Characterization of the Ionic Currents in Cultured Small Intensely Fluorescent Cells from Superior Cervical Ganglia of Neonatal Rats

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ABSTRACT

The superior cervical ganglion (SCG) is the largest of the sympathetic chain ganglia which control a number of autonomic cardiovascular reflexes via neural activity in the postganglionic nerve trunk. In addition to the large principal neurons, these ganglia contain a minority population of smaller cells, the small intensely fluorescent or SIF cells, so named because of their intense fluorescence following treatments which reveal the presence of endogenous catecholamines (mainly dopamine in the rat). The physiological functions of the SIF cells are largely unknown and various roles have been proposed including (i) dopaminergic interneuron, which modulates ganglionic transmission, (ii) endocrine function, since many of them have a close association with the vasculature and (iii) chemosensory function, similar to that of the arterial chemoreceptors which sense blood gases and pH. Understanding the physiological role of SIF cells has been hampered by their small size, sparse distribution and relative inaccessibility, all of which render microelectrode electrophysiological studies difficult. In this thesis these limitations were overcome by use of (i) dissociated cell cultures of the rat SCG, in which growth conditions favoured SIF cell survival but not that of the principal neurons, and (ii) the novel high resolution patch clamp/whole cell recording technique which is ideal for the study of the electrophysiology of small cells.

The ionic currents, which underlie many basic electrophysiological processes, were characterized in 5-16 day old cultures of SIF cells obtained from the SCG of

neonatal rats. The main methodology consisted of whole cell recording under voltage clamp conditions, which permit the study of membrane ionic currents. Five main ionic currents were identified in all of the SIF cells (> 100) studied: (i) a fast transient inward Na^+ current, sensitive to the well-known blocker of voltage-gated Na^+ channels i.e. tetrodotoxin or TTX; (ii) the delayed rectifier outward K^+ current that is found in a variety of cell types; (iii) a Ca^{2+}- activated outward K^+ current, sensitive to Ca^{2+} channel blockers; (iv) a transient inward Ca^{2+} current which appears to be carried by N-type Ca^{2+} channels and (v) a slower, sustained inward Ca^{2+} current which appears to be carried by L-type Ca^{2+} channels. In addition a third type of outward K^+ current, the fast transient K^+ current or I_A, was found in SIF cells obtained from 3-7 day old rats, but not from 1 day old rats. It therefore appears that this I_A current, which is known to modulate firing frequency in neurons develops rapidly *in vivo* during the first postnatal week. This broad repertoire of ion channels in SIF cells suggests several possible sites for modulation by various agents including neurotransmitters, neuromodulators, or other chemosensitive agents. Since SIF cells were recently proposed to have arterial chemoreceptor function similar to glomus cells, the effect of one such stimulus, i.e. an acidic (intracellular) pH, was tested. It has recently been suggested that a decrease in intracellular pH is part of the pathway responding to extracellular stimuli in the glomus cell (Stea, Alexander and Nurse, in press). Acidification of the SIF cell's cytoplasm with the K^+/H^+ ionophore nigericin resulted in a suppression of both the fast inward Na^+ current as well as the outward K^+ current. However, these effects do not appear to be

unique to SIF cells and therefore the possibility of a chemoreceptor role in the cardiovascular system requires further study.

In summary, the characterization of the various ionic currents in SIF cells resulting from this thesis provides the necessary background which should eventually resolve not only the question of the physiological role of SIF cells in autonomic ganglia, but also help to understand the underlying mechanisms responsible for SIF cell function.

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TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1

General Introduction

Along with the principal neurons and support cells found in autonomic ganglia there exists another cell type, which has variously been called the extra-adrenal chromaffin cell (Kohn, 1903), the small granule-containing cell (Matthews and Raisman, 1969) and most commonly now, the small, intensely fluorescent or SIF cell (Norberg, Ritzén and Ungerstedt, 1966). These names derived respectively from the various techniques employed when visualizing these cells, i.e., the chromaffin reaction which displayed the presence of catecholamine-containing vesicles, electron microscopy which revealed clearly these small chromaffin granules, and the formaldehyde-induced fluoresence technique (Falck, Hillarp, Thieme and Torp, 1962) which detected a strong presence of catecholamines. Developmentally, SIF cells are derived from the sympathoadrenal sublineage of the neural crest and their properties appear intermediate between those of other members of this lineage, viz the adrenal medullary chromaffin cells and the principal neurons of the sympathetic ganglia (Doupe Patterson and Landis, 1985). Ultrastructurally, SIF cells closely resemble another member of this lineage, the chemoreceptor glomus cells of the carotid body, and some workers have even suggested that these cell types are identical (Kobayashi, 1971; McDonald and Blewett, 1981; Doupe, Patterson and Landis, 1985; but see Nurse, 1990). While much is known about the functions and electrophysiological properties of the chromaffin cells (Fenwick, Marty

and Neher 1982a,b; Bossu, De Waard and Feltz, 1991a,b), principal neurons (Constanti and Brown, 1981; Galvan, 1982; Galvan and Sedlmeier, 1984; Belluzzi Sacchi and Wanke, 1985a,b; Belluzi and Sacchi, 1986; Hirning, Fox, McCleskey, Olivera, Baldomero, Thayer, Miller and Tsien, 1988; Plummer, Logothetis and Hess, 1989) and more recently of the chemoreceptor glomus cells (Duchen, Caddy, Kirby, Patterson, Ponte and Biscoe, 1988; Ureña, López-López, González and López-Barneo, 1989; Hescheler, Delpiano, Acker and Pietruschka, 1989; Stea and Nurse, 1989, 1990, 1991a; Stea, Alexander and Nurse, in press), much less is known about the electrophysiological properties of SIF cells. Indeed, prior to the commencement of this study there was only one brief report on intracellular recordings of membrane potentials from SIF cells in lower vertebrates (Dunn and Marshall, 1985).

The functional role of the SIF cell has been postulated to be similar to each of the functions of the related sympathoadrenal cell types. The earliest hypotheses concerning SIF cell function presumed an interneuronal role (Marrazzi, 1939a,b; Bülbring, 1944). This is an analogous function to that of the principal neurons whereby both cell types receive synaptic input and release neurotransmitter onto a target cell. Further studies led to the belief that SIF cells had an endocrine function similar to the adrenal chromaffin cells (Chiba and Williams, 1975; Williams, Black, Chiba and Bhalla, 1975; Williams, Chiba, Black, Bhalla and Jew, 1976). These two separate functions were not incompatible owing to the presence of two distinct types of SIF cells based on morphological criteria (Chiba and Williams, 1975; Williams, Black, Chiba and Bhalla,

1975; Williams, Chiba, Black, Bhalla and Jew, 1976): Type I SIF cells, which had axon-like processes and made synapses with post-ganglionic neurons appeared to subserve an interneuronal role, whereas type II SIF cells which had short or no processes, and were located near fenestrated capillaries or interstitial spaces, were thought to possess an endocrine function. Lastly, on the basis of an electron microscopic study of synaptic connections on SIF cells, Kondo (1977b) proposed a role for SIF cells in chemoreception, similar to that of glomus cells in the carotid body.

SIF Cells as Interneurons

Well before the SIF cell was discovered researchers were studying the structure and function of autonomic sympathetic ganglia. In 1889 Langley and Dickinson found that nicotine was able to block nerve transmission in sympathetic ganglia. This result led them to postulate the concept of a two neuron effector chain. Accordingly, a 'pre-ganglionic' neuron would transmit an impulse to a second 'post-ganglionic' neuron across a synapse present in the ganglion. Dogiel (1896) thought this system to be too simple, and without the benefit of any structural or pharmacological evidence, advanced the concept that a third cell might be present to act as an interneuron. This putative interneuron would be able to provide finer control of signal transmission between the pre- and post-ganglionic neurons. Shortly after, Kohn (1903), while mapping chromaffin-positive cells in sympathetic ganglia and paraganglia, found clusters of small cells resembling chromaffin cells of the adrenal medulla. This new cell type, then called the extra-adrenal chromaffin cell, eventually came to be known as the small intensely

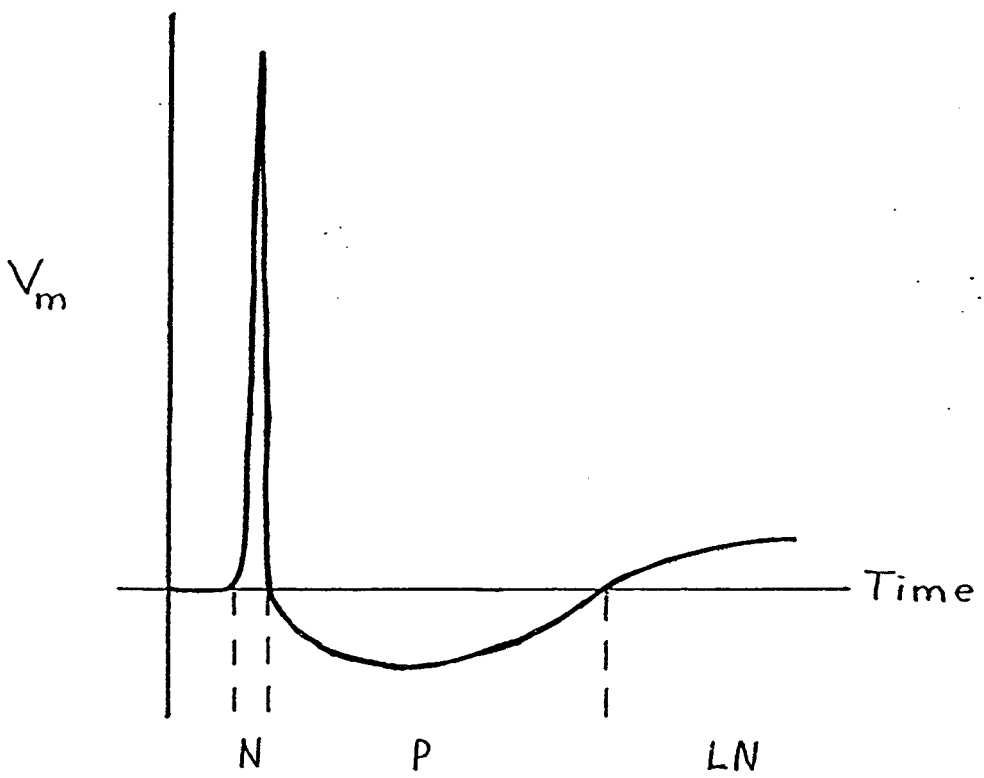
fluorescent, or SIF cell. Surprisingly, a number of years passed before anyone linked the SIF cell with the interneuronal function.

Marrazzi (1939) found that adrenaline had an inhibitory effect on sympathetic ganglionic transmission. Bülbring (1944) repeated and extended this work and found that the venous outflow collected from the ganglia had an inhibitory effect on the ganglionic transmission, and that it contained adrenaline. She then postulated that the SIF cells were the possible source of this adrenaline.

In the early to mid 1950's Eccles studied transmission of the action potential in the rabbit superior cervical ganglion, or SCG (Eccles, 1952a,b, 1955). These experiments laid the groundwork for the understanding of the various components of the post synaptic potentials (PSPs) arising in the ganglion following stimulation of the pre-ganglionic nerve trunk (FIG. 1.1). Eccles, using extracellular recording, found an early negative (N) potential, which is the primary ganglionic response. This corresponds to the intracellularly-recorded fast excitatory post synaptic potential, or fEPSP. This was followed by a late positive (P) potential, which corresponds to the slow inhibitory post synaptic potential (sIPSP). The final phase, was a late negative (LN), or slow excitatory post synaptic potential (sEPSP). These three phases of the post-synaptic potential were found in rabbit SCG but may not generally be present in all sympathetic ganglia. For example, the sIPSP was not found in guinea pig SCG (Dail and Evans, 1978). This point will be addressed later.

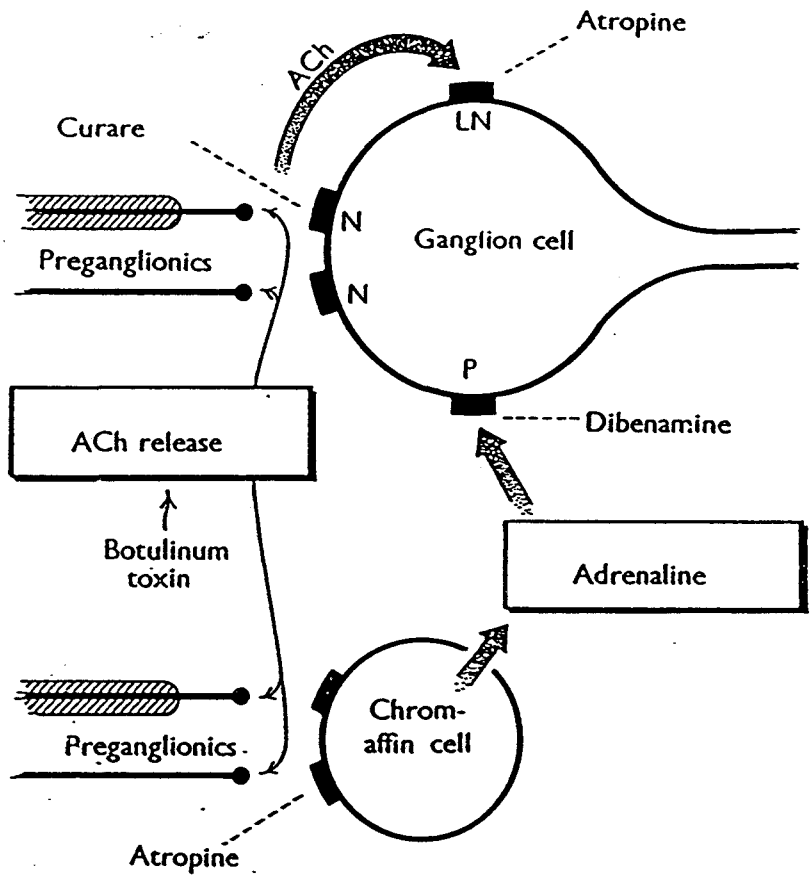
In subsequent experiments Eccles and Libet (1961) used pharmacological tools

Figure 1.1: Schematic diagram showing the three phases of the rabbit superior cervical ganglion post-synaptic potential. Symbols represent: N, negative depolarizing response or fEPSP; P, positive hyperpolarizing response of sIPSP; LN, late negative depolarizing response or sEPSP.


