and Huxley, 1952). In cells from most tissues, K currents are present at the earliest time studied, whereas the appearance of inward currents, carried by Na or Ca, varies in timing with respect to the appearance of K currents (Spitzer, 1985). Once a neuron becomes electrically excitable, with the appearance of a mechanism for producing APs, it can follow one of two general patterns of AP development.

In the first pattern, the ionic basis of the AP does not change much during development (Bader et al., 1983; Bader et al., 1985). Such a pattern is observed in chick neural crest cells (Bader et al., 1985). In these cells, once the various ionic currents appear their absolute levels increase, but no new currents appear as the neurons put out processes and increase in size.

A second pattern exists in which the ionic basis of the AP changes during development (Yool et al., 1988; McCobb et al., 1990). One of the best examples is the development of the AP in amphibian spinal neurons (see Spitzer reviews, 1979, 1985, 1991). In these neurons, there are marked changes in the relative levels of expression of ionic currents during development (O'Dowd et al., 1988; Harris et al., 1988), as well as the appearance of a new current, the rapidly inactivating IK_A current (Ribera and Spitzer, 1990). Spitzer and co-workers showed that at early stages of neuronal development the AP is Ca dependent, and repolarizing K currents are small; as a result, the APs last for almost 100 msecs. With development, either *in vivo* or in culture, the Na current develops, Ca current densities show no, or very small changes, and K current densities increase (Barish, 1986; O'Dowd et al., 1988). The large increase in repolarizing K currents results in a 10 fold decrease in the duration of the AP, and as a result truncates Ca entry into the cell. The decrease in Ca entry during the AP, and the increase in the Na current means that with development the Na current becomes primarily responsible for the upstroke of the AP.

1.4.2 Changes in the total outward K current

As discussed above, modifications of the AP can result from changes in the types and levels of K currents. Studies of the developmental expression of K currents suggest that there is no set temporal sequence for these changes. For instance, a number of neurons start to express a rapidly inactivating K current during embryonic development: In the rat superior cervical ganglion (Nerbonne et al., 1986; Nerbonne and Gurney, 1989) and amphibian spinal neurons (Barish et al., 1986; Ribera and Spitzer, 1990) this current develops later than other K currents; whereas, in Drosophila flight muscle (Salkoff and Wyman, 1981a; Zagotta et al., 1988), or cells from the quail mesencephalic neural crest (Bader et al., 1985) a rapidly inactivating current is the first K current to develop. Therefore, the temporal sequences of K current expression are particular to the type of neuron, and it appears that no general rules describe these processes.

What are the mechanisms a neuron could use to produce changes in the total outward K current it expresses? Presumably there could be changes in either the types or levels of K current a neuron expresses, or there could be modifications in the physical properties of the channel proteins. Evidence exists for all 3 of these possibilities. Developmental studies show that neurons can change the types of K currents they express. For example, in developing rat pineal cells a large slowly inactivating current present at birth disappears by 4 weeks postnatally (Aguayo, 1989). This is different from what is observed for rat neostriatal neurons, where a rapidly inactivating current is the predominant current at birth, but by 4 weeks postnatally a slowly inactivating current, not present at birth, is the major outward current (Surmeier et al., 1991).

Alternatively, neurons may continue to express the same set of K currents, but change their relative levels of expression (Bader et al., 1985; O'Dowd et al., 1988; McCobb et al., 1990). For example, as quail mesencephalic neural crest cells develop, there is a small decrease in the A-current, and a large increase in the delayed rectifier, resulting in a 2 fold increase in the ratio of delayed rectifier to A-current (Bader et al., 1985). Since the A-current inactivates rapidly, and the delayed rectifier shows little or no inactivation, changes in the relative proportions of these currents will influence neuronal firing properties.

While most of the changes in ionic currents during development probably involve changes in the number of functional channels present in the membrane, several reports show modifications in the properties of ionic channels. These include changes in the voltage-dependence and kinetics, for activation and inactivation. For example, during development, there are changes in the voltage dependence for activation (Park and Ahmed, 1991) and inactivation of Na currents (Sontheimer et al., 1991). Studies have also shown changes in the mean open time for a voltage-dependent, K selective channel in rat Purkinje cells developing in culture (Yool et al., 1988), and for the latency to first opening of a class of 30pS K channels in amphibian spinal neurons (Harris et al., 1988).

In summary, developmental studies indicate that changes in the patterns of expression of ionic channels during development can take many different forms. Since developmental changes in K channel expression will have effects on cell excitability, the control of K channel expression is an important consideration of the nervous system. Presumably, the K currents a neuron expresses are determined by the interplay between its lineage and its environment. What are the epigenetic influences? Are different factors involved in the control of different K channels? Do similar factors regulate the expression of K channels on different neurons? Are there any general rules for the extrinsic control of K channel expression? All of these questions remain to be answered.

1.4.3 Extrinsic control of K channel expression

A number of factors are postulated to act as regulators of neuronal differentiation: among these are growth factors, hormones, and neurotransmitters. These agents act on neurons both in the peripheral and central nervous systems to influence survival and neuronal differentiation. A full description of these factors and their effects on growth and differentiation is outside the focus of this thesis; however, for recent reviews on the involvement of extrinsic factors in neuronal development consult the following references (Davies, 1988; Barde, 1989; Reichardt and Tomaseli, 1991; Thoenen, 1991; Lipton and Kater, 1989). Unfortunately, only a few reports have investigated epigenetic influences on K channel expression, and, as a result, surprisingly little is known about factors that regulate K currents. However, some guidance may be offered from studies of the control of other differentiated properties of neurons, if we assume that many of the same signals are also used in the control of K current expression.

A neuron's cellular environment includes the target contacted by its nerve terminals, the presynaptic nerve terminals that contact its dendrites and soma, and the glia cells that surround it. The fluid environment includes growth factors and hormones which are found in the extracellular solution. Presumably, any of these factors could be involved in regulating K current expression.

To properly coordinate pre- and post-synaptic neural activity, it would be beneficial to have a system where presynaptic nerves could affect K channel expression in the cells they contact, either by releasing chemical factors from the nerve terminals, or indirectly, by changing the membrane potential. For example, the expression of nicotinic acetylcholine receptors on postsynaptic neurons is dependent on innervation by presynaptic nerve terminals (Schuetze and Role, 1987; Jacob and Berg, 1987). To my knowledge, the only report that shows evidence, though indirect, that synaptic inputs affect neuronal electrical excitability is one on rat hypothalamic neurons in culture (Ling et al., 1991). When hypothalamic neurons are grown in the presence of tetrodotoxin (TTX), an Na channel blocker, there is a decrease in the number of spontaneously active cells measured after the drug is washed out.

Target factors are also known to affect the differentiated properties of neurons (Purves and Lichtman, 1985). A good example, is of a factor from rat sweat glands which establishes the neurotransmitter phenotype of the sympathetic neurons that innervate the gland (Landis and Keefe, 1983; Landis et al., 1988; Stevens and Landis, 1990). Conceivably, therefore, target factors may have a role in controlling a neuron's expression of K currents. In fact, several lines of evidence suggest that the target influences the expression of a Ca-activated K conductance on sympathetic neurons. First, when rat sympathetic neurons are grown in culture with heart cells, one of their targets, evoked APs are followed by a long-lasting afterhyperpolarization (O'Lague et al., 1978). This hyperpolarization, thought to be due to a Ca-activated K conductance, is absent in pure neuronal cultures. Second, removal of bullfrog sympathetic neurons from their targets by axotomy results in a reduction of the after-hyperpolarization of the AP, supposedly due to a decrease in a Ca-activated K conductance (Kelly et al., 1986). A qualifier for these axotomy results however, and for other axotomy experiments that demonstrate effects on the electrical activity of neurons (Kuno et al., 1974; Purves, 1975; Gallego et al., 1987), is that it is hard to dissociate effects due to removal of the target, from effects due to physical injury of the neuron.

In vivo, neurons are surrounded by glial, or nonneuronal, cells. Given that nonneuronal cells can affect the neuron's expression of cholinergic receptors (Cooper and Lau, 1986; Smith and Kessler, 1988; Mandelzys and Cooper, 1992), these cells may also affect its expression of voltage-gated K channels. However, except for a report demonstrating that nonneuronal cells influence the expression of a Ca-activated K conductance on sympathetic neurons in culture (O'Lague et al., 1978), no studies have examined this possibility.

The final possibility, for which there is some evidence, is that circulating factors, including growth factors, peptides and hormones, may influence K current expression. A few studies have shown an effect of extrinsic factors on the AP duration, with the inference that changes in repolarizing K currents underlie the alterations in the AP. For instance, androgen treatment increases the AP duration in the electric organ of the fish Sternopygus (Mills and Zakon, 1991). In contrast, Nerve Growth Factor (NGF) treatment causes a 34% decrease in the AP duration of mature rat dorsal root ganglion neurons (Chalazontis et al., 1987). However, NGF treatment of pheochromocytoma (PC12) cells has no effect on the expression of K currents (Garber et al., 1989), though it does increase cellular excitability (Dichter et al., 1977).

Other reports of epigenetic influences on K current expression include one that shows that in adrenalectomized rats there is a decrease in the size of the hyperpolarizing current after the AP

(I_{AHP}) of CA1 hippocampal neurons, as compared to sham operated animals (Joels and de Kloet, 1989; Joels and de Kloet, 1992). Administration of glucocorticoids to brain slices from these animals for 30-90 min increases the I_{AHP} in a protein synthesis dependent manner, suggesting that gene regulation is involved. It is likely that both the increase and decrease in I_{AHP} are due to changes in a Ca-activated K conductance, however, it is not known whether this is an effect on intracellular Ca concentration, or on the K channels.

Estrogen treatment of estrogen-deprived rats rapidly induces in 3 hours a uterine mRNA, that encodes for a 130 amino acid protein, which is capable of inducing a slow K current when injected into Xenopus oocytes (Pragnell et al., 1990). However, because of the small size of the protein it is not clear whether it encodes for a K channel protein, or a modulator of a K channel protein. Nonetheless, these results do suggest that estrogen can have a long term influence on the functional activity of a K current. Finally, a recent abstract suggests that cAMP can down regulate the expression of a K channel gene (RCK1), in that it reduces the mRNA levels in cultured rat astrocytoma C6 cells by more than 70% (Wang et al., 1990). However, what extrinsic factor mediates such a response is unknown.

1.4.4 Functional significance of developmental changes in ionic currents

Many studies have correlated changes in K current expression with changes in cellular excitability. These changes can take two forms. The first involves modifications of the shape of the AP. As discussed earlier, there is a marked decrease in the duration of the AP as amphibian spinal neurons develop in culture. This decrease is mainly due to an increase in a voltage-dependent delayed rectifier type K current (Barish et al., 1986; Spitzer, 1985). In contrast, shortening of the AP duration of embryonic rat superior cervical ganglion neurons is due to the appearance of a rapidly inactivating K current (Nerbonne and Gurney, 1989).

The second form involves changes in the firing pattern of neurons. For instance, in rat neostriatal neurons a rapidly inactivating K current present at early postnatal stages is thought to be responsible for the ability of young neurons to fire repetitively (Surmeier et al., 1991). The appearance of a slowly inactivating K current, by four weeks postnatally, changes the firing pattern of the neurons such that most neurons will only fire once in response to a depolarizing input.

While changes in ionic currents during development are important for electrical signaling, they may also play roles in directing neuronal differentiation (see review by Spitzer, 1991). Ca, in particular, is important in that it is a co-factor in both transcriptional and posttranslational regulation of gene expression (Carafoli and Penniston, 1985). Since K currents play a role in controlling the shape and duration of the AP, they also help to regulate Ca influx into the cell; as a result, changes in K currents during development may alter gene expression. For example, the long lasting, Ca-dependent APs of embryonic amphibian spinal neurons are truncated by developmental increases in K currents (Spitzer, 1985; Barish, 1986). The transient expression of Ca-dependent APs or certain K channels (Aguayo, 1989; Surmeier et al., 1991), suggests that ion fluxes may be critical at specific times during development (Spitzer, 1991).

Studies indicate that neuronal activity is important for the patterning of connections during the development of the nervous system. Investigations in the vertebrate visual system provide good evidence that activity-dependent competition for postsynaptic neurons is responsible, in part, for organization of the primary visual cortex into ocular dominance columns (Hubel and Wiesel, 1965; also see review by Shatz, 1990). Hubel and Wiesel showed that if an eye was temporarily patched during development, the ocular dominance columns of the kitten primary visual cortex were disrupted, presumably because inputs to the cortex from the unpatched eye, in a competitive, use-dependent fashion, replaced the electrically silent inputs from the patched eye. Further experiments showed that both presynaptic and postsynaptic activity are important in making connections. Since K channels regulate both pre- and post synaptic activity, it is quite likely that the differential expression of K currents during development plays an important role in determining the connective organization of the nervous system.

1.5 Summary and conclusions

Appropriate communication at the synapse is essential for the functioning of the nervous system. The action potentials produced by neurons must be of a precise duration and shape, intensity and frequency if they are to produce appropriate functional responses in the target cells. Electrophysiological experiments, discussed in this chapter, show that ionic flow through voltage-gated K channels serves to regulate the pattern of neural signals. Molecular biological studies have revealed that a large number of different K channels may exist. The particular set of K channels a neuron expresses will determine its signaling behaviour. Which set of K channels a neuron expresses is decided during development, probably as a result of a combination of intrinsic and extrinsic regulation. For the most part the nature of this regulation, both in terms of factors involved, and their mechanism of action, is unknown.

As such, the rationale for my doctoral research was to study questions relating to the expression of voltage-gated K channels, to gain a better understanding of the control mechanisms involved in determining the firing behaviour of neurons. While other K channels such as Ca-activated K channels and inward rectifiers also play an important role in regulating the electrical activity of neurons (Rudy, 1988), we concentrated on the expression of K channels gated by voltage as a model for how the expression of a particular class of ion channels is regulated.

Questions concerning the control of K current expression are difficult to address in the adult mammalian central nervous system (CNS). To investigate epigenetic influences on K current expression it is necessary to be able to manipulate the environment of the neurons. However, given the complex organization of the brain, there are problems in isolating pure populations of

CNS neurons, their axons, and the neurons that innervate them. As such, it is difficult to test whether the expression of K currents by neurons is influenced by their presynaptic neurons, or by the neurons they contact. In addition, it is often not possible to grow pure populations of CNS neurons in culture where the external environment can be controlled.

In contrast, the peripheral nervous system (PNS) has several advantages for studies on the control of K current expression. The neurons are collected into homogeneous ganglia ensheathed by connective tissue, and as such, are easily accessible. Therefore, it is possible to properly document and characterize the K currents expressed by specific neurons, and to determine whether changes in their expression occur during development. If there are modifications in the expression of K currents during development, peripheral ganglia are a suitable preparation to examine the role of epigenetic influences in these changes; the axons of the neurons, and the axons of the neurons that innervate them, are easily accessible for axotomy and denervation, and the neurons can be cultured. A final important point is that neurons of the CNS and PNS express some of the same K channel genes (Beckh and Pongs, 1990) indicating that the information obtained by studying K current expression in the PNS will be relevant to the control of K current expression on CNS neurons, which are more difficult to study.

1.6 Overview

We have investigated the postnatal control of K current expression on sensory and sympathetic neurons of the PNS. The objectives of the experiments presented in this thesis were: First to characterize voltage-gated K currents on neonatal rat nodose sensory and superior cervical ganglion (SCG) neurons; Then to investigate possible changes in the expression of voltage-gated K currents during postnatal development; Subsequently to identify factors from the fluid or cellular environment of the neurons that are involved in developmental changes in K current expression, and; Finally, to identify the genes that encode for the voltage-gated K channels in PNS neurons, in order to compare them with those genes expressed in the CNS, and to investigate whether developmental changes occur at a transcriptional or translational level.

Chapters in this thesis present data on the expression of voltage-gated K currents on rat peripheral neurons, and the control of this expression during postnatal development.

(A) Chapter 2: This chapter is a general methods section that describes the culture, electrophysiological, and molecular biological techniques used for the experiments discussed in Chapters 3, 4, 5 and 6.

(B) Chapter 3: This chapter discusses the characterization of K currents on nodose and SCG neurons. Cooper and Shrier (1989) showed that rat nodose neurons express 3 types of voltage-gated K channels: a non-inactivating delayed rectifier (IK); a rapidly inactivating A-current (IAf); and a slowly inactivating A-current (IAs). IAf and IAs share many single channel properties, such as single channel conductance and mean open time.

The studies in this chapter were performed to answer two questions: Since IAf and IAs share several single channel properties, are they mediated by the same channel or 2 different channel subtypes? To answer this question I investigated the voltage-dependent, kinetic and pharmacological properties of IAf and IAs both at the single channel and macroscopic level. These studies show that IAf, IAs and IK differ significantly in their voltage-dependent, pharmacological and kinetic properties suggesting they are mediated by different K channels.

The second question is do neurons of the peripheral nervous system express similar voltage-gated K currents? To answer this question I investigated K current expression on rat sympathetic neurons from the SCG. My studies show that SCG neurons express IAf, IAs and IK, with very similar properties to those reported for nodose neurons. This is the first demonstration that SCG neurons express a slowly inactivating current, and the first time the properties of a slowly inactivating current have been characterized on peripheral neurons.

(C) Chapter 4: This chapter discusses studies investigating the expression of voltage-gated K currents on nodose and SCG neurons. I quantified the levels of expression of IAf, IAs and IK on nodose and SCG neurons at different times during the first two weeks postnatally. These studies show that during the first two weeks of postnatal development there is a switch from a predominantly slowly inactivating outward current at birth, to a predominantly rapidly inactivating outward current by postnatal day 14. Interestingly, there appear to be few changes in the expression of IAf, IAs and IK on nodose neurons over this same period.

The studies presented in this chapter suggest that the voltage-gated K currents expressed on neonatal SCG neurons, but not nodose neurons, undergo further modification over the first two weeks of postnatal development. In addition, these results suggest that the regulation of voltage-dependent K currents by sensory and sympathetic peripheral neurons is different.

(D) Chapter 5: This chapter discusses the results of experiments with postnatal SCG neurons, designed to identify the factors involved in changes in K current expression during development. The results from *in vivo* denervation and axotomy experiments show that preganglionic innervation, and the target are not involved in changes in K current expression during postnatal development. In addition, SCG neurons were grown in culture; the results demonstrate that neonatal SCG neurons in culture do not show the normal developmental change in K currents, suggesting that an extrinsic factor, missing in culture, is required for the developmental change to occur. In addition, in culture, SCG neurons actually lose their IAf and IAs currents. The loss is prevented by a soluble factor provided by nonneuronal cells, suggesting that a nonneuronal cell factor is required for the neurons to express IAf and IAs.

(D) Chapter 6: This chapter discusses initial attempts using PCR (polymerase chain reaction) homology screening to identify the genes responsible for the K currents expressed by SCG neurons. Preliminary results suggest that SCG neurons express a member of a known K channel gene subfamily. In addition, these studies indicate that the technique of PCR homology screening will be useful in isolating other K channel genes expressed by SCG neurons.

Chapter 2

Materials and Methods

2.1 Preparation of cell cultures

2.1.1 Animals

Nodose and superior cervical (SCG) ganglia were dissected under sterile conditions from newborn rats (C.D strain, Charles River, Canada) killed by cervical dislocation. In some experiments, ganglia were removed from rat pups 3, 7 or 14 days postnatally.

2.1.2 Dissociation

SCG and nodose ganglia were dissociated and the neurons cultured as originally described by Mains and Patterson, 1973 and Hawrot, 1980. The ganglia were dissociated at 37°C in the following enzyme containing media: modified Leibovitch-15 (L-15, Gibco) media (supplemented with vitamins and cofactors, see below), penicillin (100u./ml, Gibco), streptomycin (0.25mg/ml, Gibco), glutamine (200mM-stock solution, Microbiological Associates, 0.2mg/ml) and glucose (0.01g/ml) to which was added collagenase (1mg/ml, type I, Sigma) and a neutral protease (Dispase, grade 2, 2.4mg/ml, Boehringer Mannheim). After 15 minutes in this solution, the ganglia were gently triturated using a fire polished glass pipette. The pipette was first coated with plating media, consisting of a L-15 solution containing 10% horse serum (Gibco), to prevent the ganglia sticking to the sides. When the chunks of ganglia had settled, the solution was removed and replaced with media similar to that described above, except that the collagenase was not present. The dissociation was continued at 37°C, and every 15 mins the solution was triturated 100 times; after 3-4 hours there were no chunks visible to the eye and the dissociation was stopped.

The cell suspension was added to 5 ml of plating media to inactivate the enzymes, and centrifuged (International Clinical Centrifuge, International Equipment Co.) at 1335 r.p.m. (25g). To separate the nonneuronal cells from the neurons, the pellet was resuspended in 0.5 ml of plating media and added to the top of 6 ml of Percoll solution; this solution consisted of 33% Percoll (Pharmacia), made with L-15 Air, and buffered with 10mM HEPES. The Percoll solution was centrifuged for 15-20 min to establish a density gradient; the neurons which are denser than the nonneuronal cells are found in the pellet, while the nonneuronal cells remain in suspension. The top 70% of the solution was removed, the pellet resuspended, and 5ml of plating media was added. The cell suspension was centrifuged for 5min at the same speed as above. The cells were washed in plating media by resuspending the pellet in 5ml of plating media, and centrifuging it at 1335 r.p.m for 5 min. The pellet was resuspended in plating media (0.1 ml/culture dish), and two drops of the suspension were plated onto laminin/ collagen coated coverslips of modified culture dishes containing 1.5 ml of growth media (described below). The cell suspension was restricted to the center well of the culture dish by a sterile glass ring. The cultures were incubated in an atmosphere of 95% Air and 5% CO_2 at 37°C, and were fed with growth media every 2 days for the first 4 days, and subsequently every 3-4 days.

2.1.3 Culture media

L15-Air

L15 medium (14.9g, Flow Laboratories Inc.) powder was dissolved in 1080ml of double distilled water. To the mixture was added: 60mg imidazole, 15mg glutamic acid, 15mg proline, 10mg inositol, 15mg aspartic acid, 15mg cystine, 5mg ß-alanine, 2mg vitamin B12, 10mg choline chloride, 0.5mg lipoic acid, 0.02mg biotin, 5mg ß-aminobenzoic acid, 25mg fumaric acid (all from Sigma), and 0.4mg coenzyme-A (P-L Biochemicals, Inc.). The pH of the solution was adjusted to 7.35 with 1N HCL, and was filtered through a 0.2μm filter (Nucleopore). L15 Air was stored at 4°C.

L15-CO_2

Before filtering, 170 ml of 150 mM $NaHCO_3$ was added to 850ml of base L15 Air medium. When used in growth media, exposure of the solution to an atmosphere containing 95% air and 5% CO_2 keeps the pH of the solution at 7.4. L15-CO_2 was stored at 4°C.

Plating media

Plating media consisted of 50ml of modified L15 Air medium to which was added: 5.0 ml horse serum, 0.5 ml penicillin-streptomycin (10000 units/ml penicillin and 10mg/ml streptomycin, Gibco), 0.5 ml glucose (0.4g/ml, sterilized by autoclaving), 0.5 ml L-glutamine (200mM stock solution, Microbiological Associates), and 0.5 ml fresh vitamin mix (FVM). FVM consisted of 0.025mg 6,7-dimethyl-5,6,7,8-tetra hydropterine (Biochem-Boehring Corp.), 0.125mg glutathione (Sigma) and 2.5 mg ascorbic acid (Sigma) in 0.5 ml of sterile water (pH 5.4), which was sterilized by filtering through a 0.2 μm filter (Nucleopore).

Growth media

Growth media consisted of L15-CO_2 media, supplemented with penicillin-streptomycin, glucose, glutamine, FVM, 5% rat serum, and 7S-Nerve Growth Factor (10nM). Nerve Growth Factor (NGF) was prepared from male mouse submaxillary glands by gel filtration through Sephadex G-100 followed by DAEA-cellulose fractionation (Bocchini and Angeletti, 1969) and stored at -20°C. In one series of experiments, neonatal nodose neurons were grown in culture in the absence of Nerve Growth Factor, and in the presence of rabbit antiserum to 2.5S NGF at a 1:1000 dilution. This antiserum at 1:10000, blocks the effects of 10nM 7S NGF when added to dissociated rat sympathetic neuron cultures.

In some cultures of SCG neurons, ciliary neurotrophic factor, CNTF, was added to the growth media. Recombinant rat CNTF (kindly provided by Dr.P. Richardson) was used at a concentration of 10ng/ml, which is saturating for its effects on the neurotransmitter phenotype of sympathetic neurons (Saadat et al., 1989).

2.1.4 Culture dishes

Culture dishes were prepared by boring a hole (10mm) in the center of a plastic petri dish (Falcon #1008, 35mm) and attaching an aclar (Allied Plastics) coverslip, coated three times with adult rat tail collagen, to the outside of the dish with a silicon elastomer (Sylgard, Dow Corning). These culture dishes also served as recording chambers for the electrophysiological experiments. Laminin (kindly provided by Dr.S.Carbonetto) at a concentration of 30μg/ml (diluted in L15-Air) was added to the center well of the dishes for 12-24 hrs at 4°C before plating the neurons. Prior to plating, the dishes were washed 3 times with L15-Air to remove excess laminin.

2.2 Experimental preparations

2.2.1 Neuronal cultures

To prepare pure neuronal cultures, most of the nonneuronal cells were removed from the original ganglionic cell suspension by centrifugation through a density gradient (Percoll). In addition, cytosine-arabinoside (ARA-C, Sigma) (5-10μM) was added to the growth media for the initial 2-3 days to eliminate virtually all the remaining nonneuronal cells from the cultures. In some experiments, whole cell recordings were performed on acutely (2-48hr) dissociated neurons. Other neurons were maintained in culture and experiments were performed 3-28 days later.

2.2.2 Explant cultures

SCG were removed from postnatal day one (P1) or day 7 (P7) animals, cut in half and maintained in tissue culture for 2 weeks, as described above for dissociated neurons. Thereafter, the cultures were removed and the neurons dissociated enzymatically, as described for freshly isolated ganglia (see section 2.1.2). Whole cell recordings were then performed on these neurons within 2-24hrs of plating.

2.2.3 Co-cultures of SCG neurons with other cell types

Neurons dissociated from SCG from P1 animals were plated on top of monolayers of nonneuronal cells, and maintained in culture for 14 to 28 days. The growth media is as described above.

Ganglionic nonneuronal cells

Nonneuronal cells were obtained by spinning the ganglionic cell suspension through a Percoll density gradient. The top 70% of the supernatant was removed to recover the nonneuronal cells (the neurons can be recovered by resuspending the pellet). These cells were then plated on laminin/collagen coated coverslips, and fed with growth media consisting of L15-CO_2 medium, penicillin-streptomycin, glutamine, glucose, FVM and fetal calf serum (10%). Once the cells had formed a confluent monolayer, the cells were irradiated (^{60}Co, 5000cGy) to prevent further cell division. Freshly dissociated P1 SCG neurons were plated on top of these nonneuronal cell monolayers and cultured in the presence of growth media with 5% rat serum.

Another method for obtaining co-cultures of neurons with their nonneuronal cells was to omit the Percoll separation step during the plating of the neurons. In addition, these cultures were not treated with ARA-C. By a week in culture the nonneuronal cells completely covered the culture dish. In some experiments, neurons were isolated from 14 day old animals (P14) and were grown in the presence of nonneuronal cells from P14 animals.

Heart cells

Heart muscle was removed from P1 rats, cut into small pieces using iridectomy scissors and incubated with collagenase (5mg/ml) for 15 minutes while gently stirring. The supernatant was discarded, fresh enzyme added, and incubation continued for another 15 minutes. The supernatant, containing the isolated heart cells, was removed and 5ml of serum containing media (L15 Air and 10% fetal calf serum) was added to the supernatant to inactivate the enzyme. Meanwhile, fresh enzyme was added to the tissue, and the incubation proceeded for a final 15 minutes. The supernatant from this third incubation was removed and added to 5ml of serum containing media. The supernatants from the second and third enzyme treatments were combined and spun at 1335 r.p.m for 5 minutes. The supernatant was discarded, and the pellet resuspended in 5 ml of plating media. The suspension was spun again for 5 minutes at 1335 r.p.m, the supernatant discarded and the cells resuspended in 1-2mls of plating media. One drop of this cell suspension was plated on laminin/collagen coated coverslips. The cultures were then treated as described above for the ganglionic nonneuronal cell cultures.

Skin cultures

A piece of skin, including ectodermal tissue, was removed from the lower back of postnatal day one rats. This piece was then cut into smaller pieces using fine iridectomy scissors. These pieces were treated as described above for the heart muscle dissociation, and cultured as described for the ganglionic nonneuronal cell cultures.

2.2.4 Conditioned media cultures

To obtain conditioned media, pure cultures of nonneuronal cells from P1 animals, obtained as above, were grown to confluency. The growth media was then changed to that used for neuronal cultures: L15-$C0_2$ containing Nerve Growth Factor (10nM), 5% rat serum, penicillin-streptomycin, glucose, glutamine and FVM. Cells were fed every 3-4 days, the media was harvested, filtered through a 0.2μm filter (Nucleopore), and stored at -20°C. SCG neurons from P1 animals were grown in culture in the presence of 60% conditioned media / 40% growth media; NGF, rat serum (5%), penicillin-streptomycin, glucose, glutamine, and FVM were added in the appropriate quantities to the total volume of media as though they were not present in the conditioned media, to account for the possible loss of these factors with time in the conditioned media. In experiments with conditioned media, control cultures (no conditioned media) also had the ingredients of their growth media doubled.

2.2.5 Redissociation of 2 week old cultures

In one experimental series, neurons that had grown in culture for two weeks were re-dissociated and re-plated in order to examine voltage-gated K currents on neurons that had been in culture for 2-3 weeks but that did not have extensive process outgrowth. For these experiments, the cultured neurons were incubated with trypsin (0.5% in L15 Air) for 5 min at 37°C and then triturated with a fire-polished pipette before re-plating the neurons in growth media as described above.

2.3 Electrophysiology

2.3.1 Data recording and analysis

Voltage steps were delivered by a computer-controlled stimulator. The computer was a 386 based PC (AT class with an EISA bus and running at 33 MHz with a 64K cashe and an A/D card (Omega)); the software for stimulation, data acquisition and analysis was written by Mr. A. Sherman (Alembic Inc., Montreal, Que). Membrane currents and voltages were filtered at 3 kHz with an 8-pole bessel filter (Frequency Devices), digitized at 44 kHz by a pulse code modulation unit (PCM, Sony Corp.), sampled, displayed and stored online. For earlier experiments (those described in Chapter 3), whole cell and single channel currents were filtered at 3 kHz and digitized at 44 kHz by a pulse code modulation unit (PCM, Sony Corp.) and recorded on a Beta cassette recorder (Sony SL 2700). For off-line analysis, currents were filtered through an 8-pole Bessel filter, digitized by a 12-bit A/D converter controlled by a Digital Equipment Corporation (DEC) PDP 11/73 microcomputer and written onto data files. Data analysis was carried out on a DEC computer. For all experiments, the duration of the voltage steps were either 125ms, 6s or 10s: for 125ms steps the data were filtered at 1.5 kHz and sampled at 5-10 KHz, whereas, for the 6s and 10s steps, the data were first refiltered at 100 Hz and then sampled at 200 Hz.

2.3.2 Whole cell recording

2.3.2.1 Experimental conditions:

SCG and nodose neurons were voltage-clamped using whole-cell patch recording techniques (Hamill et al., 1981). In some experiments, recordings were done on acutely dissociated neurons that had been in culture for 2-48 hour. No changes in the properties, or current densities of the K currents expressed by nodose or SCG neurons were observed over the first 2 days in culture. Alternatively, neurons were grown in culture for 3, 7, 14, or 28 days before recording.

All experiments were done at room temperature (21-24°C) with a List EPC-7 amplifier. The pipette resistances were 2-5 MOhm and were filled with intracellular media (described below). The current signal was balanced to zero with the pipette immersed in the bathing solution. The seal resistances were 5-50 GOhm and the series resistances were usually compensated for when recording whole-cell currents and had values of about 4-10 MOhms. The neurons were continuously superfused with extracellular solution (described below) at a rate of 0.5 ml/min, during the recording session. In the pharmacological experiments the K channel blockers tetraethylammonium(TEA) Br (10mM; Sigma) and 4-aminopyridine (4-AP) Cl (1mM; Sigma) were applied by bath perfusion.

2.3.2.2 Whole-cell currents:

To study voltage-activated K currents, inward Na and Ca currents, and the Ca-dependent K current were blocked pharmacologically: Na currents were blocked using TTX (1μM) and external Na (2mM was added) was replaced with ChCl (140mM); $CoCl_2$ (2.5mM) and low Ca solutions (0.5mM) were used to block Ca and Ca-dependent currents. Only neurons with greatly reduced, or completely blocked inward currents were used in this study. Any neuron in which there was a suggestion of poor clamping, such as notches, oscillations, or delayed all-or-none inward currents was excluded.

The total outward currents on nodose and SCG neurons (characterized in detail in Chapter 3) are made up of three voltage-gated currents which differ in their kinetic and voltage-dependent properties: a non-inactivating current (IK); a fast transient A-type current (IAf) that inactivates with a time course of 10-30 msecs; and a slow transient A-type current (IAs) that inactivates with two components, one with a time constant of 100-300ms, the other with a time constant of 1-3s. The three K currents investigated differ in their voltage-dependence of inactivation. Therefore, by holding the membrane at different potentials we were able to selectively activate one or two of the currents. This enabled us to use subtraction techniques (described in full in Chapter 3) to separate individual currents for characterization. Briefly, the membrane was held at a potential (-10 or -20 mV) where depolarizing steps evoked only IK. The IK currents were subtracted from currents (IAf+IAs+IK) evoked by steps to the same depolarizing potentials from a more negative potential, -90 mV, to isolate the A-currents.

For whole cell current analysis, IAf, IAs and IK currents were isolated, uncontaminated by each other, or by leakage currents. For measurement of the current density (pA/pF), IAf, IAs and IK current amplitudes were determined from the current evoked by a voltage step to +30mV after each current was isolated from the other two, and divided by the cell capacitance (pF).

Measurements of IAf were made at the peak of the current, approximately 5-10ms after the beginning of the depolarizing voltage step. For IAs and IK, the amplitude measurements were made at the plateau, 125 ms after the beginning of the step since these currents showed little or no inactivation during the first 125 ms; any contaminating IAf current was completely inactivated by this point. Cell capacitance (pF) was obtained by integrating the capacity current evoked by a 10mV hyperpolarizing voltage step and then dividing this current by the voltage step.

2.3.3 Single channel recording

All single channel recordings were made in the cell-attached configuration (Hamill et al., 1981) on nodose neurons that had been in culture for 10-30 days. Recordings were done at room temperature with a List EPC-7 amplifier. Electrodes were sylgarded (Sylgard, Dow Corning) and were filled with extracellular media (see below). Pipette resistances were 2-6 MOhm, and the seal resistances were 5-50 GOhm. The mean resting potential was -55mV±6.7mV (SD, n=41), measured from the zero current immediately after breaking through the patch at the end of the experiment, and was consistent with values that have been measured in previous studies with intracellular electrodes. Patch potentials are expressed in this thesis as membrane potentials by subtracting the pipette potential from the resting potential. In those patches where it was not possible to break through the patch at the end of the experiment to record a resting potential, the mean resting potential was used to determine the experimental membrane potentials. All records, except where indicated, were corrected for capacity transients and leakage currents by subtracting an average of steps (n=15) to the same potential in which no channel openings occurred. Ensemble averages were produced by averaging the sum of 40-50 individual traces to a single step potential, using the DEC computer. Single channel currents were low-pass filtered (1 kHz) with an eight-pole Bessel filter, and sampled at 5 kHz, except where indicated.

2.3.4 Solutions

The ionic composition of the extracellular solution was (in mM): 140 NaCl, 5.4 KCl, 2.8 $CaCl_2$, 0.18 $MgCl_2$, 10 HEPES (pH adjusted with NaOH; British Drug House (BDH)), 5.6 Glucose. The pH was 7.3-7.4. For whole-cell recording 1.0 μM Tetrodotoxin (Sigma) was added to the media to block the Na current, and almost all the Na was replaced by 140mM ChCl. $CoCl_2$ (1-3mM) was added to block Ca currents, and $CaCl_2$ levels were also reduced to 0.5mM. The recording pipette contained (in mM): 5 NaCl, 140 KCl, 10 HEPES (pH adjusted with KOH), 1 $MgCl_2$, 10 EGTA and 0.2 $CaCl_2$ (final concentration of approximately 10^{-7} M). The pH was 7.3-7.4.

2.4 Molecular biological identification of K channels in SCG neurons

2.4.1 Total RNA isolation

Total RNA was isolated according to the method of Chomczynski and Sacchi (1987). Three ml of solution D (4M Guanidine thiocyanate, 25 mM Na Citrate, 0.5% w/v Sarcosyl, and 0.72% v/v ß-Mercaptoethanol) was added to SCG removed from P1 rat pups. Ganglia were homogenized using a polytron for 10-30s. Three hundred μl Na Acetate (2M, pH 4.0), 3ml H_2O saturated phenol and 600μl Chloroform:isoamyl alcohol (24:1) were added sequentially with vigorous vortexing between each addition. The tube was incubated on ice for 15 min, and then centrifuged (Beckman, SS24) for 20 min at 9500 rpm at 4°C. The aqueous phase was transferred to a clean tube, an equal volume of 100% isopropanol added, and the mixture left to sit for an hour at 4°C. The solution was centrifuged (Beckman, SS24) at 9500 rpm, for 20 min at 4°C to pellet the RNA. The pellet was resuspended in 3 ml of solution D, and 3 ml of 100% isopropanol was added. The solution was left at -20°C for 1 hour, and then centrifuged at 9500 rpm at 4°C for 20 min. After resuspending the pellet in 75% ethanol, the RNA was precipitated by centrifugation, and resuspended in 400μl of DEPC (diethylpyrocarbonate, Sigma) treated H_2O. The RNA was stored as a salt/ethanol precipitate at -20°C.

2.4.2 First strand cDNA synthesis

The total RNA isolated from SCG was used to produce first strand cDNA (cDNA Cycle Kit from Invitrogen). To synthesize first strand cDNA, 2.5μl of ß-mercaptoethanol (0.7M) was added to 10μg of total cellular RNA. This mixture was incubated for 2 min at 65°C in the presence of random primers provided by the kit (1μg/1μl). DNA synthesis occurred in the presence of a placental RNAse inhibitor, avian murine reverse transcriptase (5 units), and the 4 nucleotide bases (dATP, dTTP, dCTP, dGTP) each at a final concentration of 1.25mM. Radioactively labelled ^{32}P-dCTP (10μCi/tube) was used to monitor the amount of DNA produced in the reaction. Synthesis reactions were carried out for an hour at 42°C. After heating at 95°C for 3 min to denature the cDNA-RNA hybrids in order to provide fresh RNA templates, new reverse transcriptase enzyme was added, and the reaction allowed to proceed for another hour at 42°C. DNA was extracted with phenol/chloroform, precipitated with a 0.1 volume of 3M NH_4Ac (pH 5.2) and 2 volumes of 100% EtOH, washed with 70% EtOH, vacuum dried and resuspended in 15 μl of TE buffer (10mM Tris-HCl pH 8.0, 1mM EDTA) for storage.