V_m
Time
N
P
LN

to separate the various phases of the complex post synaptic potential (PSP). Rabbit SCG was curarized with a concentration of drug that just blocked the post-synaptic spike discharge, or fEPSP. Under these conditions there was no large depolarization that would create an afterpotential that masks the late PSPs; however, a small portion of the fEPSP remained such that the effect of other drugs could be tested. In some cases botulinum toxin, a presynaptic blocker of transmitter release, was also used to prevent acetylcholine (ACh) release from pre-ganglionic neurons. With ACh application that elicited all phases of the PSP, it was found that atropine, a specific muscarinic ACh-receptor blocker prevented the late P and LN responses, leaving the early N response intact. In addition, dibenamine, an α-adrenergic antagonist did not affect the N response but reduced the LN and strongly depressed the P responses. A high concentration of dibenamine suppressed all phases to some degree, but always in the order P $>>$ LN $>>$ N. The P phase was clearly dependent on an additional adrenergic component in its elicitation as opposed to the exclusively cholinergic pathway for the N phase. Eccles and Libet (1961) then proposed a model to explain these results (FIG. 1.2). Release of ACh from pre-ganglionic terminals stimulates nicotinic ACh-receptors on post-ganglionic neurons thus eliciting the fast depolarizing N response. ACh also stimulates muscarinic ACh-receptors on the chromaffin, i.e. putative interneuronal cell, causing release of an adrenaline-like substance. This, in turn, stimulates a second receptor type on the post-ganglionic neuron, sensitive to α-adrenergic blockade, and elicits the hyperpolarizing P response. A third post-ganglionic receptor, a muscarinic ACh-receptor, is also

Figure 1.2: Schematic diagram of disynaptic hypothesis of sympathetic ganglionic transmission. ACh is released from pre-ganglionic nerve endings and stimulates nicotinic cholinergic receptors on the post-ganglionic neurons eliciting the N or fEPSP response. This response is blocked by curare. Ach also stimulates muscarinic cholinergic receptors on the interneuronal cell causing release of adrenaline or an adrenaline-like substance. The effect of ACh on the interneuron is blocked by atropine. The adrenaline stimulates adrenergic receptors on the post-ganglionic neuron eliciting the P or sIPSP response. This response is blocked by dibenamine. A third effect of pre-ganglionic Ach is its diffusion to muscarinic cholinergic receptors on the post-ganglionic neuron where it elicits the LN or sEPSP response. This response is blocked by atropine. (Figure taken from Eccles and Libet, 1961)


Atropine
ACh
Curare
LN
N
Ganglion cell
Preganglionics
N
P
Dibenamine
ACh release
Botulinum
toxin
Adrenaline
Chrom-
affin cell
Preganglionics
Atropine

stimulated directly via the the pre-ganglionic input and results in the late depolarizing LN phase. This general scheme for ganglionic transmission has also been applicable in other animal systems. For example, Libet (1964) also found the same atropine effects on the P and LN components of the PSP in cat stellate ganglion. Libet and Tosaka (1966) then found adrenergic compounds could elicit the P and LN phases in bullfrog sympathetic ganglia, and atropine blocked this effect. In 1968 the work on bullfrog sympathetic ganglia was extended (Libet, Chichibu and Tosaka, 1968; Tosaka, Chichibu and Libet, 1968) and it was demonstrated that in curarized ganglia atropine did not affect the N response elicited by direct ACh application. As well, dibenamine reduced the P phase, but not very strongly. Nickerson and Nomeguchi (1950) also found that dibenamine was not very effective in frog preparations. As a result of these experiments the presence of an interneuron in autonomic sympathetic ganglia was gaining acceptance, however, the absolute identity of this cell was still undetermined. The SIF cell was a leading candidate since it was in the ganglion, it had a neuronal-like morphology (especially the type I SIF cell) and it contained catecholamine-containing vesicles.

Between 1966 and 1970, morphological evidence supporting the role of the SIF cell as an interneuron was reported. In these studies synaptic profiles or specializations were observed in the electron microscope between pre-ganglionic neurons and SIF cells (Grillo, 1966; Williams, 1967), and between SIF cells and post-ganglionic neurons (Williams, 1967; Matthews and Raisman, 1969; Williams and Palay, 1969; Matthews and Nash, 1970). While these efferent contacts from SIF cells to post-ganglionic neurons

were found in many species they were not universal. In fact, no evidence was found for synaptic connections between SIF cells and post-ganglionic neurons in guinea pig sympathetic ganglia even after an extensive search (Ostberg, 1970; Elfvin, Hökfelt and Goldstein, 1975; Furness and Sobels, 1976; Watanabe and Burnstock, 1976; Jew, 1980). This morphological evidence in the guinea pig sympathetic ganglia corroborates well with the functional studies which indicate that a slow IPSP is not present (Dun and Karczmar, 1977), suggesting the absence of an interneuronal function for SIF cells in this case. This correlation between the presence of SIF cell efferents in the electron microscope and the electrophysiological demonstration of a sIPSP in the post-ganglionic neuron pointed to the SIF cell as the likely candidate for mediation of the sIPSP in sympathetic ganglia.

The progression to acceptance that an adrenergic interneuron was present in sympathetic ganglia, and that the interneuron was indeed the SIF cell was slowed by the results of Weight and Padjen (1973a,b). They found evidence consistent with a monosynaptic control of the sIPSP in bullfrog sympathetic ganglia, where earlier studies had suggested that two synapses were involved, one mediated by a muscarinic cholinergic synapse, and the other by an adrenergic synapse (Libet and Tosaka, 1966; Libet et. al., 1968; Tosaka et. al., 1968). One notable difference in methodology was that Weight and Padjen used nicotine, rather than curare, to prevent the large EPSP. In nicotinized ganglia it was found that preganglionic stimulation elicited a hyperpolarizing response (i.e. the expected sIPSP) in the postganglionic neuron, as did direct ACh application. Under both of these conditions, the post-ganglionic response could well have been

mediated via an interneuron. To test for this, one-to-one synaptic transmission was blocked by the addition of low Ca/high Mg to the bathing solution so as to reduce transmitter release to a level that was insufficient to excite the putative interneuron. Preganglionic stimulation was not employed as no significant amount of transmitter could be released under these conditions in order to produce the sIPSP. Since ACh application produced a hyperpolarizing response in the postganglionic neuron under these conditions (Weight and Padjen, 1973b), it appeared that an interneuron was not required for the response. It should be noted that the mechanism of nicotine blockade differs from that by blockade with curare. Blockade with nicotine not only leaves muscarinic receptors present on the putative interneuron free to bind ACh and respond, but seems to increase their sensitivity, and as a result leave them able to transmit preganglionic impulses to the post-ganglionic neuron (Trendelenburg, 1966). This may account for the difference in requirement for an interneuronal step to produce the sIPSP in curarized ganglia versus the monosynaptic step seen in nicotinized ganglia.

To show that the sIPSP and hyperpolarizing response to direct ACh application were analagous, Weight and Padjen (1973a) examined the underlying ionic mechanisms. They concluded that both the sIPSP and hyperpolarizing responses resulted from an increase in membrane resistance determined from membrane potential (V_M) changes following injection of constant current pulses across the membrane before and during these responses. To account for these observations a decrease in Na^+ conductance was suggested as the mechanism underlying the hyperpolarizing response. In addition, pre-

stimulus depolarizations of the post-ganglionic membrane caused a decrease in sIPSP response and post-ganglionic hyperpolarization, while slight pre-stimulus hyperpolarizations of the membrane had the opposite effect. Again this follows the known properties of voltage-dependent Na^+ channels, that inactivate with depolarization and reactivate with hyperpolarization, that are known to be found in these post-ganglionic neurons (Belluzzi et. al., 1985a; Belluzzi and Sacchi, 1986). A final result was an abolishment of the hyperpolarization in Na^+-free Ringer's solution. If indeed the sIPSP was carried by Na^+ ions the removal of Na^+ should prevent its production. This block of the sIPSP by removing Na^+ was easily reversible upon return to normal Ringer's. The above findings that the sIPSP was accompanied by a decrease in membrane conductance contradicted earlier reports indicating that no change in membrane conductance was associated with either the sIPSP or adrenergically-elicited post-ganglionic hyperpolarization (Tosaka, et. al., 1968; Libet and Kobayashi, 1969; Kobayashi and Libet, 1970).

In a more detailed study, Libet and Kobayashi (1974) compared the effects of curare and nicotine on transmission through the bullfrog sympathetic ganglion. Curarization of the ganglion, in order to completely remove the fEPSP, coupled with a low Ca/high Mg Ringer's bathing solution to suppress pre-synaptic transmitter release, was tested first. It was found that bath-applied ACh resulted in only a very small, sub-normal hyperpolarizing response in the curarized ganglion, suggesting that the normal post-ganglionic hyperpolarizing reponse involved an additional transmitter. It seemed

that the nicotinization of the ganglia could be the cause of the unusual result of Weight and Padjen (1973a,b) indicating a monosynaptic cholinergic pathway to the sIPSP in the bullfrog sympathetic ganglia. Using nicotinized ganglia Libet and Kobayashi confirmed the results of Weight and Padjen (1973a,b) that applied ACh could directly elicit a hyperpolarizing response with nicotine present. The prevailing view then, is that in curarized ganglia a second transmitter is necessary to produce a hyperpolarizing response with ACh, while in nicotinized ganglia it is not. Neither of these two pathways renders the other insignificant; it simply indicates that under each of the pharmacological, if not physiological, conditions that a unique process is uncovered. Two pathways could exist that each produce a slow positive post-synaptic potential with similar time-course.

In the same study Libet and Kobayashi (1974) also examined the effects of adrenergic stimulation and adrenergic and cholinergic antagonists on the ganglionic hyperpolarizing response. Adrenaline produced a maximal hyperpolarization quite similar to the ACh response elicited in curarized ganglia. The same concentration of noradrenaline was also effective, but produced only half of the response, whereas dopamine, again at the same concentration, produced only a small response. The response to adrenaline was completely abolished by the α-adrenergic antagonist dihydroergotamine (DHE), while neither the nicotinic blocker dihydro-β-erythroidine (DHßE), nor the muscarinic blocker atropine affected the response. These data are consistent with the presence of the adrenergic interneuron.

Libet and Kobayashi (1974) reasoned that if there are indeed two pathways for

eliciting post-ganglionic hyperpolarization, one in curarized ganglia and mediated by release of a second adrenergic transmitter acting on α-adrenergic receptors, and the other via direct muscarinic cholinergic stimulation in nicotinized ganglia, then the competitive α-adrenergic antagonist phentolamine and the catechol-O-methyl-transferase inhibitor, U-0521, that prevents breakdown of adrenergic transmitters should produce different effects in the two preparations. In the presence of the muscarinic specific cholinergic agonist acetyl-β-methylcholine, instead of ACh, phentolamine completely abolished the hyperpolarizing response in curarized ganglia, but did not significantly affect it in nicotinized ganglia. Phentolamine was, however, not nearly as effective against ACh-induced hyperpolarization in curarized ganglia. These results support the presence of two separate pathways to the sIPSP. Application of U-0521 (used to prolong the action of adrenergic transmitters) was found to enhance the amplitude and duration of the sIPSP response in both curarized and nicotinized ganglia. The effect seen in curarized ganglia was larger than that in nicotinized ganglia. While this is expected in curarized ganglia where an adrenergic effect has been shown to exist, the result in nicotinized ganglia is more interesting as it was believed that adrenergic stimulation was not involved in eliciting a hyperpolarization in the post-ganglionic neuron (Weight and Padjen, 1973a,b). This seems to indicate that some adrenergic effect is present even in the seemingly monosynaptic cholinergic-specific response.

The main conclusion from the above discussion is that an adrenergic interneuron is indeed present in frog sympathetic ganglia, as in rabbit sympathetic

ganglia, and that it mediates a hyperpolarizing sIPSP response in post-ganglionic neurons. It also appears that there is an alternate muscarinic cholinergic pathway which also mediates a hyperpolarization in post-ganglionic neurons, but by a direct, monosynaptic connection. Libet and Kobayashi (1974) proposed that the phenomena seen in curarized ganglia are more relevant physiologically in the production of the sIPSP, while the results from nicotinized ganglia are more of a pharmacological interest.

A number of related studies examining different ganglionic preparations appeared in the following years. Hartzell, Kuffler, Stickgold and Yoshikami (1977) found that ACh also mediated the sIPSP via a monosynaptic muscarinic pathway in mudpuppy parasympathetic ganglia. The muscarinic specific agonist bethanechol elicited exclusively a hyperpolarizing response. In contrast to the conclusion of Weight and Padjen (1973a) that the sIPSP in bullfrog sympathetic ganglia was the result of a decrease in Na^+ conductance, the mechanism was found to be an increase in K^+ conductance in the mudpuppy parasympathetic ganglia. Reversal potential for the sIPSP was approximately equal to the calculated equilibrium potential for K^+, and changes in the K^+ concentrations, and therefore the K^+ equilibrium potential, resulted in concomitant changes in the sIPSP reversal potential.

Studies on nicotinized rabbit SCG (Cole and Shinnick-Gallagher, 1980, 1984) provided further evidence that an adrenergic interneuron was not necessary for the sIPSP. The α-adrenergic specific antagonist phentolamine showed no effect on the sIPSP, nor did low Ca Ringer's. Reversal of the sIPSP was also found to be near the K^+ reversal

potential in agreement with the mechanism as proposed by Hartzell, Kuffler, Stickgold and Yoshikami (1977). Horn and Dodd (1981) using curarized bullfrog ganglia, as Libet and Kobayashi (1974) did, found a monosynaptic ACh-dependent IPSP. Low Ca/high Mg prevented preganglionic stimulation of the sIPSP, however directly applied ACh did elicit a hyperpolarization. This contradicts the findings of Libet and Kobayashi (1974) where a second transmitter was found to be necessary in curarized bullfrog ganglia. Horn and Dodd also found that the mechanism for the sIPSP in bullfrog sympathetic ganglia was an increase in K^+ conductance (as in mudpuppy: Hartzell, Kuffler, Stickgold and Yoshikami, 1977; and rabbit: Cole and Shinnick-Gallagher, 1984); this contradicts with the decrease in Na^+ conductance proposed by Weight and Padjen (1973a) in the bullfrog. It has variously been found that there has been: (i) no change in post-ganglionic membrane conductance (Eccles and Libet, 1961); (ii) a decrease in post-ganglionic Na^+ conductance (Weight and Padjen, 1973a); and (iii) an increase in post-ganglionic K^+ conductance (Hartzell et. al. 1977; Horn and Dodd, 1981). It seems most likely that both a decrease in Na^+ conductance and an increase in K^+ conductance occur to various degrees in different preparations. In the case of no increase in membrane conductance associated with the hyperpolarization, the decrease in Na^+, and increase in K^+ conductance probably cancel each other out such that no significant change in total membrane conductance occurred.

The reasons why these groups have found results at such odds with each other are as yet unknown. The present state of knowledge indicates that there are two separate

pathways to production of a post-ganglionic hyperpolarization. Obviously future studies are required to clarify the mechanisms of sympathetic ganglionic transmission, including any species differences that may exist.

Meanwhile, on the assumption that the SIF cell was the interneuronal candidate, Libet and Owman (1974) examined dopamine (DA) release accompanying the sIPSP in rabbit SCG by either preganglionic stimulation or perfusion of the muscarinic-specific drug bethanechol. It was found that both of these conditions produced release of DA from SIF cells as indicated by a reduction in DA content. Long term stimulation of DA release from SIF cells was studied by perfusing bethanechol in the bathing solution for 30-60 minutes. This treatment resulted in an apparent exhaustion of DA stores in the SIF cells, and a concomitant loss of the sIPSP response. Uptake of exogenous DA by the SIF cells was then followed by a return of the sIPSP. This study further supports an interneuron function for the SIF cells in rabbit sympathetic ganglia.

A brief, but interesting study by Dunn and Marshall (1985) using bullfrog sympathetic ganglia investigated the SIF cell response to preganglionic stimulation and ACh application. It appears to be one of only a few studies where direct recordings from SIF cells were obtained. As discussed later, this thesis provides the first complete description of membrane currents in SIF cells. Dunn and Marshall first looked for evidence of afferent synaptic contact between preganglionic fibres and SIF cells. Selective horseradish peroxidase (HRP) staining of either the B or C preganglionic fibre types revealed that the C fibres formed afferent synapses with SIF cells while B fibres

did not. To test whether these were indeed functioning synapses Dunn and Marshall recorded intracellularly from SIF cells while stimulating both B and C fibres together or separately. While stimulating both fibre types EPSPs similar to those seen in principal neurons were evoked in one third of the SIF cells. The conduction velocity of fibres mediating these responses corresponded to C but not to B pre-ganglionic fibres. Microiontophoretic application of ACh also caused a depolarization in 18 of the 21 SIF cells studied, resulting in multiple spiking in many cases, though only 7 responded to preganglionic stimulation as well. The reason why not all SIF cells exhibited synaptic activity upon stimulation was unclear. Dunn and Marshall (1985) proposed that subpopulations may exist with different functional roles, or alternatively that a more widespread innervation present in developing ganglia is partially lost during maturation. While this evidence is consistent with an interneuronal role for the SIF cells there is as yet no morphological evidence for efferent synapses from SIF cells to post ganglionic neurons in the bullfrog sympathetic ganglia (Weight and Weitsen, 1977). As Dunn and Marshall (1985) pointed out, this may be because the fraction of SIF cells with synapses is small and so the probability of finding these contacts is low. An alternative theory is that there are no efferent synapses, and the SIF cells release their vesicle contents in a paracrine or neuroendocrine fashion following depolarization.

Endocrine Role of SIF Cells

The presence of a large number of SIF cells that do not display efferent synapses directly onto post-ganglionic neurons led to consideration of alternative

functions other than that of interneuron. The lack of efferent synapses coupled with similarities between the SIF cells and adrenal chromaffin cells in terms of origin, ultrastructure, and relationship to surrounding tissues contributed to the hypothesis of an endocrine role for SIF cells. Both cell types are neural crest derived, have large secretory granules (type II SIF cells: 150-300 nm; adrenal chromaffin cells: 150-350 nm; Doupe, Landis and Patterson, 1985), possess afferent input, and are often found in close proximity to blood vessels (McDonald and Blewett, 1981). The presumed exocytotic action of these SIF cells then, could be paracrine (secreting to neighbouring cells) neuroendocrine (secreting via axons to the bloodstream) or true endocrine (secreting directly into the bloodstream) as suggested by Pearse (1977). In the rest of this thesis the term endocrine will be used to describe all three cases unless it is necessary to create a distinction.

Evidence for two distinct SIF cell types in various species was obtained in morphological studies by Chiba and Williams (1975), Williams et. al., (1975, 1976). Type I SIF cells were solitary, situated near principal neurons, and their processes were in the general vicinity of ganglionic neurons. These SIF cells were the presumptive interneurons. Type II SIF cells were found in clusters, associated with blood vessels, and when present, their processes were directed towards nearby blood vessels. These SIF cells were devoid of efferent synapses, and their resemblance to adrenal chromaffin cells pointed to a secretory, or endocrine role. In rabbit sympathetic ganglia most of the SIF cells ($\approx 80\%$) exhibited type I morphology whereas the remainder were type II. In

the cow, monkey, ox and cat type II morphology predominated, varying between 70 and 100%. In the rat and guinea pig while both types of SIF cells were present, the distribution was more difficult to determine since some type I cells were interspersed among clusters of type II cells (rat: Eränkö and Härkönen, 1965; Siegrist, Dolivo, Dunant, Foroglou-Kerameus, De Ribaupierre and Rouiller, 1968; Matthews and Raisman, 1969; Eränkö and Eränkö, 1971; McDonald and Blewett, 1981; guinea pig: Jew, 1980).

In general, the capillaries associated with type II SIF cells were found to be fenestrated with only a single-layered membrane sealing the fenestrations (rat: Eränkö and Härkönen, 1965; Siegrist et. al., 1968; Matthews and Raisman, 1969; Eränkö and Eränkö, 1971; McDonald and Blewett, 1981; monkey: Chiba and Williams, 1975; galago: Williams et. al., 1976; human: Chiba, 1980; Hélen, Alho and Hervonen, 1980; rabbit and guinea pig: Jew, 1980). In some cases the short SIF cell processes were seen to extend into the perivascular space (Chiba, 1980), an arrangement similar to the adrenal medullary chromaffin cells (Coupland, 1965; Elfvin, 1965). On occasion SIF cells were seen with their plasma membrane exposed directly to the intraganglionic tissue space (Tanaka and Chiba, 1991). In the region of contact between SIF cell and capillary fenestrations the Schwann cell sheath that normally surrounds the SIF cell was often found to be partial or absent (rat: Siegrist et. al., 1968; Yokota, 1973; rabbit and guinea pig: Jew, 1980; guinea pig: Tanaka and Chiba, 1991; monkey: Chiba and Williams, 1975). These morphological relationships suggest that type II SIF cells have ready access

to the pericapillary space and that upon stimulation by the pre-ganglionic neurons catecholamine secretion is likely to occur directly into the circulation.

Consistent with this notion, the membranes of SIF cells have occasionally been shown to contain structures resembling vesicle release sites. Early studies showed inpocketings of SIF cell membrane around vesicular structures that have the appearance of released intravesicular materials (Elfvin et. al., 1975; Furness and Sobels, 1976; Taxi, Gautron and L'Hermite, 1969; Taxi, 1973; Taxi and Mikulajova, 1976; Matthews, 1976). Though exocytotic profiles were seen only rarely by Matthews (1978) in SIF cells from rat SCG, this may be related to the duration and frequency of the release event (Taxi, 1973), as well as sampling limitations inherent in electron microscopic studies.

Since the clustered type II SIF cells usually are not closely related to principal neurons a paracrine function seems unlikely in the majority of cases. The usual location of the type II cell clusters near blood vessels indicates a more likely neuroendocrine or true endocrine role. It was suggested by many authors that a portal system may be involved in the distribution of catecholamines from the SIF cells (Siegrist et. al., 1968; Chiba and Williams, 1975; Chiba et. al., 1977), though none of these authors presented any morphological evidence for such a system. The portal system theory was fueled in part by the findings of Winckler (1969) and of Lever, Santer, Lu and Presley (1973) in which the injection of dyes revealed that the vascularization surrounding SIF cell clusters had a glomerulus-like morphology. Later, using serial sections Heym and Williams (1979) and Abe, Watanabe and Yamamoto (1983) showed evidence for portal systems

in the SCG of the shrew (*Tupaia glis*), and rat, respectively. In these studies, numerous capillaries originating from networks in SIF cell clusters diverged to connect adjacent capillaries surrounding principal neurons. More recently, Tanaka and Chiba (1991) using serial sections followed the complete progress of blood vessels from artery to vein in guinea pig inferior mesenteric ganglion (IMG), which appeared to contain only type II SIF cells. The inferior mesenteric artery, which supplies the IMG, ran directly to the SIF cell clusters where they then divided into numerous coiled and looped sinusoidal capillaries before draining into a large sinus. Other capillaries formed from this sinus, and then went on to ramify amongst the principal neurons of the ganglion before draining into veins surrounding the IMG. Two separate groups of capillaries were seen in series separated by what the authors called the intraganglionic portal sinus. This appeared to be a system ideally suited for the collection of neurotransmitter released from the SIF cell clusters and its subsequent distribution to the principal ganglionic neurons.

Chemosensory Function in SIF Cells

The location and morphology of SIF cells that are supportive of, and consistent with an endocrine function are just as easily compatible with a chemosensitive function in the cardiovascular system. The chemosensitive glomus cells of the carotid body bear a striking ultrastructural resemblance to SIF cells (Kobayashi, 1971) and are stimulated by hypoxic, hypercapnic and acidic stimuli in arterial blood (Biscoe, 1971; Eyzaguirre and Zapata, 1984; Fishman, Greene and Platika, 1985; Duchen et. al., 1988; López-Barneo, López-López, Ureña and González, 1988; López-López, González, Ureña,

López-Barneo, 1989; Hescheler et. al., 1989; Stea and Nurse, 1991; Stea et. al., in press). They also occur in clusters near fenestrated capillaries, possess incomplete Schwann cell coverings at sites of apposition to these fenestrations, and short processes that invade the perivascular space (Biscoe, 1971). Though fenestrations can provide an easy route for released transmitters from SIF cells, they also provide a route for stimuli from the blood to a chemosensitive SIF cell. It has in fact been shown that while SIF cells from rat SCG do not respond to a hypoxic stimulus with a rapid decrease in outward K^+ current as is found in glomus cells (Stea and Nurse, 1991), they do respond with an increase in number and intensity of staining for tyrosine hydroxylase (TH), the first enzyme in the pathway for synthesis of adrenergic neurotransmitters from tyrosine (Dalmaz, Borghini, Pequignot and Peyrin, 1991), and both SIF and glomus cells experience a decrease in cGMP levels (Wang, Chen, Wang, Dinger, Hanson, Stensaas and Fidone, 1991). Thus, SIF cells clustered near blood vessels could function in a similar manner to the closely-related chemosensitive glomus cells of the carotid body.