To verify if cDNA synthesis reactions were successful, 1μl of each sample was counted using a liquid scintillation analyzer (1600 TR, Packard). Six thousand cpms of each reaction were run on a 1% agarose gel. The gel was dried at 65°C for 2 hours, and exposed to X-ray film (Kodak) for at least 12 hours.

2.4.3 Polymerase chain reactions (PCR)

PCR reactions were run in either 25, or 50µl volumes, on a Perkin Elmer-Cetus Thermocycler using standard PCR procedures as described by Saiki and co-workers (1985; see also Innis et al., 1990). Negative controls, where all reagents are added, but no DNA is present, were always included. For a 25µl PCR reaction, 1µl of first strand cDNA (approximately 1 ng) was added to 14.5 µl of distilled H_2O, 2.5µl of PCR buffer (consisting of 20mM Tris-HCl (pH 8.3), 5mM MgCl, 25mM KCl, 0.05% Tween 20, 100µg/ml BSA), and 1µl each of sense and anti-sense degenerate primer pools at a final concentration of 1µM. The DNA was denatured by heating the mixture at 98°C for 10 min. The mixture was allowed to cool, and was centrifuged briefly to collect condensation. Then, 2.9µl of distilled H_2O, 0.5µl of 10mM dNTPs (dATP, dTTP, dGTP and dCTP), and 1-2 units of a thermostable DNA polymerase (TAQ DNA polymerase; U.S.B) were added. Twenty-five µl of mineral oil was layered on top of the reaction to prevent dehydration. Thirty-five cycles of denaturation, annealing and extension were performed for amplification reactions.

Denaturation

The double stranded DNA was denatured for 1 min at 94°C to provide single stranded DNA templates appropriate for priming and DNA synthesis.

Annealing

Annealing was carried out for 1.5 mins at a temperature where the oligonucleotide primers hybridize to the template DNA. The use of degenerate primers, whose nucleotide contents differ, necessitated an initial five low stringency cycles at an annealing temperature of 35°C to ensure that all primers could potentially prime DNA synthesis. The remaining 30 cycles (high stringency) were performed with an annealing temperature of 55°C (for 2 min) to increase the specificity of the reaction by decreasing the amount of non-specific priming.

Extension

For the 5 low stringency cycles the temperature was ramped from 35°C to 72°C over 2.5 min. DNA extension by the TAQ DNA polymerase proceeded at 72°C for 1 min. For the 30 high stringency cycles the temperature was changed as quickly as possible between 55°C and 94°C; there is sufficient time for DNA extension given the short length of the desired nucleotide fragment.

2.4.4 Processing of DNA

2.4.4.1 Size selection:

The PCR reaction was halted by cooling to 4°C. The PCR reaction volume was carefully removed to avoid contamination with mineral oil. The DNA was precipitated with a 0.1 volume of NH_4Ac(3M) and 2 volumes of 100% EtOH. The DNA pellet was washed with 70% EtOH, vacuum dried, and resuspended in 8µl of TE (10mM Tris-Cl, 1mM EDTA, pH 8.0). The entire sample of DNA from each reaction was run on a 3% agarose gel, containing ethidium bromide (0.1µg/ml) that allows DNA fragments to be visualized under ultraviolet light. A band corresponding to the appropriate size was cut from the gel with a razor blade, under ultraviolet light.

2.4.4.2 Isolation of DNA:

The cut gel slice was placed on top of siliconized glass wool that was packed into the bottom of a picofuge tube, which had a hole poked in its bottom with a 26 gauge needle. The picofuge tube was placed in a microfuge tube. This assembly was spun for 10min at 6000 rpm. The liquid that collected in the bottom of the microfuge tube was recovered and stored at 4°C.

2.4.5 Reamplification

To increase the amount of DNA, the size selected DNA from the original amplification was used in a second amplification reaction. For reamplification, we used 1µl of the DNA isolated from the original PCR reaction. Reagents were identical to those used in the original PCR amplification, except that the sense and anti-sense primers were non-degenerate. Because non-degenerate primers were used, cycling conditions for the reamplification reaction were slightly different: The initial 5 low stringency cycles of annealing at 35°C were omitted. In addition, the temperature was slowly ramped from 55°C to 94°C, omitting the extension plateau of 72°C; given that DNA extension occurs very rapidly, and the length of the desired DNA segment is relatively short (approximately 100 bases), this ramp provided sufficient time for extension.

After 35 cycles of the PCR reaction, DNA was size selected, and isolated as discussed earlier for the original amplification reaction. To purify the DNA, the DNA was precipitated with 0.1 volume of 3M NH_4Ac and 2 volumes of 100% EtOH, washed with 70% and 95% EtOH, vacuum dried and resuspended in 10µl of TE (pH 8.0). DNA was precipitated, washed and resuspended a second time. The DNA was quantified using a spectrophotometer.

2.4.6 Subcloning

2.4.6.1 Enzymic digest:

The primers used in PCR were designed so their 5' ends contain the consensus sequence of a restriction enzyme: the sense primer has an EcoR I consensus sequence, the anti-sense primer has an Xba I consensus sequence. The amplified DNA was digested with these 2 enzymes to generate sticky ends for ligation into M13 bacteriophage. The enzyme digest was performed in a 40µl volume containing 120ng of DNA from the reamplification reaction, 4µl of 10X buffer H (Boehringer Mannheim) and brought up to 38µl with distilled H_2O. The reaction mixture was left at 65°C for 5 min to denature DNAses, and cooled to room temperature before adding 1μl each of EcoRI (1 unit/μl, Boehringer Mannheim) and Xba I (1 unit/μl, Boehringer Mannheim). The mixture was left at 37°C for 1.5 hours. A vector, bacteriophage M13mp18 RF DNA (0.25μg/μl; BRL) was cut with the same restriction enzymes, but in a 20μl reaction volume. One fifth of the M13 enzyme digest was added to the digest of the reamplification product. The DNA of this mixture was precipitated with 0.1 volume of 3M NH_4Ac and 2 volumes of 100% ethanol. The pellet was washed with 70% and 95% EtOH, vacuum dried, and then resuspended in 34μl of TE (pH 8.0).

2.4.6.2 Ligation:

The reamplified DNA was ligated into M13 bacteriophage. To the final 34μl of DNA solution from above was added 4μl of 10X ligation buffer (Boehringer Mannheim). This mixture was heated for 65°C for 5 min, and cooled before adding 2μl of T4 DNA Ligase (1 unit/μl, Boehringer Mannheim). The ligation reaction was carried out overnight at 15°C. The DNA was precipitated using 3M NH_4Ac and 100% EtOH, washed with 70% EtOH, vacuum dried, and then resuspended in 10μl of TE buffer (pH 8.0).

2.4.6.3 Cell transformation:

JM101 bacteria were transformed with the M13mp18 RF bacteriophage, using the electroporation transformation procedure (Sambrook et al., 1989): a mixture consisting of 1μl of the ligase reaction and 40μl of competent bacterial cells was subjected to 2.5 kV using an electroporation apparatus. One ml of media (NZ media: 10g NZ amine, 5g NaCl, 2g $MgSO_4$) was added to the mixture and the cells allowed to recover for 1 hour at 37°C.

2.4.6.4 Plating:

JM101 or DH5αF' bacterial cells were streaked on a 100x15mm 2xYT (16g bacterial tryptone, 10g bacterial yeast extract and 5g of NaCl) agar plate and left overnight at 37°C. An overnight culture was made by infecting 50 ml of 2xYT media with a single bacterial colony. For a 100x15mm 2xYT agar plate, 200 µl of the overnight culture was added to a mixture of 1-100µl of the transformed bacteria, 50µl of Isopropylthio-ß-D-Galactosidase (100mM, Gibco BRL), and 10µl of X-Galactosidase (2% in DMSO, Gibco BRL). To this mixture was added 3 ml of melted top loading agarose (7g agarose/l of 2xYT media). After gentle vortexing the plating solution was poured on to the agar plate. The plates were left for 4-6 hrs at 37°C for bacterial colonies to develop. The infective cycle of M13 bacteriophage does not involve cell lysis, however infected bacteria grow more slowly. As such, colonies of infected bacteria appear as plaques on a turbid background lawn of non-transformed bacteria.

2.4.6.5 ß-Galactosidase blue/ white color selection:

To select only those bacterial colonies infected with M13 containing a foreign DNA insert, we used a ß-galactosidase (ß-gal) color assay. M13 includes a short segment of E.Coli DNA within its major intergenic segment, which is part of the lac Z gene, but does not produce a functional ß-galactosidase (ß-Gal) enzyme. Alpha-complementation between the bacterial and bacteriophage lac Z gene products in bacterial transformants results in functional ß-gal activity. However, the insertion of a piece of foreign DNA into M13 disrupts the lac Z gene, so that it can no longer α-complement with the gene product in bacterial cells.

The transformed bacteria are plated with an inducer of the ß-gal enzyme (IPTG), and X-gal which is a chromogenic substrate for ß-gal. ß-gal acts on X-gal and produces a blue product. Therefore, colonies with ß-gal activity will appear as a blue plaque. Those colonies with no ß-gal activity, will appear as colourless plaques. Normally, colourless plaques are selected for further analysis as they contain M13 with foreign DNA inserts. However, the combined length of the PCR primers plus the K channel DNA fragment was designed so that the desired PCR products are ligated into M13 in frame, such that there is no disruption of the amino acid sequence of the bacteriophage Lac-Z gene product. As such, a certain degree of α-complementation with the bacterial Lac-Z product occurs, and colonies with inserts of interest are light blue in color. Therefore, we chose light blue plaques for further analysis.

2.4.7 Small scale preparations of single stranded M13 DNA

2.4.7.1 Picking bacterial colonies:

Transformed bacteria were plated at sufficiently low titre so that individual colonies were clearly resolved. The surface of a selected colony was touched with a sterilized platinum wire. The wire tip was then agitated in 5ml of 2xYT media, also containing 100µl of an overnight bacterial culture of DH5αF' or JM101. The culture was shaken at 37°C for 4-6 hours. Five hundred µl of the culture was stored at -70°C in 65% glycerol, 0.025M Tris-Cl (pH 8.0) and 0.1M $MgSO_4$. The remaining 4.5 ml of the culture was centrifuged for 15 min at 11,000g (SS42, Beckman) to remove cells and debris, and the supernatant, which contains the M13 bacteriophage, was recovered.

2.4.7.2 Isolation and purification of M13 bacteriophage:

The single stranded DNA was precipitated with 0.25 volume of 20% polyethylene glycol (PEG)/ 3.5 M NH_4Ac solution. Care was taken to remove any excess PEG from the sides of the tubes. The pellet was resuspended in 150µl of TE (pH 8.0). The DNA was extracted with phenol/chloroform (chloroform:isoamyl alcohol, 24:1) twice, and then chloroform extracted to remove any remaining phenol. The DNA was precipitated with 3M NH_4Ac and 100% EtOH, washed with 70% EtOH, vacuum dried and resuspended in 15 µl of TE buffer (pH 8.0). DNA was then quantified; approximately 2µg of DNA are required for a DNA sequencing reaction.

2.4.8 DNA sequencing

Preparation of gel assembly

Glass gel plates were cleaned with soap, rinsed with water, and left to dry. The plates were cleaned with ethanol and dried. Before cleaning, one plate was siliconized (Dimethyl-dichlorosilane, Sigma). For assembly, the plates were separated by two 0.2mm spacers between the long edges of the places. The plates were then taped together.

Preparation of polyacrylamide gel solution

A 6% polyacrylamide solution was prepared: 50.4g of urea, 18ml bis-acrylamide (Fisher), and 12ml of 10xTBE (121g Tris base, 7.4g EDTA, 53.4g boric acid in distilled H_2O, pH 8.3), was brought up to 120ml with distilled H_2O. This solution was filtered (0.45µ nucleopore filter) under vacuum and placed on ice. Just before the gel was ready to pour, 60µl of 10% ammonium persulfate, and 0.8ml TEMED were added.

Pouring the gel

The assembled plates were set at a 45° angle. The 6% polyacrylamide solution was injected between the plates using a 50ml syringe, taking care to avoid introducing air bubbles. The flat end of a shark's-tooth comb was inserted into the space at the top of the plates and clamped in place. The gel was allowed to polymerize in a horizontal position for 45 min with extra solution added to the top of the gel as it polymerized. After polymerization, the comb was removed and the area rinsed with distilled H_2O to remove unpolymerized acrylamide. The tape was removed from the bottom of the gel and the gel placed in the electrophoresis apparatus (Sigma). Buffer (TBE, pH 8.3) was added to bottom and top containers of the apparatus. A syringe with an 18-gauge needle, filled with buffer, was used to clean out the sample wells and the space at the bottom of the gel. The gel was pre-run at 65 watts for 30 min. After pre-running, the bottom and the top of the gel were cleaned again. Then the shark's-tooth comb was inserted so that the points just touched the surface of the gel and was then clamped in place.

DNA sequencing reactions

A modified version of the dideoxy sequencing method described by Sanger and co-workers (1977) was used to sequence the foreign DNA inserts (T7 Sequencing Kit from Promega).

The concentration of template DNA was adjusted so that 10µl contained 1.5-2.0µg of DNA. Two µl of the *Universal primer*, and 2µl of *annealing buffer* were added to the 10µl of template DNA, and the tube centrifuged briefly to mix. Priming proceeded at 60°C for 10 min. The tube was allowed to cool at room temperature for at least 10 min.

A mixture of 1µl of distilled H_2O, 3µl *labelling mix-dATP* (contains the 4 dNTPs; dATP, dCTP, dGTP, dTTP), 2µl of T7 DNA Polymerase diluted in *enzyme buffer*, and 1µl labelled S^{35}-dATP(10µCi/µl) was prepared. The tube was spun briefly to mix. Six µl of this mixture was added to the annealed template. DNA synthesis proceeded for 5 min at room temperature to ensure incorporation of sufficient radioactivity.

After 5 min, 4.5µl of this mixture was added to 4 separate tubes, preheated to 37°C for at least a min, each containing a different dideoxy nucleotide triphosphate(ddATP, ddGTP, ddCTP and ddTTP). The termination reaction continued at 37°C for 5 min, and was halted with a *stop solution* containing bromophenol blue and xylene cyanol. These dyes were used to monitor the migration of the DNA on the gel.

Running the gel

Before loading the gel, DNA samples were denatured at 75-80°C for 2 min. The 4 separate reactions were run adjacent to each other on a 200µm 6% polyacrylamide gel. The gel was run for 2.5 hours, at 65 watts, and removed from the gel apparatus.

Processing the gel

The gel assembly was placed horizontally so that the siliconized glass plate was on top. This plate was removed and a piece of 35x43cm filter paper (3M) was placed on top of the exposed polyacrylamide gel. The gel and filter paper were pealed off the bottom glass plate. The gel was dried for half an hour, under vacuum at 80°C. X-ray film (Kodak) was placed on top of the gel, and left to expose at room temperature overnight before developing. The sequences of K channel DNA inserts were identified on the basis of the known nucleotide length, and the presence of certain amino acids that are highly conserved in all K channels cloned to date. The sequences were then compared to the sequences of previously cloned K channel genes.

Chapter 3

Characterization of K Currents on Peripheral Neurons

3.1 Introduction

In this study, we have examined the properties of voltage-gated K currents on neonatal rat sensory neurons from the distal ganglion of the vagus (nodose ganglion). The nodose ganglion is a sensory ganglion located near the bifurcation of the carotid arteries. Nodose neurons are pseudo-unipolar cells whose axons run in the vagus nerve to provide sensory innervation to the heart, lungs and much of the viscera. They originate from the ectodermal placodes, which are thickenings of the superficial ectoderm close to the neural plate (Le Douarin et al., 1981). These neurons have been divided into 2 categories: A and C cells, which have myelinated and unmyelinated axons, respectively (Stansfeld and Wallis, 1985). A few studies have investigated voltage-gated K currents on rat peripheral sensory neurons. These studies have shown that sensory neurons express several voltage-gated K currents that differ in their rates of inactivation (Kostyuk et al., 1981; Kasai et al., 1986; Mayer and Sugiyama, 1988). This is particularly evident for sensory neurons from mammalian nodose ganglia (Stansfeld and Wallis, 1985), where some C-cells have rapidly inactivating K currents, similar to the classical transient A-current, while A-cells have a rapidly activating, but slowly inactivating K current that is involved in spike frequency adaptation (Stansfeld et al., 1986). Slowly inactivating K currents are also expressed on other mammalian neurons (Storm, 1988; Greene et al., 1990).

Cooper and Shrier (1989) demonstrated that nodose neurons express 3 voltage-gated outward K currents: a non-inactivating delayed rectifier current (IK); a slowly inactivating A-current (IAs), and; a rapidly inactivating A-current (IAf). Their single channel studies on nodose neurons showed that a single class of A-channel (based on conductance) can inactivate with considerably different time courses. In response to a depolarizing voltage step some channels opened once and inactivated rapidly in 10-30ms, other channels opened and closed 3-4 times and inactivated more slowly (100-300ms), and others opened and closed several hundred times before inactivating in 1-3 secs. These channels were shown to underlie rapidly(IAf) and slowly(IAs) inactivating macroscopic currents. Cooper and Shrier classified both rapidly and slowly inactivating channels as A-channels as they share such properties as conductance and mean open time. A-channels having two different inactivation rates have been reported for guinea pig DRG neurons (Kasai et al., 1986) and for rabbit atrial cells (Clark et al., 1988).

This chapter discusses experiments that characterize voltage-gated K currents on peripheral neurons. There were two major reasons for doing these studies: First we were interested in the question of whether one or more types of A-channel are present on nodose neurons. Cooper and Shrier's observation that channels with the same conductance can inactivate at very disparate rates could be explained by two related, but separate channels with different inactivation rates, or, by a single channel that when modulated by second messengers changes its inactivation behaviour (Strong, 1984; Kawa, 1987). To answer this question, I examined other properties of the channels besides their inactivation kinetics, to determine if different A-channel subtypes are present on these neurons. The premise was that if slowly and rapidly inactivating A-currents differ in a number of their properties, not only their kinetics of inactivation, it is likely that they are different physical channels encoded for by different messenger RNA species.

The second reason for these studies was that by characterizing the properties of K currents on nodose sensory and sympathetic neurons, it might then be possible to study the developmental expression of these currents.

In this chapter, I show that rapidly and slowly inactivating A-currents on rat peripheral nodose neurons differ in their voltage-dependence and pharmacology, as well as in their kinetics. Furthermore, comparison of the voltage-dependence of fast and slow A-channels at the single channel level with macroscopic measurements, supports the view that channels which inactivate rapidly in 10-30ms underlie the fast A-current and those that inactivate slowly underlie the slow A-current. The fact that rapidly inactivating and slowly inactivating channels differ in a number of their macroscopic properties, and yet have the same single channel conductance, suggests they are distinct subtypes of the same basic channel.

I also show that SCG neurons express voltage-gated K currents with similar properties to those observed on nodose neurons: SCG neurons express a slowly inactivating current, a fact not previously documented for sympathetic neurons, in addition to IAf and IK.

3.2 Methods

3.2.1 Recording of voltage-gated K currents on peripheral neurons

Nodose and superior cervical (SCG) ganglia were removed from neonatal rat pups. The ganglia were dissociated and the neurons cultured without other cell types, as described in Chapter 2. To study voltage-activated K currents, inward Na and Ca currents, and the Ca-dependent K current were blocked pharmacologically: Na currents were blocked using TTX (1μM) and in most cases almost all the external Na (2mM was added) was replaced with ChCl (140mM); Co (2.5mM) and low Ca solutions(0.5mM) were used to block Ca and Ca-dependent currents. SCG and nodose neurons were voltage-clamped using whole-cell patch recording techniques (Hamill et al., 1981).

The total outward currents on nodose and SCG neurons consist of three different components which differ in their kinetic and voltage-dependent properties: a non-inactivating current (IK); a fast transient A-type current (IAf) that inactivates with a time constant of 10-30 ms; and a slow transient A-type current (IAs) that inactivates with two components, one with a time constant of 100-300ms, the other with a time constant of 1-3s. These three K currents differ in their voltage-dependence of inactivation; therefore, by holding the membrane at different potentials, we were able to selectively activate one or two of the currents and use subtraction techniques to separate individual currents. Briefly, the membrane was held at a potential (-10 or -20 mV) where depolarizing steps evoked only IK. The IK currents were subtracted from currents (IAf+IAs+IK) evoked by steps to the same depolarizing potentials from a more negative potential, -90 mV, to isolate the A-currents. A hyperpolarizing step to -90mV, from the -10 or -20 mV holding potential, was added to the result to remove the leakage current. The leakage and capacitance currents were usually linear; for currents evoked from -40 mV and -20 mV holding potentials, corrections were made digitally by adding the current response from an equivalent amplitude hyperpolarizing step.

Nodose neurons that had been in culture for 10-30 days were used for single channel recordings in the cell-attached configuration. All records, except where indicated, were corrected for capacity transients and leakage currents by subtracting an average of steps (n=15) to the same potential in which no channel openings occurred. Ensemble averages were produced by averaging the sum of 40-50 individual traces to a single step potential. Patch potentials are expressed in this chapter as membrane potentials, by subtracting the pipette potential from the resting potential.

3.2.2 Data analysis

Analysis of whole-cell currents was performed on IAf, IAs and IK currents, isolated using the separation protocols discussed above. Though the analysis described below pertains to whole cell currents, similar measurements were made for the ensemble average currents of single channel recordings.

3.2.2.1 Analysis of voltage-dependent properties:

Activation:

For activation experiments, depolarizing voltage steps to different command potentials (V_m) were given from a fixed holding potential (V_H). The conductance (G) for each command potential was measured by dividing the isolated current (I) by the driving force (V_m-Ek),

$$G=I/(V_m-Ek), \qquad (1)$$

where Ek is the equilibrium potential of K, which is -85mV in nodose neurons (Cooper and Shrier, 1989). The peak conductance for each voltage step was normalized to the conductance at the potential where the channels are maximally activated (Gmax), which was usually at +50 mV.

G/Gmax was plotted against the command potential (Vm); these plots indicate the percentage of the available channels that are activated by a given depolarizing step.

Inactivation:

Steady-state inactivation was determined by stepping to +40mV from different membrane holding potentials. G/Gmax is the ratio of conductance (calculated as above by dividing the isolated current by the driving force) evoked from different holding potentials to the maximal conductance (Gmax), evoked from a holding potential of -100mV. G/Gmax is plotted against the holding potential (Vm); these plots indicate the percentage of channels that are available for activation in response to a depolarizing voltage step.

Boltzmann distribution:

Equation 2 shows the Boltzmann equation,

$$G/Gmax = 1/(1+\exp((V'-Vm)/k)), \qquad (2)$$

where k=KT/ze and is a slope factor that indicates the steepness of the voltage-dependence, z is the valency of the gating charge, e is the elementary charge, K is the Boltzmann constant, T is the absolute temperature, and V' is the voltage at half-maximal conductance. This equation describes the probability of a two state system being in one state or the other. The ratio of G/Gmax refers to the percentage of open channels relative to the total present for activation experiments, and to the percentage of inactivated channels relative to the total number of channels for steady state inactivation experiments. This equation was used to fit the activation and inactivation data, and describes the range, and steepness of the voltage-dependence of activation or inactivation.

3.2.2.2 Analysis of Kinetic properties:

Activation:

The time constants for activation were measured by fitting the activation of the isolated current with an exponential raised to the fourth power (see Equation 3).

$$[1-\exp(t/\tau_a)]^4, \qquad (3)$$

where τ_a is the time constant of activation. We chose the fourth power for two reasons: (1) Recent kinetic models of A channels seem to suggest that there are at least 5 resting closed states (Zagotta and Aldrich, 1990b). (2) The fourth power fit our data better than a third power, especially for IAf. A least-squares method was used for exponential fitting to the data (Hunter and Kearny, 1984). The overall goodness of fit was given by the variance accounted for, and the asymptotic information criterion (AIC) (Akaike, 1974) was used to determine the most appropriate fit to the data. All fits had at least 99% of the variance accounted for. Time constants were plotted against the membrane potential (Vm).

Inactivation:

For the inactivation time constant, τ, the currents were either fit by a single exponential, or were fit as the sum of exponentials, $A\ \exp(t/\tau_1) +.....+A_n\exp(t/\tau_n)$, where n=2-3. Time constants were plotted against the membrane potential (Vm).

Removal and development of inactivation:

The kinetics for the development and removal of inactivation for IAf and IAs were determined by a 2 step protocol. For development of inactivation, the membrane was held at -90mV and stepped to an intermediate potential (either -50mV or -30mV) for different durations and then stepped to +40mV. For removal of inactivation, the membrane was held at -20mV and stepped to either -70mV, -90mV or -110mV for different times before stepping to +40mV. The peak current at +40mV was expressed as a function of the time at the intermediate potential; these curves were fit with single exponential functions. The time constants of these exponentials were plotted against the membrane holding potential (Vm).

3.3 Results

3.3.1 Voltage-dependent outward currents on nodose neurons

Nodose neurons express a number of inward currents (Baccaglini and Cooper, 1982): a TTX-sensitive Na current, an Na TTX-resistant current (Ikeda and Schofield, 1987), and Ca currents (Bossu et al., 1985). Since these currents were not investigated in this study, to study outward K currents in isolation, inward currents and Ca activated currents were blocked pharmacologically (see methods).

Figure 3.1A shows the total outward K currents on a rat nodose neuron evoked by 125ms depolarizing voltage clamp steps from a holding potential (V_h) of -90mV. The outward current consists of 3 currents: a rapidly inactivating component (IAf); a slowly inactivating component (IAs) which shows little inactivation over the course of a 125ms pulse, but shows clear inactivation in response to a long 6s depolarizing step evoked from a V_h of either -90mV or -40mV, shown in Fig. 3.1B (also see Fig.3.4A); and a component that does not inactivate, at least over tens of seconds (IK). This non-inactivating component is most evident when evoked by depolarizing voltage steps from a V_h of -20mV (Fig. 3.1B), because the two inactivating currents are inactivated at -20 mV. Complete descriptions of each current are given in the sections that follow.

Neonatal nodose neurons are heterogeneous in their expression of these outward currents. In the majority of neurons examined (66/97), all 3 currents contributed to the outward current; an example of such a neuron is shown in Fig. 3.1. The total outward current (A) can be separated into inactivating and non-inactivating currents (C-E) on the basis of holding potential. The method of separating the total outward current into its component parts is described below.

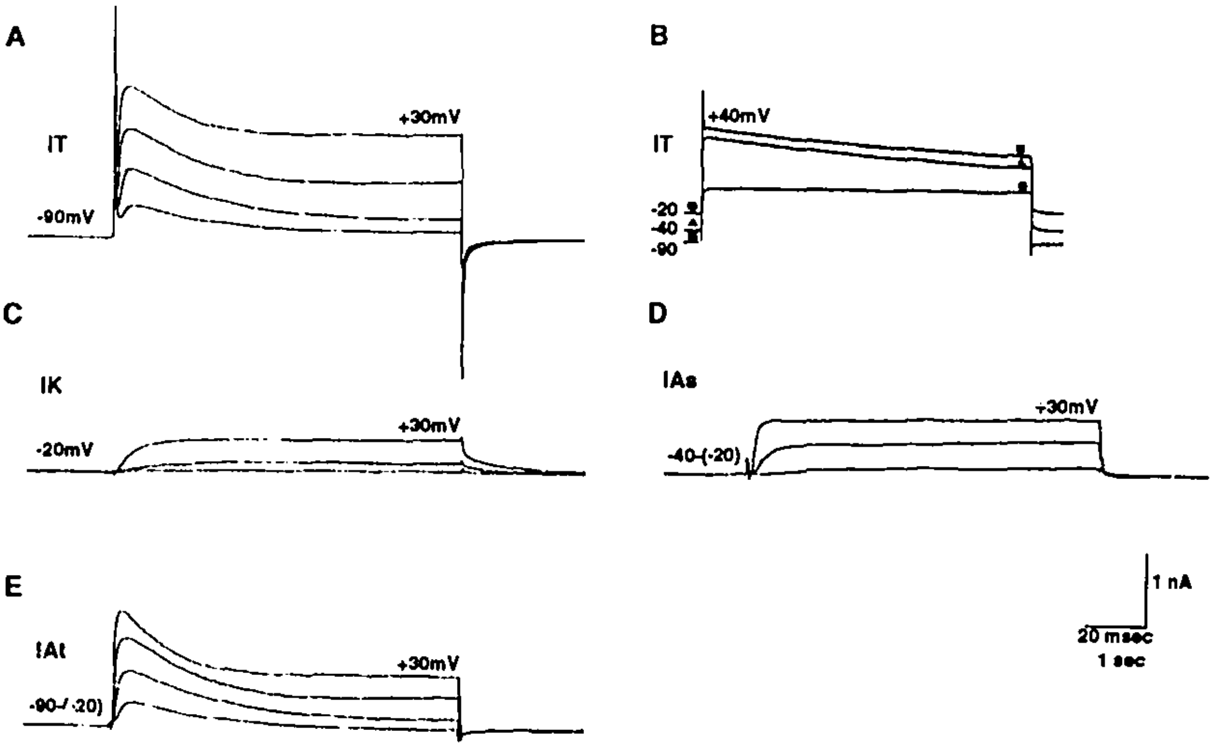

Figure 3.1: Voltage-gated K currents on neonatal nodose neuron. A,C-E: Currents evoked in the same neuron by 125ms depolarizing voltage steps, from different holding potentials, up to +30mV in 20mV increments. Records were filtered at 1.5kHz, and sampled at 5kHz. A: Total outward currents, uncorrected for leakage or capacity currents, evoked from a holding potential of -90mV. B: Three superimposed total outward currents, uncorrected for leakage, evoked by 6 sec voltage steps to +40mV from holding potentials of -90mV (top), -40mV (middle) and -20mV (lower). The traces were filtered at 100Hz and sampled at 200Hz. C-E: Isolated K currents that make up the total outward current shown in A. The leakage and capacity currents were subtracted from the records in this figure, and in all subsequent figures except where indicated. C: IK is activated in isolation when the membrane is held at -20mV. D: IAs: IK currents evoked from a holding potential of -20mV were subtracted from currents (IAs+IK) evoked by steps to the same final potential, from a holding potential of -40mV, to isolate IAs. E: IAt=IAf+IAs: IK currents evoked from a holding potential of -20mV were subtracted from currents (IAf+IAs+IK) evoked by depolarizing steps from a holding potential of -90mV, in order to isolate the A-currents.

3.3.2 Separation of outward K currents

The outward current evoked from a V_h of -20mV activates slowly, as shown in Fig. 3.1C; we refer to this current as the *delayed rectifier*, IK. This current does not inactivate over a 6 sec depolarizing pulse, and shows no steady-state inactivation, meaning that the same current is evoked from V_h's of -20 mV, -40 mV and -90 mV. To isolate IK, the membrane is held at a potential (-10 mV to -20 mV) where only the non-inactivating current is activated by depolarizing steps, as the other two currents are inactivated at this potential.

If the membrane is held at a more negative potential of -40mV, inactivation is partially or totally removed from a second population of channels. These channels, which give rise to a current we refer to as the *slow A-current* (IAs), inactivate over a 6sec depolarizing step (Fig.3.1B). Depolarizing steps from a potential of -40mV activate both IK and IAs. IAs can be seen in isolation (Fig.3.1D) by subtracting the IK current evoked from a V_h of -20mV.

Depolarizing steps from a more negative V_h of -90mV evoke a third component that activates and inactivates more rapidly than IAs and corresponds to the rapidly inactivating component in Fig.3.1A, B and E. We refer to this additional current as the *fast A-current*, IAf. The sum of IAf and IAs currents is separated from IK, as was described for IAs, by subtracting the currents evoked from a V_h of -20mV (shown in Fig. 3.1E).

Generally, in nodose neurons, IAf and IAs can not be completely separated from each other on the basis of V_h because of overlap between the voltage ranges to remove inactivation, as will be described below. However, we were able to take advantage of the fact that a significant number of neurons expressed only one inactivating current besides IK (Fig. 3.4), and in a few neurons only IK was evident (Fig. 3.3). These neurons were important to this study because they allowed individual currents to be characterized without the need for additional procedures, such as complicated subtraction protocols or pharmacology to isolate the currents.

In addition, in some neurons (n=6), it was possible to take advantage of the fact that IAs

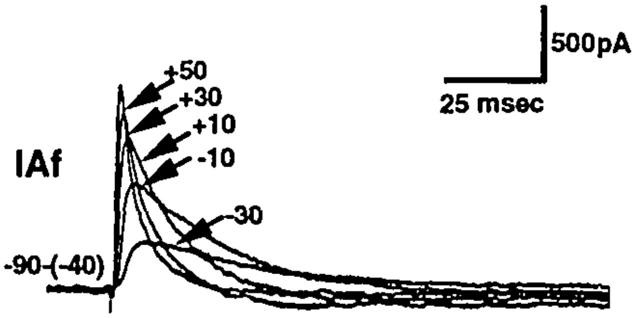

Figure 3.2: IAf isolated on a neuron that expressed all three voltage-gated K currents: IAf, IAs, and IK. IAf currents evoked by depolarizing voltage steps up to +50mV in 20mV increments, from a holding potential of -90mV. IAs in this neuron showed no steady-state inactivation between -90 and -40mV. Therefore IAf could be isolated by subtracting away the IAs and IK currents evoked by depolarizing voltage steps to equivalent potentials from a holding potential of -40mV [i.e. -90-(-40mV)]. Traces were filtered at 1.5kHz, and sampled at 5kHz.

showed no steady-state inactivation over the range of -90mV to -40mV. In these neurons, subtracting the currents evoked from a V_h of -40mV (IK+IAs), from the currents evoked from a V_h of -90mV (IK+IAf+IAs), resulted in isolated IAf currents. Figure 3.2 shows the isolated IAf current in such a neuron.

3.3.3 Properties of K currents on rat nodose neurons

In the following sections, I use the neurons which express only one or two of the K currents to analyze the kinetic and voltage-dependent properties of each current, IAf, IAs and IK. The voltage protocols used in the experiments, and the data analyses are described in the methods.

3.3.3.1 Delayed Rectifier, IK

Fig. 3.3A shows outward currents, evoked by depolarizing steps, from a neuron that expressed IK as its only outward voltage-gated K current; only five neurons of 97 examined were in this category. IK activates near -20mV and does not inactivate over 6s (see Fig. 3.3A, right). In addition, IK shows no sign of steady-state inactivation; holding the membrane at -20mV, -40mV, or -90mV did not change the amplitude, or the kinetics of the evoked currents.

Voltage-dependence

In the 5 neurons that only expressed IK, activation of IK was determined from the leak subtracted currents evoked by voltage steps from a V_h of -40mV. Maximal activation of the IK channels, as indicated by the saturation of tail currents (see Fig. 3.3A), usually occurred for a step potential to +50 mV. The steady-state currents were expressed as conductances (see Equation 1). The relationship between the conductance (G) normalized to the maximum conductance (Gmax) for the step to +50 mV, and the membrane potential (V_m) is shown in Fig. 3.3B. The data are well fit by a Boltzmann distribution (see equation 2 in methods) with V'= 16mV and k=12mV.

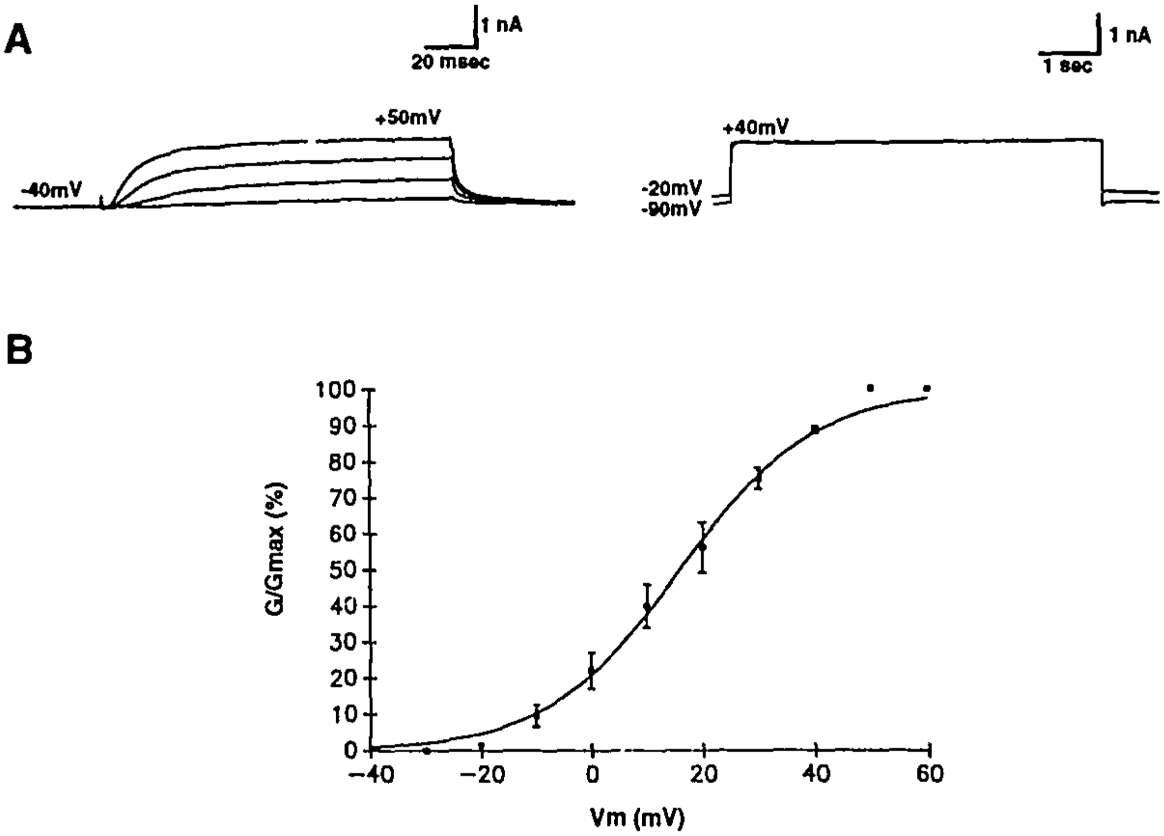

Figure 3.3: Delayed rectifier, IK. Outward currents evoked by depolarizing voltage steps from a neuron that did not have detectable A-currents. A, left: current records from a Vh of -40mV in response to 125 ms steps to -10, +10, +30, and +50 mV (bottom to top). Traces were filtered at 1.5 kHz and sampled at 5 kHz. Right: currents evoked from the same neuron, in response to 6 s steps to +40mV from -20 and -90 mV holding potentials. Traces on the right were filtered at 100Hz, sampled at 200Hz, and not corrected for leakage. B: squares show the conductance (G) at the end of a 125 ms step as a percentage of Gmax at +50mV, vs. membrane potential (mean ±SE, n=5). The conductance was determined by dividing the current by the driving force (Vm-Ek), where Ek was obtained from Cooper and Shrier (1989). The solid line is the Boltzmann distribution (see Eq.2) with V'=+16mV and k=12mV.

Kinetic properties

IK activates slowly and with considerable delay for small depolarizations (see Fig.s 3.3 and 3.6). The time constants for activation were measured by fitting the activation of the current with an exponential raised to the fourth power (see Equation 3). Figure 3.6A shows an example of an isolated IK current with the superimposed fit, and Fig. 3.6B shows the voltage-dependence of the time constants and indicates that IK activates faster when more depolarized voltage pulses are applied.

3.3.3.2 Slow A-current, IAs

In most neurons (90/97), when depolarizing voltage clamp steps were given from a V_h of -40mV, the outward current was larger than that seen at -20mV; hyperpolarization partially removes inactivation from IAs, so that depolarizing voltage steps evoke IAs besides IK (Fig. 3.1D). In 24 of these neurons, IAs was the only voltage-gated K current present in addition to IK, and no IAf currents were evident. Fig. 3.4A shows the K currents from such a neuron.

Voltage-dependence

This group of 24 neurons was used to determine the voltage-dependence for activation and inactivation. Measurements of IAs were made at the end of a 125msec depolarizing step to ensure that if these cells did express a small rapidly inactivating current it would have completely inactivated by this point. Steady-state inactivation was determined by holding the membrane at different potentials, and stepping to the same command potential (+40 mV). The relationship between the conductance (G) for a given V_h, relative to the maximal conductance (Gmax) from a V_h of -100 mV, is plotted against the holding potential (Vm) in Fig. 3.5A. The data are well fit by a Boltzmann distribution (Equation 2) with V'= -51mV and k=-14mV. This figure also demonstrates that IAs is maximally available at -90mV, is partially inactivated at -40mV, and almost completely inactivated at the V_h (-20mV) we use to investigate IK.

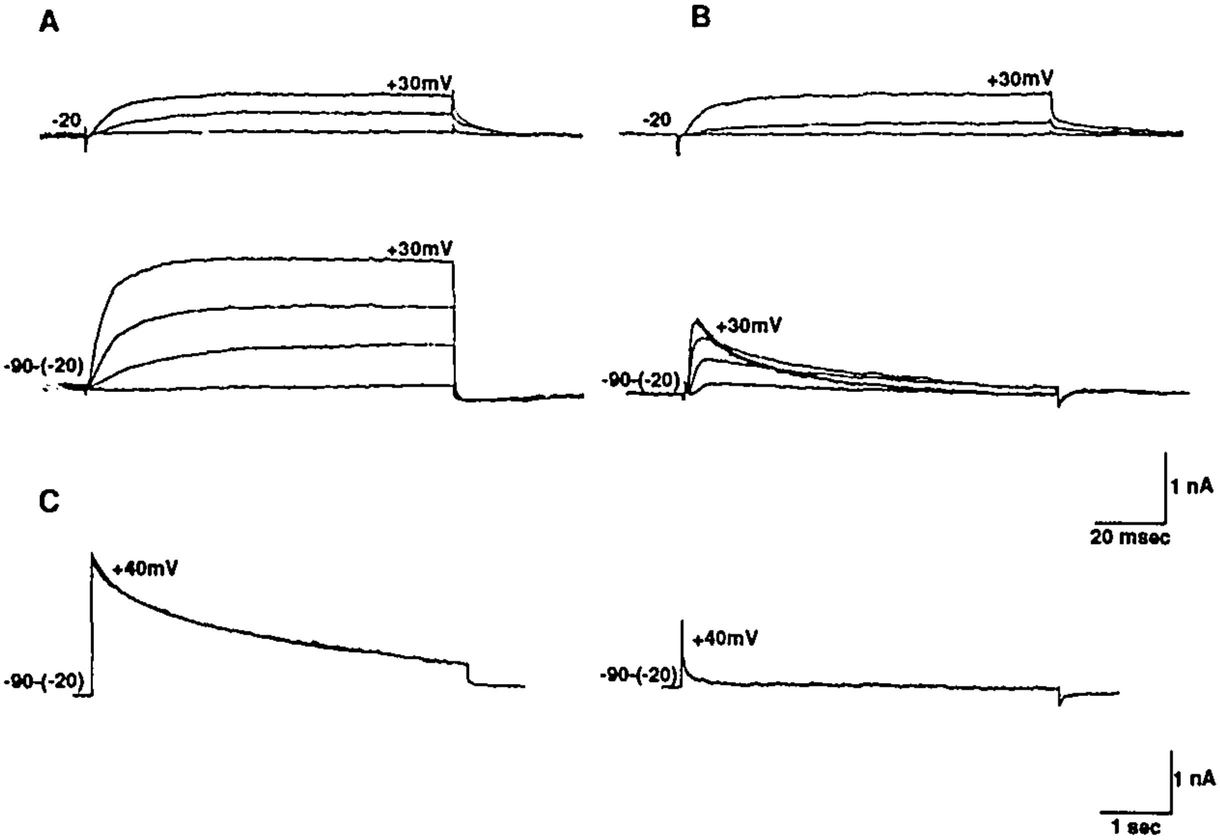

Figure 3.4: Examples of IAs and IAf on different nodose neurons. A: current traces from a neuron with no detectable IAf. B: current traces from a neuron with IAf and IK but only a small amount of IAs. For both A and B, 125 ms depolarizing voltage steps were made from 2 different holding potentials, -20 and -90 mV, and the steps were made in 20 mV increments up to +30mV. IK currents evoked from a holding potential of -20mV have been subtracted to isolate the A-currents (IAf and IAs). The currents were filtered at 1.5kHz and sampled at 5 kHz. C: the isolated, leak subtracted A-current for each neuron evoked by a 6 s depolarizing step to +40mV. Traces were filtered at 100Hz and sampled at 200Hz. The solid line in B for IAf, and in C for IAs represents the exponential fit to the inactivation of the current. IAf was fit with a single exponential with a time constant of 12 ms, and IAs was fit with a sum of 2 exponentials, the time constants of which were 496ms and 4.4s.

The voltage-dependence for activation of IAs was determined in the same manner as for IK, but depolarizing steps were given from a steady V_h of -90mV, where IAs is maximally available. For each neuron, the conductance (G) relative to the maximum conductance at +50 mV (Gmax) was plotted against the membrane potential of the voltage step (Vm), as shown in Fig. 3.5A. The data are described by a Boltzmann distribution with V'=-2mV and k=14mV. Measurements of IAs in neurons in which all three currents, IAf, IAs, and IK were present, as in Fig. 3.1, gave results that were consistent with those presented in Fig. 3.5 (data not shown). This result is important in that it appears possible to analyze IAs even in neurons that express all three currents. The difference in the voltage-dependence for activation of the slowly inactivating and non-inactivating currents, and that only IAs exhibits steady state inactivation, strongly support the idea that IAs and IK are mediated by different channels.

Kinetic properties

The activation time constants for IAs were obtained by fitting the rising portion of IAs currents evoked by depolarizing voltage steps, by an exponential raised to the fourth power (Equation 3). Figure 3.6A shows a fit superimposed on an isolated IAs current evoked from a V_h of -40 mV. Figure 3.6B shows a graph of the time constants for activation versus the step potential (Vm) and demonstrates that at -10mV IAs activates 5 times faster than IK, but the difference between the activation rates of the two is reduced with larger depolarizations. The difference in activation rate at -10mV was useful in helping to distinguish between IAs and IK.

While IAs was often not seen to inactivate over a 125ms voltage step (Fig. 3.4A), the current always showed considerable inactivation over a 6s step, as shown in Fig. 3.4C. The decay of the macroscopic IAs current on 40% of the neurons was well fit by the sum of 2 exponentials, one whose time constant was in the hundreds of milliseconds and the other with a time constant approximately 5 fold slower. Figure 3.4C shows the isolated IAs on such a neuron, along with the superimposed double exponential fit to the decay of the current. As shown in Fig. 3.7B, the

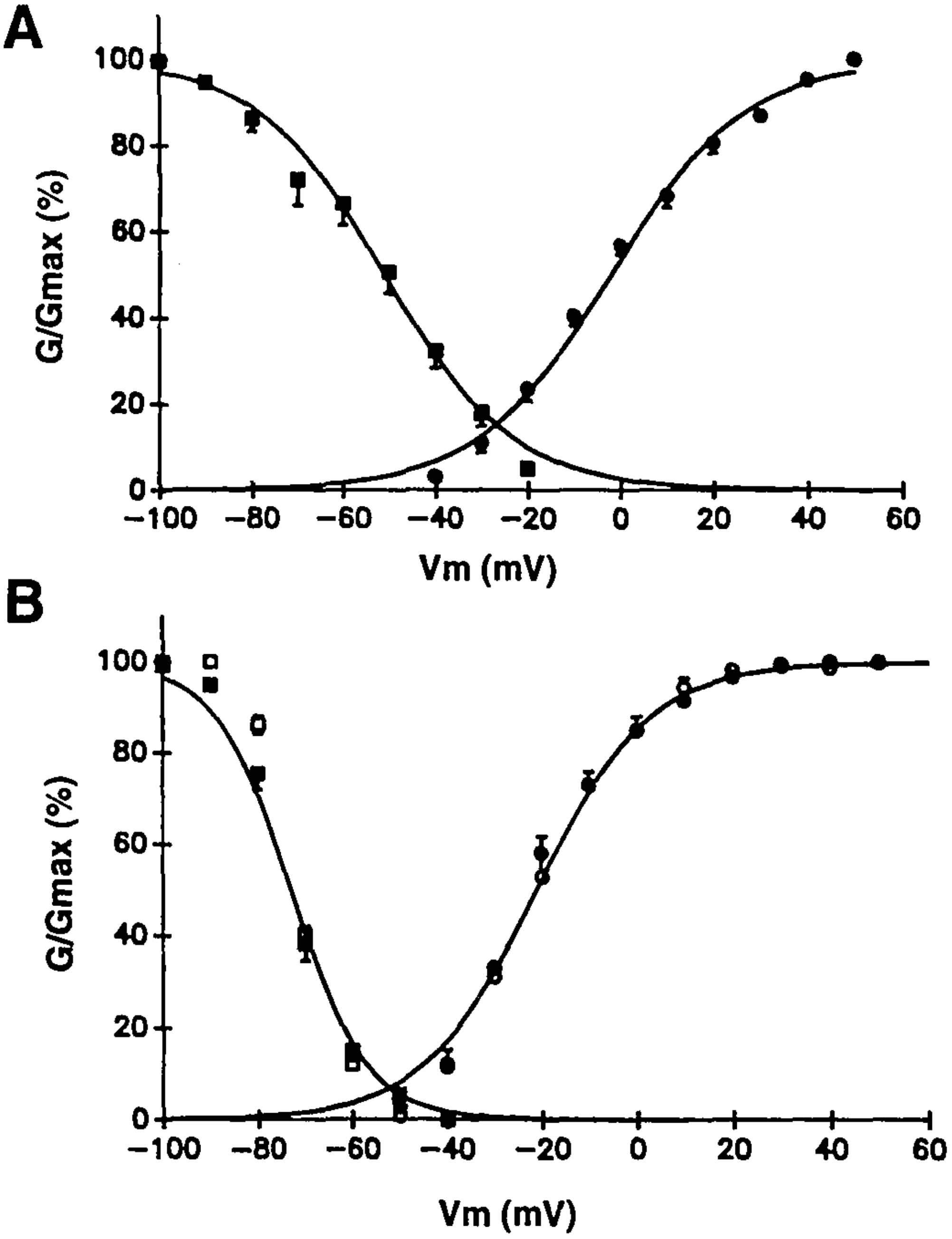

Figure 3.5: Voltage dependence of activation and inactivation for IAs, and IAf. A: IAs. Squares represent steady-state inactivation (mean, ±S.E, n=10) measured as the ratio of the conductance, measured at the plateau at the end of the voltage step, evoked by steps to +40mV from different holding potentials, to the isolated IAs conductance evoked from -100mV. The conductance was determined by dividing the current by the driving force(+40-Ek). Circles represent activation (mean, ±SE, n=13), measured from the plateau current evoked by depolarizing voltage steps from -90mV holding potential, and dividing by the driving force, as a percentage of Gmax. Solid lines are the fitted Boltzmann distributions with coefficients for activation and inactivation of V'=-2mV, k=14mV, and V'=-51mV, k=-14mV, respectively. B: IAf. Squares represent steady-state inactivation, and the circles activation, as in A, except the currents were taken as the peak isolated transient current. Solid lines in A are the fitted Boltzmann distributions for activation and steady-state inactivation with values of V'=-21mV, k=12mV and V'=-73mV, k=-8mV, respectively. Open symbols are data from the 2 neurons that had no IAs; values for activation and inactivation are in good agreement with IAf data from neurons that also had IAs (filled symbols; n=6).

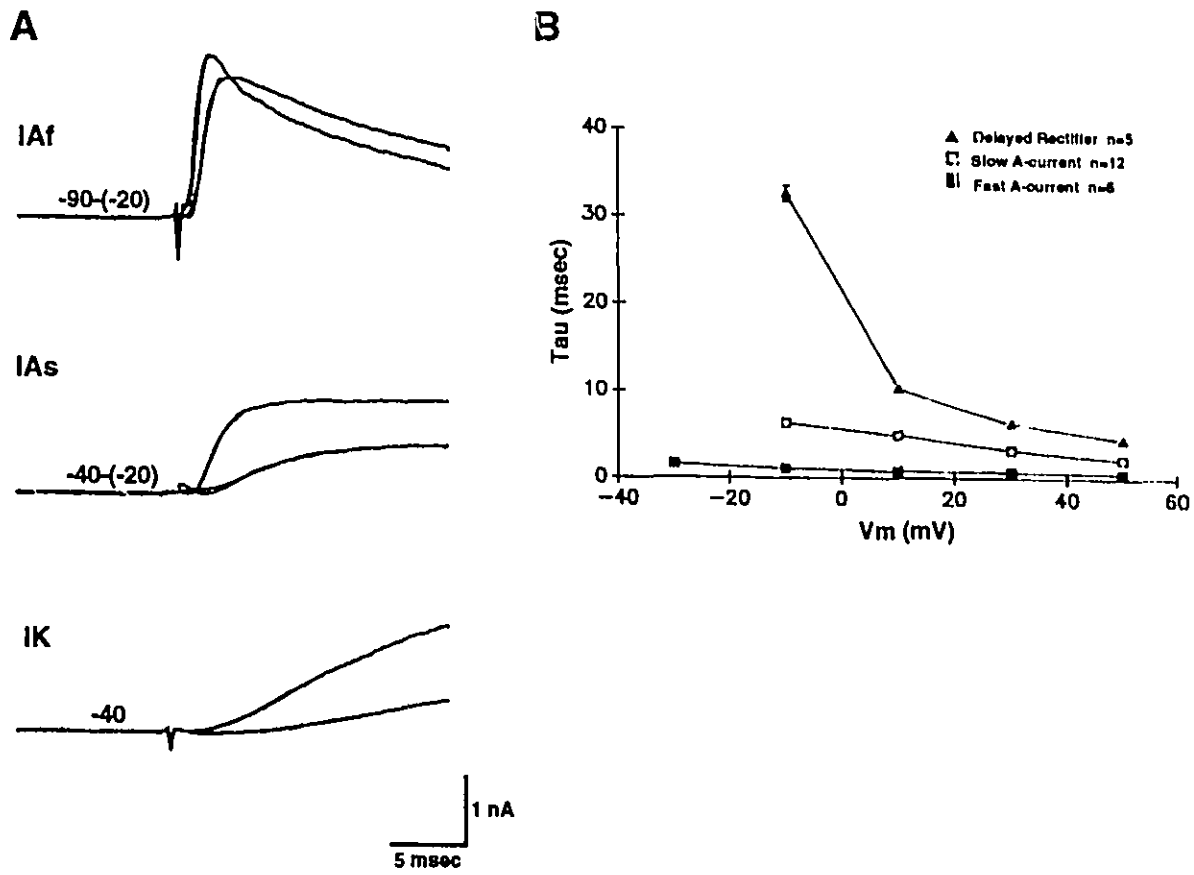

Figure 3.6: Activation Kinetics. A shows the activation of isolated IAf (top), IAs (middle) and IK (bottom) currents evoked by voltage steps to +10mV (lower) and +30mV (upper). For the top and middle set, steps from a Vh of -20mV have been subtracted from the outward currents, to isolate the A-currents. The solid lines are fits superimposed on the data and represent curves drawn as rising exponentials to the fourth power. Traces were sampled at 20kHz and filtered at 1.5 kHz. B shows the activation time constants (mean ± s.e.m.) versus step potential for IK (triangles), IAs (empty squares) and IAf (solid squares). (At most potentials, the error bars are smaller than the symbols)

inactivation time constants for both components are voltage-dependent, with inactivation becoming more rapid at more depolarized potentials. In 5% of the neurons only the faster component was apparent, and in the remaining 55%, only the slower component was evident.

The kinetics for development and removal of inactivation for IAs were determined by a 2 step protocol (see methods for details). For development of inactivation, the membrane was held at -90mV and stepped to an intermediate potential for increasing durations and then stepped to +40mV. For removal of inactivation, the membrane was held at -20mV and then hyperpolarized for different periods of times before stepping to +40mV. The time dependence for removal and development of inactivation for IAs was best described by one or two time constants. Figure 3.8 plots the time constants against the conditioning potential; both time constants are voltage-dependent, being largest near -70mV and decreasing significantly with depolarization or hyperpolarization.

3.3.3.3 Fast A-current, IAf:

In many neurons (68/97), voltage clamp steps from a V_h of -90mV evoked an additional current, not present at -40mV, that activated rapidly and inactivated in 10-30 ms, as shown in Fig.s 3.1, 3.2 and 3.4B. This current resembles the classical A-current first characterized in molluscan neurons (Connor and Stevens, 1971a; Neher, 1971), and we refer to it as the fast A-current (IAf).

Voltage-dependence

In 2 neurons, IAf appeared to be the only current present besides IK. Figure 3.4B shows an example of the K currents expressed by such a neuron. Because IAf undergoes steady-state inactivation the current could be studied in isolation by subtracting the IK currents evoked from a potential where IAf was not activated (for eg. -20mV). In another 6 neurons, the steady-state inactivation of IAs was shifted 20mV more positive so that the IAs currents evoked from -90mV

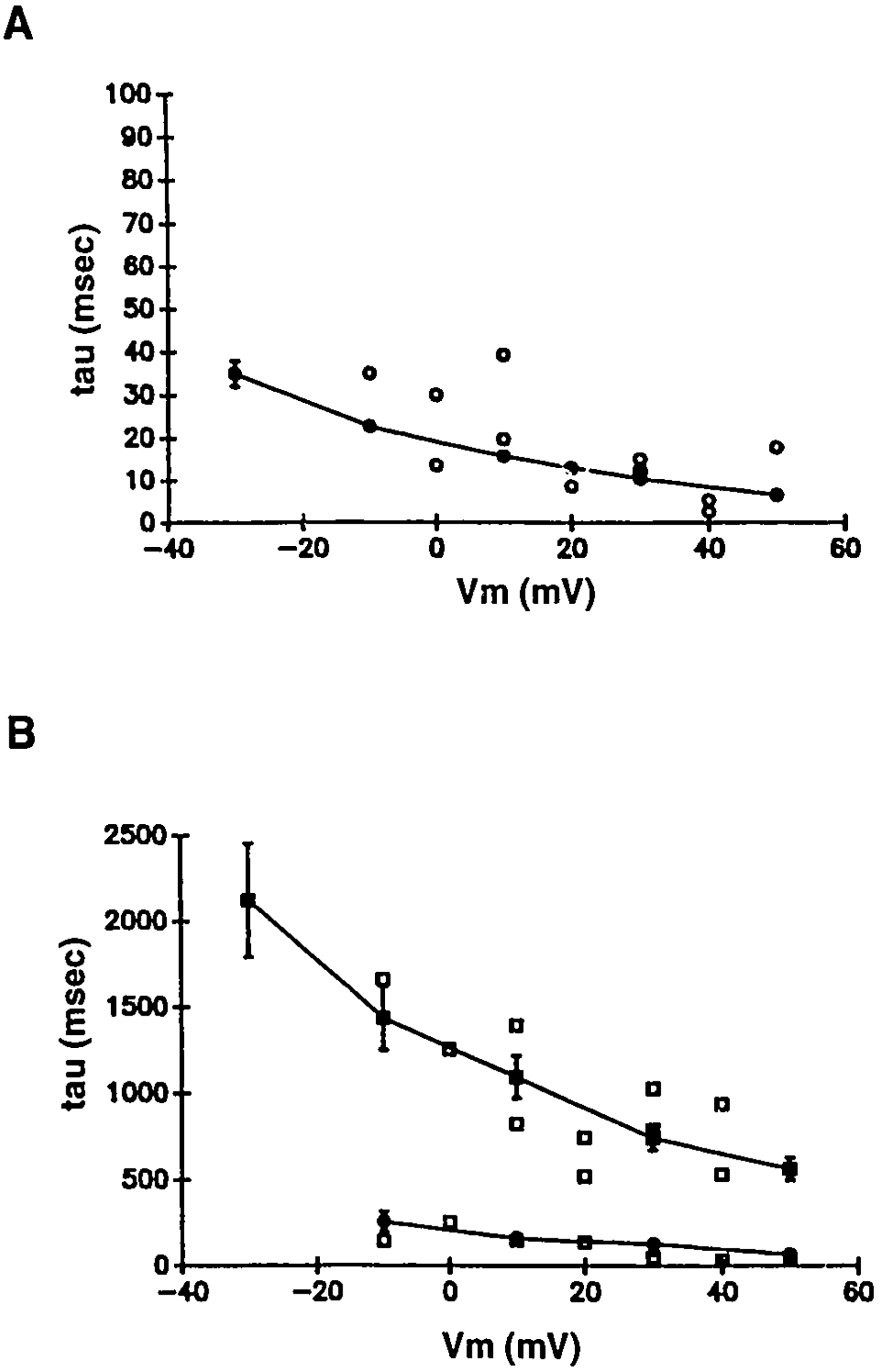

Figure 3.7: Kinetics for inactivation. A: IAf. The decaying phase of the whole cell current was fit with a single exponential and closed circles represent the time constants of decay (mean ± s.e.m., n=8) plotted as a function of the step potential. B: IAs. The decaying phase was fit by a single exponential in some neurons and as the sum of two exponentials in others. The time constants are plotted as a function of step potential as in A. The closed circles represent the mean of one time constant (n=6) and the closed squares represent the mean of the slower time constant (n=6). The open symbols superimposed on the data are examples of the time constants for inactivation of ensemble averages of Af and As channels.

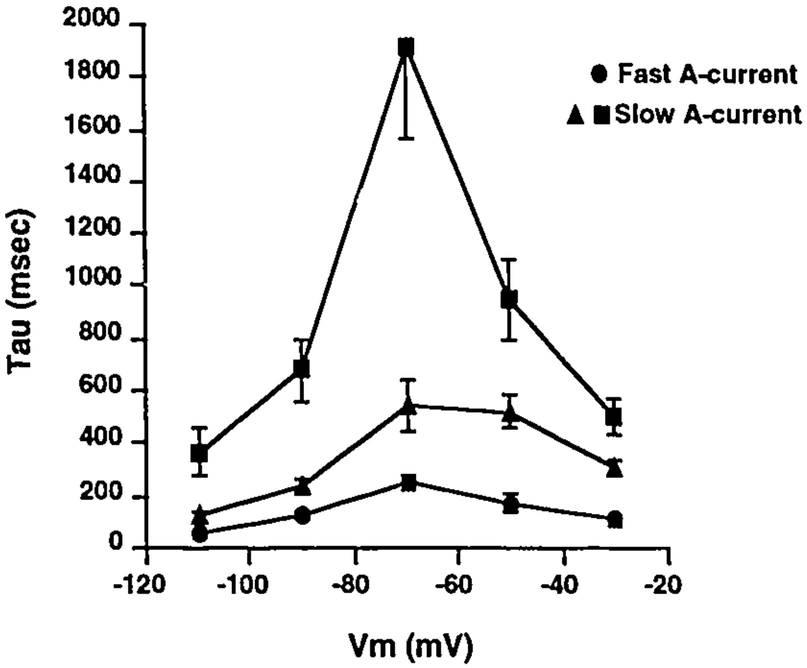

Figure 3.8: Voltage and time dependence of development and removal of inactivation: The development and removal of inactivation for IAf and IAs were determined by a two step protocol and the time constants(tau) in msec are plotted as a function of membrane potential (Vm). The time dependence for removal and development of inactivation of IAf was best described by a single time constant (circles), while that of IAs was best described by two time constants (squares, triangles).

and -40mV were the same, which allowed for isolation of IAf by subtraction of the current evoked from a V_h of -40 mV (see Fig. 3.2). The reason for this shift is unclear, although IAs currents with a similar voltage-dependence for inactivation are common on sympathetic neurons (see below). In addition, neostriatal neurons are reported to express more than one type of rapidly inactivating current, which differ in their voltage-dependence (Surmeier et al, 1989). The data for activation and steady-state inactivation of IAf were determined in the same way as for IAs. For the 8 neurons, and shown in Fig. 3.5B, the activation and inactivation data are well fit by Boltzmann distributions; V'=-21mV, 19mV more negative than IAs, and k=12mV for activation, and V'=-73mV, 20mV more negative than that for IAs, and k=-8mV for inactivation. Fig. 3.5B also shows that there is good agreement between the activation and inactivation data for IAf from the two groups of neurons used in the analysis.

Kinetic properties

The activation kinetics for IAf were determined, as for IK and IAs, by fitting the rising portion of the isolated currents evoked by depolarizing steps from a V_h of -90mV, with an exponential raised to the fourth power (Equation 3). Figure 3.6A shows a fit superimposed on the rising portion of an isolated IAf current. The voltage-dependence of the time constants for activation of IAf are shown in fig. 3.6B, and indicate that IAf activates approximately 5 times faster than IAs. The inactivation of IAf was well fit by a single exponential, an example is shown superimposed on the IAf current in Fig 3.4B. Figure 3.7A shows the relationship between the time constants and the membrane potential, and demonstrates that the time constant for inactivation changes from 33ms at -10mV to 7ms at +50mV.

The time constants for the development and removal of inactivation for IAf are shown in Fig. 3.8. The kinetics are voltage-dependent, being slowest near -70mV, and are at least an order of magnitude faster than those measured for IAs. This difference in kinetics for IAf and IAs was useful in detecting IAf in neurons that only expressed a small IAf current; 125 msec hyperpolarizing steps to -90 mV, from a V_h of -20 mV were sufficient to remove inactivation of IAf but not IAs. For the 24 neurons used to characterize IAs, this procedure failed to reveal any IAf, which supports our contention that measurements in these neurons were uncontaminated by IAf.

3.3.4 Pharmacology of voltage-gated K currents

As mentioned in Chapter 1, A-currents and delayed rectifier currents are often distinguished from one another by their selective sensitivity to two K channel blockers, 4-aminopyridine (4-AP), which blocks A-currents, and tetraethylammonium (TEA), which blocks IK (Thompson, 1977). Therefore, we investigated the pharmacological sensitivity of the three outward K currents to 4-AP and TEA.

IK

In 6/6 neurons examined (two of which had IK as their only outward current), IK was significantly reduced (73±6% block, n=6) by 10mM TEA. However, IK was not blocked by 1mM 4-AP (n=12) (Fig. 3.9A). In a different series of experiments, 5mM 4-AP was unable to block IK (n=5).

A-currents

Originally we had hoped to use 4-AP or TEA as pharmacological tools to isolate IAf, IAs and IK. Unfortunately, however, the relative sensitivities of these three currents to either of the drugs are such that neither TEA or 4-AP could be used to separate the K currents. Nonetheless, the pharmacology of IAf differs from that of IAs (Fig. 3.9B). IAf was almost completely blocked (94±5% block, n=11) by 1mM 4-AP in all neurons studied, whereas IAs was only partially blocked (56±10%, n=9) at the same concentration (the block of IAs by 4-AP tended to be somewhat variable). In 5 neurons, 4-AP concentrations of up to 5mM were used, however 40% of IAs remained unblocked. This is in contrast to the pharmacology attributed to a slowly

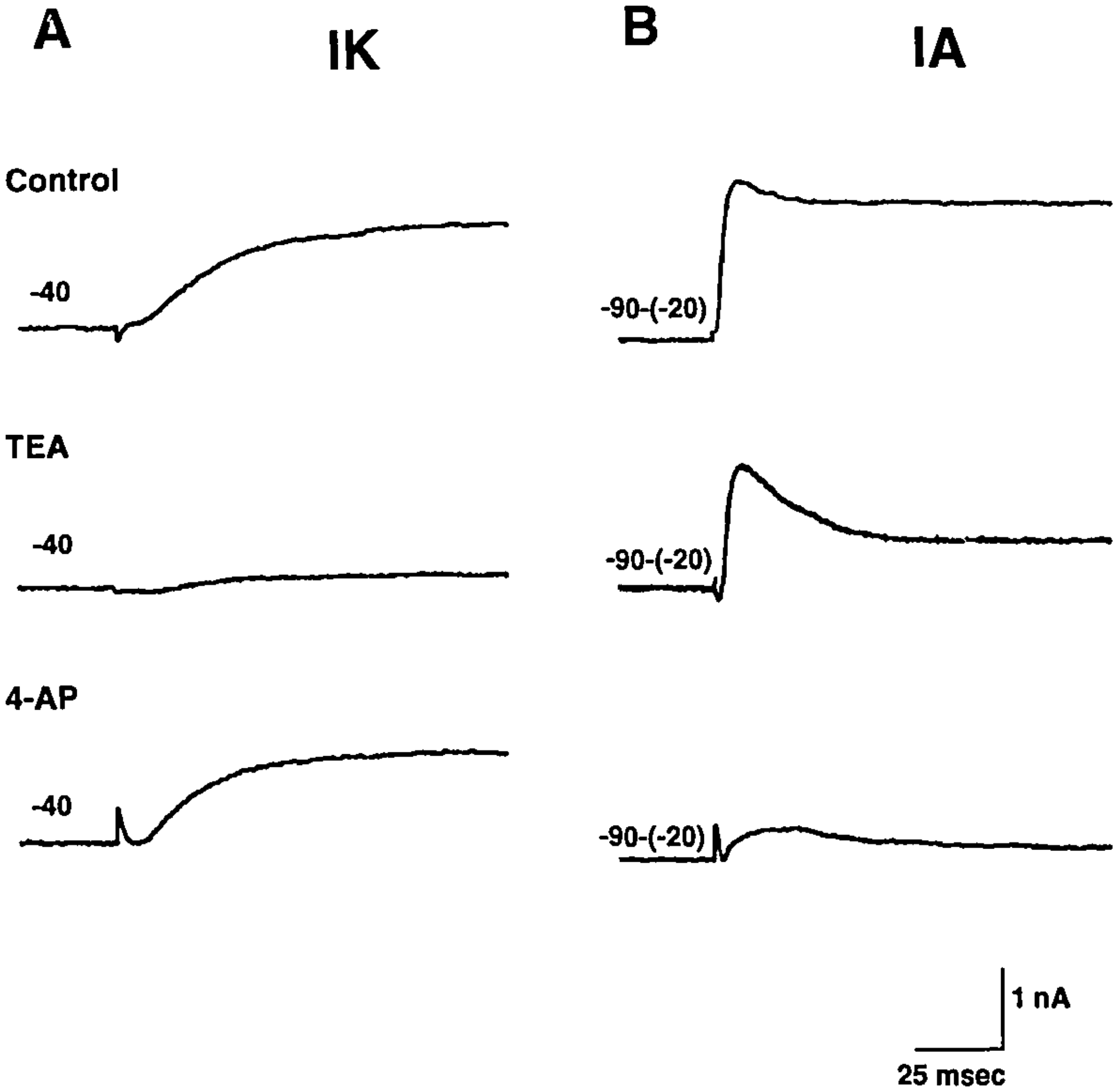

Figure 3.9: Pharmacology of Outward K Currents: Pharmacology of the outward K currents to two K channel blockers, 4-AP (1mM) and TEA (10mM). Traces are isolated IK(A) and A-currents(B) evoked by a depolarizing step to +30mV from the indicated holding potential. A: IK: In a neuron which only expressed IK (top:control), TEA (middle) decreased the current to an amplitude of 18% of control, but 4-AP (bottom) was seen to have no effect on the outward current. B: IAf and IAs: A-currents evoked from a holding potential of -90mV were isolated by subtracting the IK current evoked by a depolarizing step to +30mV, from a holding potential of -20mV (top:control). Application of TEA (middle) blocks most of IAs, but does not block IAf. Application of 4-AP in the same neuron, 20 minutes after TEA removal, is seen to block IAf (bottom). In this particular neuron 4-AP also blocked most of IAs, however in other neurons IAs was blocked to a lesser extent. Traces were filtered at 1.5kHz and sampled at 5kHz.

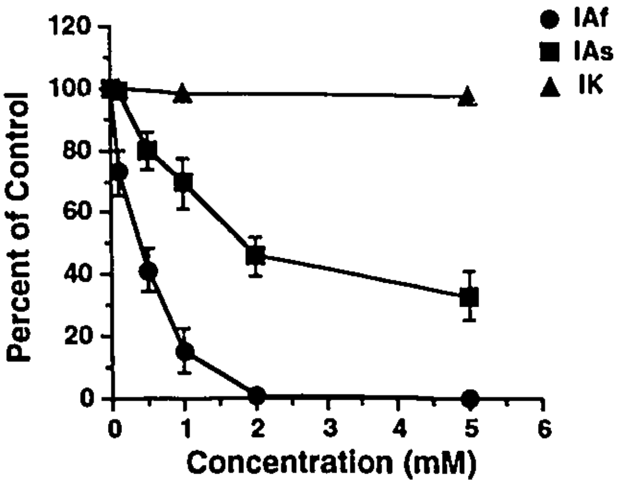

Figure 3.10: 4-Aminopyridine Dose Response Curve: IAf, IAs and IK currents in the presence of different concentrations of 4-aminopyridine (4-AP), are represented as a percentage of the corresponding control currents, and plotted against the concentration of 4-AP in mM.

inactivating K current in A-cells of adult nodose neurons, which is reportedly blocked by 100 µM 4-AP, whereas the rapidly inactivating current in C-cells of the same ganglion was unaffected (Stansfeld et al., 1986). This may suggest that IAs is different from the slowly inactivating K current in adult A-cells.

Figure 3.10 shows the 4-AP dose-response curves for IAf, IAs and IK. The sensitivity of IAf and IAs to 4-AP differs 4-5 fold: the IC_{50} (concentraion for 50% block) for IAf is 300µM, and for IAs is approximately an order of magnitude greater. More importantly, for this study, was the observation that a low concentration of 4-AP was not able to completely block IAf without affecting IAs, and as such 4-AP cannot be used as a tool to separate IAs from IAf in nodose neurons.

A difference was also observed in the sensitivity of IAf and IAs to TEA; 10mM TEA did not block IAf (n=10), whereas IAs was partially blocked by TEA (55±5%, n=11).

3.3.5 Single channels that underlie IAf and IAs

Cooper and Shrier (1989) demonstrated that on nodose neurons a single class of A-channel, with a conductance of 22pS, can inactivate in different ways. Some channels inactivated in 10-30ms suggesting that they could underlie IAf, while other channels inactivated in 100-300ms, and sometimes in 1-3s, suggesting that they could underlie IAs. However, the differences in the voltage-dependent, kinetic and pharmacological properties of the macroscopic IAf and IAs reported here, might suggest that the single channels that underlie these two currents are different, even though they share the same single channel conductance. To investigate this we measured the kinetics for inactivation and the voltage-dependence of IAf and IAs at the single channel level.

Ensemble average currents

For these experiments, we used cell-attached patches that only contained A-channels as previously described (Cooper and Shrier, 1985, 1989); that is, 22pS channels that activated and inactivated with membrane depolarization, and required hyperpolarizing steps to remove inactivation. To activate these channels, depolarizing steps were delivered from a V_h of -90 mV; steps to the same potentials from a -20 mV holding potential failed to activate the channel (see Cooper and Shrier, 1989). Of 51 patches, 35 contained only A-channels. Patches used for the analysis of voltage-dependent and kinetic properties contained 2-5 channels, all with the same conductance. In 12 of these, all the channels in the patch inactivated with the same time course. An example of a patch containing only rapidly inactivating channels is shown in Fig. 3.11A. The channels opened briefly in response to a depolarizing step and then inactivated. Ensemble averages, produced by repeatedly stepping the voltage to different levels, decayed with single exponential time courses (fit is shown superimposed), whose time constants were from 10-30ms, similar to what is seen for the macroscopic IAf (Fig 3.7A).

The patch in Fig. 3.11B contained channels that inactivated with a slow time course, such that the channels and ensemble average currents show little inactivation over the course of the 200msec step, similar to the macroscopic IAs. Most patches (23/35), however, contained channels of both types (see Fig. 3.11C). The decaying phase of the ensemble averages for this patch is well described by the sum of two exponential components (a double exponential fit is shown superimposed), with time constants corresponding to those observed for the two macroscopic A-currents.