Kondo (1977b, 1980) found SIF cells in rat SCG with innervation patterns that diverged from both the interneuronal (pre-ganglionic to SIF to post-ganglionic) or endocrine (pre-ganglionic to SIF to capillary vasculature) pattern seen in many earlier studies (Grillo, 1966; Williams, 1967a,b; Matthews and Raisman, 1969; Williams and Palay, 1969; Matthews and Nash, 1970). These SIF cells had both afferent and efferent synapses and in most cases a single axon formed both *en passant* and bouton synapses with many different SIF cells. The occurrence of efferent synapses was greater than that

of afferent synapses indicating that the main direction of transmission was from SIF cell to axon. The origin of the axon appeared to be via the pre-ganglionic trunk since severing the latter resulted in the loss of most of the synapses on these SIF cells. This innervation pattern was similar to that seen by McDonald and Mitchell (1975) and Kondo (1976, 1977b) in the chemosensory organ, the rat carotid body where sensory axons formed both afferent and efferent synapses on the glomus cells, again with efferent synapses predominating. Kondo (1977b) also found this same innervation pattern for SIF cells in the nodose ganglion, a sensory ganglion that had previously been shown to have chemosensitive properties since it could be stimulated by various chemicals introduced into its circulation (Borison and Fairbanks, 1952; Chai and Wang, 1966; Jacobs and Comroe, 1971). It is still unclear whether SIF cells are true arterial chemosensors like glomus cells of the carotid body. In fact, while it was shown that the glomus cells of the rat carotid body responded to a hypoxic stimulus with a reduction in outward K^+ current, and cultured SIF cells from neonatal rat pups were unresponsive (Stea and Nurse, 1991) it was recently shown that SIF cells responded to hypoxic stimuli in different ways. SIF cells demonstrated both an increase in number and intensity of TH-staining after exposure to long-term normobaric hypoxia that was not dependent on either pre- or post-ganglionic neuronal input (Dalmaz, Borghini, Pequignot and Peyrin, 1991), and as did glomus cells, exhibited a decrease in cGMP levels after short-term hypoxia (Wang et. al., 1991). The lack of neuronal input in the former of these studies suggests that the SIF cell directly sensed the hypoxic stimuli, while the latter report indicates a

further similarity between SIF cells and the chemosensitive glomus cell. It is conceivable that hypoxia is not the adequate stimulus for developing SIF cells, or that they do not respond rapidly with a decrease in outward current upon depolarization, though this requires validation with older, mature SIF cells. The obvious similarity between the innervation, and ultrastructural features of the chemosensitive glomus cells and SIF cells suggests that a possible chemosensitive role for the latter should be further investigated.

Goals Of This Thesis

Compelling evidence exists for each of the interneuronal, endocrine and chemosensory functions hypothesized for the SIF cell. However, all of these functions are still far from being proven. A greater understanding of the electrophysiological properties of the SIF cell would be a key step in elucidating the function or functions of this cell type. A background knowledge of the membrane ionic currents in SIF cells could be used as a basis to study the effects of different stimuli, such as neurotransmitters, neuromodulators or chemosensory stimuli.

At the commencement of this project the only known record of SIF cell electrophysiology was an intracellular recording of action potentials in SIF cells from an *in vitro* preparation of whole-mounted bullfrog sympathetic ganglia by Dunn and Marshall (1985). They found that both pre-ganglionic stimulation and direct ACh application elicited action potential waveforms in SIF cells that appeared similar to those seen in the other closely related cell types of the sympathoadrenal lineage (principal neuron: Belluzzi and Sacchi, 1986; adrenal chromaffin cell: Biales, Dichter and Tischler,

1976; Kidikoro and Ritchie, 1980; and glomus cell: Duchen et. al., 1988). This indicated that the SIF cells possessed a variety of voltage-activated ion channels responsible for the spiking behaviour. An examination of the individual currents underlying this excitable behaviour, that would represent a major step forward in understanding the functional properties of the SIF cell, had not been performed prior to this thesis.

While the sympathetic principal neurons and adrenal chromaffin cells are found at high densities in the sympathetic ganglia and adrenal medulla respectively, the SIF cells are only sparsely located in ganglionic tissue. Attempts to record from SIF cells, are made difficult by this low density. While in certain ganglia, such as the rat SCG, the SIF cells number in the hundreds, there are many more principal neurons, contributing tens of thousands of cells, as well as numerous Schwann and endothelial cells (Eränkö and Soinila, 1981). Dispersion of the ganglionic cells, and plating for the purpose of immediate recording has the same problem in that only a small percentage of the plated cells will be SIF cells. A method for long-term selective culturing of SIF cells was required. Doupe, Patterson and Landis (1985) were the first to describe conditions for the dispersal and culturing of rat sympathetic ganglionic cells that resulted in selective survival of SIF cells, with principal neurons and most of the other cells dying. Finally there was a technique for isolation of SIF cells for voltage-clamp recording. The problem at this point is in recording from such small cells.

SIF cells are approximately 15-20 μm in diameter (Doupe, Patterson and

Landis, 1985; Chapter 2, this thesis), making them quite difficult to impale with an electrode to perform intracellular voltage clamp recording. The high-resolution patch clamp technique for whole cell recording was an attractive alternative (Hamill, Marty, Neher, Sakmann and Sigworth, 1981). This method uses microelectrodes with tip sizes of 1-2 μm that allow for the current- or voltage-clamping of small cells such as the SIF cell. As well, the recent nystatin perforated-patch method (Horn and Marty, 1988) that simply permeabilizes the membrane patch instead of creating a large whole, allows for more stable, long-term recordings of membrane currents without loss of the cell's natural homeostatic mechanisms as a result of diffusion of necessary proteins and second messengers out of the cell. This is especially important in the recordings of Ca^{2+} currents and effects of intracellular acidification in SIF cells, both of which rely on cellular homeostasis mechanisms for their control. Without these techniques of cell culturing and patch-clamp recording the characterization of membrane currents in SIF cells would be virtually impossible.

Using the techniques just mentioned the goals of this thesis were to: (i) demonstrate the suitability of Doupe, Patterson and Landis's (1985) culture methods for the electrophysiological study of SIF cells; (ii) perform a characterization of the individual membrane ionic currents present in cultured SIF cells isolated from one-day-old rat superior cervical ganglia; (iii) follow the development of a fast transient outward K^+ current (I_A) over the first postnatal week of life in SIF cells from rat SCG; and (iv) determine the effects of intracellular acidification on the membrane ionic currents of

these SIF cells.

CHAPTER 2

Characterization of the Ionic Currents in Cultured Small Intensely Flourescent Cells from Superior Cervical Ganglia of Neonatal Rats

Introduction

The small intensely fluorescent (SIF) cell is found in autonomic ganglia and adrenal medullary tissue. It was named due to its size and intense fluorescence resulting when the endogenous catecholamines are revealed by the formaldehyde-induced fluorescence method (Falck et. al., 1961). SIF cells share a common neural crest origin with the principal neurons of the sympathetic ganglia, the adrenal medullary chromaffin cells, and the glomus cells of the carotid body; together these cells form the sympathoadrenal lineage (Doupe, Landis and Patterson, 1985). While numerous comprehensive studies have been published on the electrophysiological properties and function of these other members of the sympathoadrenal lineage (principal neurons: Constanti and Brown, 1981; Galvan, 1982; Galvan and Sedlmeier, 1984; Belluzzi et. al., 1985a,b; Belluzi and Sacchi, 1986, 1989; chromaffin cells: Fenwick et. al., 1982a,b; Bossu et. al., 1991a,b; glomus cells: Duchen et. al, 1988; Ureña et. al., 1989; Hescheler et. al, 1989; Stea and Nurse, 1989, 1990, 1991a; Stea et. al., in press;) there are relatively few studies on the electrophysiology of SIF cells (Dunn and Marshall, 1985; Stea and Nurse, 1991, Stea et. al., in press).

The functional role of the SIF cell has been the subject of much contention. Synaptic connections have been shown between pre- or post-ganglionic neurons and SIF cells (Grillo, 1966; Williams, 1967; Matthews and Raisman, 1969; Taxi et. al., 1969, Matthews, 1978), while other studies indicate that the SIF cell is required for production of the slow inhibitory post-synaptic potential in sympathetic ganglia (Eccles and Libet, 1961; Libet et. al., 1968; Libet and Owman, 1974; Libet and Kobayashi, 1974), suggesting a possible role as an interneuron. However, an interneuron has been shown to be unnecessary for the production of the sIPSP in sympathetic ganglia in other studies (Weight and Padjen, 1973a,b; Horn and Dodd, 1981; Cole and Shinnick-Gallagher, 1984). SIF cells have also been shown to have regions of membrane devoid of Schwann cell covering closely apposed to fenestrated capillaries (Eränkö and Härkönen, 1965; Siegrist et. al., 1968; Matthews and Raisman, 1969; Eränkö and Eränkö, 1971; Chiba et. al., 1976; Chiba and Williams, 1975; Chiba, 1980; Hélen et. al., 1980; Jew, 1980). In addition, evidence for vesicular release has been obtained for SIF cells (Taxi et. al., 1969; Taxi, 1973; Taxi and Mikulajova, 1976; Matthews, 1978), indicating an endocrine role similar to that of the adrenal medullary chromaffin cell. The proximity of SIF cells to fenestrated capillaries could also provide a direct route for blood-borne stimuli to modulate SIF cell function. This, coupled with the ultrastructural resemblance between SIF cells and glomus cells (Kobayashi, 1971; McDonald and Blewett, 1981; Doupe, Patterson and Landis, 1985) and their similar innervation patterns (Kondo, 1977b, 1980, Biscoe, 1971) had lead to the proposal of a chemosensitive role.

After many years and numerous indirect studies the function of the SIF cell is still far from being understood. A greater understanding of the electrophysiological properties of SIF cells would provide an important basis for further study of the responses of SIF cells to different stimuli. Cell culture techniques were utilized to provide a dispersed cell population enriched in SIF cells from neonatal rat SCG; these were used in electrophysiological experiments to determine the whole-cell currents recorded under voltage clamp. The methods of conventional whole-cell and perforated-patch whole-cell recording were employed in order to isolate and characterize the individual ionic components of membrane current present in SIF cells. Separation of currents was aided by using a combination of ion substitution and pharmacological experiments. As well, development of a fast I_A current was followed over the first postnatal week in a series of SIF cell cultures prepared from rat pups of different ages.

Materials and Methods

Culture Conditions

The procedures for growing small intensely fluorescent (SIF) cells in culture were similar to those described by Doupe patterson and Landis (1985). Wistar rat pups (Charles River, Quebec), 1-6 days old, were sacrificed by a sudden blow to the head and the superior cervical ganglia removed, cleaned of surrounding tissue and placed in L-15 plating medium. This medium consisted of Leibovitz's L-15 medium supplemented with 1% of 2 mM L-glutamine, 0.6% glucose and 1% penicillin-streptomycin (Gibco). Dispersed cells were obtained by combined enzymatic and mechanical dissociation. First, the ganglia were incubated in enzymatic solution containing: Ca-Mg-free Hank's BSS, 0.1% collagenase and 0.1% trypsin (Gibco), 0.01% deoxyribonuclease (Millipore Corp. Freehold, N.J.) and 1% penicillin-streptomycin (Gibco) for 1 hr at 37°C. The enzyme was then removed and the tissue rinsed and stored in growth medium containing F-12 nutrient medium supplemented with 5% fetal bovine serum, 0.6% glucose, 2mM L-glutamine, 1% penicillin/streptomycin, 2.5 μM dexamethasone (which selects for type II SIF cells; Doupe et al. 1985), and 10^{-5}M cytosine arabinoside (Ara C; Sigma Chemical Co.), to reduce proliferation of the background cells. The tissue was then mechanically teased apart, and triturated with a sterile pipette. For electrophysiological experiments the resulting cell suspension was plated on a thin layer of Matrigel (Collaborative Research, Bedford, Mass.) applied to the central area (ca. 1cm diameter) of 35 mm Nunclon culture dishes. This central area was marked on the underside of the dish with

a needle, prior to coating with the substrate. The stock Matrigel was diluted with phosphate buffered saline 1:1 before use. The dishes and pipettes were pre-cooled in a refrigerator at 4°C before application of Matrigel since the latter changes from a fluid state to a gel as the temperature is raised from 4°C to room temperature. The Matrigel vial was kept on ice during these manipulations. After coating the central area with 3-4 drops of Matrigel solution the dishes were kept at 4°C for approximately 3 hr before the excess fluid was removed and stored; this excess Matrigel solution was reused for 3 separate applications before being finally discarded. The dishes with the film of Matrigel were washed 3 times with deionized, distilled water (Gibco) and left to dry. For immunofluorescence, polylysine-coated glass coverslips covered with a thin layer of Matrigel were used instead of the plastic bottom of the Nunclon dishes. The coverslips were attached with silicone to seal the bottom of Nunclon culture dishes that had central holes of ca. 1 cm diameter drilled. Approximately 0.15 ml of polylysine (0.125 mg/ml) was applied to the coverslip and left to dry overnight at room temperature. The dishes were then rinsed 3 times with distilled water, and air-dried before application of Matrigel as described above. Typically, the plating density was one to two ganglia per dish. After the first two days in culture the old medium was removed and the dish was flooded with 2 mL of growth medium. This process was repeated every 5-6 days. The cultures were incubated in a humidified atmosphere of 95% air/5% CO_2 at 37°C for 5-16 days before they were used in patch-clamp experiments.

Identification of SIF Cells by Immunofluorescence

SIF cells were identified by the presence of tyrosine hydroxylase (TH) immunoreactivity. The cultures were first rinsed with phosphate buffered saline (PBS) and fixed at room temperature for 1 hr in 0.1 M phosphate buffer (pH 7.2) containing 4% paraformaldehyde plus 0.01% glutaraldehyde. The fixative was then removed and the culture incubated in PBS containing 0.1 M glycine for 5 min at room temperature. After 3 x 10 min washes in PBS, the cells were covered and incubated overnight (18-24 h) with a solution containing 0.1 M phosphate buffer, 0.3% Triton X-100, 1% goat serum and rabbit anti-TH antiserum (Chemicon, El Segundo, CA) at a final dilution of 1:500 to 1:1000. Following removal of the antiserum, cultures were washed 3 x, for 10 min each, with 0.1 M PBS containing 0.3% Triton X-100 and 1% goat anti-rabbit IgG serum (PBS-GS; Cappel, Malvern, PA) at a 1:50 dilution. After two rinses in PBS-GS and 3 x 5 min washes with 0.1 M phosphate buffer, the cells were covered with a 1:1 solution of 0.2 M sodium carbonate (pH 9.0): glycerol, to which 2mg/10ml p-phenylenediamine was added to reduce photobleaching of fluorescein (Johnson and Nogueira, 1981). Immunofluorescence was visualized and photographed with a Zeiss S16 standard microscope, equipped with epifluorescence and automatic exposure control.