Inactivation kinetics

There is good agreement between the inactivation kinetics for the rapidly-inactivating channels (Af) and the macroscopic IAf, as well as between the slowly-inactivating channels (As) and IAs. This can be seen in Fig. 3.7 where the time constants for inactivation of the ensemble averages (open symbols) are superimposed on data obtained for the macroscopic A-currents. The inactivation kinetics of both channels, Af and As, are voltage-dependent, becoming faster with increasing depolarization. Furthermore, as was the case for the macroscopic IAs, the inactivation

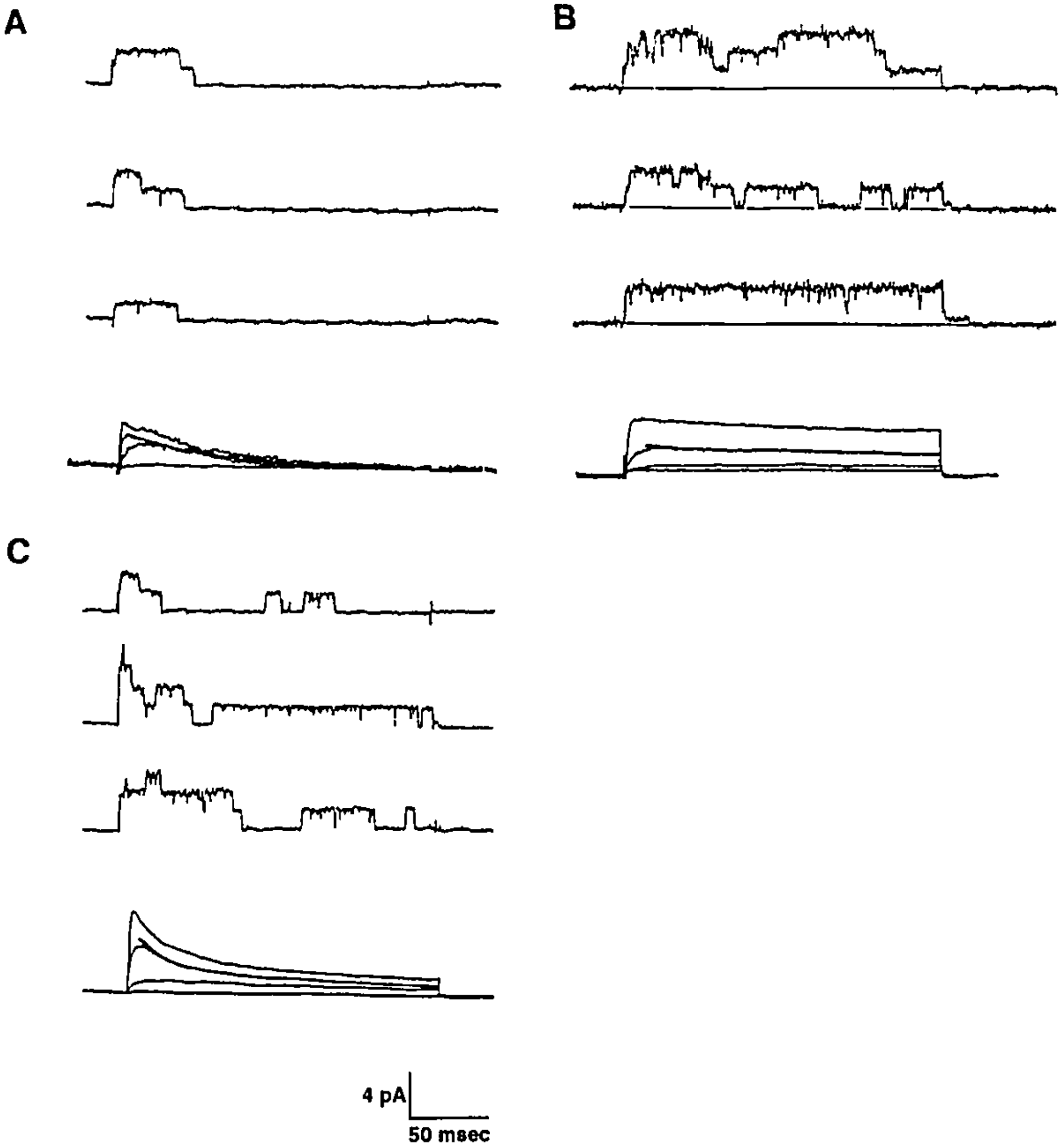

Figure 3.11: Single-channel records of As and Af channels on nodose neurons. A, B, and C show single channel recordings in the cell-attached configuration from 3 different neurons. A shows a patch with only Af channels, B shows a patch with only As channels, and C shows a patch with both Af and As channels. The upper three records in each show representative records evoked by 200 ms depolarizing steps to +40 mV from -90 mV (A), -50 mV (B) and -70 mV (C) holding potentials. (Patch potentials are expressed as membrane potentials by subtracting the pipette potential from the resting potential). The lower records are superimposed ensemble averages of 50 steps to four different potentials, -20 (lower), 0, +20, and +40 mV (upper), from a holding potential of -90 mV. The current traces were filtered at 1.5 kHz and sampled at 10 kHz, and the leakage currents and capacity transients have been subtracted. The solid lines for voltage steps to + 20 mV in A and B are single exponential decays with time constants of 21 ms, and 520 ms respectively. In C, the solid line is the sum of 2 exponentials, one with a time constant of 12.9 ms and the other 139 ms.

kinetics of the ensemble averages for the As channels could be described by a single exponential in some cases, while in others, double exponentials were needed to describe the decay.

It was difficult to measure the activation kinetics of the ensemble averages reliably because the initial parts of the ensemble averages were too fast to resolve; nonetheless, qualitatively the activation kinetics for the ensemble averages of As channels are slower than those for Af channels (compare Fig. 3.11A and B), which is consistent with our macroscopic measurements of IAf and IAs (see Fig. 3.6).

Voltage-dependence

In addition to differences in their kinetics, IAf and IAs differ in their voltage-dependence for activation and inactivation. Therefore, we measured the voltage-dependence for activation and inactivation of the rapidly-inactivating Af channels, and of the slowly-inactivating As channels. Activation experiments involved holding the membrane at -90mV and stepping repeatedly (n=50-100) to different membrane potentials. Inactivation experiments involved holding the membrane at different membrane potentials and stepping repeatedly (n=50-100) to the same membrane potential. We used the peak of the ensemble average, from patches with only fast A-channels (n=5), as a measure of the rapidly inactivating channels, and the plateau value after 150ms, in patches with only slowly inactivating channels (n=6), as a measure of the As channels. These amplitudes were converted to conductances (G) by dividing by the driving force (see equation 1). For activation, the ratio of G/Gmax was the conductance for a given step potential (G) divided by the maximal conductance (Gmax), taken as the conductance for the voltage steps to +40-+60 mV. Whereas for inactivation, the ratio of G/Gmax was the conductance for a given V_h, divided by the maximal conductance (Gmax) evoked from a V_h of approximately -90mV. The G/Gmax data for activation and inactivation are plotted vs the membrane potential (Vm) in Fig. 3.12. This figure also includes the Boltzmann distributions used to describe the data from the macroscopic currents in Fig. 3.5, to demonstrate the agreement between the single-channel data and the

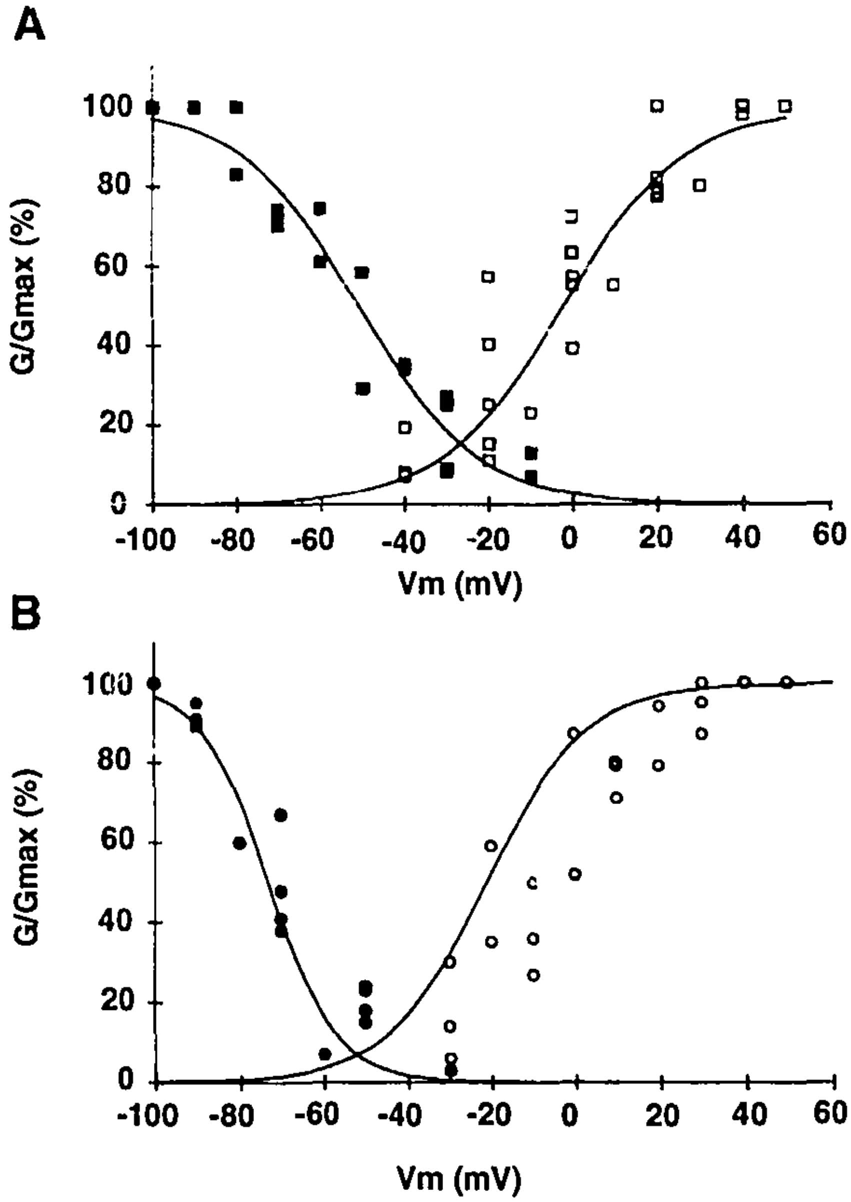

Figure 3.12: Voltage-dependence of activation and inactivation for Af and As channels. A: As channels. Filled and open squares represent steady-state inactivation and activation data, respectively, from 5 patches containing only As channels. For inactivation, each point represents the ratio of the ensemble average measured at the plateau at the end of the step to that of the maximum. For activation, each point represents the percentage of the plateau of the ensemble average divided by the driving force, to the maximum. B: Af channels. Filled and open circles represent steady-state inactivation and activation data, respectively, as in A, except that the measurements were made at the peak of the Af ensemble averages. Solid line in A and B are the fitted Boltzmann distributions for activation and inactivation of the macroscopic IAs (A) and IAf (B) as shown in Fig. 3.5.

corresponding macroscopic currents.

The Boltzmann distributions for activation (V'=-6mV, k=+12mV) and inactivation (V'=-70mV, k=-8mV) for Af ensemble averages are in good agreement with those seen for the macroscopic IAf, except that the ensemble averages activated 15mV more positive. The reason for the shift is unclear to us, however we have repeated these observations several times (n=10). Others have reported similar discrepancies between single channel data and macroscopic data (Zagotta and Aldrich, 1990b; Fernandez et al., 1984). In addition, activation experiments were always performed after inactivation experiments, and in such a way that the largest voltage steps were delivered first. If there was a decrease in channel activity during an experiment then the ensemble averages produced for subsequent voltage steps would be reduced. This would be seen as a depolarized shift in the G/Gmax vs Vm graph. In this case, one would have to postulate that the activity of Af channels is more likely to change with time than As channels.

The data for the voltage-dependence for activation (V'=-3mV, k=14mV) and inactivation (V'=-53mV, k=14mV) of As channels (shown in Fig. 3.12B) were fit with similar Boltzmann distributions as used to fit the activation and inactivation of the macroscopic IAs (see Fig. 3.5B). The agreement between single-channel and macroscopic data suggests that the differences in IAf and IAs are due to differences at the single-channel level.

Channels identified as A-type always inactivated with depolarization and exhibited steady-state inactivation, and therefore we conclude that they are different from the channels that underlie IK. In none of our patches did we identify a channel that could account for the kinetic and voltage-dependent properties of IK. In addition non-inactivating K channels seem to have smaller conductances than Af and As channels (SK, 8pS, Marty and Neher, 1985; Kx, 7pS, Hoshi and Aldrich, 1988b; DRG neurons, 5-10pS, Stansfeld and Feltz, 1988; RCK1 clone, 9pS, Stuhmer et al, 1988).

3.3.6 K currents on Superior Cervical Ganglion neurons

The results discussed above, are the first characterization of the voltage-dependent and kinetic properties of a slowly inactivating current (IAs) on sensory neurons. We were interested in determining whether IAs was a current specific to sensory neurons, or whether it was expressed on other peripheral neurons. As such, we investigated the expression of voltage-gated K currents on sympathetic neurons of the rat superior cervical ganglion (SCG) which are known to express a rapidly inactivating A-current (Galvan and Sedlmeir, 1984; Belluzzi et al., 1985; Marrion et al., 1987). The voltage-activated outward current (Fig. 3.13A) on postnatal rat superior cervical ganglion (SCG) neurons, as for neonatal nodose neurons, consists of IAf, IAs and IK. To isolate IAf and IAs on SCG neurons we did not have to take advantage of neurons that only expressed one or two of these currents, as the voltage-dependencies of IAf and IAs were sufficiently different to allow separation by holding potential. Otherwise, the methods used to isolate these currents on postnatal SCG neurons are identical to those used to separate the currents on nodose neurons.

The properties of these 3 currents are very similar to the currents expressed on nodose sensory neurons. Table 3.1 lists the properties of IAf, IAs and IK on SCG neurons, and compares them to the currents expressed on nodose neurons.

IK

IK on SCG neurons shows no steady-state inactivation, and is the only current activated by depolarizing steps when the membrane is held at depolarized potentials of -10mV to -20mV (Fig. 3.13B). This current is slowly activating, and requires quite depolarized potentials for activation. The relationship between the steady-state conductance relative to the maximum (G/Gmax), and the membrane potential was fit by a Boltzmann distribution with V'=22mV and k=10mV (see equation 2). IK does not inactivate appreciably over 6 secs, and tetraethylammonium (TEA; 10mM) blocks IK by 70% (n=4).

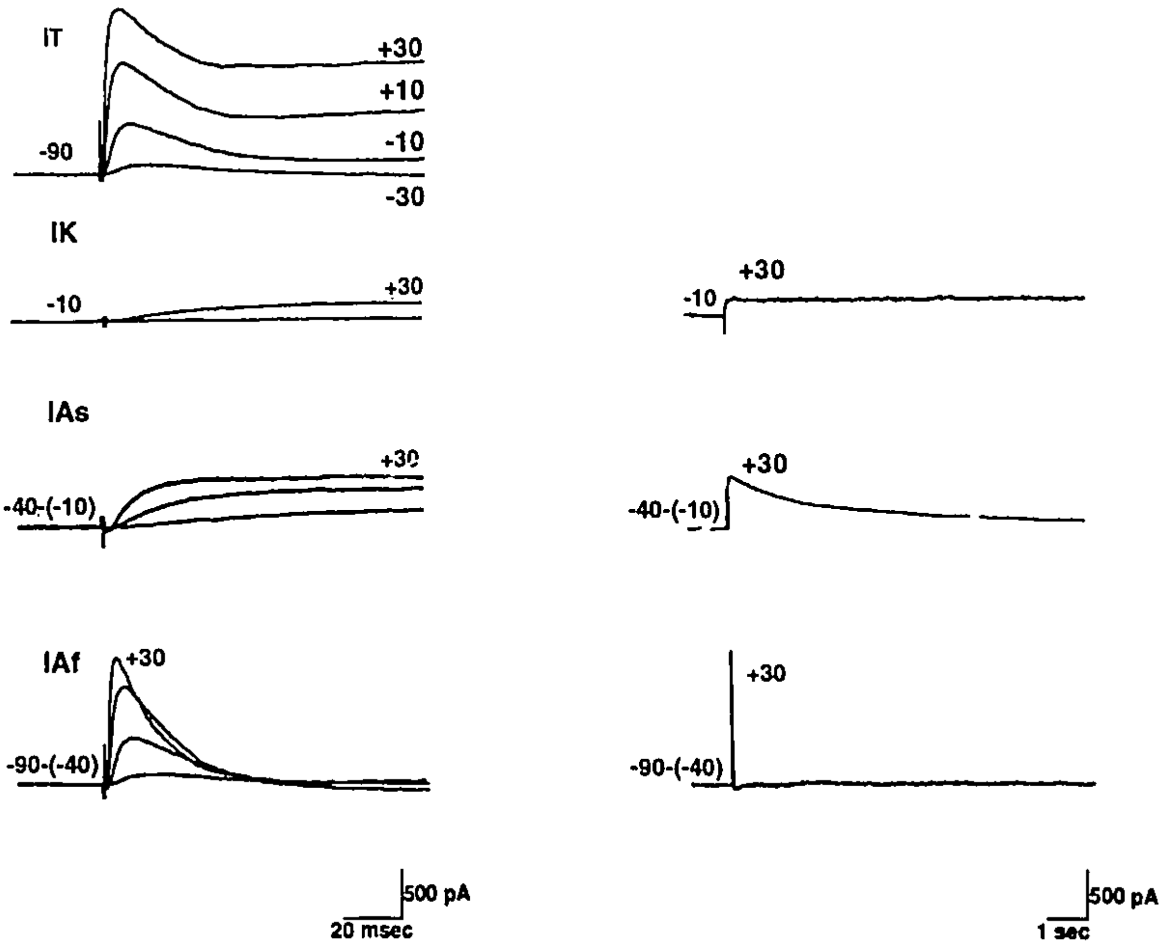

Figure 3.13: Voltage-dependent K currents on SCG neurons from a 3 day old rat: A: Total outward currents evoked by depolarizing voltage steps from a holding potential (Vh) of -90mV. B: IK: Depolarizing voltage steps from a Vh of -10mV activate only a slowly activating, non-inactivating current (IK). C: IAs: IAs is isolated by subtracting the currents evoked by depolarizing steps from a Vh of -10mV (IK) from the corresponding currents evoked from a Vh of -40mV (IK + IAs). D: IAf: IAf is isolated by subtracting currents from a Vh of -40 mV (IAs+IK) from the corresponding currents evoked from a Vh of -90mV (IAf+IAs+IK). For A-D, current traces on the left were evoked by 125ms depolarizing voltage steps in 20mV increments, up to +30mV and traces on the right are currents evoked by a 10sec depolarizing voltage step to +30mV. The leakage and capacity currents have been subtracted from all the traces. For the 125ms steps, the currents were filtered at 1.5kHz and sampled at 5kHz, and for the 10s steps, the currents were filtered at 100Hz and sampled at 200Hz.

	Nodose				SCG			
	Activation		Inactivation		Activation		Inactivation	
	V'	k	V'	k	V'	k	V'	k
IAf	-21 (6)	12	-73 (6)	-8	-1.8 (8)	12	-65 (7)	-7
IAs	-2 (13)	14	-51 (10)	14	10 (8)	11.5	-40 (10)	-7.3
IK	16 (5)	12	none		22 (4)	10	none	

Table 3.1: Comparison of the voltage-dependent properties of IAf, IAs and IK on SCG and nodose neurons: The table gives the parameters of the Boltzmann distributions (V' and k) used to describe the voltage-dependent properties for inactivation and activation, of K currents expressed on acutely dissociated neurons. from one day old animals. V' is the voltage required for either half maximal inactivation, or half maximal activation of the current, and k is a slope factor. The numbers in brackets refer to the number of neurons.

IAs

The slowly-inactivating IAs has not been previously described in mammalian sympathetic neurons, although from examination of published records it appears that IAs is present (Galvan and Sedlmeir, 1984; Belluzzi et al., 1985), and in fact, as discussed in Chapter 1, may have been mistaken for IK. Our results from whole-cell experiments on SCG neurons indicate that neonatal rat SCG neurons express IAs. In fact, IAs is the predominant voltage-gated K current in the majority of the neurons from one day old animals (see Chapter 4).

Figure 3.13C shows IAs isolated by V_h in an SCG neuron which expressed all 3 currents. IAs is more rapidly activating than IK, and inactivates slowly with a single time constant of 4s±0.3s (mean±s.e.m, n=6) for a step to +30 mV. In addition, IAs activates at more hyperpolarized potentials than IK, and shows steady-state inactivation. The voltage-dependent data for activation and inactivation were fit with Boltzmann distributions and are compared in Table 3.1 to the values obtained for the Boltzmann fits of the voltage-dependence data for IAs on nodose neurons.

IAf

IAf was fairly easy to separate from IAs on SCG neurons. For the majority of neurons, IAs shows little steady-state inactivation over the range of potentials between -90mV and -40mV, whereas IAf is completely inactivated at a potential of -40mV. Thus, subtraction of currents evoked from a potential of -40mV, from currents evoked by steps to equivalent potentials from a V_h of -90mV isolates the fast A-current (Fig. 3.12D). This is different from what is observed for IAf and IAs on nodose neurons, where there is considerable overlap of their ranges of steady-state inactivation, therefore making isolation by subtraction more difficult.

The IAf current on SCG neurons is more rapidly activating than IAs and inactivates rapidly with a time constant of 15ms±1ms (mean±s.e.m, n=6) for a step to +30 mV. The current has a lower threshold for activation than IAs, and is over half inactivated at the resting potential of these cells (-50 to -60 mV). Activation and inactivation data for IAf were fit by Boltzmann distributions, and the values of V' and k are compared to those obtained for nodose neurons in Table 3.1.

3.4 Discussion of Results

3.4.1 Summary

In this chapter, I have characterized three K currents on neonatal rat nodose neurons: a slowly inactivating current (IAs); a rapidly inactivating current (IAf); and, a non-inactivating current (IK). These three currents are also present on neonatal SCG neurons.

Most nodose and SCG neurons express all three currents. In nodose neurons, the relative amount of each current varied considerably. A significant number of nodose neurons lacked one or two of the currents facilitating the characterization of each current in isolation. In addition, because these currents differ in their steady-state inactivation properties, it was often possible to separate them by holding the membrane at different potentials and using subtraction protocols. We also isolated IAf and IAs by recording the single channels that underlie these currents.

SCG neurons also express these three voltage-gated K currents. However, there is less variability in the relative levels of expression of the currents as compared to nodose neurons: IAs is the predominant voltage-gated K current for the majority (67%, n=46) of neurons from one day old animals. On SCG neurons, steady-state inactivation of IAs was shifted to more depolarized potentials as compared to nodose neurons, and as such, we could isolate each one of the currents using holding potential and subtraction protocols, even in neurons where all 3 currents were present.

3.4.2 Properties of voltage-dependent currents on peripheral neurons

Delayed Rectifier, IK:

The voltage-dependence for activation of IK is similar to that on other peripheral neurons (Kostyuk et al., 1981). IK on nodose neurons activates at -10mV and is half maximal at +16mV.

The current activates slowly; its rate is approximately two orders of magnitude slower than IAf. In addition, IK on nodose neurons is blocked by 10mM TEA, but is not sensitive to 1mM 4-AP (Thompson, 1977). In terms of its voltage-dependent, pharmacological and kinetic properties, IK expressed by SCG neurons is almost identical to that on nodose neurons.

As discussed in Chapter 1 there is some discrepancy in the literature about whether IK inactivates (reviewed by Rudy, 1988). In some studies, IK has been reported to undergo slow (1-2 secs) inactivation (Segal and Barker, 1984; Belluzzi et al., 1985; Sah et al., 1988), whereas in others, IK does not inactivate, at least over tens of seconds (Kostyuk et al., 1981). Our results illustrate that IK on mammalian peripheral neurons is a non inactivating current; we feel that the slow inactivation (τ=1-3s) that is apparent from some records in the literature, may be explained by a contamination of IK by IAs (for example, see Fig. 3.4A). In fact, while Nerbonne and Gurney (1989) described a delayed rectifier on neonatal SCG neurons, our data from neonatal SCG neurons indicates that most of the outward voltage-gated K current is IAs, and that IK makes up very little of the outward current (see chapter 4).

A-currents; IAf and IAs:

IAf on nodose neurons has kinetic and voltage-dependent properties similar to fast A currents on other mammalian neurons (Kostyuk et al., 1981; Segal and Barker, 1984; Zbicz and Weight, 1985; Surmeier et al., 1989). The currents activate rapidly, inactivate with a single exponential time course whose time constant is 10-30ms, and are blocked by 4-AP.

IAs differs from IAf and IK in its kinetics, voltage-dependence and its sensitivity to 4-AP and TEA. IAs is only partly blocked by 1mM 4-AP, and unlike IAf, it is also partly blocked by 10mM TEA. IAs activates rapidly compared to IK, but at a rate that is about 5 times slower than IAf. Inactivation of IAs occurs with 2 components, one with a time constant of 150-300ms and the other with a time constant of 1-3 seconds. In most neurons, both inactivation components are apparent, but in some neurons, IAs inactivates with only one component. This may suggest that IAs is actually made up of 2 similar channels that differ in their rate of inactivation. This is supported by the observation that only the slower exponential is required to describe the inactivation of IAs on SCG neurons. However, a recent report showed that a single rat brain K channel mRNA (RBK-1) injected into Xenopus oocytes results in the expression of a K current with 2 components of inactivation (50-100ms; 5-10s) (Christie et al., 1989), suggesting that one channel subtype can have 2 rates of inactivation.

On nodose neurons, IAs activates at more depolarized voltages than IAf: it is only half activated at -2mV, compared to -21 mV for IAf. SCG neurons show a similar difference in the voltage-dependence for IAf and IAs. However, the voltage-dependence for activation of IAf and IAs on neonatal SCG neurons is shifted by 15-20mV to more depolarized potentials as compared to nodose neurons: IAs is half activated at 10mV, and IAf is half activated at -2mV. Thus the threshold for activation of A-currents on neonatal SCG neurons is higher than that observed for similar currents on nodose neurons.

IAs and IAf on peripheral neurons also differ in their voltage-dependence for steady-state inactivation. IAs on nodose neurons appears to be less steeply dependent on voltage than IAf (k=-14mV vs k=-8mV) and the voltage range for steady-state inactivation is shifted approximately 20mV more positive than for IAf. IAs on SCG neurons shows a similar shift in its voltage-dependence of inactivation as compared to IAf. However, the voltage-dependencies of inactivation on SCG neurons are shifted by about 10 mV to more positive potentials as compared to those for IAf and IAs on nodose neurons.

3.4.3 Functional significance of A-currents on peripheral neurons

The differences in the voltage-dependencies for IAf and IAs indicate that the two inactivating currents will be available at different potentials, and as such could serve different roles. In SCG neurons, IAf and IAs are available at the resting potential (-55 to -60 mV). SCG neurons receive synaptic inputs on to their cell bodies, and as such, IAs may decrease cellular excitability by acting as a partial shunt for membrane depolarizations. Slowly inactivating currents, similar to IAs, have been described for other neurons and are thought to be important in regulating neuronal excitability (Stansfeld et al., 1986; Lukasiewicz and Werblin, 1988; Storm, 1988). Whereas, IAf on sympathetic neurons is involved in controlling the rate of repolarization of the AP (Belluzzi et al., 1985; Nerbonne and Guerny, 1989).

It is unclear what the functions of IAf, IAs and IK are for nodose neurons, whose cell body receives no synaptic contacts. If one makes the assumption that the K currents expressed on the cell body reflects what is seen at the nerve terminals, then these currents may play an important role in the initial encoding of sensory information. It is important to note that at the resting potential of nodose neurons (resting potential around -55mV) IAf is mainly inactivated, whereas, only a small proportion of IAs is inactivated, which suggests that only IAs plays a role in controlling the excitability of resting nodose neurons.

The results discussed in this chapter suggest that there is a large variation in the proportion of IAs and IAf expressed on different nodose neurons. Similar results are observed for adult nodose neurons (Stansfeld and Wallis, 1985) and for transient K currents expressed on identified molluscan neurons (Serrano and Getting, 1989). It seems likely that the variation in K current expression is related to the neurons' function in vivo: Nodose neurons provide sensory innervation to a number of different peripheral targets, such as the heart, the lungs, the trachea, and other visceral structures. It may be that the different targets of nodose neurons influence which K currents they express in their membrane. For example, the terminals of some neurons act as rapidly adapting receptors, while others act as slowly adapting receptors (Paintal, 1973). One would expect that different K currents are needed to serve these two functions.

A study of the firing behaviour of three types of neuron from the nucleus tractus solitarius

(NTS) (Dekin et al., 1987) supports the idea that variability in K current expression on neurons reflects the need for different excitable behaviours. Neurons from the NTS exhibit different firing behaviours, dependent on the relative proportions of at least 3 different K currents in their membranes. In this light, it is interesting that considerably less variation is observed for the proportions of IAf, IAs and IK on neonatal SCG neurons: IAs is the predominant current in two thirds of the neurons. This may indicate that electrically SCG neurons are more homogeneous. This idea is supported by data that demonstrates that neurons of a given sympathetic ganglion behave similarly in response to a depolarizing input; the majority of neurons of the lumbar sympathetic chain fire transiently, while neurons of the inferior mesenteric ganglion fire tonically (Cassell et al., 1986).

3.4.4 Single channels underlying IAf and IAs

The most frequently observed K channels in our patches were A-channels (Af and/or As channels). This observation is consistent with our macroscopic whole-cell current data indicating that most of the outward current consists of IAf and IAs and that IK usually represents only a minor component of the outward current on these neurons. Similar observations have been made for A-channels (A_1) and delayed rectifier type channels (Kd) in Drosophila muscle (Zagotta et al., 1988).

In this study, we showed that some patches only contained channels that inactivated rapidly (Af channels) and whose ensemble averages decayed with kinetics that corresponded to those of IAf, and we showed that other patches only contained slowly inactivating channels (As channels) whose ensemble averages kinetics corresponded to those of IAs. Furthermore, we showed that the voltage-dependence for activation and inactivation of Af channels are similar to those for IAf, and the voltage-dependent properties of As correspond to those for IAs. These results indicate that Af channels are different from As channels, even though they have the same open channel conductance.

One possible explanation for the different properties of IAs and IAf could be that a single channel type is subject to modulation by second messengers. There is considerable evidence for modulation of channel function by second messengers (see review by Kaczmarek and Levitan, 1987; Levitan, 1988; Rudy, 1988). However, because the channels differ in their pharmacology, kinetics and voltage-dependence a more likely explanation for these findings is that Af channels and As channels are separate, but related channels. In this case, it may be that the region of the protein responsible for the channel pore is conserved between the two channels, and those domains that are responsible for channel kinetics and voltage-dependence are different (see review by Miller, 1991; also Chapter 1).

A number of other lines of evidence support the idea that Af and As are separate channels: First, some neurons were found to express only Af channels, or As channels; Second, channels that resemble the As channel on rat nodose neurons, in terms of single channel conductance and kinetic behaviour, are also present on bovine adrenal chromaffin cells (FK channel, 18-20pS, Marty and Neher 1985); Third, recent molecular experiments show that two A-channel subtypes sharing the same single channel conductance can differ in a number of their physical properties (Stocker et al., 1990). Drosophila Shaker cDNAs that differ only in their amino termini (ie. share the same core region and carboxyl termini) when expressed in Xenopus oocytes were shown to share the same single channel conductance, but differed in their kinetics for inactivation, in their sensitivity to blockade by 4-AP, TEA and charybdotoxin, and their voltage-dependence for activation; Finally, evidence presented in Chapter 4 indicates that IAf, IAs and IK are differentially regulated during development, supporting the idea of separate, but related K channels.

Chapter 4

Developmental expression of IAf, IAs and IK on postnatal peripheral neurons

4.1 Introduction

A number of different voltage-gated potassium channels are present in neuronal membranes (Rudy, 1988). These channels play a central role in controlling a neuron's excitability (see reviews by Thompson and Aldrich, 1980; Adams and Galvan, 1986). Changes in the relative number and spatial distribution of the voltage-activated K channels expressed by a neuron will have important implications for its electrical behaviour.

Such changes are known to occur during embryonic neuronal development (Spitzer, 1979; Bader et al., 1985; Barish, 1986; Nerbonne and Gurney, 1989; O'Dowd et al., 1988). However, it is not known how the specific temporal, spatial and quantitative changes in the expression of different K channels are regulated: Is the control intrinsic to the neuron, or do extrinsic factors play a role? Are there set patterns for the changes in K channel expression, or do the patterns differ for the various types of neuron? Does any of the developmental control of K channel expression continue postnatally?

Neonatal SCG neurons have several advantages for studies investigating developmental control of K current expression. First, there is already some evidence that rat SCG neurons regulate their expression of voltage-gated K channels during development (Nerbonne et al., 1986; Nerbonne and Gurney, 1989). Nerbonne and co-workers showed that while a delayed rectifier type current is present at embryonic day 14, the rapidly inactivating K current in these cells does not appear until embryonic day 16.

A second advantage with rat SCG neurons is that several researchers have studied developmental changes in their morphology, presynaptic innervation and target innervation

(Ruben, 1985 a,b,c; Smolen and Raisman, 1980; Purves, 1975), and it is known that considerable rearrangement and refinement of both pre and post synaptic contacts occurs over the first few postnatal weeks: there is a significant increase in synapse formation by the preganglionic nerve terminals (Smolen and Raisman, 1980), from 10% to 80% of the adult synapses over the first 3 weeks postnatally; dendritic arborization increases 400 fold (Voyvodic, 1987; Bruckenstein et al., 1989); target innervation increases (Black and Mytilineou, 1976), and; ganglionic nonneuronal cells proliferate (Hendry and Campbell, 1976). Possibly, the expression of voltage-gated K currents changes over this period in response to the alterations in the environment of the neurons.

A third advantage is that the preganglionic and postganglionic nerves are readily accessible for experimental manipulation which makes it possible to examine the effects of preganglionic innervation and target factors on the expression of K currents, either by denervating or axotomizing the SCG neurons. The final advantage is that these neurons grow well in tissue culture (Mains and Patterson, 1973; O'Lague et al., 1978; Hawrot, 1980). This makes the SCG particularly attractive for studying factors affecting the expression of K currents during development.

In this chapter, I discuss experiments that investigated the postnatal expression of voltage-gated K currents on rat sympathetic and sensory neurons. Using the subtraction protocols established in Chapter 3, IAf, IAs and IK on SCG and nodose neurons were separated, and their levels of expression quantified during the first two weeks of postnatal development. I show that for SCG neurons there is a switch from an IAs to an IAf dominated outward current, whereas, there is little or no change in the expression of the three voltage-gated K currents on postnatal nodose neurons.

4.2 Methods

Nodose and superior cervical (SCG) ganglia were removed from rat pups 1, 3, 7 or 14 days postnatally. The ganglia were dissociated and the neurons cultured as described in Chapter 2. Voltage-gated K currents were recorded on acutely dissociated neurons (2-24 hours in culture), using whole-cell voltage-clamp techniques (Hamill et al., 1981), after inward Na and Ca currents, and the Ca-dependent K current were blocked pharmacologically.

The current densities of the three K currents were measured on nodose and SCG neurons at different times during the first 2 weeks of postnatal development. IAf, IAs and IK currents evoked by a 125 ms voltage step to +30 mV were isolated using the subtraction protocols established in Chapter 3. Measurements of IAf were made at the peak of the current, approximately 5-10ms after the beginning of the depolarizing voltage step. For IAs and IK, the amplitude measurements were made at the plateau, 125 ms after the beginning of the step since these currents showed little, or no inactivation during the first 125 ms; any contaminating IAf current was completely inactivated by this point. In some neurons, IK currents evoked from a holding potential of -20 mV or -10 mV were contaminated by some remaining IAs that was not inactivated at this potential. In these neurons, measurements of IK were made at the end of a 6-10sec depolarizing pulse to +30mV, at which point IAs had completely decayed and only the sustained IK current remained. For current density (pA/pF) measurements the isolated currents (pA) evoked by a depolarizing step to +30mV were divided by cell capacitance (pF), obtained by integrating the capacity current evoked by a 10mV hyperpolarizing voltage step.

4.3 Results

4.3.1 IAf, IAs and IK on postnatal SCG neurons

4.3.1.1 Switch from IAs to IAf dominated outward current

Figure 4.1A shows the total voltage-gated outward current from a postnatal day one (P1) SCG neuron evoked by 125 ms (left) or 6 sec (right) depolarizing steps from a holding potential (V_h) of -90mV. As discussed in chapter 3, the voltage-gated outward K current is made up by 3 distinct currents: a non-inactivating, delayed rectifier-like current(IK), and slowly(IAs) and rapidly(IAf) inactivating A-currents. This figure demonstrates that on the P1 neuron most of the outward current inactivates slowly and only a small rapidly inactivating component is evident. Of the three currents, IAs is the predominant current on P1 neurons and makes up, on average 67% (n=42) of the total outward current.

In contrast, on neurons from postnatal day 14 (P14) animals, IAf is clearly evident; this is shown in Fig. 4.1B. This figure shows that a large fraction of the outward current on P14 neurons inactivates rapidly, but a smaller slowly inactivating component is also present.

4.3.1.2 Current density measurements of IAf, IAs and IK on postnatal SCG neurons

To quantify the changes in the expression of the outward current over the first two postnatal weeks, we measured the current densities (pA/pF) for IAf, IAs and IK on SCG neurons at 4 different postnatal times: P1, P3, P7 and P14. Figure 4.2A shows the distributions of IAf, IAs and IK current densities on 47 neurons from P1 animals and 62 neurons from P14 animals. The distribution of IAf current densities on P1 neurons is skewed to the right, and in over 50% of the neurons IAf is less than 20pA/pF. By comparison, IAf current densities on P14 neurons have increased significantly; fewer than 10% of the neurons have current densities less than 20pA/pF,

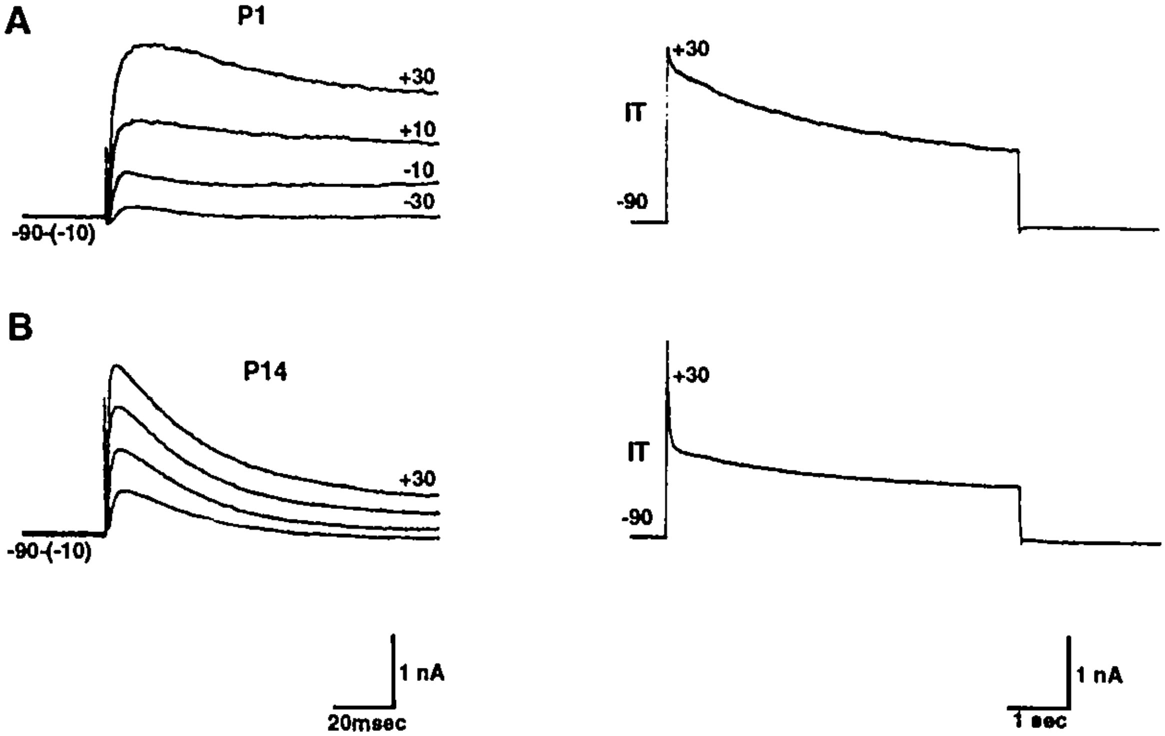

Figure 4.1: Changes in IAf and IAs expression during postnatal development: K currents on SCG neurons from P1 (A) and P14 (B) rats. The inactivating currents evoked from a Vh of -90mV are shown for acutely dissociated SCG neurons. The traces on the left show the total IAf and IAs currents (IK is subtracted) evoked by 125ms depolarizing pulses up to +30mV, in 20mV increments. The traces on the right show the total outward currents, uncorrected for leakage, evoked by a 6s depolarizing pulse to +30mV. IAs is the predominant voltage-dependent K current for P1 neurons, whereas IAf is clearly evident on P14 neurons. For the 125ms steps, the currents were filtered at 1.5kHz and sampled at 5kHz, and for the 6s steps, the currents were filtered at 100Hz and sampled at 200Hz.

and over 80% have IAf current densities greater than 20pA/pF, of which >20% have IAf expressed at >100pA/pF.