Conventional Whole Cell Recording

Formation of gigaohm seals for patch-clamp, whole-cell recording requires both clean cell surfaces and pipette tips, as well as specific pipette dimensions (Hamill, Marty, Neher, Sakmann and Sigworth, 1981). Use of neonatal rat pups in which connective

tissue was not well-developed, combined with the enzymatic dissociation produced isolated SIF cells which readily formed gigohm seals. Prior to recording the cultures were rinsed and then bathed with a physiological salt solution (see below) and mounted on the stage of an inverted phase-contrast microscope (Leitz Diavert). The reference electrode consisted of a polyethylene tube (PE160) filled with 0.9% saline/2% agar connecting the bathing solution to a pool of 150 mM KCl which was grounded via an Ag/AgCl electrode. The tube made contact with the bath via an insertion in a stainless steel block which was fitted into the dish. Patch pipettes were pulled from Corning 7052 glass (1.5mm O.D.) with a Narishige two stage vertical puller (model PP-83). Heating coil settings for the two stages were varied until tips of appropriate size and shape were formed, as determined by observing under a microscope (100X) and examining resistance measurements (see below). Once appropriate settings were found, they were maintained, and tip parameters were highly reproducible. The tips of the pipettes were then fire polished in order to smooth the edge of the glass and facilitate sealing with the cell membrane (Hamill et. al., 1981). This was accomplished by bringing the tips close to a glass-coated V-shaped tungsten filament, and then passing current throught the filament for about 3 s until it glowed dull red. The current was then discontinued and the pipette was ready for filling. When viewed at approximately 100X magnification no visible difference could be seen at the pipette tips after fire polishing. Filling of the pipettes was accomplished in 2 stages. First, a syringe was connected to the pipette shank, via a length of polyethylene tubing, and the tip of the pipette was filled by gentle suction

following immersion in a small pool of filtered intracellular solution and slow withdrawal of the plunger in the syringe. The rest of the pipette was then filled by inserting a syringe with a 24 gauge needle, that is filled with intracellular solution, into the shank of the pipette and depressing the plunger while slowly removing the needle. Any air bubbles formed during the pipette filling were removed by gentle tapping. After filling, pipette resistances were typically in the range of 2-10 MΩ. SIF cells with clean surfaces, clear nuclei and nucleoli, and lacking visible processes (to minimize space clamp error; Jack, Noble and Tsien, 1975; Johnston and Brown, 1983; Rall and Segev, 1985) were chosen for patch recording. Gigaohm seals with resistances typically 2-10 GΩ were first formed prior to whole cell recording using the procedures of Hamill et. al. (1981). To help prevent debris from interfering with the sealing process solutions were filtered with 0.2 μm filters (Gelman) before use. The pipette tip was carefully positioned on the cell surface under visual control with the aid of a micromanipulator (Leitz). Once contact was made slight negative pressure was applied to the interior of the pipette. A sudden, large increase in seal resistance from the megaohm to the gigaohm range, as measured by monitoring the current response to a 0.2 mV voltage pulse, indicated successful formation of a Giga-seal. In earlier experiments with the WPI S7050A amplifier, compensation of the cell-attached capacitance was not possible; however, in later experiments with the Dagan 3900 amplifier it was possible to compensate the cell-attached capacitance immediately after formation of the Giga-seal. To produce the whole-cell configuration further negative pressure was applied. This resulted in rupture

of the membrane patch encircled by the pipette tip, usually without causing damage to the seal between the pipette and cell. A large increase in the capacity transient current signalled successful break-through to the whole-cell configuration. The input resistance, measured via the 0.2 mV pulses, also greatly decreases as a result of this rupture. A portion of this resistance is a result of restriction of current flow through the pipette and contributes a resistance in series with the cell's input resistance. This series resistance (R_S, ca. 5-10 MΩ) was mostly compensated for in all experiments. Since this compensation involves a positive feedback current , if the resistance is overcompensated instability can occur, leading to oscillations in the membrane potential and destruction of the preparation (Jones, 1990). After series resistance compensation whole-cell recordings were initiated. All recordings were carried out at room temperature.

Perforated Patch Recording

In many experiments the perforated-patch, whole-cell recording technique was used (Horn and Marty, 1988). This technique permits more stable long-term recordings of whole-cell currents and membrane potential by limiting diffusive exchange to small monovalent ions, and thus causing minimal disruption of the cell's cytoplasmic components. In addition it is useful for studying Ca^{2+} currents which tend to 'run down' rapidly with conventional whole cell recordings. Perforated-patch recordings were obtained from SIF cells using techniques similar to those described by Horn and Marty (1988). In these studies the pore-forming antibiotic nystatin was dissolved in dimethylsulphoxide (5mg/ml) instead of methanol (Horn and Marty, 1988) and

ultrasonicated for 5 min. This stock was diluted to 500ug/ml in the pipette solution (see below) just before use. As nystatin is sensitive to both light and temperature, deterioration of stock solutions was prevented by covering with aluminum foil and freezing after use. In addition, fresh solutions were used for 5 days before being discarded. Solutions for experiments were covered to prevent exposure to light, and used for only 3-4 h until discarded. The method for forming giga-seals is essentially the same as described above for whole-cell recording with some slight differences. Though the intracellular solution used for backfilling pipettes contained 500 μg/ml nystatin, that used for filling the pipette tip was devoid of nystatin. Thus after formation of the giga-seal there was a delay in the decrease in access resistance due to diffusion of nystatin into the membrane patch. Pipette resistances were similar to those seen without nystatin, typically 2-10 MΩ. Upon establishment of a giga-seal in the cell-attached mode further suction is not applied as with conventional whole cell recording; the capacitance is quickly compensated for, and then time is allowed for insertion of the nystatin into the membrane. After 5-15 minutes of exposure to nystatin in the cell-attached mode the capacitative transient increased and eventually the series resistance stabilized at typically 10-15 MΩ. The series resistance was compensated in all experiments before recording of whole-cell currents. All experiments were performed at room temperature.

Data Acquisition and Analysis

Whole-cell currents were recorded with either a Dagan 3900 patch clamp amplifier or a World Precision Instruments (WPI) patch-clamp module (S7050A) and a

probe equipped with a 1 GΩ headstage feedback resistor. Records were simultaneously displayed on an oscilloscope (Tektronix, 5111A), digitized with an Axolab 1100 computer interface (Axon Instruments, Burlingame, CA) and stored on disk in an IBM-AT-compatible computer using PCLAMP version 5.0, or 5.5, software (Axon Instruments). Data were analyzed using PCLAMP, and voltage clamp traces shown in the text were plotted on a Hewlett Packard Laserjet III after subtraction of leakage and capacitative currents, and Gaussian filtering at 1000 Hz. Membrane potential traces recorded under current clamp were plotted in a similar manner. Junction potentials, resulting from diffusion of ions across the boundaries of differrent ionic compositions were usually in the range of 2-4 mV and were cancelled at the beginning of the experiment.

Solutions

The extracellular solution used in most experiments consisted of (in mM): NaCl,135; KCl,5; $CaCl_2$,2; $MgCl_2$,2; glucose,10; N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (HEPES),10 at pH 7.4 . The pipette solution used for conventional whole-cell recording contained (in mM): NaCl,5; KCl,135; $CaCl_2$,1; ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA),11 ($pCa^{2+} \approx 8$); HEPES,10 at pH 7.2 ; for perforated-patch recording 500μg/ml nystatin was added. To eliminate inward Na^+ currents in some experiments, Na^+-free solutions were used where equimolar choline chloride replaced NaCl in the bathing solution while K^+ replaced Na^+ in the pipette solution; in other experiments 500 nM tetrodotoxin (TTX) was used. Similarly,

to block outward K^+ currents, K^+ free solutions were used in several experiments where the external 5 mM KCl was replaced by 5mM NaCl (total $[NaCl]_o$ = 140mM) and the internal 135mM KCl by 105mM CsCl, 25mM TEA-Cl and 5mM NaCl. Alternatively, in some experiments 10 mM tetraethylammonmium (TEA) or 4-aminopyridine (4-AP) was used to block various components of K^+ currents. Following elimination of outward K^+ currents and inward Na^+ currents a residual inward Ca^{2+} current was unmasked. To study the possible contribution of L-type Ca^{2+} channels to the overall Ca^{2+} current the dihydropyridine (DHP) antagonist nifedipine (50 μM) was used in some experiments. All drugs were obtained from Sigma Chemical Company. In most cases, solutions were filtered with 0.2 μm filters before use. Extracellular solutions were introduced into the recording chamber by gravity and withdrawn by vacuum suction.

Results

Appearance of SIF Cell Cultures

After 1-2 days in culture, cells with SIF-like morphology were seen to be attached to the central area of the dishes. Since nerve growth factor (NGF) was omitted from the growth medium the larger principal neurons did not survive and contributed to the debris of broken and dying cells seen during the first two days of culture. This debris was removed during subsequent feeding of the cultures. The SIF cells had a slightly oval to round shape with the occasional process projecting from one end of the long axis of the cell when viewed under phase contrast microscopy (Fig. 2.1A). After 6 days in culture, about the time cultures were first used for whole cell recording, the mean cell diameter ($\pm$ S.E.) estimated from the average of the perpendicular major and minor axes of oval SIF cells was 13.7 $\pm$ 0.2 μm (n=55; range 11.0 to 17.3). With longer time in culture the SIF cells seemed to increase slightly in size. It was found that the average cell diameter ($\pm$ S.E.) had increased to 15.7 $\pm$ 0.3 μm (n=43; range 11.6 to 21.2 μm) after 12 days and 15.3 $\pm$ 0.4 μm (n=42; range 10.6 to 20.6) after 19 days in culture. When first observed in culture (after 1-2 days) the cytoplasm of SIF cells exhibited a slightly granular appearance while the nuclei remained clear. The nuclei were round, eccentrically situated and occupied about one-third of the cell. One dark nucleolus was situated, also eccentrically, in the nucleus of each cell (Fig. 2.1A). The SIF cells exhibited strong immunoreactivity for tyrosine hydroxylase (Fig. 2.1B), the rate-limiting enzyme in catecholamine biosynthesis (see Doupe, Landis and Patterson,

Figure 2.1A: Phase contrast micrograph of cultured small intensely fluorescent (SIF) cells. Clear and nuclei are visible in a number of these cells. These are representative of SIF cells approached for electrophysiolgical recordings. Bar equals 20 μm.

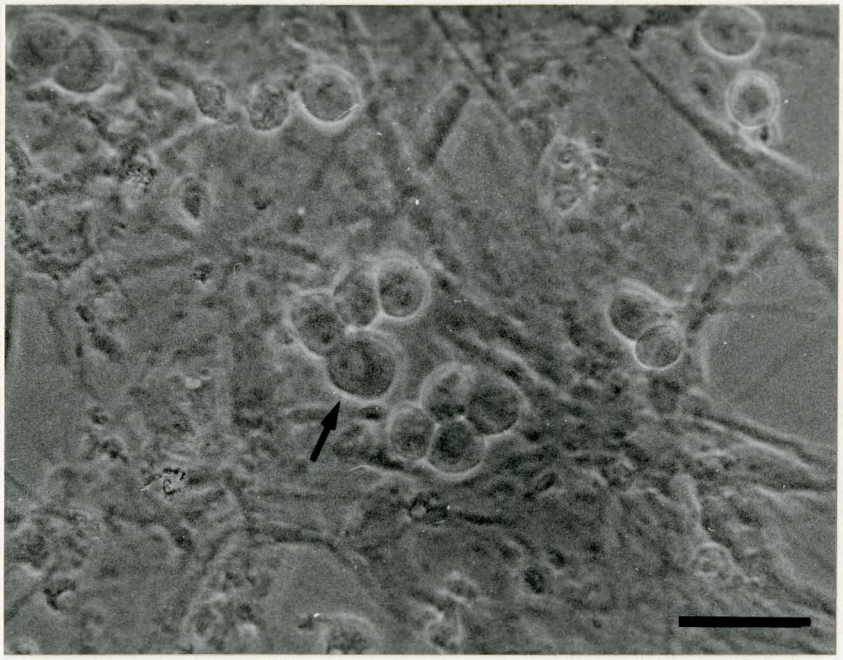
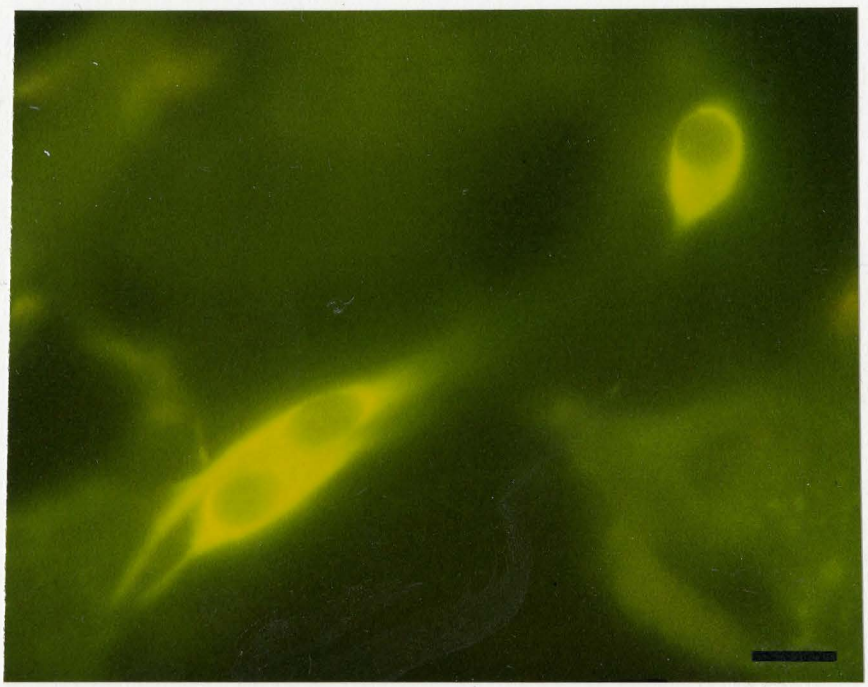


Figure 2.1B: Small intensely fluorescent (SIF) cells showing TH-immunofluorescence. The cytoplasm shows strong staining for TH, with cells at lower left showing short processes. Bar equals 10 μm.

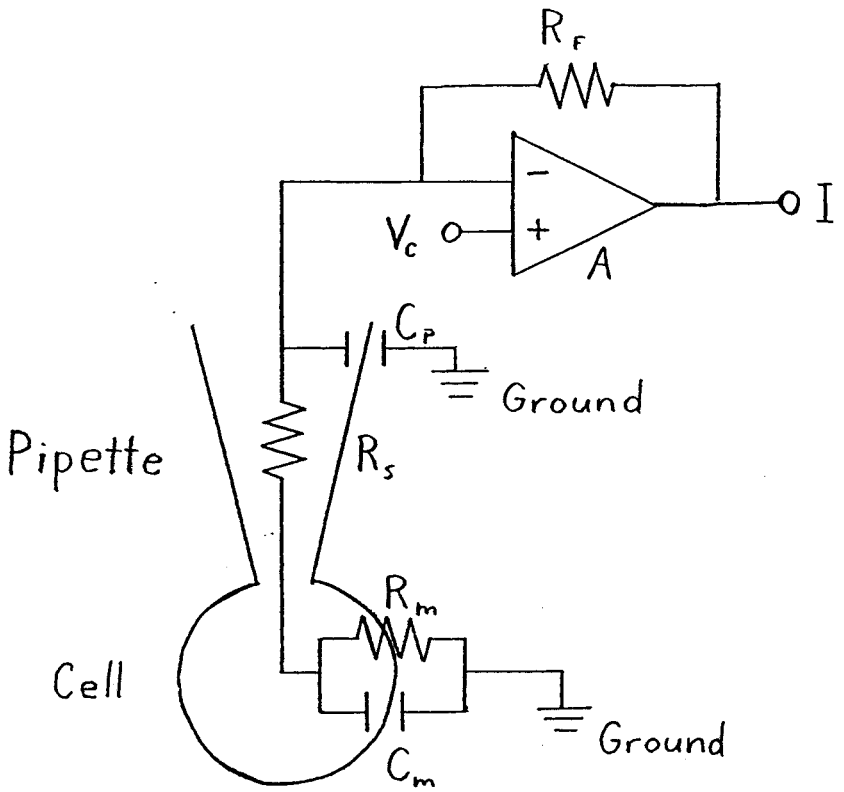


1985), and responsible for production of DOPA from the amino acid tyrosine. DOPA is the direct precursor of dopamine, which is known to be the primary neurotransmitter in SIF cells of the rat superior cervical ganglion.

Passive Membrane Properties

The input capacitance of SIF cells was determined by integration of the capacity transient under voltage-clamp. In earlier experiments, with the WPI S7050A amplifier, electrode capacitance could not be compensated for. When calculating cell capacitance using this amplifier the cell attached trace was subtracted from the corresponding whole-cell trace for the same voltage step. This left a trace where the capacity transient represented only the whole-cell capacitance excluding that of the electrode. In later experiments utilizing the Dagan 3900 amplifier electrode capacitance was compensated for prior to whole-cell recording and this permitted direct measurement of whole cell capacitance. The patch clamp set-up can be represented by a simplified circuit diagram (Fig. 2.2). Current is measured across the feedback resistor (R_f) and the amplifier injects another current in order to maintain the command potential (V_C). Both the pipette and cell have a resistance and capacitance associated with them. Without compensation of the pipette capacitance (C_P) and resistance (R_S) the speed and accuracy of the clamp is limited. With each voltage step, current is flowing into the capacitance and resistance i.e. the RC circuit. Injected current flows into the circuit with a time constant equal to the product of the capacitance and resistance such that after each period of time equal to the time constant, the voltage moves 1-1/e, or 63% of the way to the new potential.

Figure 2.2: Simplified circuit diagram of whole cell patch clamp experiment. The symbols represent: A, amplifier; R_F, follower resistance; I, injected current; V_C, command voltage; C_P, pipette capacitance; R_S, pipette or series resistance; R_m, membrane resistance; C_m, membrane capacitance.


R_F
−
+
V_c
A
I
C_P
Ground
Pipette
R_s
R_m
Cell
Ground
C_m

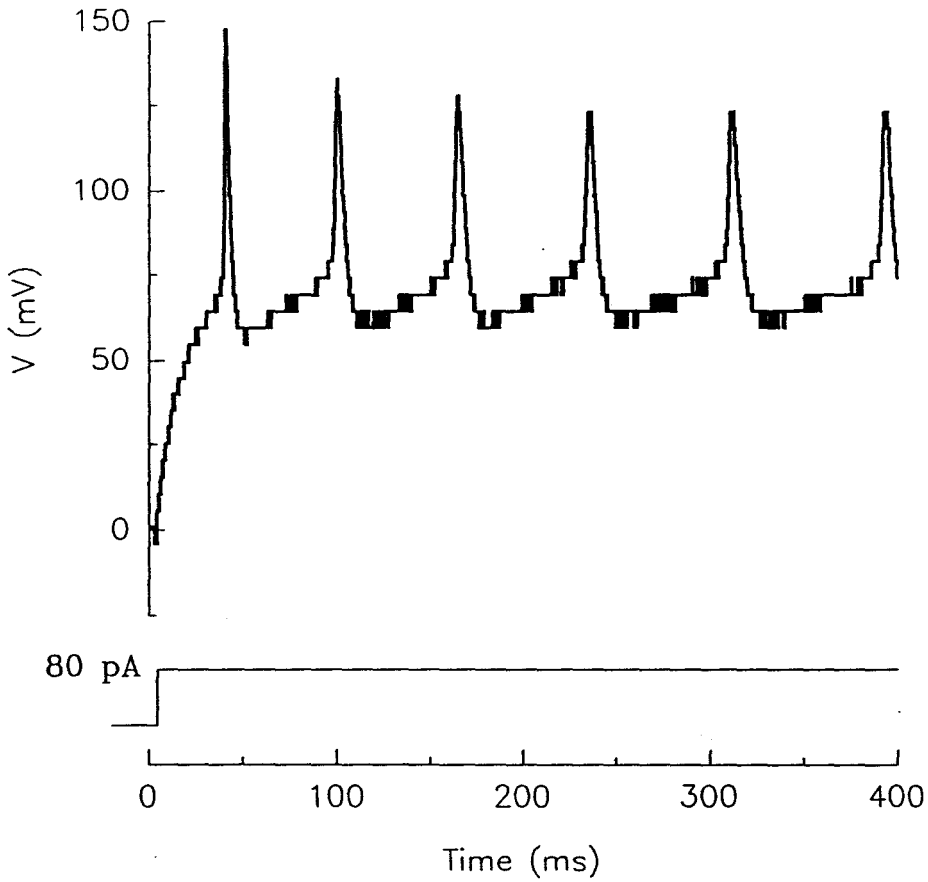
With large values for C_P and R_S the step becomes slower and results in unrepresentative recordings. The compensation of C_P and R_S are important in obtaining true representations of channel properties. The calculated average whole-cell capacitance was 10.19 ± 0.42 pF (mean ± S.E.; n=95) in SIF cells cultured from 5 to 16 days. This corresponds to a cell size of about 18μm in diameter assuming a specific membrane capacitance of 1μF/cm^2 and a spherical shape. This size is similar to that for cultured SIF cells measured in this study (see above), as well as in previous studies (Doupe Patterson and Landis 1985; Stea and Nurse, 1991; Stea et. al., in press). While the mean capacitance did increase slightly with time in culture there was no significant difference between those cultured for 5-7 days (10.8 ± 0.78; mean ± S.E.; n=54) and those cultured for 9-16 days (12.4 ± 1.1; mean ± S.E.; n=41).

The average input resistance was determined from the I/V curve generated during voltage clamp experiments. Only that portion of the curve that showed linear leakage currents, i.e. the region generated by hyperpolarizing pulses was used. The slope of this portion of the I/V curve is a measure of the input conductance (G_I), and its inverse gives the average input resistance (R_I). With this method the average input resistance of cells cultured for 5-16 days was 0.92 ± 0.08 GΩ (mean ± S.E.; n=100). As with capacitance measurements no significant difference was seen in R_I in SIF cells cultured for 5-7 days (1.00 ± 0.15; mean ± S.E.; n=57) and those cultured for 9-16 days (0.79 ± 0.10; mean ± S.E.; n=43).

Spiking Behaviour of SIF Cells

Unless otherwise noted, all electrophysiological experiments were carried out on SIF cells isolated from one-day-old pups. Since action potentials have previously been recorded from SIF cells in bullfrog sympathetic ganglia (Dunn and Marshall, 1985), as well as from the closely-related members of the sympathoadrenal lineage, i.e. adrenal medullary chromaffin cells (Fenwick et. al., 1982a) and type I cells of the carotid body (Duchen et. al., 1988) it was of interest to test whether this property was also present in cultured SIF cells of the rat. This was examined in the whole-cell configuration under current-clamp conditions. It was found that in response to a long depolarizing current pulse, multiple spikes, or action potentials were elicited in 5 of 5 SIF cells (e.g. Fig. 2.3). A typical example, taken from a 5 day-old culture is shown in Fig. 2.3. The spikes exhibited a characteristic threshold and resembled those seen in electrically excitable cells. In all cases the second and subsequent spikes had smaller peak amplitudes than the first spike, and it appeared that the spike duration was longer for the second and later spikes. Multiple spiking was also observed with intracellular recording from SIF cells in bullfrog sympathetic ganglia (Dunn and Marshall, 1985). Microiontophoretic application of ACh elicited a series of spikes in SIF cells (four spikes shown in their Fig. 2B), however amplitudes and durations were not discussed, and while the durations appeared similar it was not possible to conclude this from the figure provided.

Figure 2.3: Multiple spikes from a one-day-old SIF cell in culture for 5 days. The cell was current clamped at -80 pA and then stepped to 0 pA for 400 ms. The second and subsequent spikes had lower peaks than the first, and exhibited slower activation and inactivation, resulting in wider spikes.


150
100
50
0
V (mV)
80 pA
0
100
200
300
400
Time (ms)

Membrane Currents in SIF Cells

The occurrence of action potentials in these cultured SIF cells suggested that they contained a variety of voltage-activated ion channels, including a fast transient inward Na^+ current and a delayed rectifier outward K^+ current. To separate the various components of ionic currents, SIF cells were investigated under voltage clamp conditions. The absence of processes from the majority of SIF cells studied meant that space clamp errors were unlikely to be significant in this study. A typical example of a series of membrane currents recorded in a SIF cell under voltage-clamp conditions with normal extracellular and pipette solutions (see methods), and the corresponding I/V relation are shown in Fig. 2.4. The cell was held at -60 mV and a series of voltage-clamp steps lasting 40 ms was applied over a range of -170 to +50 mV in 10 mV increments (every 20 mV shown in Fig. 2.4A,B). The hyperpolarizing steps (-170 through -70 mV; Fig. 2.4A) showed no active currents, with only capacitative and linear leakage currents present (Fig. 2.4A,C). In Fig. 2.4A, the capacitative currents appear as transient vertical deflections in the current trace occurring at the beginning and end of the voltage steps, indicated on the lower trace; after the initial voltage step the capacitative current was followed by a prolonged steady state current corresponding to current flow through the cell's input resistance. Depolarizing steps over the range -30 to +50 mV always resulted in currents which displayed both time and voltage dependence, superimposed on the capacitative and leakage currents (Fig. 2.4B). At a step to -30 mV a fast transient inward current carried by Na^+ ions (see below) was elicited. This current always

Figure 2.4A: Response of a one-day-old SIF cell cultured for 5 days to hyperpolarizing voltage steps. The cell was held at -60 mV and then stepped to various hyperpolarized potentials (-170 to -70 mv in 20 mV steps shown). Capacitance and leakage currents were elicited upon these steps, while no active currents were seen.