In addition, Fig. 4.2A indicates that on P1 neurons, peak IAs current densities are larger than for IAf, but decrease over the next two weeks. This is particularly evident in the proportion of neurons with current densities greater than 100pA/pF: 25% of the P1 neurons have IAs current densities greater than 100pA/pF, as compared to 2% of the P14 neurons.

While IAf and IAs expression changes during the first two weeks of postnatal development, IK current densities on P1 neurons are similar to those on P14 neurons, as shown in Fig. 4.2A. Of the 3 currents, IK was the smallest; almost 80% of the neurons had IK current densities less than 20pA/pF.

The mean current densities obtained from the distributions for IAf, IAs and IK for P1 and P14 neurons (shown in Fig. 4.2A), as well as from the distributions for P3 and P7 neurons (not shown), are plotted in Fig. 4.2B. On P1 neurons, the mean IAs current density is 3 fold greater than IAf, and 5 fold greater than the mean IK current density. Over the next 3 days, IAf increases 2 fold, while IAs decreases by 30-40%. There is no significant change in IK. By 2 weeks, the mean IAf current density is now 2 fold greater than the mean IAs current density, resulting in a 6 fold change in the IAf/IAs ratio over the two week period. This change in the ratio is evident in comparing the K currents shown in Fig. 4.1A and Fig. 4.1B.

4.3.1.3 Properties of K currents on developing SCG neurons

Whereas IAf and IAs current densities change considerably over the first two weeks of postnatal development, we did not observe any developmental changes in the kinetics of activation, or inactivation, of these currents. Table 4.1 compares the Boltzmann parameters (V' and k) obtained by fitting Boltzmann distributions to the data for the voltage-dependence of activation and inactivation for P1 and P14 SCG neurons (see methods of Chapter 3 for details of

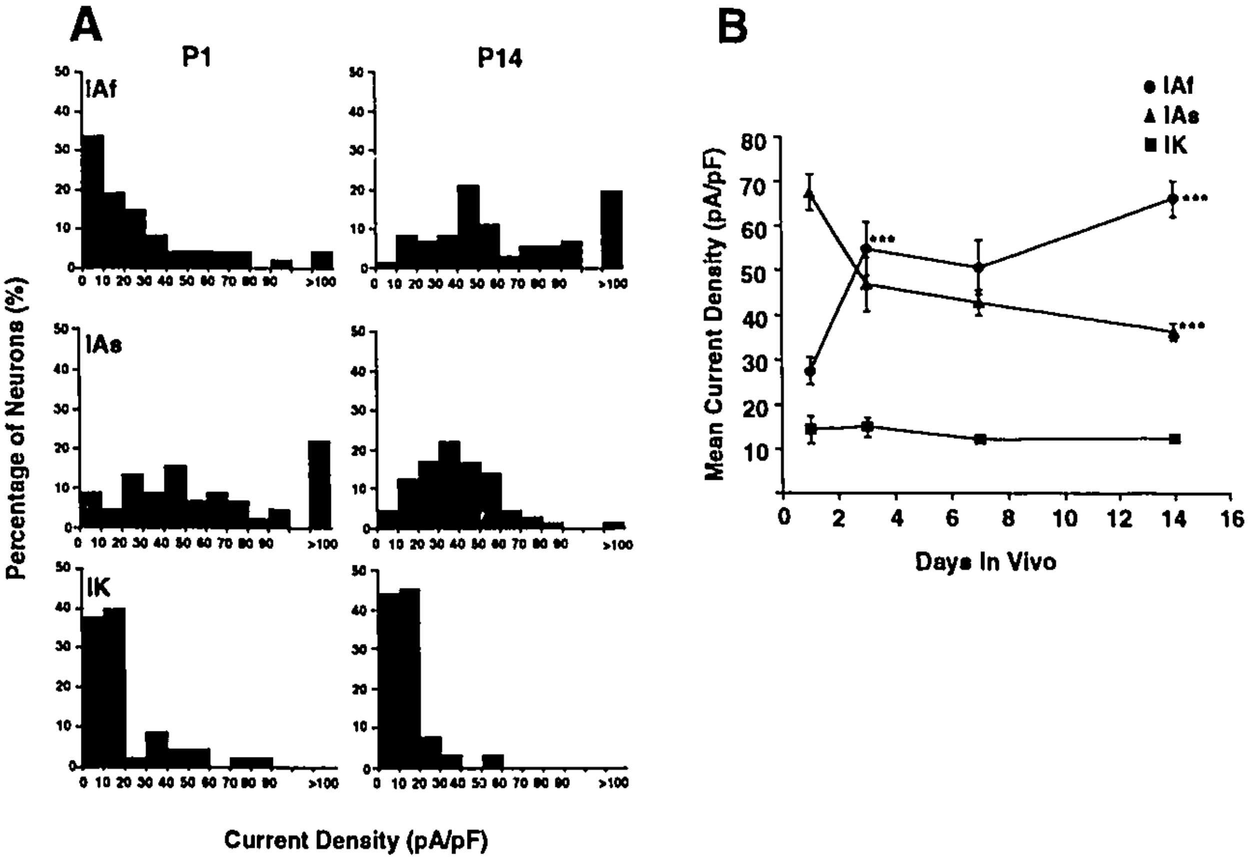

Figure 4.2: K current densities on SCG neurons change during development in vivo: A: shows the current density distributions for IAf (top), IAs (middle) and IK (bottom) for 47 P1 neurons (left), and 62 P14 neurons (right). Current density (pA/pF) was measured as the peak isolated K current (pA) divided by the membrane capacitance (pF). B: shows the mean current densities, as calculated from the density distributions, of IAf, IAs and IK from P1 (n=47), P3 (n=32), P7 (n=40) and P14 (n=62) SCG neurons. Error bars are standard error of the mean (s.e.m). In vivo, there is a significant increase (***, $p<0.001$) in IAf by the third day, and a significant decrease in IAs (***, $p<0.001$) by 2 weeks. There is no significant difference ($p>0.2$) between the mean densities of IK from neurons at different postnatal ages.

	P14				P1			
Current	Inactivation		Activation		Inactivation		Activation	
	V'(mV)	k(mV)	V'(mV)	k(mV)	V'(mV)	k(mV)	V'(mV)	k(mV)
IAf	-59 ± 0.2(7)	-6.1 ± 0.1	-4 ± 1(6)	12 ± 0.4	-65 ± 1 (7)	7 ± 0.6	-1.8 ± 1(8)	12 ± 1
IAs	-24 ± 0.5(8)	-9.2 ± 0.4	15 ± 0.8(7)	11 ± 0.5	-40 ± 1 (10)	-7.3 ± 0.5	10 ± 1(8)	11.5 ± 0.4

Table 4.1: Activation and inactivation properties of IAf and IAs: The table gives the values for the parameters of the Boltzmann distributions (V' and k) used to describe the voltage-dependent properties for inactivation and activation of K currents expressed on acutely dissociated P1 and P14 SCG neurons. V' is the voltage required for either half maximal inactivation, or half maximal activation of the current, and k is a slope factor. The numbers in brackets refer to the number of neurons.

experimental protocols). There were no significant changes in the voltage-dependencies for activation or steady-state inactivation of IAf and IAs over the 2 week period, except that the inactivation curve of IAs on P1 neurons was shifted 15-20mV more negative; this shift, however, had no effect on the measurement of IAs current density because measurements were always made of the maximal IAs.

4.3.2 Expression of IAf, IAs and IK on postnatal nodose neurons

The levels of expression of IAf, IAs and IK were measured on nodose neurons over the first two postnatal weeks. Figures 4.3A and B show the voltage-gated K currents expressed by a representative P14 nodose neuron; A shows the total A-current, evoked by 125 ms depolarizing voltage steps from a V_h of -90mV, while B shows the total outward current evoked by a 6 s depolarizing step to +30mV from the same V_h. Comparisons of K current densities between P1 and P14 neurons are complicated somewhat by the variability in K current expression among different nodose neurons. Figure 4.4 compares the mean current densities for IAf, IAs and IK observed on P1 and P14 nodose neurons. IAf and IAs levels were not significantly different ($p>0.1$, t-test) between P1 and P14 neurons, although there was a small but significant ($p<0.01$, t-test) decrease in IK. However, unlike SCG neurons, there was no striking qualitative change in the outward current profile during the first two weeks of postnatal development (compare Fig. 4.3 with Fig. 3.1).

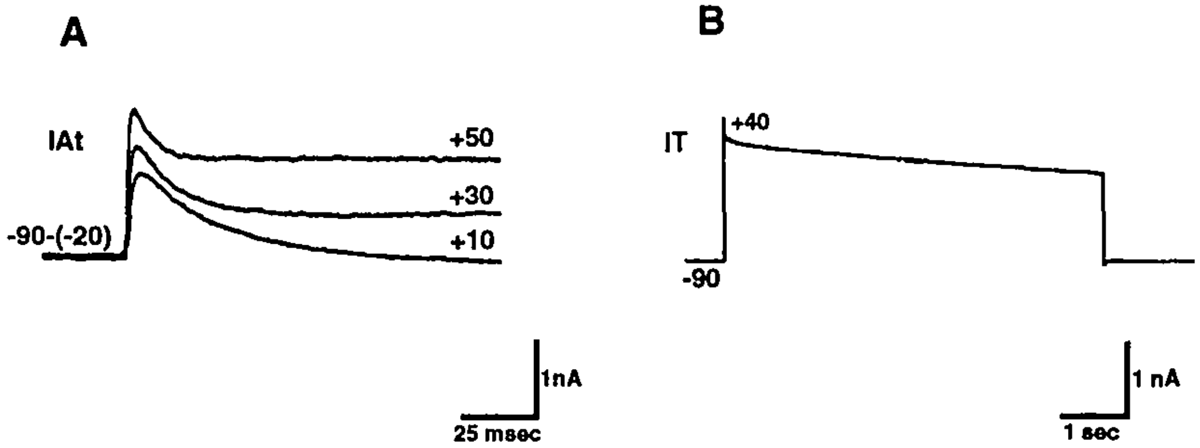

Figure 4.3: K currents expressed on a P14 nodose neuron: A: shows the total A-currents evoked by depolarizing voltage steps up to +50 mV in 20 mV increments, from a Vh of -90mV. IK currents evoked by depolarizing steps to the equivalent potentials, from a Vh of -20mV were subtracted from the traces to isolate the total A-current. A -70mV hyperpolarizing step from a Vh of -20mV was added to the traces to remove capacity and leakage currents. Traces were filtered at 1.5 kHz, and sampled at 5 kHz. B: non-leakage subtracted total outward current evoked by a 6 sec depolarizing voltage step to +40mV. The current was filtered at 100Hz and sampled at 200Hz.

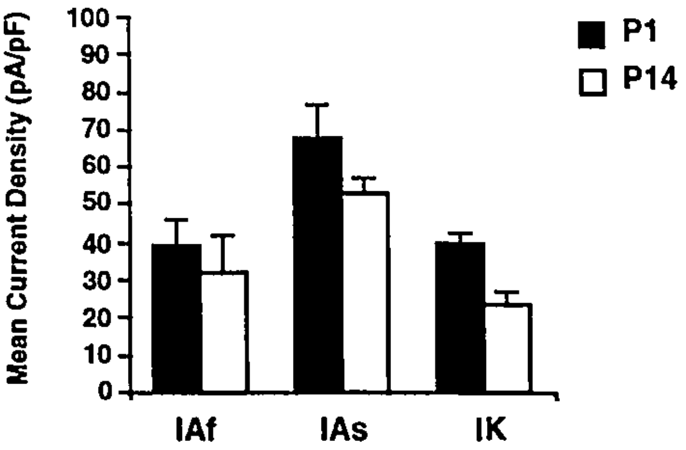

Figure 4.4: Developmental expression of IAf, IAs and IK on nodose neurons in vivo: shows the mean current densities (pA/pF) for IAf, IAs and IK expressed on P1(n=30) and P14(n=12) neurons. There is no significant difference between the IAf and IAs current densities (p>0.1, t-test) measured for P1 and P14 neurons. All recordings were performed 2-48 hours after plating. Errors are s.e.m.

4.4 Discussion of Results

4.4.1 Switch in inactivating K current types during postnatal developmental of SCG neurons

The results in this chapter demonstrate that there is a change in the pattern of expression of IAf and IAs on rat SCG neurons during the first two weeks of postnatal life. All three voltage-gated K currents are found on P1 neurons, however, IAs is the predominant current; it is expressed at a density 3 fold greater than IAf and 5 fold greater than IK. IAf expression increases 2 fold within the first 3 postnatal days and continues to increase more gradually over the next 10 days. IAs, on the other hand, decreases over the first week to 50% of its P1 value. As a result, on P14 neurons, IAf is the predominant current and the ratio of IAf to IAs has increased 6 fold from the P1 value. IK is expressed at the lowest density, and furthermore, we observed no change in expression of IK over the 2 week period.

These findings are consistent with a previous developmental study of K currents on embryonic SCG neurons (Nerbonne et al., 1986). Their study demonstrated that IAf develops 2 days later than non-rapidly inactivating K currents, which may explain our finding that IAf is smaller than IAs on P1 neurons. Developmental changes in IAf continue postnatally as IAf levels increase over the first two weeks after birth. This result is consistent with several studies which showed that adult rat SCG neurons express a large rapidly inactivating A-current that has properties similar to IAf (Galvan and Sedlmeir, 1984; Belluzzi et al., 1985; Marrion et al, 1987).

The pattern of expression of fast transient currents on rat SCG neurons appears different from that of Drosophila flight muscle (Salkoff and Wyman, 1981a), or neurons from the quail mesencephalic neural crest (Bader et al., 1985), where a fast A current develops before other K currents. In fact, in the delayed appearance of IAf, the pattern for SCG neurons seems more similar to that of amphibian spinal neurons (Ribera and Spitzer, 1990). Slowly inactivating currents in other systems are also developmentally regulated; a slowly inactivating current present on P1 rat pineal cells disappears by the fourth postnatal week (Aguayo, 1989). However, on rat neostriatal neurons a slowly inactivating current first appears and then increases over this same time period (Surmeier et al., 1991).

4.4.2 Peripheral neurons differ in their control of IAf, IAs and IK during postnatal development

In addition, our results indicate that there are clear differences observed in the developmental expression of voltage-gated K currents in sensory and sympathetic neurons of the rat peripheral nervous system. During the first two weeks of postnatal development there are few, if any, qualitative or quantitative changes in the K currents expressed on nodose neurons. These results from nodose neurons suggest that much of the regulation of K current expression is occurring embryonically, whereas, for SCG neurons at least part of the regulation occurs postnatally. The K currents on SCG and nodose neurons, and on neostriatal neurons (Surmeier et al., 1991) appear similar in terms of their macroscopic properties, and yet, their expression on each type of neuron is regulated differently. These results indicate that changes in K current expression can occur embryonically and/or postnatally, and that neurons show distinct developmental sequences of appearance and disappearance of similar voltage-gated K currents.

4.4.3 Effects of developmental changes in IAf, IAs and IK on the electrical properties of SCG neurons

Since IAf and IAs have different kinetic and voltage-dependent properties, changes in the K current profile might result in changes in the electrical behaviour of neurons during development. This is true of other systems (Barish, 1986; Yool et al., 1988; Spitzer, 1985; Surmeier et al., 1991). IAf is half inactivated at the resting potential of SCG neurons (-60mV), whereas IAs channels are almost all available for activation. On P1 neurons a large slowly inactivating current could serve to shunt out synaptic depolarizations. Slowly inactivating currents have been implicated in such a role in other systems (Storm, 1988; Lukasiewicz and Werblin, 1988). On P14 neurons however, one might expect that the decrease in IAs would make the cells more responsive to depolarizing inputs. In addition, a large IAf would serve to quicken the repolarization of the AP, a role IAf reportedly serves in SCG neurons (Belluzzi et al., 1985; Nerbonne and Gurney, 1989).

4.4.4 Possible mechanisms involved in changes in K current densities during development

While these studies show that K channels on SCG neurons change during postnatal development, it is not known how these changes come about. A number of possible mechanisms can be invoked to explain the switch in A-current type during postnatal development: (1) regulation of the turnover of the different channel proteins; (2) posttranslational modification of the K channel protein, and; (3) regulation at a genetic level, involving either transcriptional or translational control.

The first step probably involves activation of 2nd messenger systems, as demonstrated by the role of Protein kinase C in the maturation of the delayed rectifier current of amphibian spinal neurons (Desarmenien and Spitzer, 1991). It is possible that second messengers act directly on the K channel protein to cause changes in channel activity: this could involve changes in channel properties, or interconversions between non-functional and functional forms of a channel. Evidence exists for modulation of channel properties by intracellular factors (Kaczmarek and Levitan, 1987; Levitan, 1988). For example, in bag cell neurons of Aplysia, the inactivation rate of a fast A-current is altered in response to an increase in intracellular cAMP levels (Strong, 1984).

However, given the slow time course of developmental changes in postnatal SCG neurons, and that the changes are probably long lasting if not permanent, it is more likely that if second messengers are involved in controlling these changes, that they act indirectly by triggering events at the level of the genome. One mechanism of producing long term changes in functional channel expression could be to alter the levels of a protein that modulates K channel activity (Greenberg et al., 1986). Another possibility is that changes in the outward current could result from insertion, or loss, of channels in the membrane. This control of channel number could be at the transcriptional level, involving the up or down regulation of gene expression. Alternatively, changes in the number of channels in the membrane could involve changes in translation of the messenger RNA (mRNA), changes in rates of mRNA turnover, or changes in posttranslational processing of the channel proteins. However, determining at what level SCG neurons regulate their expression of K currents must wait until the genes encoding the ionic channels for IAf, IAs and IK on SCG neurons have been identified.

Chapter 5

Control of voltage-gated K currents on peripheral neurons developing in vivo and in culture

5.1 Introduction

Many studies have examined the properties of K currents expressed by different neurons (see reviews by Rudy, 1988; Llinas, 1988), and from these studies it is clear that the particular set of K currents a neuron expresses determines much of its electrophysiological behaviour, particularly the frequency and pattern of action potential firing. In view of the importance of K currents, it is surprising how little is known about the factors that govern their expression such that different neurons acquire diverse sets of K currents during their development. The set of K currents a neuron expresses could be controlled by a program intrinsic to neurons, however, there is evidence that epigenetic influences have a role (as discussed in Chapter 1). This chapter presents results of experiments that investigate the role of extrinsic factors in the control of K current expression on rat SCG neurons during postnatal development *in vivo* and in culture.

These sympathetic neurons provide several advantages for studies on the regulation of K current expression: (1) In Chapter 4, I showed that on postnatal SCG neurons, there are changes in K current expression that occur over a short (days), well defined period. (2) SCG neurons are easily accessible and grow well in culture (Mains and Patterson, 1973; Hawrot, 1980), so that the environment of the neurons can be manipulated to investigate the role of specific factors in the control of K current expression. (3) The postganglionic axons of SCG, and the nerves of the preganglionic neurons that innervate them (sympathetic trunk), are readily accessible for axotomy and denervation experiments respectively, in order to examine target and preganglionic influences on K current expression.

In the previous chapters, I showed that SCG neurons express 3 voltage-activated K currents: a non-inactivating current (IK), a slowly inactivating A-current (IAs), and a rapidly inactivating A-current (IAf). During the first 2 weeks of postnatal *in vivo* development the expression of IAf increases three fold, the expression of IAs decreases 2 fold, and IK densities remain constant. Is the *in vivo* switch from an IAs to IAf dominated outward current under intrinsic and/or extrinsic regulation? If the switch is under the control of extrinsic influences, what is the nature of this influence? Are IAf and IAs regulated independently, or does regulation of one current depend on the expression of the other? Is there a restricted window during development during which these changes can occur? The studies discussed in this chapter address these points.

As neurons differentiate during development they are subject to several environmental influences that could potentially affect the particular K channels they express (as reviewed in Chapter 1). To ensure that the electrical activity of the ganglionic neurons produces the appropriate response from the target, one might expect that the target (Black and Mytilineou, 1976; Patterson, 1978; Furshpan et al., 1982; Bunge et al., 1978; Purves and Lichtman, 1985), or pre-ganglionic inputs (Black et al., 1971; Black, 1982) play a role in determining the particular K currents neurons express In addition, several reports indicate that the nonneuronal cells that surround the neurons can influence their differentiated properties (O'Lague et al., 1978; Patterson, 1978; Zurn, 1987; Smith and Kessler, 1988; Nawa and Sah, 1990; Clendening and Hume, 1990). Finally, hormones, growth factors, or peptides, from the circulation may have effects on expression of K currents by the neurons (Joels and de Kloet, 1992).

In this chapter, I discuss experiments designed to identify factors that control the developmental changes in K current expression on SCG neurons. I show that preganglionic inputs and target contact are not responsible for the postnatal switch from an IAs to an IAf dominated outward current. In addition, I demonstrate that nonneuronal cells provide a soluble factor(s) that is required by P1 SCG neurons to express A-currents in culture. Furthermore, our results suggest that neurons have intrinsic control mechanisms to coordinate their expression of the 3 voltage-gated K currents.

5.2 Methods

Superior cervical (SCG) ganglia were removed from rat pups 1, 7, or 14 days postnatally. In addition, nodose ganglia were removed from neonatal rat pups (P1). The ganglia were dissociated and the neurons maintained in culture for 3, 7, 14 and 28 days as described in Chapter 2. In some experiments, SCG neurons were cultured in the absence of other cell types, while in sister cultures SCG neurons were grown in the presence of nonneuronal cells from either the ganglion, heart or skin (methods for establishing co-cultures are described in Chapter 2). Alternatively, undissociated P1 and P7 SCG were cut in half, and maintained in culture for 2 weeks as explants, before dissociating the neurons and plating them in culture dishes. To further investigate the effects of nonneuronal cells on the expression of IAf, IAs and IK, P1 SCG neurons were grown for 2-4 weeks in the presence of a mixture of 60% media conditioned (C.M) by nonneuronal cells from the ganglia/ 40% growth media. In some experiments, neurons were grown for 14 days in the presence of ciliary neurotrophic factor (CNTF) at a concentration of 10ng/ml. Whole-cell voltage clamp techniques (Hamill et al., 1981) were used to record voltage-gated K currents on neurons in the different culture conditions (see Chapter 2). Recordings were from single neurons and from neurons in clusters. No significant difference was observed in the degree of clustering for neurons in the different cultures, and clustering had no effect on the voltage-gated K currents expressed by the neurons.

Preganglionic denervation and postganglionic axotomy

Denervation

In one series of experiments, we examined the effects of preganglionic denervation of neonatal sympathetic neurons on the developmental appearance of voltage-gated K currents. In a second series, we examined the effects of postganglionic axotomy. Our surgical methods were similar to those of Voyvodic (1987). Briefly, for denervation experiments, postnatal day one rat pups were anaesthetized by cooling; the preganglionic cervical sympathetic trunk on one side was exposed and ligated approximately halfway between the SCG and the first rib, and then the nerve was transected distal to the ligation; the ligated proximal stump was directed in to the overlying muscle to impede regeneration. After the surgery, the pups were returned to the litter for one to two weeks. We observed that all denervated animals exhibited pronounced ptosis of the eye on the operated side two weeks later. (We could not use this test on animals that survived for shorter times because the eyes remain closed for the first 12 days postnatally). In some animals, we confirmed that the denervation was complete by electrophysiologically recording from the postganglionic nerve with extracellular electrodes while stimulating the preganglionic stump. In all control animals (n=6) a compound action potential (C.A.P) was recorded, but no C.A.P could be recorded in any of the denervated animals (n=6). Results from tested denervated neurons were not any different from those that were not tested before dissociation.

For recording C.A.Ps, the rat pups were anaesthetized with 40mg/kg body weight sodium pentabarbitol (Somnotol, MTC Pharmaceuticals), the preganglionic and postganglionic nerves were exposed and placed on silver hook electrodes; the recording electrodes were connected to AC preamplifier (made by Mr. J. Knowles, Faculty of Medicine Electronic Workshop, McGill University) and the signals stored and displayed on an AST 286 IBM compatible computer with the use of Assistant software (MacMillan software). The stimulating electrodes were connected to a Grass stimulator (model #SD9).

Axotomy

For postganglionic axotomy, the SCG was isolated from surrounding connective tissue and the main postganglionic nerves were transected near the exit of the ganglion. This procedure in

P1 animals resulted in a progressive decrease in the number of neurons in the ganglion that was more severe in ganglia that survived for two weeks after surgery than after one week; this finding is consistent with results of Hendry and Campbell (1976) who reported that 50% of the neonatal SCG neurons are lost one week after axotomy and 90% by one month.

To measure the K currents on the denervated or axotomized neurons, the ganglia were removed from the operated animals after either one or two weeks and dissociated as described above. All neurons were examined electrophysiologically using whole-cell patch clamp techniques between 2-48 hr after plating.

Data Analysis

The current densities (pA/pF) of IAf, IAs and IK were measured on nodose and SCG neurons under different experimental conditions. Details of the analysis are provided in Chapter 2 and the methods of Chapter 4. Briefly, IAf, IAs and IK currents evoked by a 125 ms voltage step to +30 mV were isolated using the subtraction protocols established in Chapter 3. To measure current density (pA/pF), the isolated currents (pA) were divided by the whole cell capacitance (pF), obtained by integrating the capacity current evoked by a 10 mV hyperpolarizing voltage step.

5.3 Results

5.3.1 A-channel expression is largely independent of preganglionic influences

We were interested in whether the postnatal increase in IAf and decrease in IAs expression on SCG neurons were a result of innervation by the preganglionic nerves. During the first 3 weeks of postnatal development there is a large increase in the synaptic innervation of SCG neurons (Black et al., 1971), rising from 10% to 80% of the number of synapses observed in the adult (Smolen and Raisman, 1980); we tested whether this increase in preganglionic innervation was responsible for the changes in K current expression over this period.

To test the effects of preganglionic innervation, the preganglionic nerve to the ganglion was cut on postnatal day 1 and denervated neurons examined 1 and 2 weeks later. An example of outward currents from a P14 neuron that has been denervated for 2 weeks is shown in Fig. 5.1A and B. The outward currents on this denervated neuron were similar to those of innervated SCG neurons from 2 week old animals (see Fig. 4.1B).

Figure 5.1E shows the mean IAf and IAs current densities for neurons that were chronically denervated for 1 (n=38) and 2 weeks (n=46). There was no significant difference ($p>0.2$, t-test) when comparing mean IAf and IAs current densities between the chronically denervated neurons and control innervated neurons, except for a small (approximately 20%) reduction in IAf after 2 weeks of denervation ($p<0.01$). Denervated neurons still showed the change from a predominantly slowly inactivating outward current at P1 to one composed of predominantly IAf by P14, indicating that developmental changes in inactivating currents are unlikely to be a result of presynaptic innervation.

5.3.2 A-channel expression is largely independent of target factors

Another possible explanation for the postnatal changes in IAf and IAs on SCG neurons is that the expression of these currents is influenced by a target-derived factor(s). During SCG postnatal development there is a large increase in the number of synaptic target contacts (Black and Mytilineou, 1976). In addition, axotomy in other preparations results in changes in the electrophysiological properties of neurons (Kuno et al., 1974; Purves, 1975; Kelly et al., 1986; Gallego et al., 1987).

To test the effects of target-derived factors, we axotomized neurons on P1 by cutting the main postganglionic trunk that exits the ganglia and then examined the neurons 1 and 2 weeks later. These experiments are complicated by the fact that almost 50% of neonatal SCG neurons do not survive more than 1 week after axotomy and almost all die by 1 month (Hendry and Campbell, 1976). However, as we have shown that the most significant changes in IAf and IAs expression occur during the first week of postnatal development, it was possible to measure the effects of axotomy on those neurons that survive during that time.

An example of the outward currents from a neuron that has been axotomized for 2 weeks is shown in Fig. 5.1C and D. The outward currents on this neuron were similar to those of control SCG neurons from 2 week old animals. Figure 5.1E shows the mean IAf and IAs current densities for neurons that were axotomized 1 week (n=43) and 2 weeks (n=41) earlier. There was no significant difference ($p>0.2$, t-test) in IAs between the neurons axotomized for 1 or 2 weeks and control neurons from the same age animals, except for a slight decrease (20%) in IAf after 2 weeks ($p<0.01$). Axotomy, failed to prevent the increase in IAf or the decrease in IAs observed during *in vivo* development, suggesting that innervatior of the target does not significantly influence the change in expression of IAf and IAs during the first two postnatal weeks.

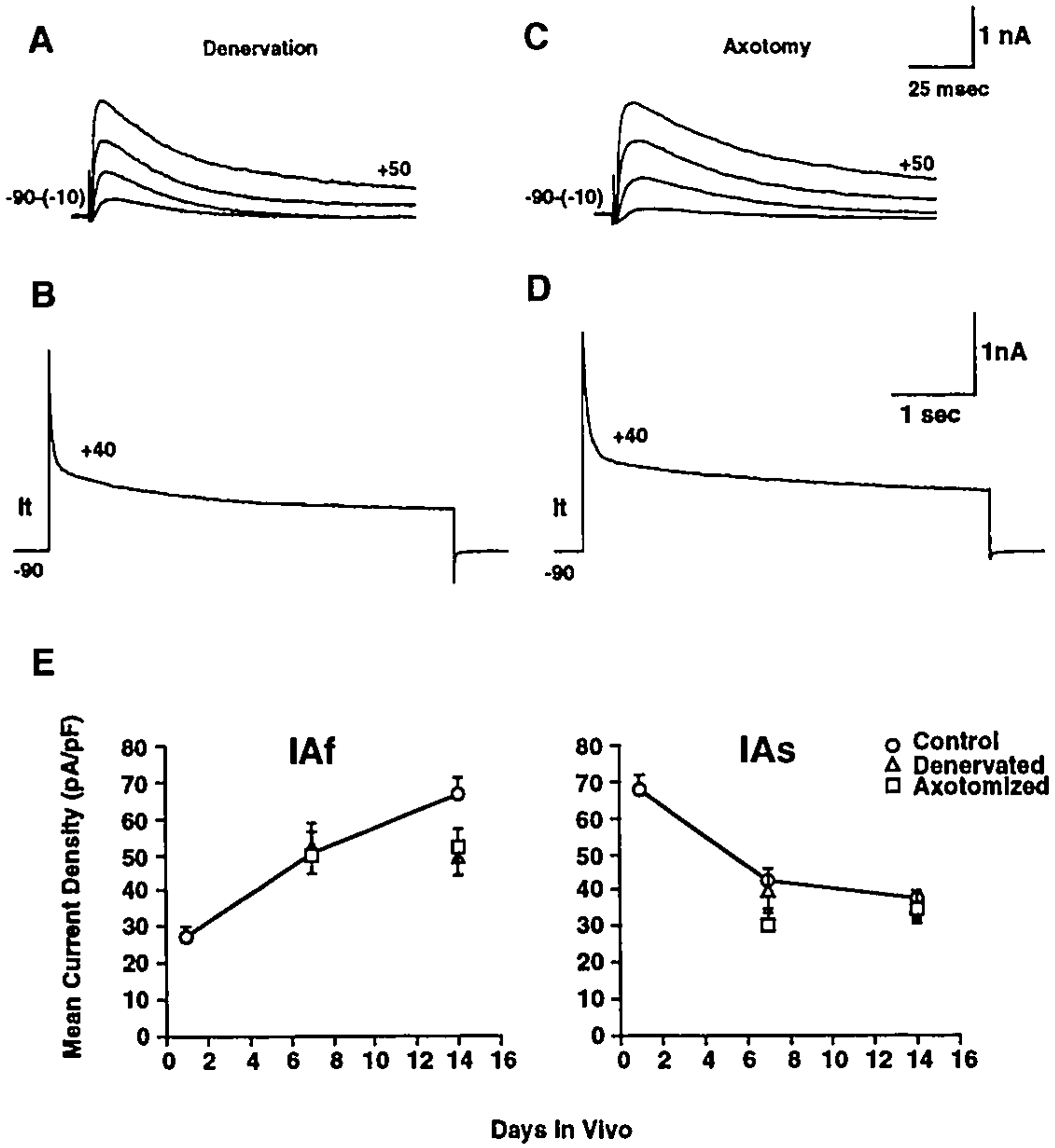

Figure 5.1: Effect of denervation and axotomy on K current expression: A-B. Denervation: A. shows total IAf and IAs currents from a P14 SCG neuron denervated on P1. The currents were evoked by depolarizing voltage steps up to +50mV (in 20mV increments), from a Vh of -90mV. Traces were leak corrected, filtered at 1.5kHz and sampled at 5kHz. The lower trace shows the total uncorrected outward current evoked by a 6 sec depolarizing step to +40mV. The trace was filtered at 100Hz and sampled at 200Hz. C-D. Axotomy: C shows the total IAf and IAs currents, as in A, and D shows the total uncorrected current, as in B, from a P14 SCG neuron axotomized on P1. E shows the mean IAf (left) and IAs (right) current densities (pA/pF) for control, denervated and axotomized SCG neurons examined on P7 and P14. All surgery was performed on P1. Error bars represent the s.e.m.. For control: n=47, P1; n=40, P7; n=62, P14. For denervation: n=38, P7; n=46, P14. For axotomy: n=43, P7; n=41, P14.

5.3.3 SCG neurons lose their expression of IAf and IAs in culture

Because factors from preganglionic nerves and target organs do not appear to influence the expression of K currents during the first 2 weeks of postnatal development, we were interested in investigating other factors that might explain the changes in the expression of IAf and IAs. Therefore, we studied the development of P1 SCG neurons in tissue culture: tissue culture offers the advantage that one can control much of the external environment as neurons develop, and in this way one can examine the role of various extrinsic factors in the developmental control of K current expression.

Several studies have shown that rat SCG neurons grow well in culture and acquire many of their *in vivo* properties (Mains and Patterson, 1973; O'Lague et al., 1978; Hawrot, 1980; Bruckenstein et al., 1989). Figure 5.2 shows a phase micrograph of a typical culture of P1 SCG neurons that have developed in culture for 3 weeks in the virtual absence of other cell types. SCG neurons in long term culture can fire overshooting APs and continue to express neuronal nicotinic acetylcholine receptors (Mandelzys and Cooper, unpublished observations).

Interestingly, the outward currents expressed by P1 neurons grown in culture are quite different from those expressed by neurons developing for the same time *in vivo*: neurons in culture longer than two weeks have significantly smaller ($p<0.001$, t-test) IAf and IAs than those *in vivo*. Figure 5.3 shows examples of the total outward current from 3 P1 SCG neurons grown in culture for different times. Figure 5.3A shows currents from a P1 neuron that had been in culture for less than 24 hours. The predominantly slowly inactivating outward current is typical of currents observed for acutely dissociated P1 neurons. Figure 5.3B shows the outward current from a neuron that developed in culture for 7 days (C7). The large increase in IAf that occurs *in vivo* does not occur, and, in fact, by a week in culture most neurons express no detectable IAf. Figure 5.3C shows the outward current from a neuron in culture for 28 days (C28). By C28, the outward

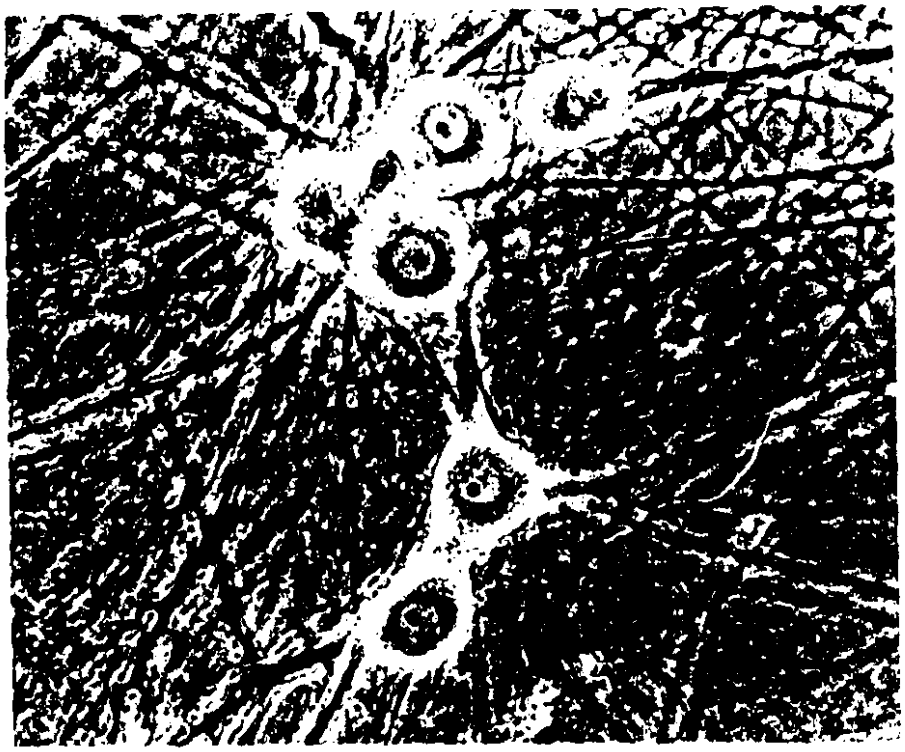

Figure 5.2: Phase micrograph of living P1 SCG neurons in culture for 3 weeks in the virtual absence of nonneuronal cells. Scale represents 50μm.