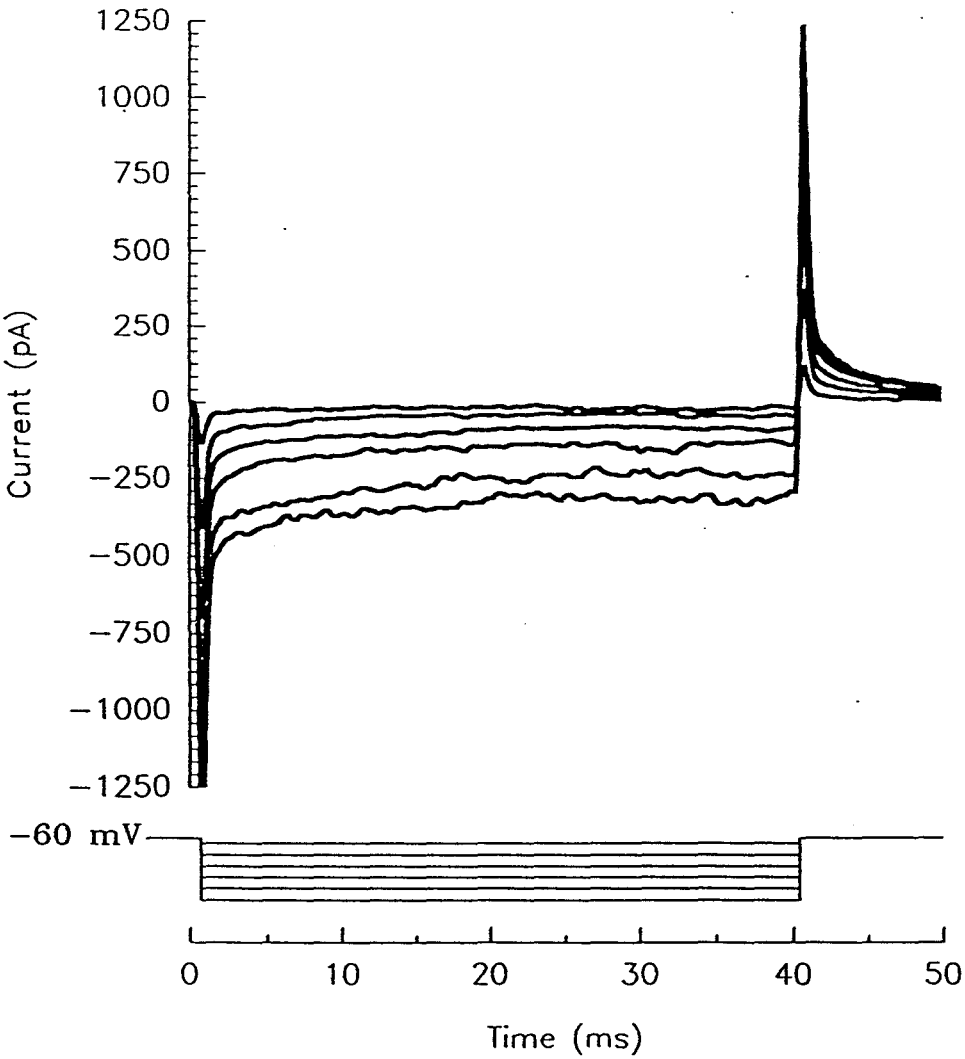

1250
1000
750
500
250
0
−250
−500
−750
−1000
−1250
Current (pA)
−60 mV
0
10
20
30
40
50
Time (ms)

Figure 2.4B: Response of a one-day-old SIF cell cultured for 5 days to depolarizing voltage steps. The cell was held at -60 mV and then stepped to various depolarized potentials (-10 to +50 mV in 20 mV steps shown). Capacitance and leakage currents were elicited as in the hyperpolarizing steps. As well, active currents were also evoked, consisting of a fast inward transient current seen upon depolarization to $\geq$ -30 mV, which was followed by a noticeable sustained outward current at depolarizations of $\geq$ -20 mV.

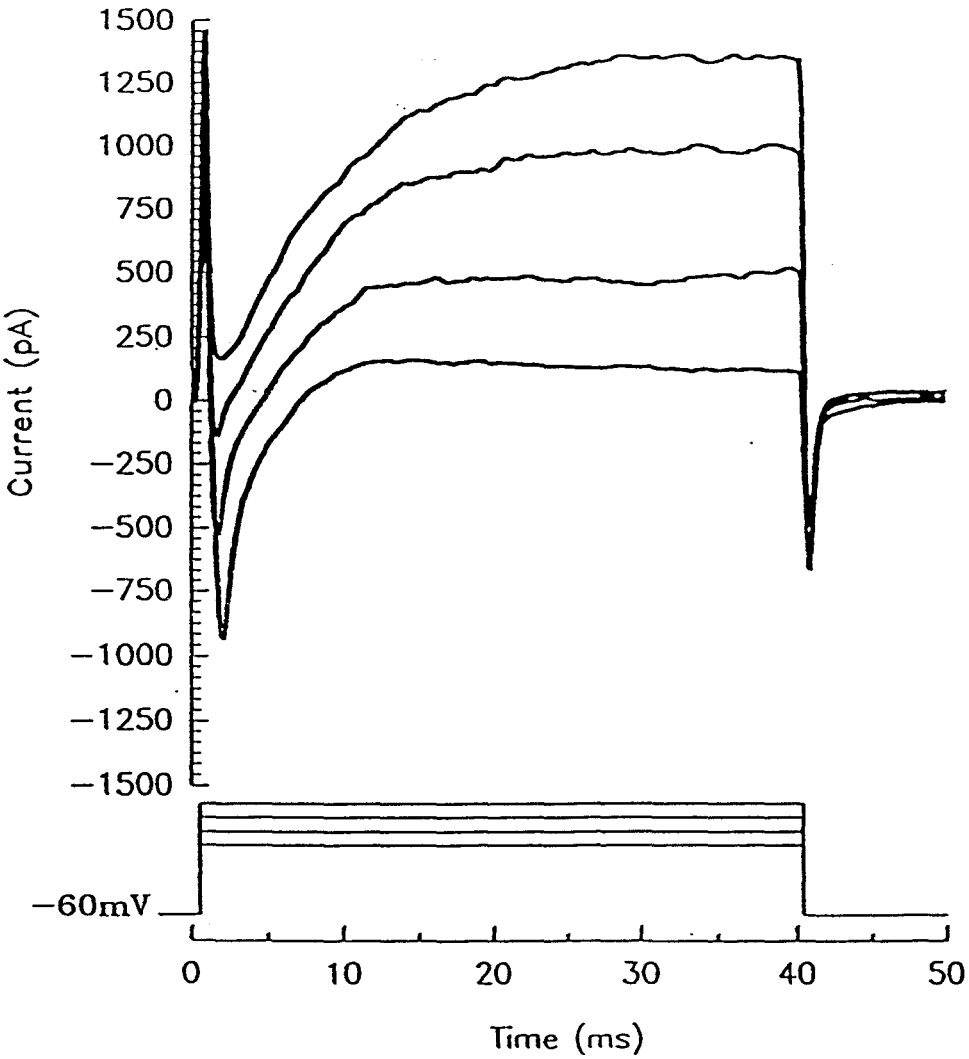
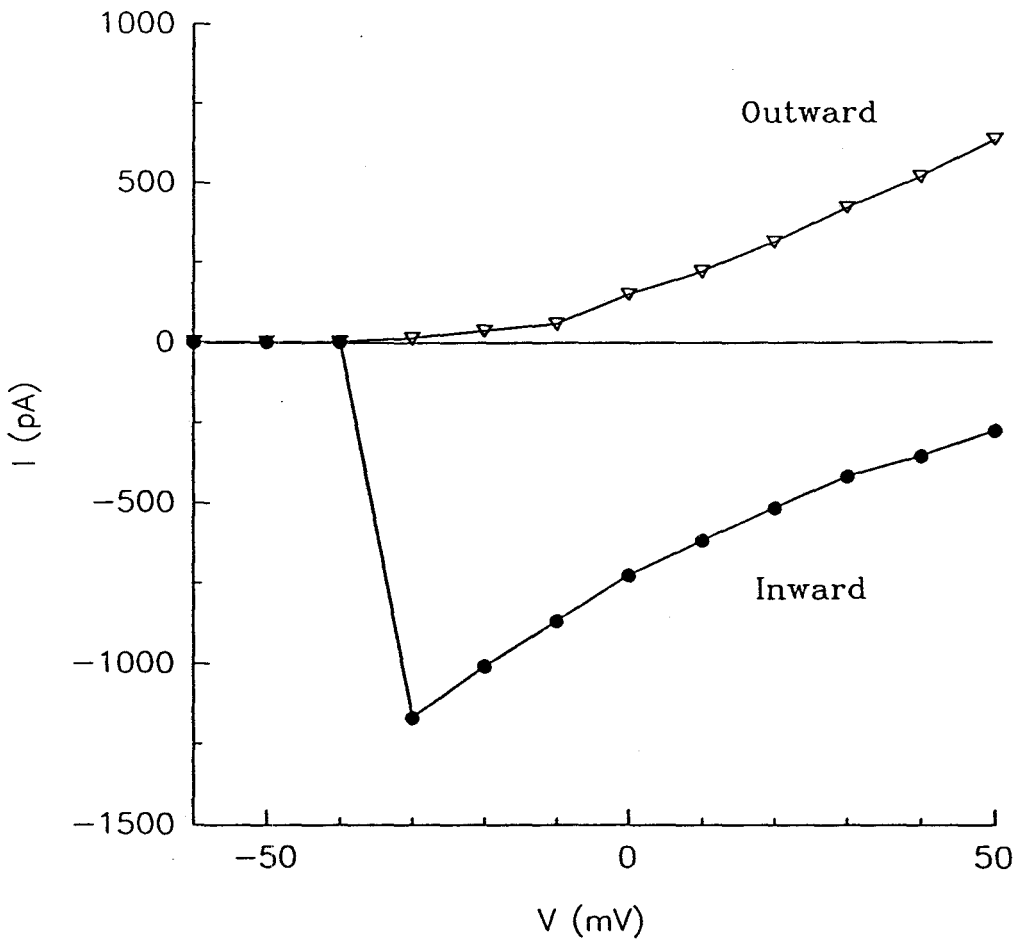

1500
1250
1000
750
500
250
0
−250
−500
−750
−1000
−1250
−1500
Current (pA)
−60mV
0
10
20
30
40
50
Time (ms)

Figure 2.4C: I/V relationship for the cell shown in figure 3A and B. Cell was held at -60 mV and stepped to test potentials from -170 to +50 mV in 10 mV steps. Capacitance and leakage currents were subtracted, by the method described in the text, to show only the voltage-activated currents. Inward current points represent the peak inward currents for each step while outward current was measured just before the end of the voltage step and return to the holding potential. Inward current was first activated over a short voltage range between -40 and -30 mV with each subsequent step producing a smaller peak current, while outward current slowly activated at $\geq$ -30 mV and increased in magnitude with each step $>$ -30 mV.


1000
500
0
−500
−1000
−1500
I (pA)
Outward
Inward
−50
0
50
V (mV)

inactivated completely. With more positive test potentials, a briefer duration of the total inward current occurred (Fig. 2.4B). Depolarizations to -20 mV, or more positive, produced the inward Na^+ current followed by a prolonged outward current carried mainly by K^+ ions (see below). In most cases the outward current reached a plateau and maintained this level over tens of milliseconds, though as discussed later, a component of this outward K^+ current (I_A) was present under certain conditions and showed rapid inactivation. In addition, following blockade of the K^+ currents, Ca^{2+} currents were revealed in SIF cells and these also had both inactivating and sustained components. Evidence for each of these different types of voltage-activated currents in SIF cells is presented below.

Inward Na^+ Currents

As seen in figure 2.4C, following a voltage step from a holding potential of -60 mV, the peak of the early transient inward current occurred at a depolarization between -30 and -40 mV. The transient inward current could sometimes be evoked at depolarizations as low as -50 mV, but usually was first seen at a test potential of $\approx$-40 mV. In order to determine the magnitude of the transient inward current, capacitative and leakage currents were subtracted from the total membrane current elicited during depolarizing steps. As mentioned in the previous section, hyperpolarizing steps produced no active currents, only capacitative and leakage currents. Since both of these passive currents are symmetrical, subtraction of the current trace elicited during a hyperpolarizing step from that elicited by a depolarizing step of the same size results in

a trace equivalent to the active currents produced by the depolarization. Using this method, the average peak inward current elicited from a holding potential of -60 mV was 1186 ± 75 pA (mean ± S.E.; n=100). Normalizing this value to unit capacitance to account for variability in cell size or surface area gave a value of 119 ± 7 pA/pF (mean ± S.E.; n=100).

As indicated in Fig. 2.4B the fast inward current was transient, and inactivated within a few msec. This is reminiscent of voltage-gated Na^+ currents in electrically-excitable cells (Hodgkin and Huxley, 1952b), where the proportion of channels present in the conducting state varies with the level of the holding or steady state potential. To test whether the transient inward current in SIF cells behaved similarly, the steady state inactivation curve was generated from data on the peak inward currents elicited during steps from different holding potentials (V_m). In Fig. 2.5A, the degree of inactivation of the inward current 'h' is plotted against the steady state holding potential V_m; a value of h=1 implies that the maximum number of Na^+ channels available is in the open, conducting state, whereas a value of h=0 implies that the Na^+ channels are completely inactivated, and therefore non-conducting. The smooth curve in Fig. 2.5A was fitted to the data from 7 cells according to a Boltzmann function of the form:

$$h = \frac{I_{Na}(V_m)}{I_{Namax}} = \frac{1}{\exp\frac{(V_m + V_{1/2})}{S_{1/2}} + 1}$$

Figure 2.5A: Inactivation curve for fast transient inward Na^+ current. Data points are mean $\pm$ S.E. from 7 cells collected by stepping from various holding potentials (-100 to -20 in 10 mV steps, and 0 mV) and measuring maximum I_{Na} upon depolarization (10 mV steps up to +50 mV). Error bars are present for all points, but are blocked from view by the filled circles in some cases. The smooth curve was fitted to the data using a modification of the Boltzmann equation (see text for equation and explanation of symbols). The curve had a half-inactivation of -55.0 mV.