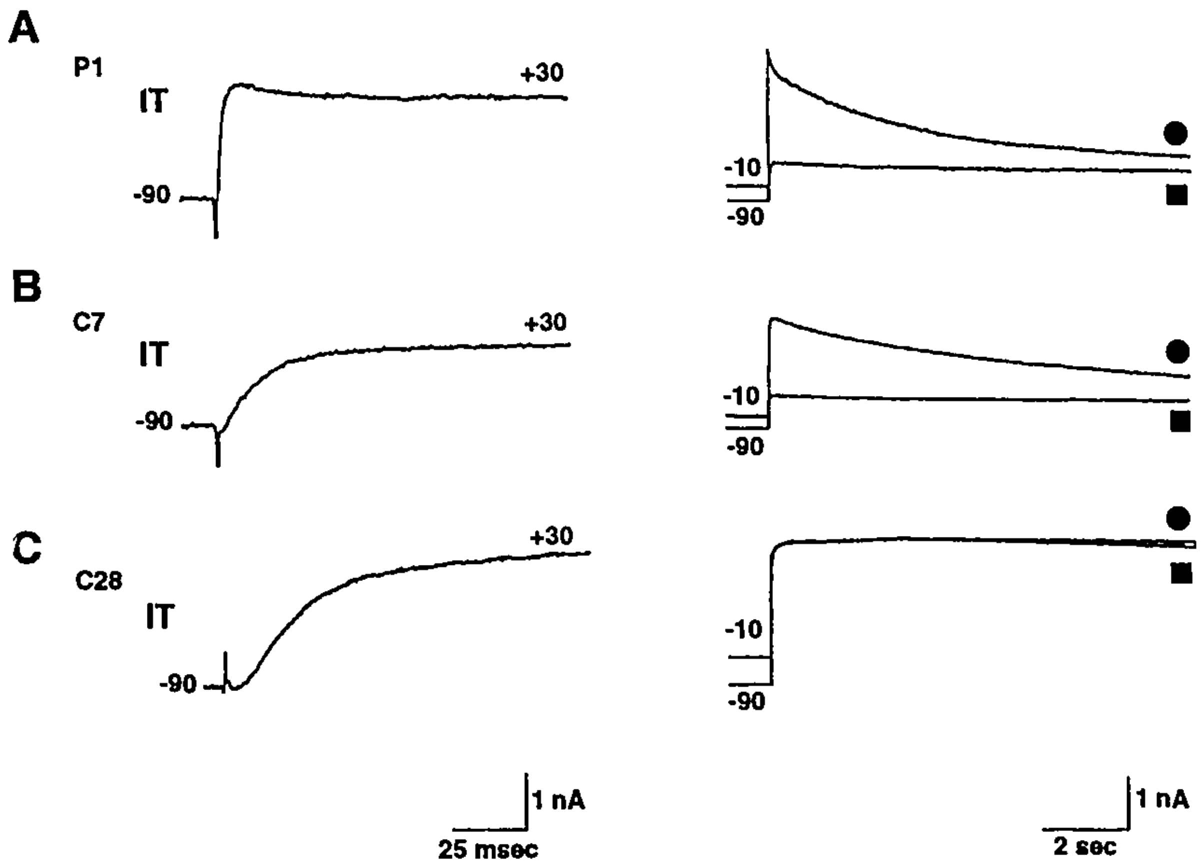

Figure 5.3: Developmental expression of K currents on SCG neurons in culture: K currents expressed on a P1 neuron in culture for less than 24hrs (A), on P1 neurons grown in culture for one week (B), and in culture for four weeks (C). Left: shows the total, leak subtracted outward currents evoked by a 125ms depolarizing voltage step to +30mV, from a Vh of -90mV. Traces were filtered at 1.5kHz and sampled at 5kHz. Right: shows the total outward currents, uncorrected for leakage, evoked by 10sec depolarizing voltage steps to +30mV from a Vh of -90mV (circle) and a Vh of -10mV (square), filtered at 100Hz and sampled at 200Hz. The difference between the IK currents evoked from a Vh of -10mV, and the outward currents evoked from a Vh of -90mV reflects the inactivating IAf and IAs currents.

currents exhibited almost no inactivation, and could be blocked by 74%±5% (mean ±s.e.m) with 10mM tetraethylammonium, consistent with the pharmacology of delayed rectifier currents (Thompson, 1977). These results indicate that the outward current of P1 neurons in culture for 28 days consists almost entirely of IK, and that the inactivating currents (IAf and IAs) are gradually lost on P1 SCG neurons in culture.

To quantify the change in expression of K currents in culture, we measured the mean current densities for IAf, IAs and IK on P1 neurons that developed in culture for different times. Figure 5.4 shows the mean current densities for IAf, IAs and IK measured on neurons for 3, 7, 14 and 28 days in culture. This figure demonstrates that over the first few days in culture there is little change in the expression of the 3 voltage-gated K currents from their P1 levels. The large increase in IAf and decrease in IAs observed on neurons during the first week *in vivo* (see Fig. 4.1 and 4.2) does not occur. Over the next week in culture, there is a significant decrease in the expression of both IAf and IAs, which continues for an additional 2-3 weeks. By 4 weeks in culture, none of the neurons expressed detectable IAf, and the mean IAs current density was only 10% of the P1 value. Interestingly, as demonstrated in Fig 5.4, there was a significant increase in the expression of IK over the time that IAf and IAs were decreasing (see also Fig. 5.3); by 4 weeks in culture the mean IK current density was 5 fold greater than that observed on P1 neurons. While IK levels increased over this period, there were no changes in the kinetic, pharmacological, or voltage-dependent properties of this current (data not shown).

The results shown in Fig. 5.4 indicate that there is a decrease in the levels of IAf and IAs on neurons that develop in culture for several days. These results suggest that there is a factor(s) needed for the maintenance of expression of IAf and IAs *in vivo*, and that this factor is missing from the cultures. However, another possibility is that neurons in culture continue to express IAf and IAs, but these channels are preferentially located at sites remote from our recording electrodes,

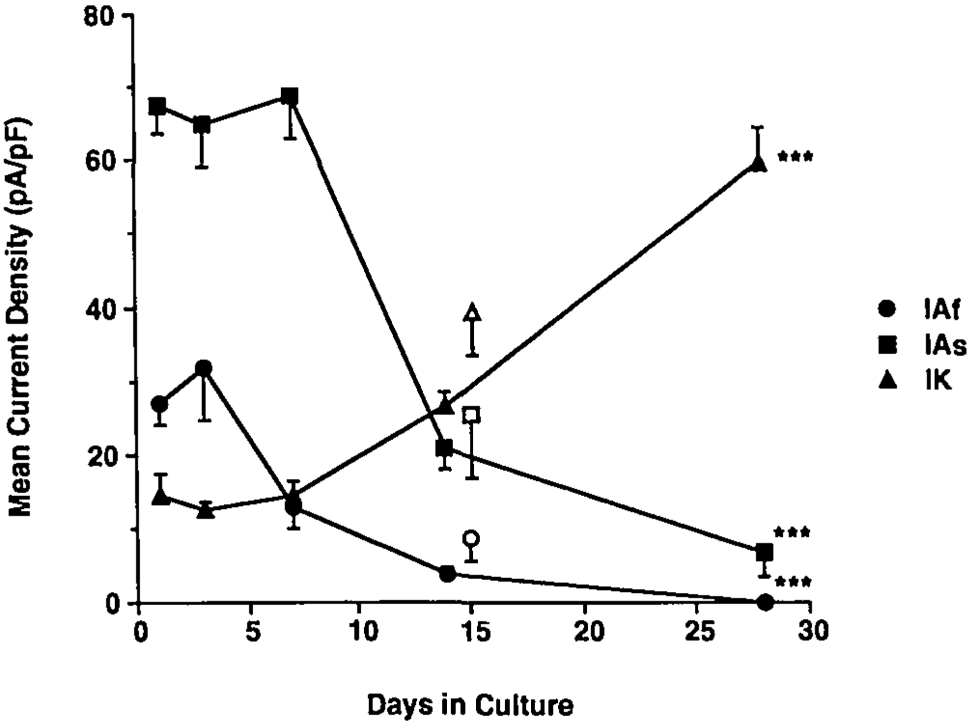

Figure 5.4: Time course of changes in K current expression in culture: The mean current densities (pA/pF) for IAf, IAs and IK measured on P1 neurons grown in culture for 3 (n=17), 7 (n=27), 14 (n=62) and 28 (n=35) days in culture. The error bars represent s.e.m.. With time in culture IAf and IAs decrease significantly (***, p<0.001) with respect to the levels expressed on P1 neurons, whereas IK is significantly greater (***, p<0.001) than on P1 neurons. The open symbols at day 16 represent the current densities on 17 neurons cultured for 14 days and then replated and examined 2 days later. These replated neurons had current densities that were not significantly different (p>0.1) from neurons in culture for 14 days.

for example at the nerve terminals, and therefore go undetected; also, by the same argument, IK could presumably become larger if it is preferentially located on the soma. This hypothesis is less likely in that it constrains the redistribution of K channels only to neurons that develop in culture, because, as shown in Chapter 4, neurons that develop *in vivo*, which also have extensive process outgrowth, express K channels on their somas and proximal processes.

However, to test this possibility, P1 neurons were allowed to develop in culture for 2 weeks by which time IAf and IAs were expressed at low levels, then the neurons were harvested and replated. These neurons were examined 2-3 days later before extensive process outgrowth occurred. If in culture there is no change in the expression of IAf, IAs and IK, we would expect the replated neurons to express these currents at levels similar to those on P1 neurons. However, as indicated by the open symbols in Fig. 5.4, we observed that these neurons had small IAf and IAs current densities, comparable to those expressed by neurons after 14 days in culture. These results support the conclusion that the expression of IAf and IAs decreases when neurons develop in culture.

5.3.4 Nonneuronal cells affect K current expression on cultured SCG neurons

K currents on SCG neurons co-cultured with ganglionic nonneuronal cells

When P1 SCG neurons develop in culture in the absence of other cell types, they lose both IAf and IAs, suggesting that some factor(s) necessary for the expression of these currents is missing in the cultures. The fact that denervated or axotomized P1 neurons continue to express IAf and IAs if they develop *in vivo*, suggests that the loss of preganglionic and target influences is not responsible for the loss of A-currents in culture.

One possibility is that nonneuronal cells from the ganglion provide a factor(s) that affects

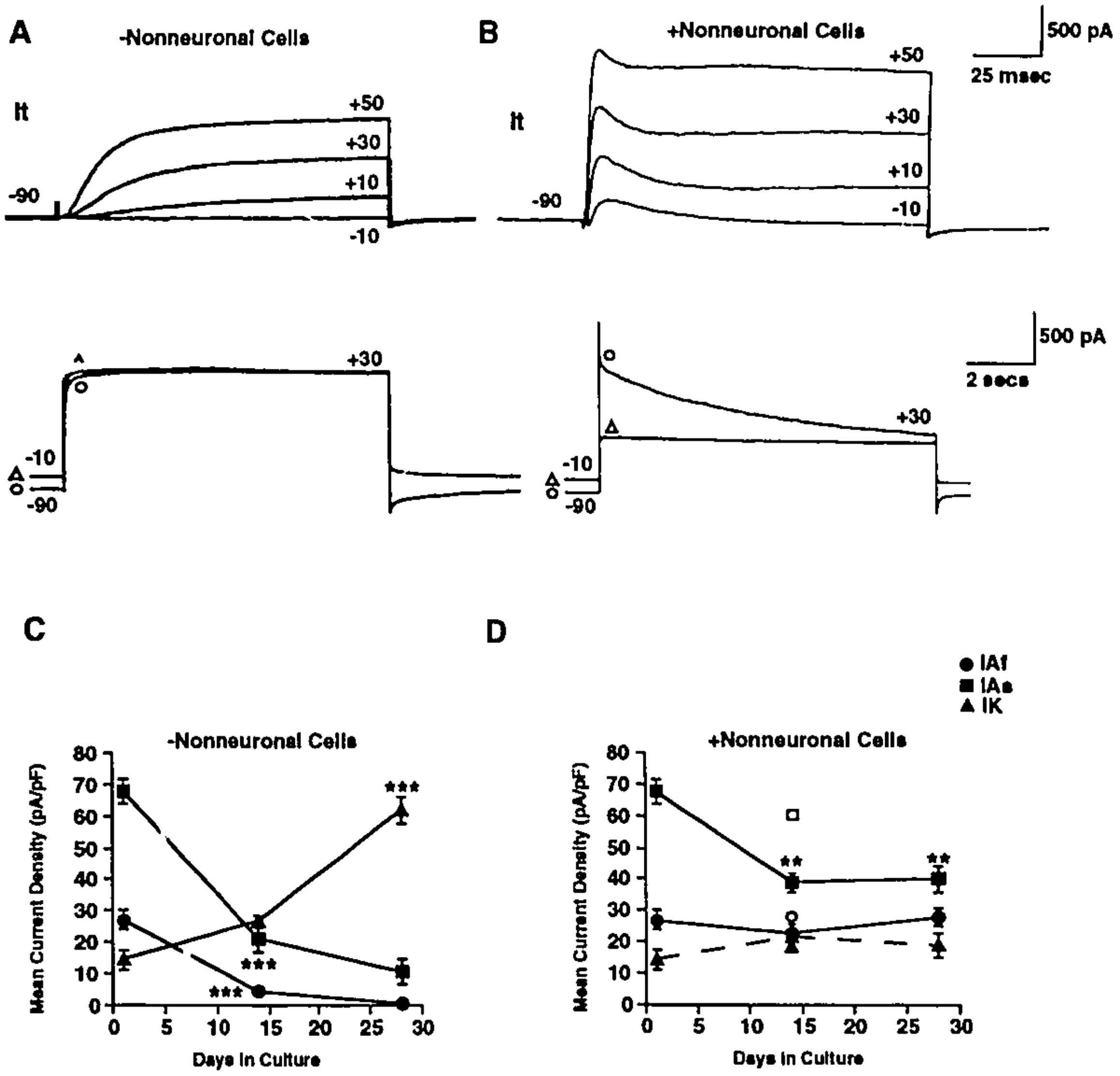

Figure 5.5: Nonneuronal cells influence K current expression in culture: A-B: Outward K currents expressed on P1 SCG neurons grown in culture for 28 days, in the absence and presence of other cell types from the ganglion. The neuron cultured in the absence of other cell types (A) expresses only IK, while the neuron in co-culture (B) expresses all 3 K currents. A and B: Top: leakage subtracted outward currents evoked by 125 msec depolarizing steps in 20 mV increments up to +50 mV, from a Vh of -90 mV. Currents were filtered at 1.5 KHz and sampled at 5KHz. Bottom: non-leakage corrected outward currents evoked by 10 sec depolarizing steps to +30 mV from Vh's of -90 mV and -10 mV. Traces were filtered at 100 Hz and sampled at 200 Hz. C-D: mean current densities (pA/pF) for IAf, IAs and IK expressed on P1 neurons after 1, 14 and 28 days in culture. Neurons were grown in the absence (C), or presence (D) of nonneuronal cells from the ganglion: -nonneuronal cells, P1 (n=46), C14 (n=62), C28 (n=35); +nonneuronal cells, C14 (n=58), C28 (n=54). Mean IAf and IAs current densities fall (***, $p<0.001$, t-test), while IK increases significantly ($p<0.001$) for P1 neurons grown in the absence of other cell types. When P1 neurons are grown in culture in the presence of nonneuronal cells from the ganglion there is no loss of IAf ($p>0.2$) and no increase in IK ($p>0.1$). IAs drops by 30-40% (**, $p<0.01$), but is 4 fold greater ($p<0.001$) than on neurons without nonneuronal cells. Open circles represent the mean current densities for IAf, IAs and IK measured for P1 neurons grown in explant cultures for 14 days (n=38). Errors are s.e.m.

A-current expression. To test this, we measured K current expression on P1 SCG neurons grown in culture either in the presence, or absence, of nonneuronal cells from the ganglion. Figure 5.5A shows voltage-gated K currents expressed by a P1 SCG neuron in culture for 28 days without nonneuronal cells, while Fig. 5.5B shows the voltage-gated K currents expressed by a neuron grown in the presence of nonneuronal cells for the same amount of time. Unlike neurons developing without nonneuronal cells, the neurons grown in the presence of nonneuronal cells continue to express both IAf and IAs.

We measured the current densities (pA/pF) of IAf, IAs and IK on SCG neurons grown in the presence and absence of nonneuronal cells. For each condition, measurements were made for 30-60 neurons at 3 different times in culture; 1, 14 and 28 days. Figures 5.5 C and D show the mean current densities (pA/pF) for IAf, IAs and IK for SCG neurons grown in the absence (C), or presence (D) of nonneuronal cells for 2 and 4 weeks. As demonstrated earlier and shown here for reference, in the cultures without nonneuronal cells IAf and IAs drop to low levels (<10pA/pF), whereas IK increases 5 fold. In the co-cultures however, IAf and IK densities do not change with time in culture (p>0.1, t-test). IAs densities drop by 30-40% (p<0.01) after 2 weeks, but are maintained thereafter at levels significantly greater (p<0.001) than the levels of IAs on neurons grown in the absence of nonneuronal cells. These results demonstrate that in the presence of other cell types from the ganglion, SCG neurons continue to express IAf and IAs.

Further evidence that nonneuronal cells from the ganglion exert an influence on K current expression comes from our experiments with explant cultures; we grew P1 ganglia as explants, dissociated the neurons after 2 weeks in culture, and recorded their K currents. The mean current densities for IAf and IK currents of P1 SCG explant cultures are not significantly different (p>0.1, t-test) from those of P1 neurons grown in the presence of nonneuronal cells, as shown by the clear symbols in Fig. 5.5C. In the explant cultures, IAs densities are 30% larger than for neurons from co-cultures with nonneuronal cells (p<0.001), and in fact, are comparable to IAs densities on P1 neurons (p>0.2).

Nonneuronal cells from other tissues affect IAf and IAs expression on cultured SCG neurons

We were interested in whether the effect of nonneuronal cells on A-current expression is specific to nonneuronal cells from the ganglion. To test this possibility, we grew P1 SCG neurons for 2 weeks on monolayers of cells from other tissues. Figure 5.6 shows the mean current densities (pA/pF) for IAf and IAs on P1 SCG neurons grown in the absence and presence of nonneuronal cells from the ganglion, shown here for reference, and on P1 neurons grown in the presence of cells from either heart or skin. Heart and skin cells are just as effective as nonneuronal cells from the ganglion at maintaining IAf expression in culture; IAf is not significantly different (p>0.2) on neurons in the three co-culture conditions.

However, the different cell types are not equally effective at maintaining the expression of IAs in culture. The mean IAs current density is significantly higher on neurons grown with other cells from the ganglion (p<0.01) or from heart (p<0.001), than on neurons cultured without other cell types. These results suggest that cells from heart also provide a factor(s) that can influence the expression of IAs in culture. In contrast, skin cells do not appear to provide such a factor as after 2 weeks in culture IAs was expressed at low levels (<20pA/pF), comparable to the levels on neurons grown without other cell types.

Nonneuronal cell conditioned media affects the expression of K currents on SCG neurons in culture

These results suggest that nonneuronal cells provide a factor(s) that can influence the expression of K currents on SCG neurons. To test whether nonneuronal cells exert their effects

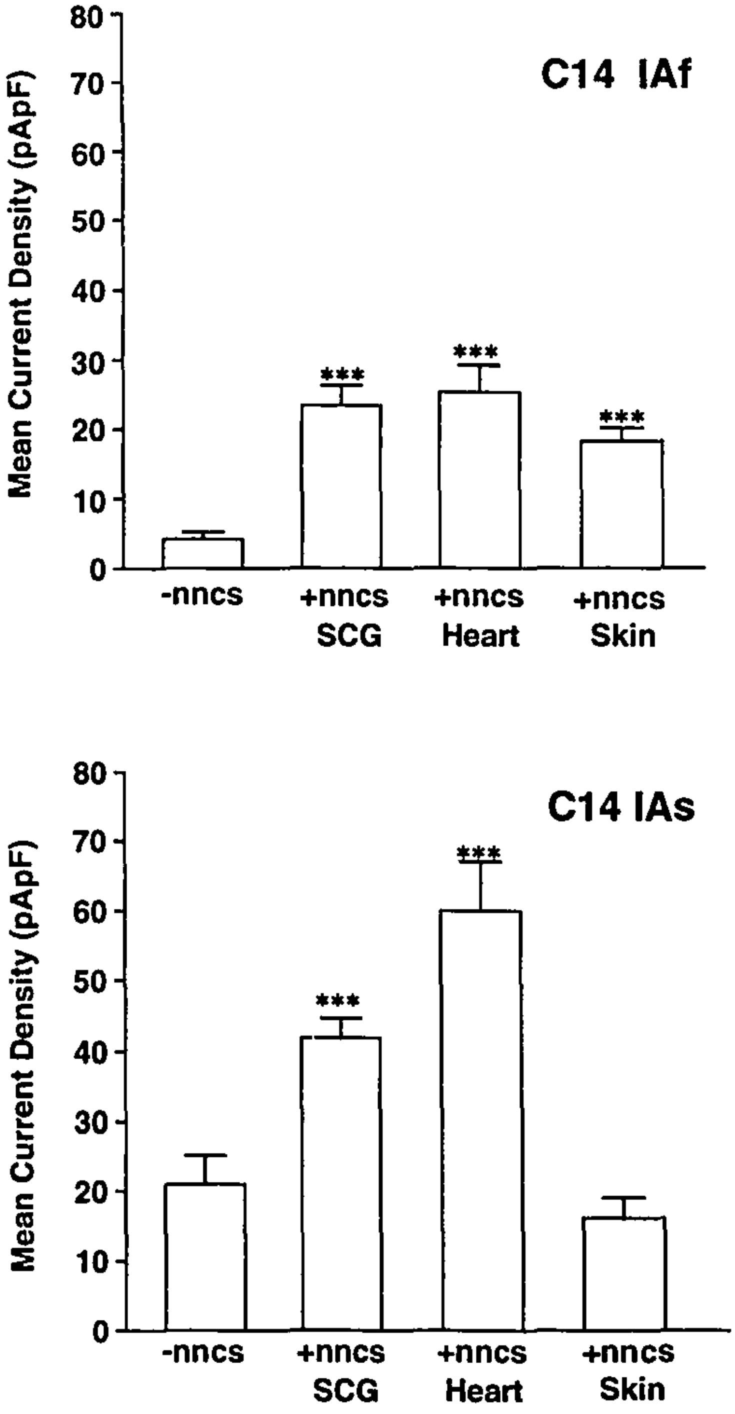

Figure 5.6: The nonneuronal cell effect on IAf expression is not specific to nonneuronal cells from the ganglion: Mean current densities (pA/pF) for IAf and IAs on P1 SCG neurons grown in culture for 2 weeks in the absence of nonneuronal cells (n=62) and in the presence of nonneuronal cells from either SCG (n=58), heart (n=39) or skin (n=18). IAf is expressed at significantly higher levels(***, $p<0.001$) when grown in the presence of other cell types, regardless of their source. IAs is significantly larger ($p<0.001$) when SCG neurons are grown in the presence of nonneuronal cells from the ganglion or from heart as compared to its expression on SCG neurons in the absence of other cell types. Skin cells have no effect on the expression of IAs in culture, as the mean current density is not significantly different from that on SCG neurons grown in the absence of other cell types ($p>0.2$). Errors are s.e.m.

via a factor they secrete into the media, we grew P1 SCG neurons without other cell types, but in the presence of medium which was first conditioned by nonneuronal cells from the ganglion.

Figure 5.7 shows the mean current densities (pA/pF) for IAf, IAs and IK on P1 neurons grown in culture for 28 days in several different conditions; absence of nonneuronal cells, presence of nonneuronal cells, or presence of nonneuronal cell conditioned media (C.M). There is no significant difference ($p>0.1$, t-test) between the levels of IAf, IAs and IK on P1 SCG neurons grown either in the presence of nonneuronal cells, or in the presence of media conditioned by these cells. Similar results are obtained for neurons grown in culture in the presence of C.M for 14 days (data not shown). These results suggest that nonneuronal cells provide a soluble factor that is capable of influencing the expression of K currents on cultured P1 SCG neurons.

CNTF treated cultures

Ciliary neurotrophic factor (CNTF) was isolated on the basis of its effect on the survival of chick ciliary ganglion neurons (Varon et al., 1979), and is found in high concentrations in the nonneuronal cells of the rat sciatic nerve (Lin et al., 1989). Since CNTF is known to affect the expression of differentiated properties of sympathetic neurons (Saadat et al., 1989), we tested whether CNTF, or a CNTF-like molecule, could be the soluble nonneuronal cell factor that affects the expression of inactivating K currents on P1 SCG neurons. Figure 5.8 shows the mean current densities of IAf and IAs on P1 neurons grown in culture in the absence or presence of CNTF (10ng/ml). Mean K current densities on P1 neurons grown in the presence of nonneuronal cell conditioned media are shown here for reference. IAf mean current density is over 5 fold greater in CNTF treated cultures than in untreated cultures ($p<0.001$), whereas IAs levels are 70% greater ($p<0.02$) in the CNTF treated cultures. In fact, IAf and IAs mean current densities for CNTF treated cultures are not significantly different ($p>0.2$) from the levels on SCG neurons grown in

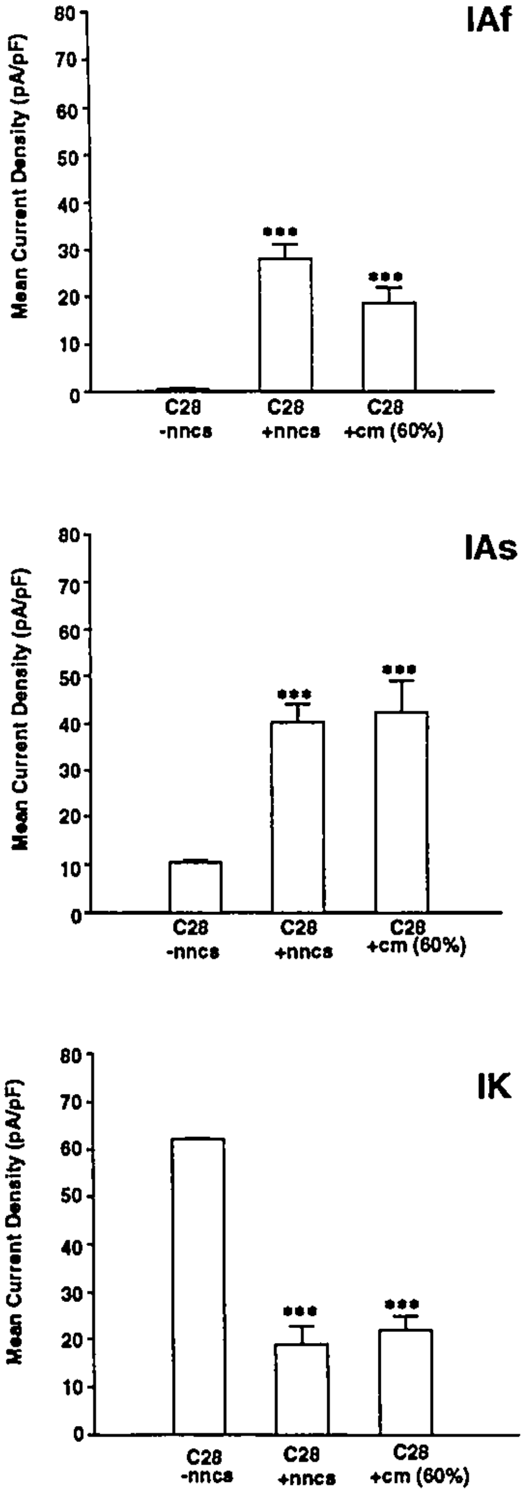

Figure 5.7: Conditioned media from nonneuronal cells mimics the effects of nonneuronal cells: Mean current densities (pA/pF) for IAf, IAs and IK on P1 neurons grown in culture for 28 days in the presence (n=54) and absence(n=35) of nonneuronal cells from the ganglion, and on P1 neurons grown in the presence of conditioned media harvested from nonneuronal cell cultures (n=44). There is no significant difference (p>0.2, t-test) between the levels of IAf, IAs and IK expressed on P1 neurons grown either in the presence of nonneuronal cells, or in the presence of media conditioned by nonneuronal cells. In both cases IAf and IAs are significantly greater (***, p<0.001) and IK significantly smaller (p<0.001) than the currents on neurons in culture without other cell types. Errors are s.e.m.

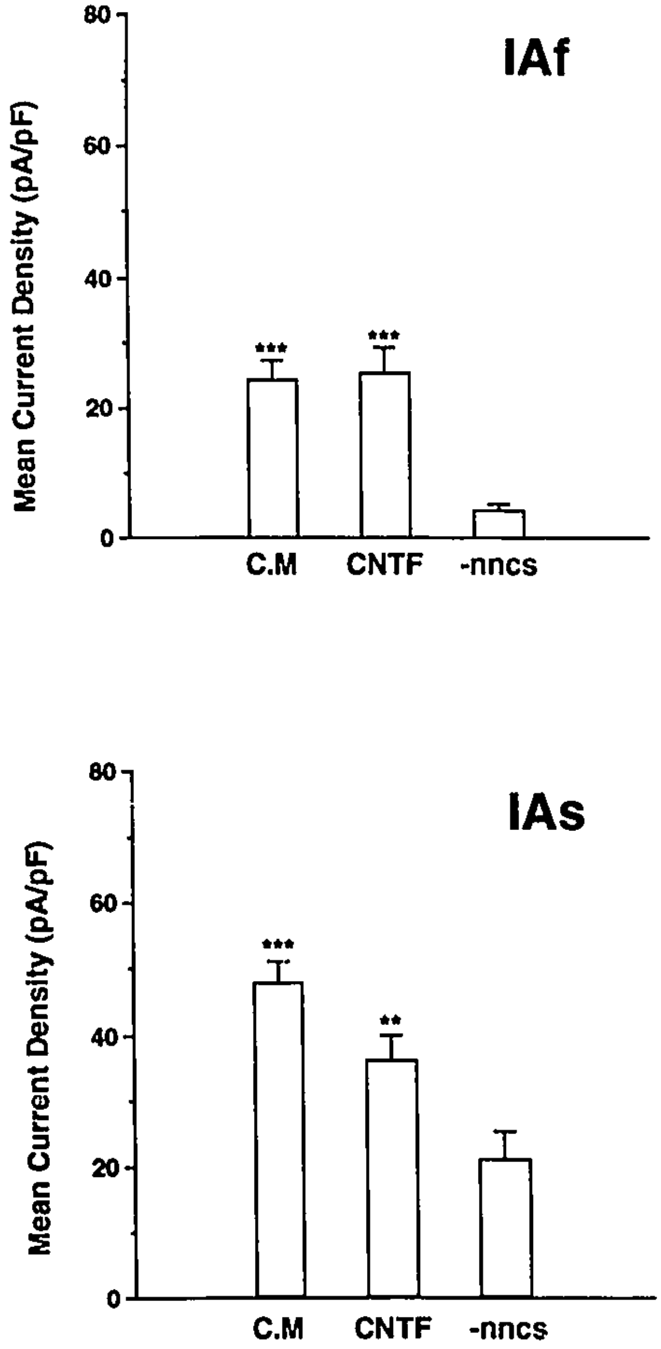

Figure 5.8: Ciliary neurotrophic factor (CNTF) affects the expression of inactivating K currents on P1 SCG neurons developing in culture: Mean current densities (pA/pF) for IAf and IAs on P1 neurons grown in culture for 14 days in the absence (n=62) of nonneuronal cells, in the presence of nonneuronal cell conditioned media (CM, n=35), or in the presence of CNTF (10ng/ml) (n=39). The levels of IAf and IAs are not significantly different on neurons grown in the presence of CNTF or in the presence of nonneuronal cell conditioned media ($p>0.2$), but are significantly greater (***, $p<0.001$; **, $p<0.01$) than the mean densities on neurons grown in the absence of nonneuronal cells. Errors are s.e.m.

the presence of media conditioned by nonneuronal cells from the ganglion.

5.3.5 P14 neurons continue to express IAf in culture

P14 neurons grown in the absence of other cell types

When P1 neurons are grown in culture without other cell types they lose their IAf and IAs currents. P14 neurons, however, express IAf at 3 fold higher and IAs at 2 fold lower levels than on P1 neurons, similar to the outward currents expressed by adult rat SCG neurons (Galvan and Sedlmeir, 1984; Belluzzi et al., 1985). As such, we were interested in whether P14 neurons are still influenced by factors that affect developmentally younger neurons. To test this possibility, we grew P14 SCG neurons in culture for 2-4 weeks in the absence of other cell types. Figures 5.9 A and B show the outward currents for a P14 neuron (A), and a P14 neuron that has developed in culture for 28 days (B). P14 neurons express all three K currents, while cultured P14 neurons express IAf and a large IK, but do not express a detectable IAs.

Figure 5.9C shows the mean current densities (pA/pF) for IAf, IAs, and IK on P14 neurons grown in culture for 1, 14, and 28 days without other cell types. IAf decreases only by about 30% over the first two weeks in culture, and thereafter shows no significant change ($p>0.2$) even after 4 weeks in culture. This is in contrast to the loss of IAf that is observed when P1 SCG neurons are grown without nonneuronal cells, and suggests that over the first 2 postnatal weeks *in vivo*, the expression of IAf loses most of its dependence on extrinsic factors. In comparison, after 2 weeks in culture, the mean IAs current density on P14 neurons drops by 60%, and continues to decrease, so that after 4 weeks neurons express little or no IAs (<10pA/pF). This is similar to what is observed for IAs on P1 neurons grown in culture in the absence of other cell types, and suggests that unlike IAf, IAs expression is still dependent on an extrinsic factor. The

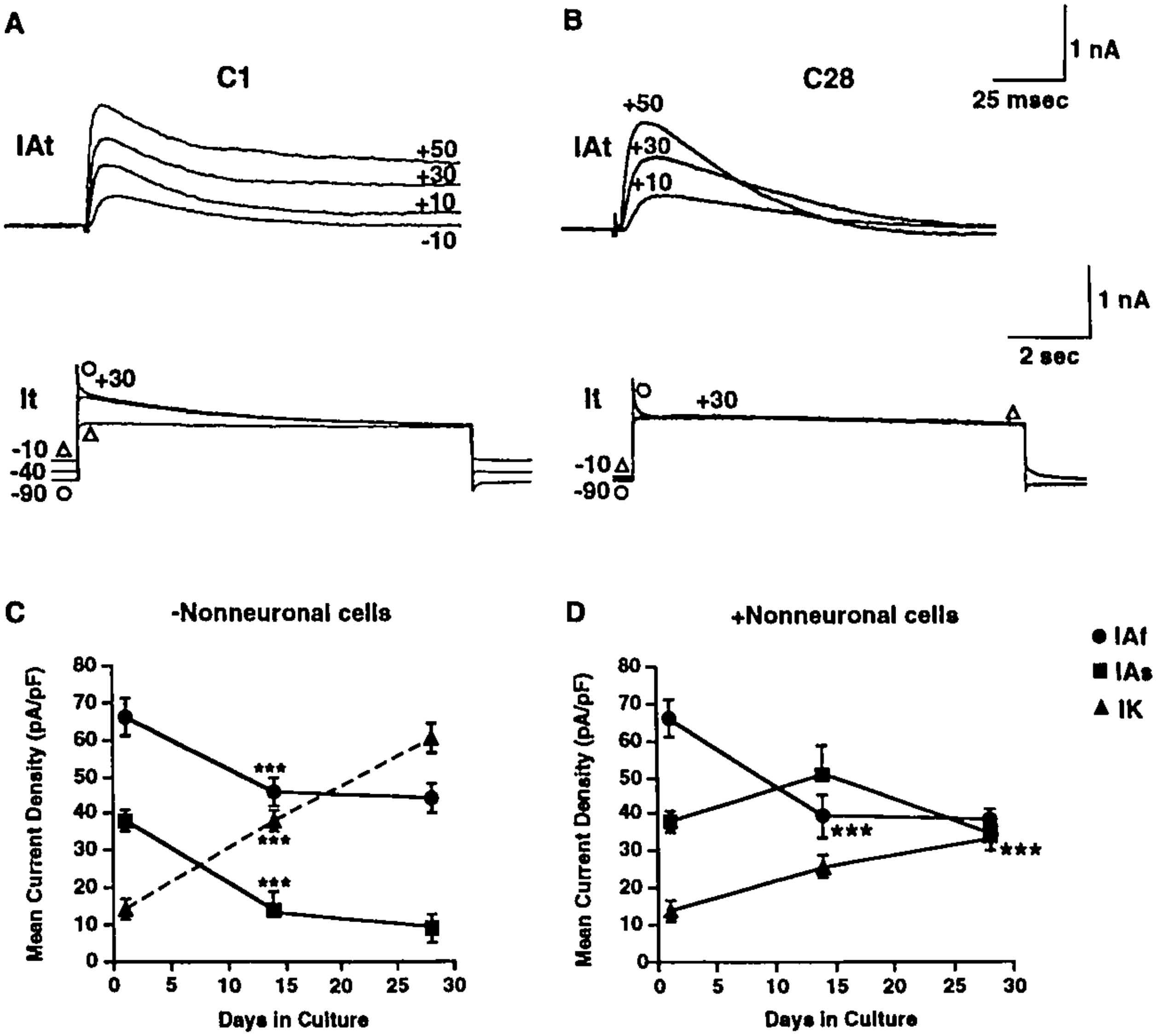

Figure 5.9: Expression of K currents on P14 SCG neurons in culture: A,B show outward currents from a representative P14 neuron(A), and a representative P14 neuron that has grown in culture for 28 days in the absence of other cell types(B). Top: Total A-current (IAt): currents were evoked by depolarizing voltage steps in 20 mV increments up to +50 mV from a Vh of -90 mV. IK currents evoked by depolarizing steps to the same command potentials, from a Vh of -10 mV, have been subtracted to isolate the total A-current. Currents were filtered at 1.5 kHz and sampled at 5 kHz. Bottom: non-leak subtracted total outward currents evoked by 10 sec depolarizing steps to + 30 mV from Vh's of -90 mV, -40mV and -10 mV. Currents were filtered at 100 Hz and sampled at 200 Hz. C-D: mean current densities for IAf, IAs and IK on P14 neurons grown in culture for 2 to 4 weeks in the absence (C) or presence (D) of nonneuronal cells. When P14 neurons are grown in the absence of other cell types IAf drops by a third (***, $p<0.001$), IK increases 5 fold ($p<0.001$), while IAs on most neurons is barely detectable. (C1, n=62; C14, n=31; C28, n=35). When P14 neurons are grown in the presence of nonneuronal cells from the ganglion IAf levels drops by a third ($p<0.001$) after 2 weeks in culture, and thereafter the levels remain constant. IAs levels are maintained, and IK levels increase 2-3 fold. (C14, n=28; C28, n=50). Errors are s.e.m.

same appears true of IK expression, as the IK mean current density increases 5 fold over 4 weeks in culture, similar to what is observed for IK on P1 neurons cultured without their nonneuronal cells.

P14 neurons grown in the presence of nonneuronal cells from the ganglia

When P1 SCG neurons are grown in the presence of nonneuronal cells from the ganglion they continue to express IAf and IAs, which they lose if grown without other cell types. We were interested to know whether a factor from nonneuronal cells could similarly influence IAf and IAs expression on P14 neurons. Therefore, we grew P14 neurons in culture with their nonneuronal cells for 2-4 weeks. In two platings (n=50), 50% of the neurons that developed in culture for 4 weeks expressed IAs at levels comparable to those found on P14 neurons *in vivo* (>30 pA/pF); IAs was expressed at significantly higher levels ($p<0.001$) than in sister cultures where the neurons were grown without other cell types. These results suggest that P14 neurons respond to a factor provided by nonneuronal cells, as do P1 neurons. For reasons that are unclear to us, in a third plating (n=21) only 9% of the neurons expressed IAs at >30 pA/pF. In fact, the neurons in this plating expressed the 3 K currents at levels that were not significantly different ($p>0.2$) from those on P14 neurons grown in culture without nonneuronal cells.

Figure 5.9D shows the mean current densities (pA/pF) for IAf, IAs and IK for the two platings where neurons responded to a factor provided by nonneuronal cells. When neurons are grown with their nonneuronal cells the mean IAs current density is >30 pA/pF, even after 4 weeks in culture, suggesting that IAs expression by P14 neurons in culture, as for P1 neurons, is sensitive to a factor provided by nonneuronal cells. Changes in IK expression also differ when P14 neurons are grown with nonneuronal cells as compared to when they are grown in their absence; the mean IK current density is 2 fold lower in the 28 day old co-cultures ($p<0.001$). In contrast, P14 neurons grown with or without their nonneuronal cells show a similar 30-40% drop in their mean IAf current density with time in culture (p>0.2), supporting the idea that IAf expression is no longer sensitive to a nonneuronal cell factor.

5.3.6 Nodose neurons continue to express IAf and IAs in culture

When P1 nodose neurons develop *in vivo* they show little or no change in their expression of IAf, IAs and IK (see Chapter 4). This suggests that the postnatal control of K current expression on sensory neurons may be different from that on SCG neurons. To investigate this idea, we grew P1 nodose neurons in culture for 2-3 weeks. Figure 5.10A compares the mean current densities (pA/pF) for IAf, IAs and IK on P1 nodose neurons, and on P1 neurons developing *in vivo* or in culture for 2 weeks. There is no significant difference (p>0.1) between the levels of expression of the 3 currents whether nodose neurons develop *in vivo*, or in culture, suggesting that nodose neurons do not require a factor provided by nonneuronal cells to express voltage-gated K currents.

Nerve Growth Factor (NGF) increases the density of functional neuronal nicotinic acetylcholine receptors on cultured nodose neurons (Mandelzys et al., 1990; Mandelzys and Cooper, 1992). To test whether NGF has effects on K current expression, we grew P1 nodose neurons in culture for 2 weeks in the absence of NGF, and in the presence of antiserum to NGF (concentration 1:1000). It was not possible to do these experiments with SCG neurons, because they die in the absence of NGF, whereas nodose neurons do not need NGF for survival (Mandelzys and Cooper, 1991). Figure 5.10B shows the mean current densities for IAf, IAs and IK when nodose neurons are grown in the absence of NGF, and demonstrates that the currents are expressed at levels comparable to those of +NGF cultures (p>0.1). This result indicates that while NGF has effects on the expression of ligand-gated channels on nodose neurons, it does not

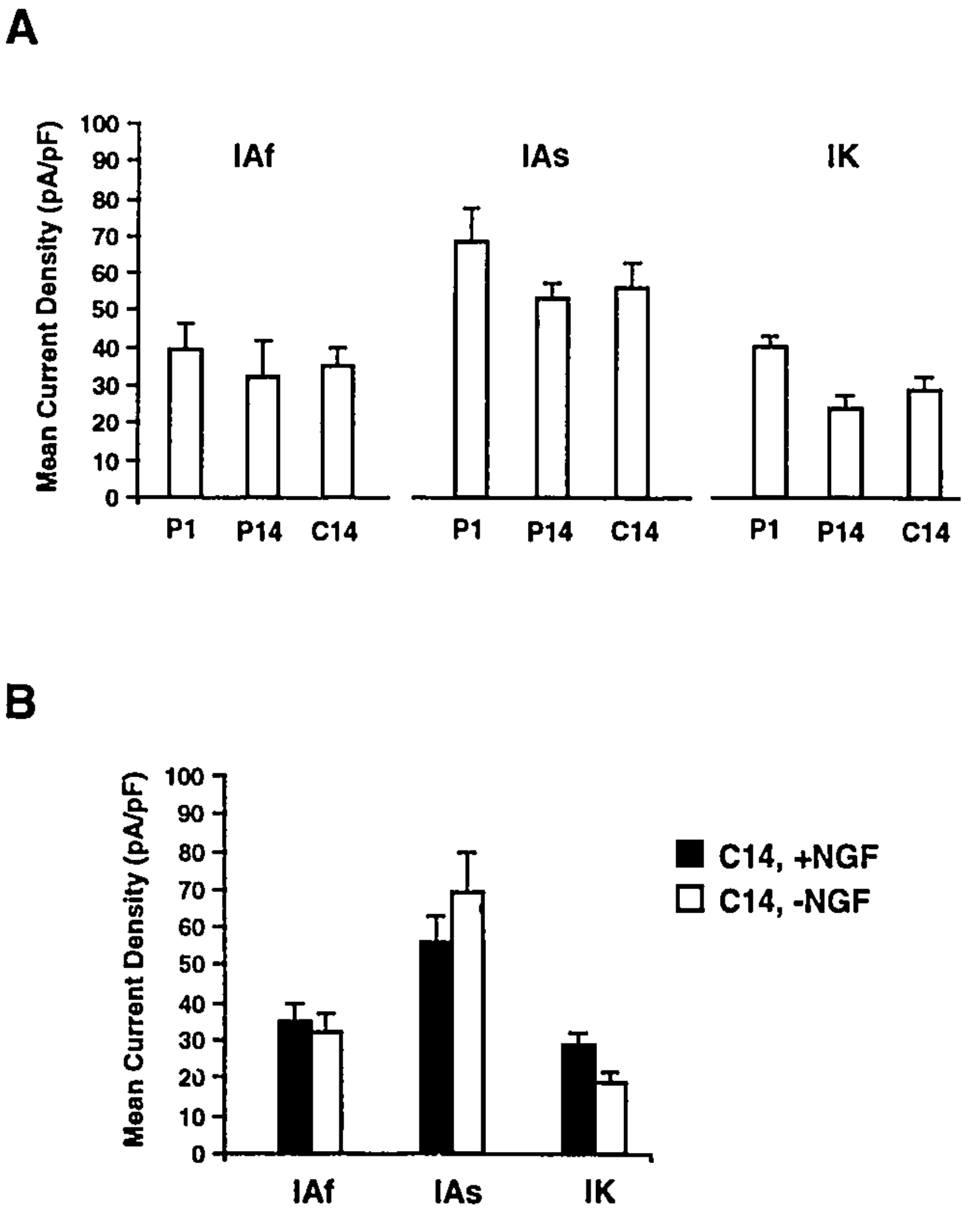

Figure 5.10: Comparison of the expression of K currents on nodose neurons developing in vivo, and in culture: A: shows the mean current densities (pA/pF) for IAf, IAs and IK expressed on P1 neurons (n=30), P14 neurons (n=12), and on P1 neurons grown in culture (n=30). There is no significant difference ($p>0.1$, t-test) in the levels of the 3 K currents on P14 neurons, and P1 cultured neurons. B: shows the mean current densities for IAf, IAs and IK on P1 nodose neurons that have grown in culture in the presence (n=30) or absence (n=22) of NGF for 2 weeks. There is no significant difference ($p>0.1$, t-test) in the levels of the 3 K currents on P1 neurons cultured with or without NGF.

influence the expression of voltage-gated channels in the same neurons.

5.3.7 Peripheral neurons coordinate their expression of IAf, IAs and IK

Figure 5.11 shows representative examples of the outward K currents expressed by P1 and P14 SCG neurons maintained in culture for different periods of time, and demonstrates that the relative proportions of the 3 voltage-gated K currents differ for SCG neurons in various conditions: some postnatal SCG neurons express all three K currents (P1 and P14) but do so in different proportions, other neurons express only 2 of the voltage-gated K currents (P1 in culture for 7 days, and P14 in culture for 28 days), while a third group only expresses IK (P1 in culture for 28 days). Since the relative proportions of IAf, IAs and IK on SCG neurons will influence their electrical behaviour, we are interested in whether the expression of these separate currents might be coordinated. Our results from P1 and P14 neurons developing *in vivo* or in culture, suggest that the expression of IAf, IAs and IK are coordinately regulated by the neuron. Figure 5.12A summarizes these findings. For SCG neurons developing *in vivo*, we observed a reciprocal relationship in the expression of IAf and IAs; initially IAs is high and decreases with postnatal development, whereas IAf is initially low and increases over the same period. In contrast, when P1 or P14 neurons develop in culture for several days, IK levels increase significantly as if to compensate for the loss of one or more of the inactivating currents. These results suggest that the neuron coordinates its expression of IAf, IAs and IK so that changes in one K current are compensated for by reciprocal changes in one or more of the other K currents.

Figure 5.12B plots the A-currents as a percentage of the total outward K current versus IK current density, for SCG neurons in different conditions in which the relative expression of the K currents varies: freshly isolated P1 and P14 neurons; P1 and P14 neurons grown in the presence and absence of nonneuronal cells; and P1 neurons grown in the presence of conditioned media.

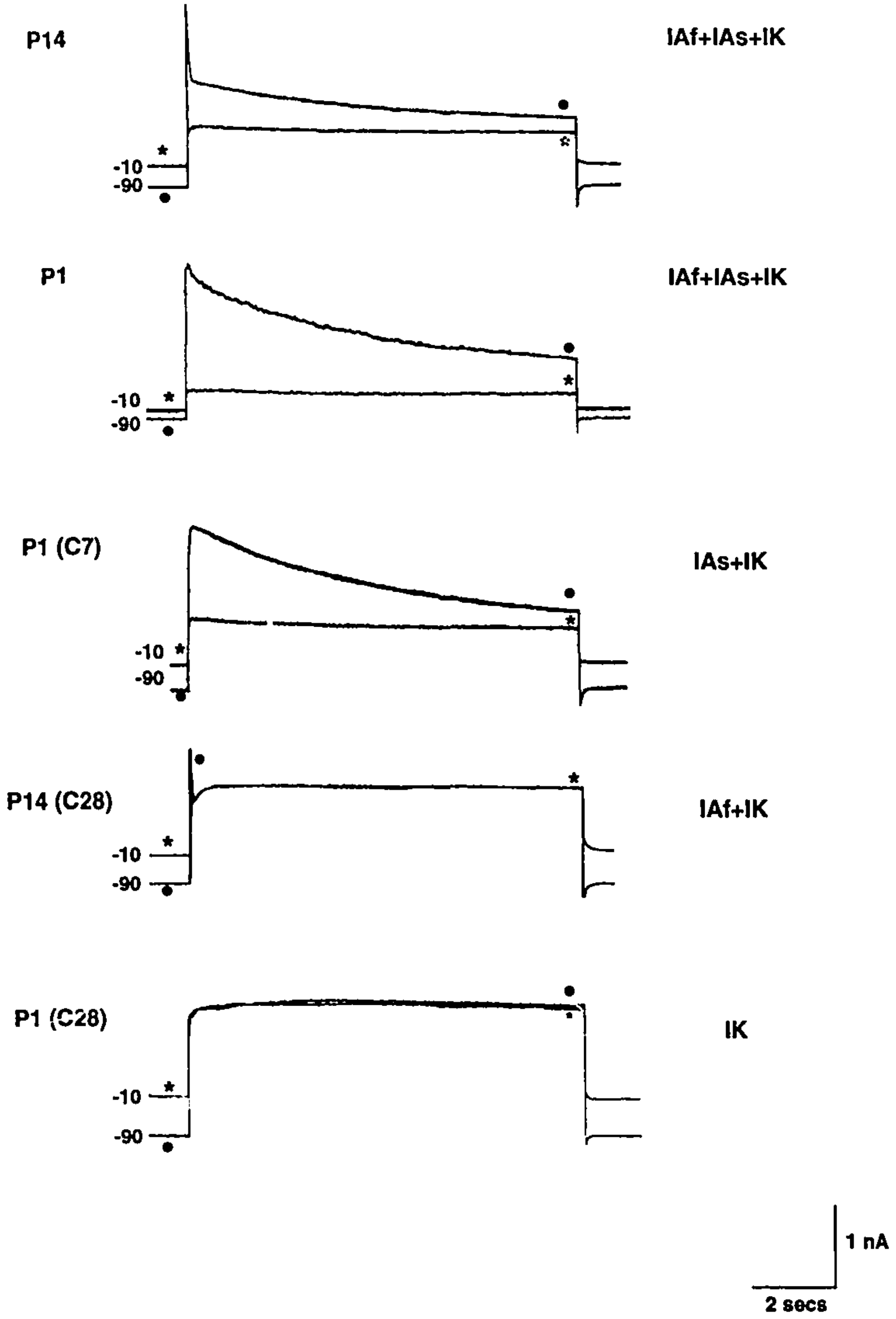

Figure 5.11: Representative examples of voltage-gated K currents expressed on neurons in different conditions. Ten second voltage steps to +30 mV were given from a Vh of -10 mV and -90 mV. Currents are not corrected for leakage, and were filtered at 100 Hz and sampled at 200 Hz.

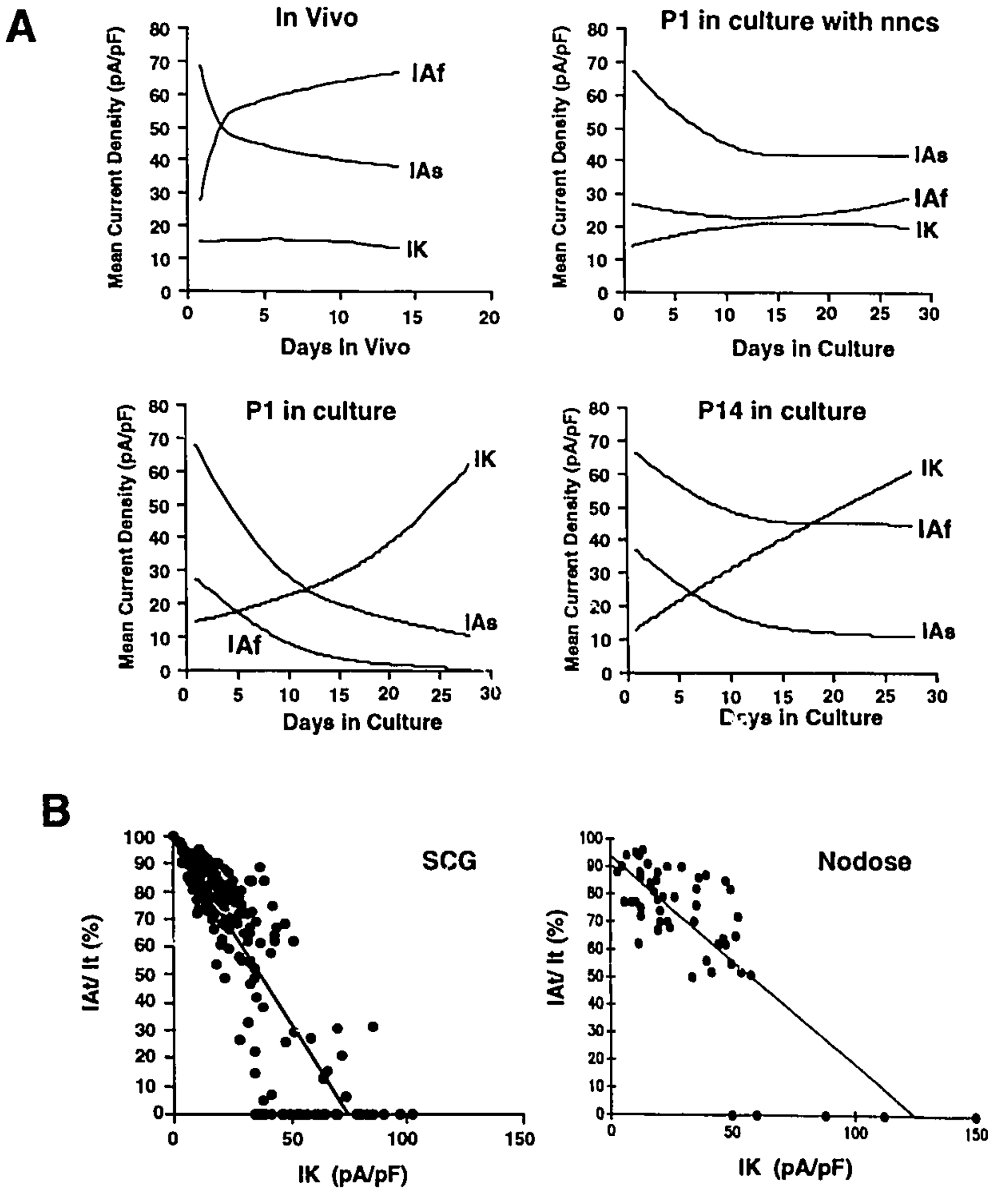

Figure 5.12: SCG and nodose neurons coordinate their expression of IAf, IAs and IK during postnatal development in vivo and in culture. A: shows schematic diagrams of the changes in IAf, IAs and IK mean current densities on P1 SCG neurons developing in vivo (top, left), in culture with their nonneuronal cells (top, right), in culture without their nonneuronal cells (bottom, left), and on P14 SCG neurons developing in culture without nonneuronal cells (bottom, right). SCG neurons coordinate their expression of the different voltage-gated K currents, so that a decrease in one of the currents is coupled to an increase in one of the other currents. B: For each neuron the size of the A-currents (IAf and IAs) as a percentage of the total outward current is plotted against the size of IK (pA/pF). Right: SCG neurons: shows the results of over 200 P1 and P14 neurons, either acutely dissociated, grown in culture in the absence or presence of nonneuronal cells, or grown in the presence of media conditioned by nonneuronal cells from the ganglion. Left: Nodose neurons: shows the results of over 50 P1 nodose neurons. For both plots the solid lines represent linear regression with correlation coefficients (r) of 0.71 for nodose neurons, and 0.73 for SCG neurons.

If neurons are not coordinating their expression of IAf, IAs and IK one would expect that any given IK current density (pA/pF) could make up a large or small proportion of the total outward current density. However, the plot in Fig. 5.12B demonstrates that the majority of neurons with large IK current densities express a much smaller total A-current. This observation is not restricted to SCG neurons, but is also true for sensory neurons (see Fig. 5.12B, right). Our results for SCG and nodose neurons support the idea that there is an intrinsic control mechanism that coordinates the appearance of different K channel types in their membranes.

5.4 Discussion of results

5.4.1 Summary

In Chapter 4, I presented results that indicate that during the first two weeks of *in vivo* postnatal development there is a switch in the relative levels of IAf and IAs expression by rat SCG neurons; on P1 neurons the outward current is dominated by IAs, whereas on P14 neurons it is dominated by IAf. In this chapter, I show that influences from the preganglionic nerves or the target do not play a role in the switch in inactivating K current type. Our data also suggest that a soluble factor provided by nonneuronal cells can influence the expression of voltage-gated K currents on neonatal rat sympathetic neurons.

5.4.2 Denervation or axotomy of SCG neurons does not affect the in vivo switch from IAs to IAf

While previous studies have shown that preganglionic innervation can affect the differentiated properties of sympathetic neurons (Black et al., 1971; Voyvodic, 1987), our results suggest that it is unlikely that the changes in IAf and IAs expression are due to the increase in preganglionic innervation observed postnatally (Smolen and Raisman, 1980). P1 neurons that were denervated for 1-2 weeks before removal from the animal still showed the normal developmental increase in IAf expression and decrease in IAs expression.

Our results also argue against the involvement of a target-derived factor taken up by the sympathetic nerve terminals and then retrogradely transported back to the cell body. Such a factor would be eliminated by axotomy, yet, our experiments indicate that axotomy has little effect on the change in postnatal expression of IAf, IAs and IK. This contrasts with some studies that show that axotomy can produce electrophysiological changes in neurons (Kuno et al., 1974; Kelly et al., 1986; Gustaffson, 1979), and may suggest that the factors that govern the expression of voltage-gated ionic channels on different neurons are not the same.

5.4.3 SCG neurons lose their A-currents in culture

One mechanism for developmental changes in K current expression on SCG neurons is that the types and levels of K currents they express are predetermined by an intrinsic program, and are not influenced by external factors. This could explain why axotomy and denervation do not affect K channel expression. However, our results from SCG neurons developing in culture argue strongly against this possibility. We observed that IAf fails to increase on neurons in culture, and in fact, disappears over a period of 4 weeks. Our observations on IAs expression in culture were similar to those for IAf. For the first week in culture, IAs is maintained at a density found on P1 neurons; however, over the next 3 weeks, IAs decreases to approximately 10% of the value found on P1 neurons. These results clearly demonstrate that extrinsic factor(s) can impinge on the machinery controlling K channel expression, and therefore, the mechanisms regulating the developmental expression of IAf and IAs are not entirely intrinsic to the neurons. In addition, they suggest that there must be some factor(s) essential for the expression of these currents that is missing in culture. Alternatively, it is conceivable that our cultures contain a factor that acts to suppress the expression of IAf and IAs. This seems less likely to us because all the ingredients in our growth media (see Chapter 2) should also be present *in vivo*. As an aside, these results also indicate that one should be cautious in interpreting electrophysiological data from neurons in long-term cultures, because the ionic currents may not necessarily be expressed in the same way as on the neurons *in vivo*.

It is important to note two points concerning the loss of IAf and IAs with time in culture. The first is that the loss of IAf and IAs is not due to a loss of cell integrity. SCG neurons in culture continue to express overshooting APs, and actually increase their expression of IK. The second point is that the lack of expression of voltage-gated K currents on cultured neurons does not appear to be a generalized effect on the differentiation of these neurons; in culture, neonatal SCG neurons differentiate many of their adult characteristics (O'Lague et al., 1978; Bruckenstein et al., 1989). Thus the loss of A-currents appears to be a specific phenomenon. Our results clearly demonstrate that there is some factor(s) that is essential to maintain IAf and IAs expression on these neurons.

Long-term changes in K current expression on cultured SCG neurons could most easily be explained by changes in the relative number of functional channels expressed in the membrane, or by changes in the spatial distribution of K currents in the membrane. In this light, it is possible that with development *in vitro*, SCG neurons are targeting their A-channels preferentially to axonal and/or dendritic regions; channels in these regions may be missed by our recordings and therefore go undetected. However, our results obtained by redissociating and replating neurons that have been in culture for 2 weeks argue against this possibility; in these neurons, processes are removed and therefore, any newly expressed A-channels should be located in the cell body region. However, this was not the case; replated and control cultured neurons expressed small, or no A-currents.

Nerbonne and Gurney (1989) reported that SCG neurons from embryonic E14 rats could acquire IAf when they developed in culture. However, they also observed that the density of this current decreased when SCG neurons from neonatal animals developed in culture. Our results are consistent with theirs in that we also observe that IAf and IAs current densities on P1 SCG neurons decrease over 2 weeks in culture. A more direct comparison between the study of Nerbonne and Gurney and ours is difficult because in their study measurements were only made over the first 6 days in culture, and because they did not separate the non-transient outward current into IAs and IK.

5.4.4 Factors affecting K current expression on SCG neurons developing in culture

Cultured neurons are without their *in vivo* targets or the preganglionic spinal neurons that normally innervate them. However, it is unlikely that either provides the factor(s) that is needed to maintain A-current expression. *In vivo*, denervated or axotomized neurons continue to express A-currents long after cultured neurons show significant decreases in their levels. We can also safely rule out a role for NGF, a factor necessary for survival of sympathetic neurons (Hendry and Campbell, 1976; Levi-Montalcini and Calissano, 1986), as a factor needed to maintain IAf and IAs expression on these neurons in culture because NGF was continually present in our cultures.

Nonneuronal cells from the ganglion influence K current expression on SCG neurons developing in culture

Our results from P1 SCG neurons co-cultured with their nonneuronal cells indicate that nonneuronal cells can influence the expression of voltage-gated K currents. In the presence of nonneuronal cells, cultured P1 neurons continue to express IAf and IAs. IAf mean current density does not change from P1 levels, and while IAs drops by a third over the first two weeks in culture, after 28 days in culture IAs densities are 7 times those of neurons in culture without nonneuronal cells. In addition, IK is maintained at low levels, similar to IK on P1 neurons.

The fact that nonneuronal cells in our cultures can prevent the loss of IAf and IAs, and moreover prevent the increase in IK, indicates that the nonneuronal cells are providing some factor(s) that can affect K current expression on cultured P1 SCG neurons. The expression of A-currents by SCG neurons cultured with other cell types is consistent with results of Freschi (1983), who observed small rapidly inactivating currents in SCG neurons co-cultured with nonneuronal cells. Several studies have demonstrated that ganglionic nonneuronal cells influence many of the differentiated properties of peripheral neurons, including neurite outgrowth (Roufa et al., 1983; De Koninck et al., 1992), muscarinic receptor expression (Smith and Kessler, 1988), and nicotinic acetylcholine receptor expression (Cooper and Lau, 1986; Mandelzys and Cooper, 1992). In addition, studies on rat sympathetic neurons developing in culture indicate that when neurons are grown in the presence of nonneuronal cells their AP's are followed by a long afterhyperpolarization, due to a Ca activated K conductance, which is absent when neurons are grown without other cell types (O'Lague et al., 1978). Coupled with our results, it appears that nonneuronal cells can influence the levels of many of the K currents expressed by SCG neurons. Such regulation may be bi-directional in that neurons can affect the developmental expression of K currents by amphibian myocytes (Ribera and Spitzer, 1991).

Specificity of the nonneuronal cell influence

Nonneuronal cells from various tissues were all able to control K current expression in culture, however, not all nonneuronal sources are equally effective. Heart cells appear to provide a factor(s), possibly the same factor as from ganglionic nonneuronal cells, that maintains K current expression on SCG neurons; IAf and IAs mean current densities for SCG neurons grown in the presence of heart cells are not significantly different from levels on P1 neurons. Since heart cells are targets for a small population of SCG neurons (Skok, 1973), it may be that the target influences the neuron's expression of K currents. However, our axotomy experiments suggest that the target does not significantly influence the expression of IAf, IAs and IK on SCG neurons *in vivo*. It may be that in the axotomy experiments, nonneuronal cells from the ganglion provide a factor that prevents the loss of A-currents in the absence of the target.

There does appear to be some specificity to the effect of nonneuronal cells. While nonneuronal cells obtained either from heart or the ganglion were effective at maintaining IAf and IAs expression in culture, skin cells were only capable of maintaining IAf expression. IAf levels were comparable on neurons grown in the presence of skin, heart or ganglionic nonneuronal cells. However, in skin co-cultures IAs current densities dropped to levels not significantly different from those expressed on neurons grown without other cell types. The fact that skin cells influence IAf, but not IAs expression, might suggest that the factors provided by the 3 cell types differ, and as such, have differential effects on IAf and IAs expression. Alternatively, it is possible that different factors control the expression of the two types of inactivating current; skin cells only provide one of the factors, whereas heart and ganglionic nonneuronal cells provide a second factor(s) that influences IAs expression.

A soluble factor from nonneuronal cells prevents the loss of IAf and IAs in culture

We investigated the nature of the factor provided by nonneuronal cells from the ganglion. If neurons are grown in the presence of media conditioned by nonneuronal cells, IAf, IAs and IK levels are not significantly different from those expressed on neurons cultured with their nonneuronal cells. Since conditioned media has no effect on the survival of sympathetic neurons in culture (Patterson, 1978; Furshpan et al., 1982), it seems unlikely that a population of neurons with A-currents is preferentially selected for under these conditions. Rather, our results suggest that the effect of nonneuronal cells on K current expression is mediated by way of a secreted soluble factor; direct cell-cell contact does not appear to be required. Several studies have shown that factors secreted by nonneuronal cells influence the neurotransmitter (Patterson, 1978; Zurn, 1987) and peptide (Nawa and Sah, 1990) phenotypes of cultured sympathetic neurons.

In co-cultures and conditioned media cultures, sympathetic neurons form functional cholinergic synapses (Furshpan et al., 1982). However, it is unlikely that electrical activity could provide a signal for controlling A-channel expression as neurons in 1-2 week co-cultures express A-currents even though electrical connectivity has not been properly established.

CNTF prevents the loss of IAf and IAs in culture

Our results demonstrate that ciliary neurotrophic factor (CNTF) is able to prevent the loss of IAf and IAs on P1 neurons in culture. This suggests that CNTF, besides influencing sympathetic neurons to express a cholinergic phenotype (Saadat et al., 1989), can affect the voltage-gated K currents expressed by these neurons. In culture, CNTF has similar effects on the levels of IAf, IAs and IK as does the soluble factor from nonneuronal cells. In addition, CNTF is found in high concentrations in the nonneuronal cells of the rat sciatic nerve (Lin et al., 1989). This might suggest that the factor provided by the ganglionic nonneuronal cells is CNTF. However, it has been shown that the primary structure of CNTF does not contain a signal sequence and therefore it is an unresolved question whether CNTF is secreted by the cells in which it is expressed (Lin et al., 1989). In addition to CNTF (Saadat et al., 1989), two other factors have been identified that influence the neurotransmitter phenotype of sympathetic neurons; cholinergic differentiation factor or leukemia inhibitory factor (Yamamari et al., 1989), and a soluble factor from rat sweat glands (Rao and Landis, 1990). It would be interesting to know whether another property of these factors is their ability to influence the K currents expressed by sympathetic neurons.

Explant cultures

When P1 ganglia are left intact, IAf and IK densities are not significantly different from when the various cell types of the ganglia are co-cultured in dissociated form. However, IAs levels are 30% higher in explant cultures than in dissociated cultures. One explanation for this difference is that IAs may be more sensitive to the concentration of the nonneuronal cell factor, and in the explant, where nonneuronal cells completely surround the neurons, more factor may be available. The variability in the expression of IAs from one dissociated co-culture to another supports this idea: in some cultures, IAs was maintained at levels comparable to those for P1 neurons, whereas in other cultures IAs was expressed at much lower levels. A similar variability is true of the response of sympathetic neurons to environmental influences that determine their neurotransmitter phenotype (Black, 1982; Wolinsky et al., 1985). Black (1982) has suggested that the diversity of neuronal phenotype may be partly due to the differential responsiveness of neurons to a given set of extrinsic factors. Alternatively, it may be that an additional factor(s) from the ganglion, other than nonneuronal cells, can affect IAs expression. One possibility for such a factor is cell density. Neurons grown in high density cultures show higher levels of choline acetyltransferase and substance P than when cultured at low density (Adler and Black, 1985). Explant cultures are essentially high density cultures and the high levels of IAs expression may be due to density dependent regulation of IAs expression.

It is interesting that when P1 SCG are maintained as explant cultures, IAf, IAs and IK levels are comparable to those on P1 neurons. However, it is important to note that while SCG neurons co-cultured with nonneuronal cells continue to express IAf and IAs, they do not exhibit the *in vivo* developmental switch from an IAs to an IAf dominated outward current. This might imply that when SCG neurons are removed with their nonneuronal cells on P1, K current development is arrested at this stage and requires some trigger to allow it to proceed to a more adult stage. Other factors, possibly circulating factors, may act as the trigger for the changes in inactivating currents: growth factors (Snider, 1988; Haugen and Letourneau, 1990; Mandelzys and Cooper, 1992) and hormones (Recio-pinto et al., 1986; Gould and Butcher, 1989) have effects on many of the differentiated properties of neurons, including their electrical excitability (Desnuelle et al., 1987; Mills and Zakon, 1991; Joels and de Kloet, 1992).

5.4.5 P14 SCG neurons in culture continue to express IAf but not IAs

During the first two weeks of postnatal development, SCG neurons increase their expression of IAf and decrease their expression of IAs, so that by P14 the outward currents appear very similar to those reported for adult SCG neurons (Galvan and Sedlmeir, 1984; Belluzzi et al., 1985). As such, we are interested in whether P14 neurons are still influenced by factors that affect developmentally younger neurons. Our results suggest that over the first 2 weeks of postnatal development IAf loses its dependence on a factor provided by nonneuronal cells: when P14 neurons are grown in culture without other cell types, IAf dropped by only 30-40% after 14 days in culture, and then was maintained at this level for at least another 2 weeks. Hence, a nonneuronal cell factor appears to be required by cultured P1 neurons, but not P14 neurons, to express IAf. These results also imply that SCG neurons have a finite period in which they respond to the loss of a nonneuronal cell influence; IAf expression by SCG neurons loses its susceptibility after 2 weeks of postnatal development.

The 30% drop in IAf is also seen if P14 neurons are grown with their nonneuronal cells, and as such, it seems likely that some other factor(s) plays a role in regulating the expression of IAf on SCG neurons. In this regard, it is interesting that P14 SCG neurons, axotomized or denervated on P1, also continue to express IAf, but at 20% lower levels. It is possible therefore, that a factor from either the preganglionic nerves and/or the [illegible]et has some role in regulating the level of IAf expression on SCG neurons. The absence of either of these influences in cultures of P14 neurons may explain the 30% drop in mean IAf current density. Alternatively, it is possible that the drop in IAf reflects the absence of a circulating factor in the adult rat serum used in our cultures, that is present in neonatal serum.

When P1 or P14 SCG neurons are grown in the absence of nonneuronal cells, IAs mean current densities drop to low levels (<10pA/pF), suggesting that IAs expression by P14 neurons is still dependent on a factor provided by nonneuronal cells. This idea is supported by the fact that IAs expression is maintained when P14 neurons are grown in culture in the presence of nonneuronal cells from the ganglion: in two platings almost 70% of the P14 neurons grown with nonneuronal cells continued to express IAs, and 50% expressed IAs at levels >30pA/pF, comparable to the mean IAs current density on P14 neurons.

Nonneuronal cells also affect the expression of IK by P14 neurons in culture. In the absence of other cell types, IK expression increases 5 fold over 28 days in culture to levels comparable to those on P1 neurons in culture. Whereas, when P14 neurons are grown in the presence of nonneuronal cells, the mean IK current density increases only 2-3 fold. If, as we suggest, the expression of IAf, IAs and IK on SCG neurons is coordinated, then this small increase in IK may reflect the small decrease in IAf that is observed in co-cultures. Our results indicate that the expression of IAs and IK, but not IAf, continue to be dependent on a nonneuronal cell factor.

5.4.6 No change postnatally in K current expression on nodose neurons

P1 nodose neurons that develop *in vivo* (see Chapter 4), or in culture, express IAf, IAs and IK at similar levels to those observed for P1 neurons. This result suggests that the nonneuronal cell factor required for IAf and IAs expression on P1 SCG neurons in culture, is not required by nodose neurons to continue to express A-currents. In addition, because K current levels on P1 nodose neurons that have developed *in vivo*, or in culture, are comparable to those on P1 neurons it appears that there is little or no postnatal control of K current expression on nodose neurons.

One possible explanation for the differences observed for postnatal K current expression on SCG and nodose neurons in culture, is that sensory and sympathetic neurons are responsive to extrinsic factors during different developmental periods. It may be that P1 nodose neurons have already acquired the set of K channels they express in the adult, and therefore, removal of nodose neurons from the influences they would normally see *in vivo* may no longer affect their expression of K channels. In contrast, the fact that there are changes in the *in vivo* postnatal expression of IAf and IAs on SCG neurons implies that SCG neurons are not yet fully committed with respect to the K currents they express, and therefore, they might be more vulnerable to the removal of external influences. Alternatively, it may be that different factors regulate the expression of very similar K currents on different neurons of the peripheral nervous system.

5.4.7 Intrinsic regulation of K current expression

Finally, our results suggest that the expression of IAf, IAs and IK is subject to an intrinsic control mechanism that coordinates their expression. For neurons developing *in vivo*, we observed a reciprocal relationship in the expression of IAf and IAs; initially IAs is high and decreases with postnatal development, whereas IAf is initially low and increases over the same period. On the other hand when P1 or P14 SCG neurons develop in culture for several days, IK levels increase significantly as if to compensate for the loss of one or more A-currents. These findings are consistent with observations on nodose neurons of an inverse relationship between the expression of A-currents and the expression of IK. These results suggest that neurons have intrinsic mechanisms to coordinate their expression of voltage-gated K currents, presumably so as to regulate their total outward K current. One possible mechanism for such regulation is that somehow the neuron senses that there are changes in its total outward current, perhaps by changes in its membrane potential, and makes adjustments to the levels of K currents it expresses.

Alternatively it is possible that there is an established 2nd messenger system through which an extrinsic factor acting on the neuron controls its expression of all three K channel proteins. One would then have to hypothesize that the various extrinsic factors use alternate systems to differentially regulate the expression of IAf, IAs and IK.

Chapter 6

Molecular identification of voltage-gated K channels of SCG neurons

The aim of the studies described in this chapter is to identify the K channel genes expressed by SCG neurons. I have initiated these experiments over the last 9 months, and have promising preliminary data. The results presented in this chapter demonstrate the validity of a procedure designed by Dr. Larry Salkoff and colleagues, involving PCR (polymerase chain reaction) homology screening, for isolating K channel genes in SCG. Regrettably, many of the specific details concerning the experiments must await publication by Salkoff and colleagues. The future goal of these experiments, is to use the sequences of the identified K channel genes to design SCG specific probes for use in RNAse protection assays, to determine whether changes in mRNA levels can explain the changes in K current expression during postnatal development *in vivo* and in culture.

6.1 Introduction

Little is known about the mechanisms used by individual cells to express different types of K channel, or about how they change their expression of K currents during development and in the adult. In Chapters 4 and 5, I showed that the levels of expression of voltage-gated K currents on SCG neurons change during postnatal development *in vivo* and in culture. *In vivo*, there is a switch from an IAs to an IAf dominated outward current, whereas in culture without other cell types, the levels of IAf and IAs on the neurons decrease significantly and IK levels increase 5 fold.

The developmental changes in K currents must involve changes in the number of functional channels in the surface membrane. One possibility is that the channels are preexisting and that second messenger modulation of the channel protein changes the functional state of the channel (Kaczmarek & Levitan, 1987; Rossie and Catteral, 1987). For example, in Aplysia bag cell neurons, modulation of A-currents by cAMP results in an increased rate of inactivation of the current (Strong, 1984), whereas activation of α_1-adrenoreceptors decreases an A-current on serotonergic neurons (Aghajanian, 1985).

However, because the time course for the changes in K current densities during postnatal development is slow (days to weeks), it is more likely that the neuron controls its expression of K currents at the level of the genome. For example, the increase in Na and K current densities on amphibian spinal neurons during development are dependent on mRNA synthesis (O'Dowd, 1983; Ribera and Spitzer, 1989). In this chapter, I present experiments designed to identify the K channel genes expressed in SCG neurons as the first step towards addressing the possibility that developmental changes in K currents involve direct alterations of mRNA levels for specific K channels: this would imply that there are changes either in gene transcription or mRNA stability during development.

Studying the regulation of K channel expression at the genetic level is now possible with recent advances in the molecular biology of K channels. However, such studies have been limited by the fact that for most neurons it is not known which specific K channel genes they express. A number of labs have recently attempted to identify the genes encoding the native K channels of a specific tissue (Ribera, 1990; Hardie et al., 1991; Baker and Salkoff, 1990; Kaang et al., 1992). For example, Hardie and co-workers (1991) used PCR techniques to identify 4 Shaker-type mRNA transcripts expressed in Drosophila retina. In addition, they measured at the single channel and macroscopic levels a single rapidly inactivating A-type channel in Drosophila photoreceptors. They suggest that multiple Shaker transcripts, different from those expressed in muscle, may be responsible for this A-type channel, although they have not confirmed, using in situ hybridization for example, that a single Drosophila photoreceptor expresses more than one of the mRNA K channel transcripts.

Recently, Salkoff and co-workers have developed an approach for identifying the K channel genes expressed in specific cell types. They use primers designed to recognize 2 stretches of amino acids highly conserved in all K channel genes to amplify, using PCR, first strand cDNA synthesized from cell specific RNA templates. The use of K channel specific primers will preferentially amplify K channel DNA, which can be subcloned and sequenced. The advantage of their approach for my study is that one can use RNA specific to SCG neurons, thereby amplifying only those K channel genes which are expressed in these neurons. Using these techniques we hope to isolate and identify the K channel genes expressed in SCG neurons.

As discussed in Chapter 1, four voltage-gated K channel gene subfamilies have been identified; Shab, Shal, Shaw (Butler et al., 1989), and Shaker (Papazian et al., 1987; Kamb et al., 1987; Pongs et al., 1988). Within the membrane spanning regions, there is 40% conservation at the amino acid level between members of different subfamilies. Within each subfamily however, the degree of conservation is closer to 70%, even across species. We are interested in first classifying the channels present in SCG neurons according to these 4 subfamilies. As such, the primer sites for PCR were chosen to flank a region which is not very well conserved between different subfamilies, but is fairly well conserved within a subfamily. On the basis of the nucleotide sequence of this region, the isolated K channel subclones can be classified as either Shab, Shaw, Shal or Shaker-like. Because the PCR primers recognize all known K channel genes, we do not have to make any assumptions as to what K channel genes are expressed by SCG neurons. In addition, because the primer regions are highly conserved in all K channels, we are not precluding the fact that novel K channel genes are expressed in SCG neurons.

The SCG specific K channel DNA sequences we isolate could then be used to design probes for RNAse protection assays, to determine whether changes in gene expression are responsible for the developmental changes in the levels of K currents observed electrophysiologically. Future research could involve studying at what level extrinsic factors, such as the factor from ganglionic nonneuronal cells, exert their effects.

6.2 Determination of the genotype of K channels expressed by SCG neurons by PCR homology screening

The approach used to isolate and identify K channel genes is one developed by Salkoff and co-workers. I will discuss the strategy behind their approach in some detail, but first to give a brief overview: First strand cDNA, synthesized from total P1 SCG RNA, is used as a template in a PCR reaction to preferentially amplify K channel DNA sequences. The amplified DNA is subcloned, and the nucleotide sequences of the subclones compared to known K channel sequences for identification purposes.

6.2.1 Amplification with degenerate oligonucleotide primers

We used first strand cDNA synthesized from total cellular RNA to obtain DNA copies of the genes expressed by P1 SCG neurons. Figure 6.1 shows an autoradiograph of the ^{32}P-dCTP labelled reaction products run on a 1% agarose gel. Since random primers were used in the cDNA synthesis reactions, cDNA fragments varying in size from 1-3kB, as shown on the autoradiograph, can potentially include the sequences of interest.

The first strand cDNA was used in a PCR reaction, with oligonucleotide primers specific for K channels, to selectively amplify K channel DNA. Salkoff and co-workers designed "universal" oligonucleotide primers against 2 regions which are highly conserved in all K channels cloned to date. Primers designed to recognize highly conserved K channel sequences will preferentially amplify K channel DNA. These two highly conserved primer sites flank a region which is poorly

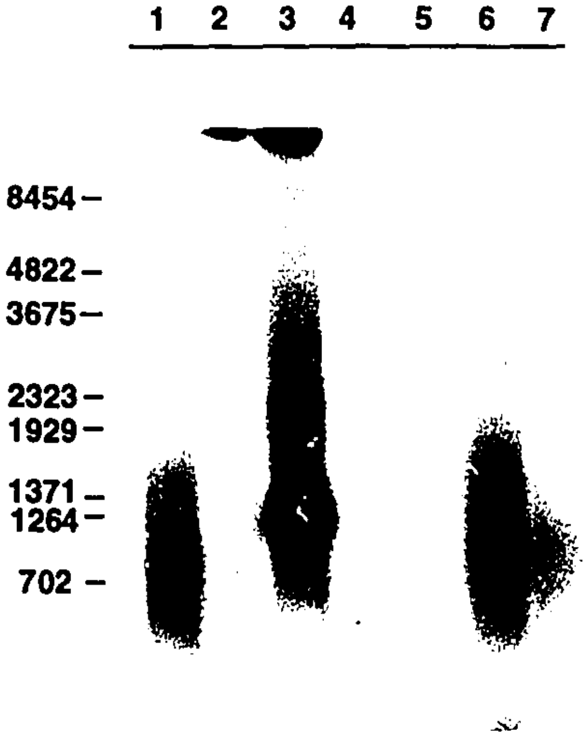

Figure 6.1: First strand cDNA synthesis: shows the size range of ^{32}P-labeled first strand cDNA products, resolved by running the products on a 1% agarose gel, and visualized by autoradiography. cDNAs range in size from 1-3 kB. Lanes 1, 6 and 7 show products from 3 separate synthesis reactions using total cellular RNA isolated from neonatal rat pups. Lane 3 shows the reaction product from the test sample provided with the kit.

conserved between K channel subfamilies, however there are certain amino acid residues which are highly conserved which allow one, with a fair degree of certainty, to identify this fragment as being part of a K channel gene. Another advantage of this region is that while it is poorly conserved between different K channel subfamilies, it is fairly well conserved within any particular K channel subfamily. As such, the sequence of this DNA fragment will identify to which K channel subfamily the subclone belongs.

While the sequences of the 2 primer regions are highly conserved at the amino acid level, at the nucleotide level there is a fair amount of degeneracy. Therefore, we used primers degenerate at the nucleotide level, whose sequences encompass all possible nucleotide substitutions. With degenerate primers (i.e. many slightly different primers) the effective concentration of any single primer is reduced, which decreases the efficiency of the amplification procedure. Therefore, to reduce the number of degenerate primers in the pool, the primers designed by the Salkoff lab are degenerate only at the 3' end. Thus, at the primer end where DNA synthesis commences, binding between primer and template DNA is maximized. The degeneracy stops at the 5' end of the primer; this reduces the total number of possible primers, but allows for some additional partial nucleotide complementation to help in the binding of the primer. Different sets of degenerate primers were used in 2 separate amplification reactions. In one series of amplification reactions, we used primers whose codon usage was biased towards the higher G/C content of mammalian genes.

The PCR reaction products were run on a 3% agarose gel to size select for a band corresponding to the two primers and the intervening DNA segment. Because only K channels should have these primer sites separated by the known length of the DNA fragment, size selection will eliminate much of the non-specifically primed DNA products. Figure 6.2 A and C show examples of photographs of PCR reaction products run on a 3% agarose gel, stained with ethidium bromide, and visualized with ultraviolet light. We found that amplification of cDNA synthesized

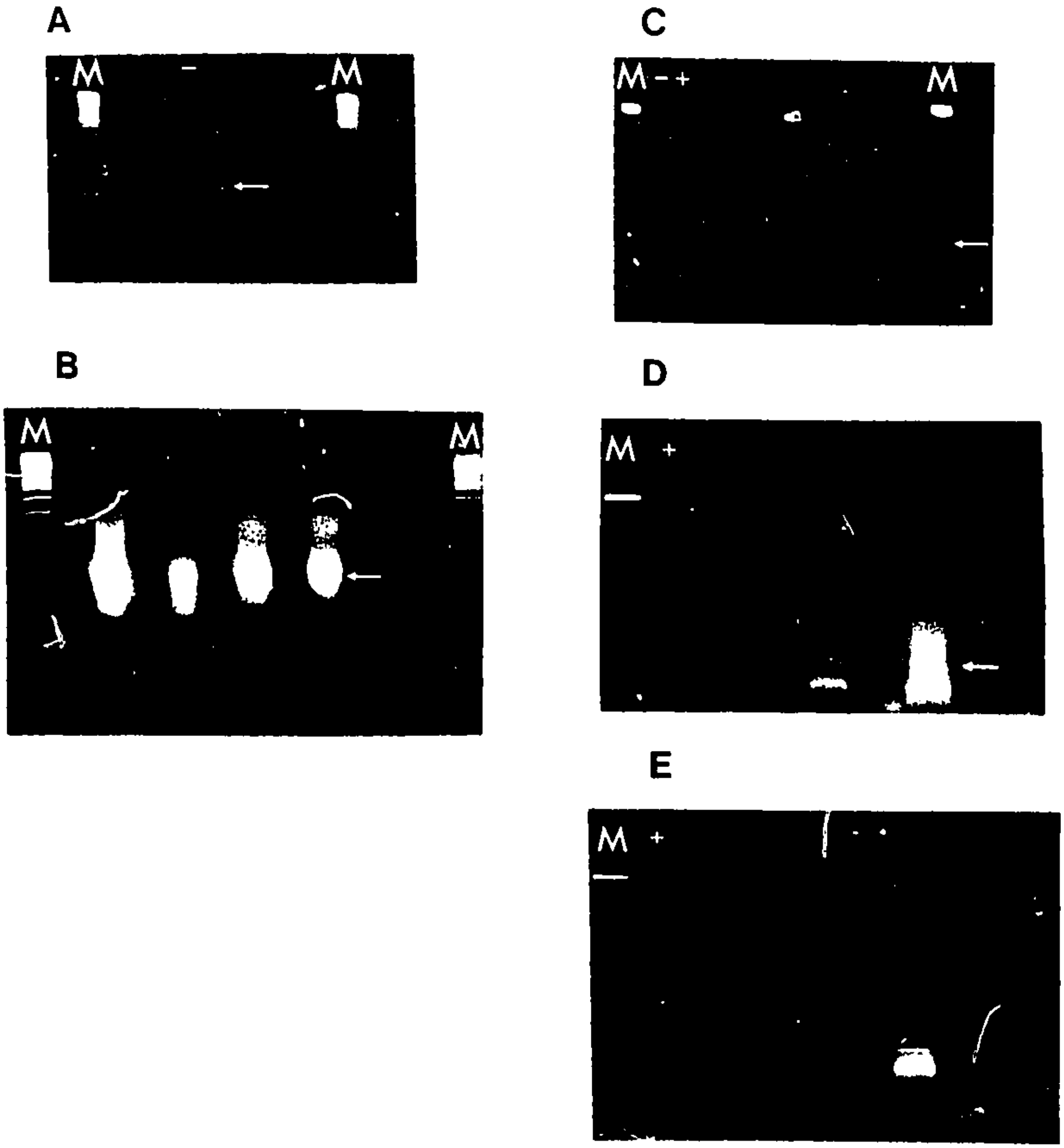

Figure 6.2: Amplification with degenerate and non-degenerate primers: A-E show polaroid photographs of PCR amplification products run on a 3% agarose gel, and visualized by ethidium bromide staining. M designates the size markers; for A and B the size markers are a 100 base pair ladder, while for C-E a 123 base pair ladder was used. The (-) designates the negative control, while the (+) indicates a positive control which contained mouse Shab cDNA which was originally amplified using the same sets of primers. A-C: shows two separate examples of the reaction products from the amplification with the degenerate primers. The arrows indicate a faint band of the appropriate size. B-D: the bands stained in A and C were cut out of the gel, the DNA extracted and a second round of amplification was done using non-degenerate primers. B and D show the respective reaction products for DNA isolated from the gels in A and C. E: shows the agarose gel in D after the band was cut out.

from total cellular RNA resulted in very faint bands of the appropriate size. This is presumably because mRNA makes up a very small percentage (<5%) of the total cellular RNA used in first strand cDNA synthesis, and in turn mRNA for K channels represents probably only about 0.1% of total mRNA. As such, the K channel RNA templates, and as a result, the cDNAs synthesized from them, are present in very small copy number.

To increase the yield and the specificity of the amplified DNA product, a new PCR reaction (reamplification) was performed. The appropriate size band was cut out of the agarose gel, and DNA isolated from the gel slice. Size selection of the DNA from the first amplification reaction increases the specificity of the second amplification reaction: Instead of using total first strand cDNA as templates, only DNA of the appropriate size serves as a template. In addition, the primers were changed to non-degenerate, "nested" primers (described below), which allowed the annealing reaction to occur at a higher temperature (55°C), thereby increasing the specificity of the reaction.

6.2.2 Amplification with non-degenerate primers

The nucleotide contents of the degenerate primers differ, therefore a fairly low annealing temperature must be used to ensure annealing of all the primers. However, with low annealing temperatures the amount of non specific binding between the primer and template DNA is increased. In addition, with a pool of degenerate primers, the effective concentration of any given primer is diluted, resulting in a lower efficiency for the amplification reaction. Therefore, to obtain optimum specificity and efficiency for the second round of amplification, we used a set of nested, non-degenerate primers. These primers are truncated versions of the primers used in the original amplification reaction so that they do not include the degenerate regions at the 3' end of the original primers. Because they contain the non-degenerate 5' ends of the original primers they will bind, with high specificity, all of the original primers that are now part of the amplified DNA. The use of non-degenerate primers allows annealing to occur at high temperatures, which increases the specificity of the reaction. In addition, because only 2 primers have to be added, the effective concentration of the primers is not diluted, and the efficiency of the reaction is optimized.

Figures 6.2 B and D show the reaction products from the second round of amplification on DNA extracted from the agarose gels shown in Fig.s 6.2 A and C. Ethidium bromide staining of the appropriate size band is much brighter, indicating that more of the amplified product was formed. This band was cut from the agarose gel (see Fig. 6.2E) and the DNA extracted. To insert this size selected DNA into M13 bacteriophage for subcloning, the DNA was cut with two restriction enzymes, EcoR I and Xba I, whose consensus sites form part of the 5' end of the sense and anti-sense primers respectively. The M13 bacteriophage were cut with the same enzymes, and a ligase reaction was used to insert the DNA fragments into M13. Bacteria were transformed with M13, and bacterial colonies infected by bacteriophage with DNA inserts identified using a color selection ß-galactosidase (ß-gal) assay. Single stranded M13 DNA was isolated and purified from small scale bacterial preparations of these selected colonies.

6.2.3 Isolation of an SCG K channel subclone

We used the dideoxy sequencing method (Sanger et al., 1977) to sequence the M13 DNA insert of a particular bacterial colony. Sequences of interest were identified by the sequences of the primer restriction sites (EcoR I and Xba I), the primers sequences, and by the known length of the DNA insert. We performed PCR amplification and subcloning of K channel specific DNA fragments on two separate occasions. In the first series, 2 novel K channel subclones were isolated. Unfortunately, these subclones were PCR contaminants of K channel DNA from another species studied in the Salkoff lab. This lab is presently working on cloning and expressing these K channel clones.

A second series of amplifications were performed on a new batch of cDNA synthesized from total cellular SCG RNA isolated from neonatal rat pups. This work is in progress, but to date 2 K channel subclones have been isolated. One of these appears to be a contaminant, even though much care was taken to avoid contamination with DNA from other sources. We are presently screening our subclones with a probe made from this contaminant subclone to eliminate those colonies which have this DNA as their insert. Colonies which do not react with the probe will be selected for further analysis.

The second subclone, through comparison at the amino acid level, is a member of one of the known K channel subfamilies. When the amino acid sequence of our subclone is compared with the corresponding region of other K channel proteins, our subclone is 100% homologous to a channel cloned from rat brain, which I will refer to as RBx. It also has high homology (79-95%) with other members of the subfamily to which RBx belongs. At the nucleotide level, our subclone differs by only 2 nucleotides with RBx. In both cases, the substitutions are at the third position of the codon and do not result in an amino acid substitution. The probability of having 2 nucleotide substitutions over a short stretch of nucleotides, both in the third position of the codon, without altering the amino acid sequence is very low; this suggests that these nucleotide differences are real and not simply artifacts of PCR amplification.

6.3 Discussion of results

By PCR homology screening with primers designed to recognize sequences that are highly conserved in all K channel genes, we have isolated and sequenced a putative K channel subclone, RSCG1, from total SCG RNA from postnatal day one rats. At the amino acid level, RSCG1 is 100% homologous to the region of interest in RBx. However, if one compares the kinetic, voltage-dependent, and pharmacological properties of the RBx channels expressed in oocytes, to those of all 3 currents on SCG neurons, RBx currents do not entirely correspond to any of the SCG K currents. Nonetheless, it is possible that an RBx homologue encodes for one of the K channels on SCG neurons. The differences in the electrophysiological properties could be explained by differences in the properties of channels expressed in oocytes and in neurons. Kaang and co-workers (1992) showed that an Aplysia Shaker channel (AK01a) produces K channels with different properties when transfected into identified molluscan neurons than when expressed in Xenopus oocytes (Kaang et al., 1992). Alternatively, differences might result from alterations in the amino acids at those specific sites determining voltage-dependency and pharmacological sensitivity. As discussed in Chapter 1, mutational analysis studies demonstrate that changes in single amino acid residues are sufficient to modulate the electrophysiological and pharmacological properties of K channels.

It would be interesting if a homologue of RBx encoded for one of the SCG K channels, and that the differences in properties of RSCG1 and the RBx channels were attributable to alterations in selective amino acid residues. Mutational analysis studies are important in helping to elucidate the structure-function relationships of K channel proteins. However, there is always the worry that mutation of a single amino acid indirectly alters the functional properties of a channel, by causing conformational changes in the channel protein complex. If RBx and RSCG1 are naturally occurring functional variants, analysis of their amino acid sequences might provide information about which regions of the protein are responsible for the differences in the properties of these 2 channels.

It is also possible that RSCG1 may correspond to another member of the K channel subfamily to which RBx belongs; the postulated RSCG1 protein shows 79-95% homology at the amino acid level with other members of the K channel subfamily.

There are problems in attempting to determine which genes encode for which K channels on the basis of comparisons of the electrophysiological properties of specific neuronal K channels with those of K channel mRNAs expressed in oocytes. Given the large number of K channel genes, and the diversity in their properties, it is difficult to assign a gene to a particular K current; even in the case of RSCG1 where the subfamily is identified. In addition, as mentioned above, it is always possible that the properties of the channel when expressed in the neuron are not the same as when expressed in the oocyte (Kaang et al., 1992).

Immunocytochemical studies (Schwarz et al., 1990), northern blot analysis of the mRNA from different tissues (Beckh and Pongs, 1990; Swanson et al., 1990), and in situ hybridization experiments (Baldwin et al., 1991; Hwang et al., 1992) have revealed that different K channels have different patterns of expression in the brain. These types of study provide information about what tissues express which K channel transcripts, and whether their levels change during development (Swanson et al., 1990; Beckh and Pongs, 1990; Luneau et al., 1991). However, for the most part, they do not disclose which mammalian K channel genes correspond to the K currents characterized electrophysiologically in identified neurons. The subcloning of a rat SCG K channel demonstrates the validity of the approach of PCR homology screening for identifying the K channels expressed in specific neurons. Research in progress will attempt to confirm that RSCG1 is a subclone of a K channel gene, and to determine which K current of SCG the protein encoded by this gene underlies. In addition, using this approach we hope to identify other K channel genes expressed in SCG neurons. In future research, we want to use the K channel DNA sequences we subclone as probes for studies on the control of K channel expression during development, and in response to the influence of extrinsic factors.

Beckh and Pongs (1990), taking an alternate approach, demonstrated, using Northern blot analysis with specific K channel probes, that RCK1 is expressed in peripheral sensory dorsal root ganglion neurons, and in tongue nerves, which presumably include nodose neurons which innervate tongue. By nature of the similarity of the K currents in nodose and SCG neurons, it is also possible that SCG neurons express RCK1. However, the voltage-dependent, kinetic and pharmacological properties of RCK1 expressed in oocytes do not correspond in entirety to any of the K channels expressed on nodose or SCG neurons; for example, the channels have a small conductance of 9 pS, as opposed to our 22 pS A-channels, and; RCK1 is sensitive to dendrotoxin (12 nM), whereas IAf and IAs are not (data not shown). The properties of RCK1 expressed in oocytes correspond fairly well to the reported properties of a small conductance, dendrotoxin sensitive channel in dorsal root ganglion neurons (Stansfeld and Feltz, 1988).

The discovery that SCG neurons might express K channel genes highly homologous to those expressed in rat brain is interesting in that it suggests that the knowledge we gain from studying control of K current expression in neurons of the peripheral nervous system will be applicable to neurons of the central nervous system as well. Because of the difficulty of isolating and culturing specific neurons of the brain, it is simpler to study K current expression in a peripheral ganglion, like the SCG.

Chapter 7

General Discussion

The experiments presented in this thesis investigate the expression of voltage-gated K currents on neonatal rat peripheral neurons, and their regulation during development. I have already discussed several of the results in previous chapters, so this discussion will concentrate first on questions concerning the nature of the genes encoding for K channels on peripheral neurons, and second on the differences in the expression and postnatal control of K currents on sensory and sympathetic neurons.

Expression of voltage-gated K currents on peripheral neurons

IAs expressed on peripheral neurons

I have characterized 3 voltage-gated K currents expressed on sensory and sympathetic peripheral neurons: a non-inactivating IK current, and fast (IAf) and slow (IAs) A-currents. A major component of my doctoral research was to describe IAs on peripheral neurons. A slowly inactivating current had been described previously for nodose neurons (Stansfeld et al., 1986; Cooper and Shrier, 1989), but not fully characterized. Whereas, rat SCG neurons were reported to express rapidly inactivating and non-inactivating K currents, but no slowly inactivating current (Nerbonne et al., 1986; Nerbonne and Gurney, 1989; Belluzzi et al.. 1985). However, we show that neonatal rat SCG neurons express a large slowly inactivating current that is distinct from the non-inactivating delayed rectifier in these neurons. Presumably, because slowly inactivating and delayed rectifier currents share many of the same properties, IAs on neonatal SCG neurons was overlooked by Nerbonne and Gurney, and mistakenly included with the delayed rectifier as a single homogeneous macroscopic current. Whereas, IAs may have been missed by Belluzzi and co-workers, who studied K current expression on adult rat SCG neurons, because IAs decreases during postnatal development and already by P14 is expressed at low levels. Voltage-gated K currents, similar to those found on nodose and SCG neurons, are found on sensory neurons from the trigeminal ganglion (Spigelman and Puil, 1989; our observations), and dorsal root ganglion (Kostyuk et al., 1981; Kasai et al., 1986; Mayer and Sugiyama, 1988). Therefore IAf, IAs and IK appear to be part of the general ionic makeup of most peripheral neurons.

Af and As are separate, but related channels

Single channel studies of inactivating channels on nodose neurons indicate that a single conductance channel of 22pS underlies both slowly and rapidly inactivating A-currents (Cooper and Shrier, 1989). However, a number of pieces of evidence argue against the possibility that the channels that underlie IAf and IAs are second messenger modulated forms of each other. First, the experiments characterizing IAf and IAs at the macroscopic and single channel level demonstrate that Af and As channels differ in their voltage-dependencies for activation and inactivation, and their kinetic properties, suggesting that they are not the same channel. Second, some SCG and nodose neurons express only one of the 2 inactivating currents. Third, on postnatal SCG neurons, the control of IAf expression is different from that for IAs. For example, when P14 SCG neurons are grown in culture, IAf levels drop only slightly, but IAs disappears over a period of 4 weeks. Because these changes in IAf and IAs occur slowly, it is hard to imagine a scheme whereby direct modulation by second messengers could result in a slow loss of one of the forms of the channel with relatively few changes in the alternate form. Finally, during 10-30 min whole-cell recording experiments, which dialyse the intracellular contents, IAf was never seen to convert to IAs and vice versa.

Though we did not record the channels that underlie IK, the same points argue strongly against the possibility that the channels that underlie IAs, also underlie IK; IK and IAs differ in their voltage-dependence for activation, kinetics for activation, pharmacology and inactivation properties. In addition, during *in vivo* postnatal development, IAs levels decrease, but there is no change in IK expression. The simpler explanation for the differences in K channel properties is that Af and As channels, and the channels that underlie IK, are all separate, but related, channels.

Given the hypothesis that IAf, IAs and IK are mediated by separate channels, what is the nature of the genes that encode for the channel proteins? Unfortunately, it is not possible to identify the genes encoding for K channel proteins simply by comparing their physiology with those of cloned K channels expressed in Xenopus oocytes. The cloned mammalian K channels when expressed in oocytes simply share too many of the same properties to be able to differentiate between them. In addition, there can be differences in the properties of K channels when expressed in oocytes as compared to those in neurons (Kaang et al., 1992).

Molecular nature of genes that encode K channels on peripheral neurons

Therefore, answers to questions that arise concerning the nature of the genes encoding for voltage-gated K channels in the peripheral nervous system, must await the actual isolation of K channel genes expressed by the neurons. Nonetheless, it is worthwhile to touch on some of points pertaining to K channel gene expression by peripheral neurons. First, what is the genetic mechanism for producing three voltage-gated K currents which share some properties, but differ in others? It is possible that there is a single K channel gene, whose primary gene product is alternatively spliced to produce variant transcripts, as for the Shaker locus of Drosophila (Timpe et al., 1988a,b; Iverson et al., 1988). However, alternative splicing does not seem to be a common mechanism used in the mammalian K channel family (review by Salkoff et al., 1992). More likely, is that the different K channels on peripheral neurons are encoded for by separate genes. Several separate mammalian K channel genes have been identified (review by Salkoff et al., 1992; also see Chapter 1), with the current hypothesis being that they arose during evolution by gene duplication, and subsequent divergence, resulting in related genes which encode for K channels which share some properties, but differ in others.

Given the assumption that IAf, IAs and IK are separate channels, encoded for by separate genes, how related are the proteins that make up these 3 channels? It is possible that all three K channels belong to the same K channel subfamily. For example, different mammalian Shaw genes encode for both rapidly (Schroter et al., 1991) and slowly inactivating K channels (Yokoyama et al., 1989; McCormack et al., 1990; Luneau et al., 1991). In fact, single nucleotide changes in the genes encoding for K channel proteins can result in significant changes in the functional properties of the channels (see Chapter 1). Given the large number of mammalian K channel genes, and that neurons can express more than one K channel gene, a more likely possibility is that IAf, IAs and IK channels belong to different subfamilies. In Drosophila, mRNAs from each subfamily, when expressed in oocytes result in currents that differ in their kinetic and voltage-dependent properties (Wei et al., 1990).

How many K channel genes are expressed in peripheral neurons? For simplicity, in discussions of the results in this thesis, I have assumed that one channel is responsible for IAf, one for IAs and one for IK, with the corollary that each channel is encoded for by a single gene. However, it may be that several separate genes encode for the proteins that are responsible for a given macroscopic current; for example, Af channels could be heteromultimers consisting of possibly 4 different K channel proteins subunits; alternatively, IAf could be made up of several A-channels, encoded for by separate genes and differing only slightly in their properties. Some support for the idea that more than one gene may be responsible for a macroscopic current comes from a study by Hardie and co-workers (1991), who show that 4 separate Shaker mRNA transcripts are apparently responsible for the rapidly inactivating current of Drosophila photoreceptors. Given this scenario, it is impossible to predict just how many different K channel genes are expressed in peripheral neurons.

Do sensory and sympathetic peripheral neurons express the same set of K channel genes? SCG and nodose neurons express similar IAf, IAs and IK currents, however the voltage-dependent properties of the A-currents are not identical in the 2 types of neuron. Nonetheless, given the overall similarity of the voltage-gated currents in SCG and nodose neurons, it is likely that these neurons express a very similar set of K channel genes. The differences in the currents between nodose and SCG neurons could be explained by slight alterations in those regions of the protein responsible for voltage-dependent properties (see Chapter 1).

The experiments discussed in Chapter 6 are a first attempt at identifying the genes encoding the channels expressed on SCG neurons. Preliminary results indicate that the approach of PCR homology screening will most likely be successful in isolating K channel genes. However, given the complexity of the molecular biology of voltage-gated K channels, it is hard to know how much of the picture we will be able to elucidate. For example, how will we know whether we have isolated all the K channel genes? In addition, it may be difficult to determine which genes correspond to which currents, especially if heteromultimeric channels occur (Isacoff et al., 1990; Ruppersberg et al., 1990; MacKinnon, 1991; Christie et al., 1990). Nonetheless, the identification of K channel transcripts from the SCG is essential for us to be able to answer questions regarding the control of K channel expression raised by the electrophysiological experiments presented in this thesis.

Control of K channel expression on peripheral neurons

Given the role of voltage-gated K currents in controlling cellular excitability (Rudy, 1988; Thompson and Aldrich, 1980; Adams and Galvan, 1986), it is not surprising that the expression of K currents is regulated during development (see Chapter 1). The astonishing thing is that so many different patterns of expression occur, raising the question of how a neuron controls what channels it expresses and at what levels? In the peripheral nervous system, sympathetic and sensory neurons do not control their postnatal expression of voltage-gated K currents in the same manner, as indicated by the fact that SCG neurons show significant changes in their K currents, whereas nodose neurons show no changes. How is it that postnatal control of K currents on nodose and SCG neurons is so different? One explanation is that P1 sensory and sympathetic neurons are not at the same developmental stage; nodose neurons are developmentally more mature and have stabilized the levels of K currents they express. Alternatively, and the hypothesis that I will spend some time discussing, is that the 2 types of neuron use different mechanisms to regulate the K currents they express. Additional support for this idea is provided by the fact that nodose and SCG neurons show differences in how much variability there is in IAf, IAs and IK expression. There is considerable variation in the relative proportions of IAf, IAs and IK expressed on nodose neurons. In contrast, the majority of P1 SCG neurons express a large IAs, while the majority of P14 neurons express a large IAf, indicating that there is considerably less variation in the expression of K channels on SCG neurons.

If sympathetic and sensory neurons do not control their K currents in the same manner, what is involved in the regulation of K currents in these neurons? Potentially, K channel expression could be under intrinsic control, meaning that a genetic program, inherent to the neuron, is followed during development. In this case, the particular set of K channels a neuron expresses would be entirely dependent on its genetic program, presumably with SCG and nodose neurons possessing different programs.

Evidence for extrinsic control of K channel expression in peripheral neurons

While it is likely that peripheral neurons have some intrinsic mechanisms to regulate their expression of K channels, a number of pieces of evidence presented in this thesis, either indirectly or directly, support the idea that epigenetic factors are also involved. First, nodose neurons show considerable variability in their expression of K currents, even though the neurons are collected into a supposedly homogenous population of sensory neurons: nodose neurons have all had similar genetic histories, originating from the neural placodes, and yet, within the constraint of a fixed set of voltage-gated K channels, the outward currents on different nodose neurons take on many diverse forms. A similar variability is observed in their biochemical properties as indicated by the differential expression of various neurotransmitters.

Second, in culture, P1 SCG neurons do not show the switch from an outward current predominantly made up of IAs to one made up of IAf, which occurs *in vivo* by the third day postnatally; because the loss of A-currents in culture occurs only after 1-2 weeks, it should not obscure the switch. In addition, if the switch is triggered during embryonic development, we would still have expected to observe the change from an IAs to an IAf dominated outward current. These results suggest that a factor, which we have not identified, is required postnatally for developmental changes in K current expression.

Finally, we show that nonneuronal cells from the ganglion, and from other tissues such as heart and skin, influence the expression of K channels on neurons in culture. The conditioned media experiments indicate that it is a soluble factor, released by the cells, that is involved.

Factors involved in the control of K current expression on peripheral neurons

If epigenetic factors play a role in controlling K current expression on peripheral neurons, then if there are differences in the expression of a similar set of K currents on sensory and sympathetic neurons, it would imply that different factors are involved in the control of K current expression on the two neuron types. What would be the nature of the factors for SCG and nodose neurons?

Nodose neurons

For nodose neurons, one likely possibility is that the targets these cells innervate influence their expression of K channels. Nodose neurons provide sensory innervation to the heart, lungs and much of the viscera. As such, their nerve terminals act as rapidly or slowly adapting sensory receptors (Paintel, 1973). To serve these diverse roles, presumably individual nodose neurons would have to express IAf, IAs and IK at different proportions. In fact, there is some evidence that the electrical properties of peripheral sensory neurons are related to the type of receptor formed by their peripheral endings (Belmonte and Gallego, 1983). Extending this argument, it may be that the target influences the K channels expressed by sensory neurons. Peripheral axotomy produces different effects in the electrical properties of sensory neurons innervating the larynx and carotid bodies (Gallego et al., 1987). While indirect, these results suggest first, that these targets have effects on the electrical properties of the neurons that contact them, and second, that targets differ in their effects.

SCG neurons

Potassium current expression by nodose neurons may be influenced by factors which differ for the various neurons, for example, factors from the innervated tissues, and/or the brainstem neurons they contact. In contrast, the fact that the relative proportions of IAf, IAs and IK on SCG neurons is less variable than on nodose neurons, implies that K currents on SCG neurons may be controlled by factors which are common to most of the neurons in the ganglion: factors such as ganglionic nonneuronal cells, and/or circulating factors. This idea, though speculative, is supported by our experiments on the postnatal control of K current expression on SCG neurons. The axotomy and denervation experiments suggest that postnatal SCG K channel expression is hardly influenced, if at all, by the target, or by the preganglionic nerves. In addition, nonneuronal cells from the ganglion provide a soluble factor which influences the expression of K channels on P1 SCG neurons, but not nodose neurons. Since all SCG neurons are surrounded by the nonneuronal cells of the ganglion, and would therefore see the same factor, it may partially explain why there is less variability in the expression of the K channels on these neurons.

At least one other factor is involved in regulating the expression of K channels on SCG neurons. This factor, whose source has not been identified, is required for the *in vivo* developmental switch from an IAs to an IAf dominated outward current. The factor is not provided by the targets, by the preganglionic nerves, or by the nonneuronal cells, which leaves the possibility that a circulating factor(s) is triggering the switch. Many hormones are known to increase transiently during development (Gould and Butcher, 1989), and as such may provide the trigger for the switch in outward current. Additional factors are probably involved in the postnatal control of K current expression on SCG neurons, as suggested by the differences in IAs expression observed for the explant and dissociated P1 SCG cultures, and that a non-nonneuronal cell dependent portion of IAf expression is lost on P14 SCG neurons in culture.

Extrinsic factors act during a critical period of development

Our results also suggest that there are critical periods of development where neurons are sensitive to these epigenetic influences; apparently once a neuron expresses the same set of K currents it does in the adult, the currents lose their sensitivity to the factors acting during development. For instance, control of K current expression continues postnatally on SCG neurons, and possibly as a result, K current expression on P1 neurons is sensitive to the loss of a nonneuronal cell factor. As postnatal development continues however, and SCG neurons start to express the set of K currents they do in the adult, their expression of IAf becomes independent of the nonneuronal cell factor. In contrast, because nodose neurons show no postnatal control of their K current expression *in vivo* or in culture, presumably at birth they already express the same K currents they do in the adult. These results imply that plasticity in K current expression in the adult must either involve a different set of factors, or a reactivation of the responsiveness of adult neurons to the developmental factors.

Intrinsic control of K current expression

I would not like to leave the impression that K current expression on peripheral neurons is entirely under the control of extrinsic factors, in fact, our data suggests that the neurons also have intrinsic mechanisms to control their expression of K currents. Presumably the different K current systems act in an interactive fashion to determine the electrical excitability of the neuron. As such, it is not surprising that our results indicate that the neuron has mechanisms by which to coordinate its expression of the different K currents. These results also imply that during postnatal development IAf and IAs are not independently regulated, but that the expression of one current is dependent on the other. What mechanism could the neuron use to coordinate its expression of IAf, IAs and IK, given our hypothesis that the three currents are encoded for by separate genes? One possibility is that the levels of the different K channels proteins are in equilibrium, so that an increase in the levels of one of the gene products will feed back in a negative fashion and decrease the expression of a different K channel protein. This feedback could occur at the level of gene transcription, or might result from stabilization of one K channel mRNA at the expense of another. However, determining at what level SCG neurons regulate their K channel expression must await the identification of the genes expressed in the neurons.

Functional Significance of developmental changes in K current expression

What is the role of the changes in K current expression in the functional development of the postnatal SCG neurons? The particular K channels expressed by a neuron will determine how it transmits the information from the neurons that synapse on its cell body. As such, an SCG neuron that switches its outward current from one that is largely slowly inactivating to one that is rapidly inactivating is going to change the way it processes the information it receives from preganglionic inputs. The large slowly inactivating current on P1 SCG neurons should serve to decrease the excitability of the neurons, by shunting out incoming depolarizations. However, if the stimulus is large enough so that the neuron fires, IAs provides a mechanism to allow the neuron to continue to fire, by hyperpolarizing the membrane sufficiently to prevent Na channel inactivation. Thus, a P1 neuron should fire only in response to a sufficiently large input, but once the neuron is excited it may fire a burst of APs. In contrast, a P14 neuron expresses a large IAf, and a smaller IAs than the P1 neuron. Because of the smaller IAs, the P14 neuron should be more excitable than the P1 neuron, requiring less summation of synaptic inputs before it fires.

What would be the advantage of having a system which is less excitable at birth, but with development becomes more excitable? One possibility is that until the targets they innervate start to function SCG neurons need not be very active electrically. For example, SCG neurons innervate the iris and the eyelid, however, in rat the eyes stay closed until 2 weeks after birth. Perhaps the increase in excitability of SCG neurons over the first 2 postnatal weeks reflects the fact that with development the target needs them to be more active.

Studies indicate that neuronal activity is important for the patterning of connections during the development of the nervous system (discussed in Chapter 1). Neurons compete for targets in an activity-dependent fashion. Since K channels regulate presynaptic activity, their expression during development could have an important role in establishing the innervation of the targets. For example, it might be that parasympathetic and sympathetic innervation of a target, such as heart, occurs asynchronously. If sympathetic nerves are the first to arrive, and the neurons are highly excitable, then the sympathetic connections could dominate. Whereas, if the activity of the sympathetic neurons is attenuated until such time as all connections are made, subsequent competition and rearrangement will ensure appropriate innervation of the target. Changes in K current expression during development, such as observed for SCG neurons, may be a general mechanism used by neurons to establish their proper connections. In addition, similar mechanisms may be functioning in long term changes in synaptic strength and connectivity in the adult CNS.

One of the major reasons for studying the control of K current expression on peripheral neurons is the ease by which the environment of these neurons can be manipulated for studies identifying extrinsic factors that influence K currents; as discussed previously, CNS neurons are much more difficult to study than PNS neurons. Fortunately, because several K channel genes expressed in the CNS are also expressed in the PNS (Beckh and Pongs, 1990), much of what we learn about control of expression on peripheral neurons will hopefully apply to CNS neurons as well. Identifying factors, such as hormones or growth factors, that control K current expression, may help in determining what is entailed in certain CNS pathologies that involve changes in the excitability of neurons. For example, Epilepsy, a disease where large ensembles of neurons discharge in an abnormally synchronous fashion, may involve a misfunction of K channel expression, resulting in a decrease in inhibition in the cortex and over excitability of CNS neurons.

Continuing on this line of thought, it is interesting that SCG neurons in culture without other cell types differentiate in a normal fashion with respect to properties other than their expression of K currents: the neurons extend axons and dendrites (Bruckenstein et al., 1989), fire action potentials (O'Lague et al., 1978), produce the correct neurotransmitter (Furshpan et al., 1982), and express nicotinic acetylcholine receptors (Mandelzys and Cooper, unpublished observations). The neurons seem normal, and it is only by recording K currents that a defect becomes apparent. Conceivably, previously unexplained CNS pathologies, where no morphological or biochemical deficit is apparent, may be explained by defects in K currents, due to the deficiency or overabundance of a factor that influences their expression. This is an important area of research which needs further attention, however, using electrophysiological techniques, it would be difficult to measure alterations in K channel expression in pathological conditions. With recent advances in the molecular biology of K channels, and identification of K channel genes expressed in specific neurons, measuring mRNA levels may be a relatively easy way to determine if there are changes in K channel expression in disease states.

In summary, a neuron has a choice perhaps of hundreds of different K channels to express, and the set and the levels of channels which do get expressed can change during development. The permutations and combinations are enormous, and must be under exquisite control if the nervous system is going to function properly. Studies such as those presented in this thesis will hopefully shed some light on what is involved in the control of K current expression, leading to a better understanding of the involvement of K currents in development, plasticity and different disease states.

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