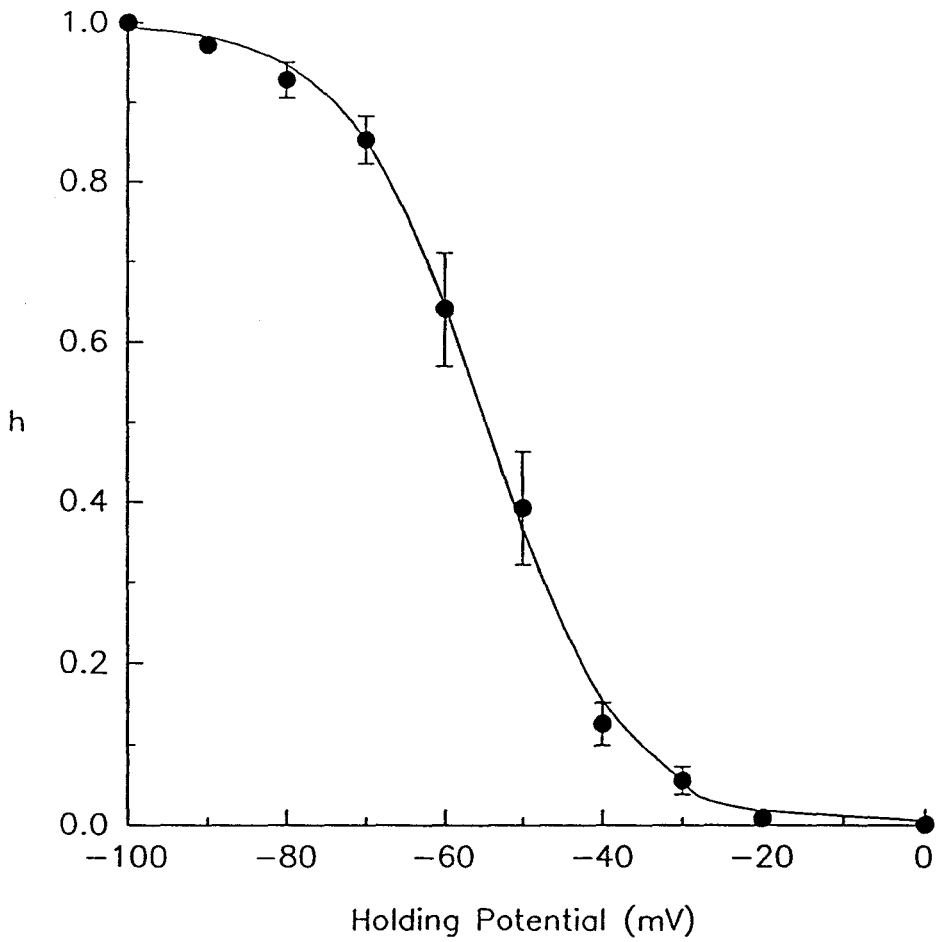

1.0
0.8
0.6
0.4
0.2
0.0
h
−100
−80
−60
−40
−20
0
Holding Potential (mV)

Figure 2.5B: Effect of replacement of external Na^+ with choline on the fast inward transient current in SIF cells. The cell was held at -60 mV and stepped to -20 mV. Inward current was almost completely abolished, with the residual inward current probably a result of activation of a small amount of inward Ca^{2+} current (see later).

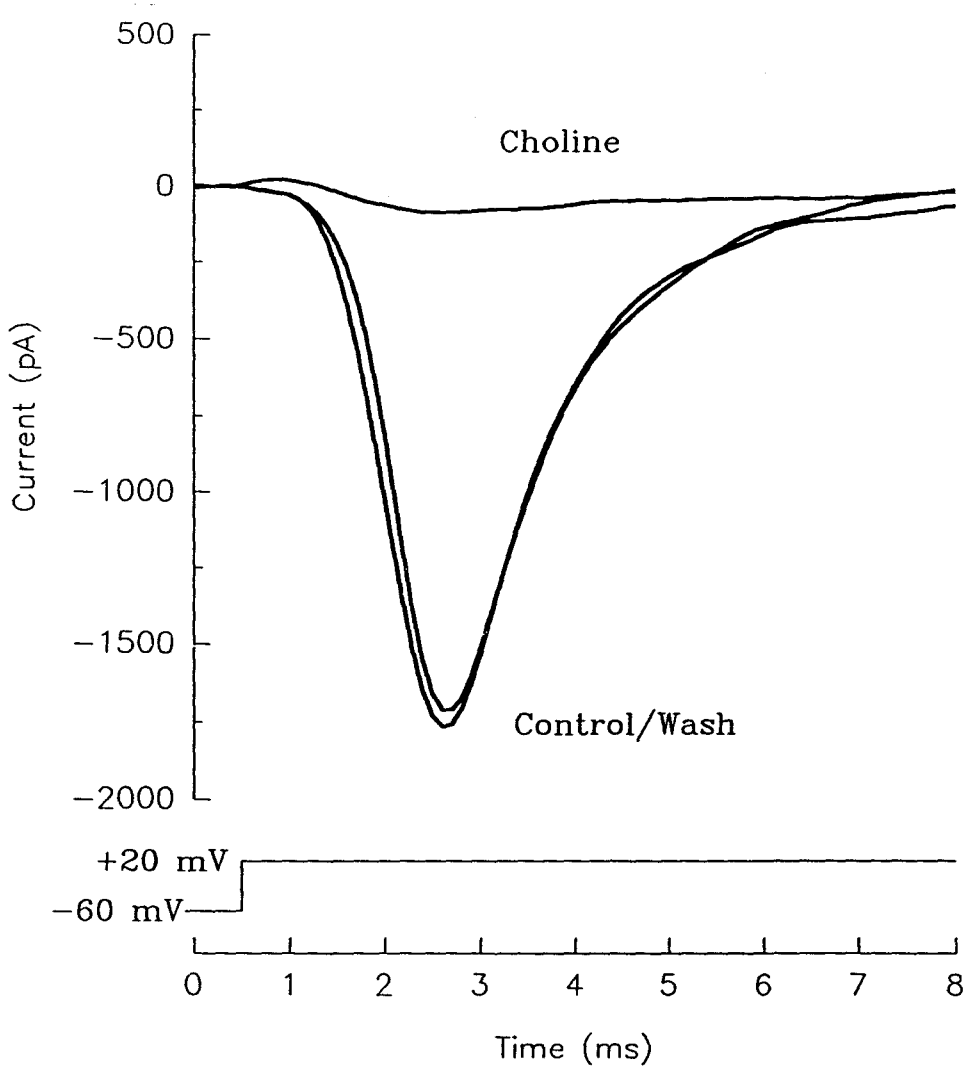
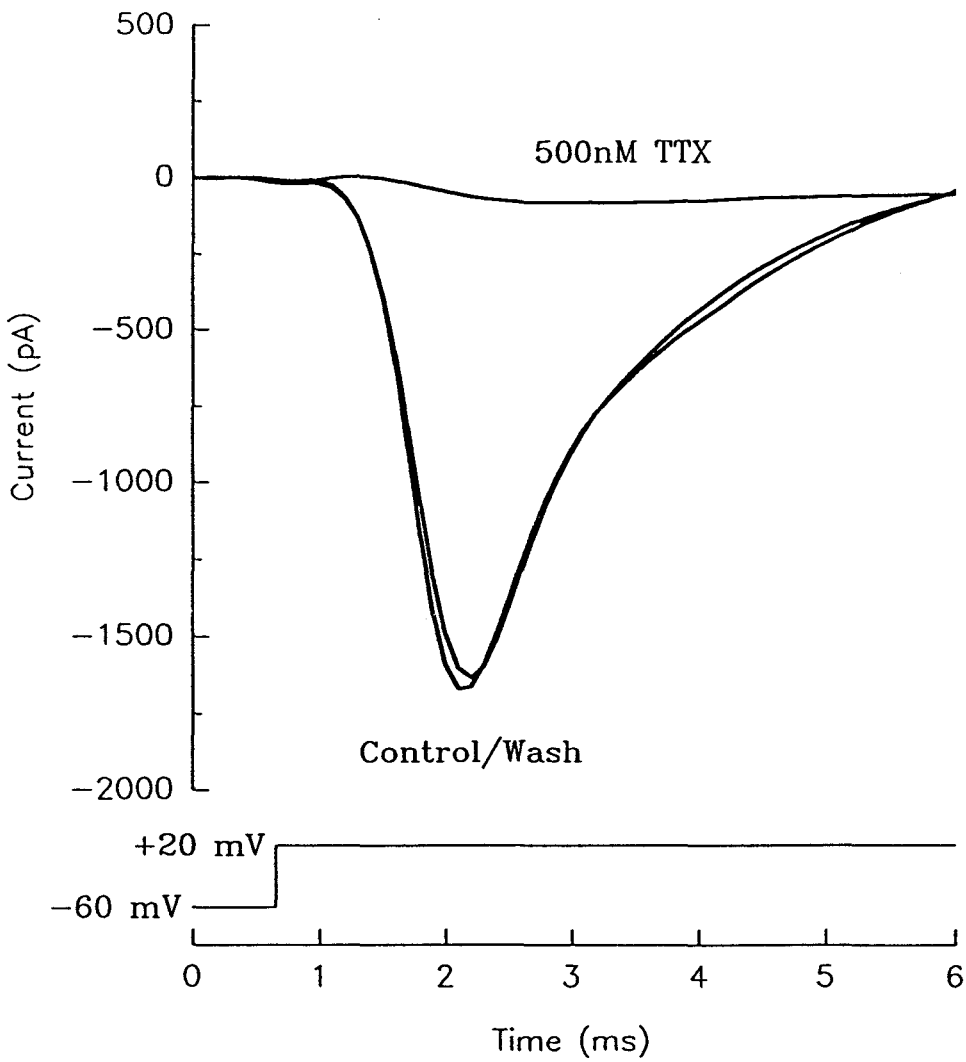

500
0
−500
−1000
−1500
−2000
Current (pA)
Choline
Control/Wash
+20 mV
−60 mV
0
1
2
3
4
5
6
7
8
Time (ms)

Figure 2.5C: Effect of addition of 500 nM TTX to the bathing solution on the fast inward transient current in SIF cells. The cell was held at -60 mV and stepped to -20 mV. As with Na^+ replacement the inward current was virtually abolished with only a small amount of Ca^{2+} current remaining.


500
0
−500
−1000
−1500
−2000
Current (pA)
500nM TTX
Control/Wash
+20 mV
−60 mV
0
1
2
3
4
5
6
Time (ms)

where $I_{Na}(V_m)$ was peak I_{Na} at holding potential of V_m, $I_{Na,max}$ was the maximum I_{Na} of all holding potentials, $V_{1/2}$ was the potential at which I_{Na} was half-maximum, and $S_{1/2}$ was a slope factor at $V_{1/2}$. The data in Fig. 2.5A are well described with the Boltzmann function and in these 7 experiments the half-maximal current occurred at -55 +/- 0.8 mV, (mean +/- S.E.). These characteristics are typical of voltage-gated transient Na^+-currents in other neural crest (sympathoadrenal) cells. Direct evidence that Na^+ ions were largely responsible for the fast transient inward current was obtained by using Na^+-free solutions or pharmacological blockade of voltage-gated Na^+ channels with 500nM tetrodotoxin (TTX). In 15 experiments (e.g. Fig 2.5B), choline substituted, Na^+-free external solution (see Methods) reversibly abolished virtually all of the transient inward current. Further, in nine experiments, addition of 500nM TTX to the bathing solution rapidly and reversibly abolished this current (Fig. 2.5C). Any residual inward current was presumably caused by Ca^{2+} (see later). The outward current was not noticeably affected in either of these treatments (not shown).

Prolonged Outward K^+ Currents

Depolarizations to -20 mV from a holding potential of -60mV evoked a sustained outward current (Fig. 2.4B) that increased with further depolarization. In most cells the current reached a plateau at approximately 15 to 20 ms after the voltage step and did not inactivate over at least a 50 ms test pulse (see later, however). The average peak outward current (step from -60 to +50 mV) was 1158 $\pm$ 60 pA (mean $\pm$ S.E.; n=89). Normalizing this value to unit capacitance to correct for variability in size or

surface area gave an average of 118 ± 55 pA/pF (mean ± S.E.; n=84).

Evidence that K^+ ions were responsible for the outward currents in SIF cells was obtained in a variety of ways. First, substitution of K^+ by Cs^+ and TEA in the pipette and by Na^+ externally (see methods) abolished all outward currents (n=11; not shown). This procedure did not affect the fast transient inward Na^+-current and unmasked a residual prolonged inward Ca^{2+}-current that will be discussed below. Second, the use of known K^+-channel blockers in the external solution confirmed that the outward currents were due largely to K^+ ions. For example, in Figure 2.6A, 10 mM tetraethylammonium (TEA), which blocks a variety of K^+ channels (Lorente de No, 1949; Smart, 1987; Hagiwara, Kusano and Saito, 1961), reversibly eliminated most of the outward current. The reduction in K^+ current was evident at all test potentials above -20 mV as indicated in the I/V curve for this experiment (Fig. 2.6B). The average reduction in outward current by 10mM TEA was 75.9 ± 4.6% (mean ± S.E.; n=14), for steps to a test potential of +50 mV from a holding potential of -60 mV (see Figure 5A,B). In addition, bath application of 10 mM 4-aminopyridine (4-AP) which blocks various K^+ channels (Thompson, 1977) also reduced the outward current (Fig. 2.6C), but it was not as effective as 10 mM TEA. In the case of 4-AP, voltage steps from -60 to +50 mV reduced the outward current by about 48.5 ± 4.5% (mean ± S.E.; n=13). The smaller effect of 4-AP is explained by the fact that it blocks fewer K^+ channels types than TEA, and one that it doesn't block effectively, the Ca^{2+}-activated K^+ current, composes a large percentage of the outward current in these SIF cells (see below).

Figure 2.6A: Effect of addition of 10 mM TEA to the bathing solution on the sustained outward current in SIF cells. The cell was held at -60 mV and stepped to +50 mV. Outward current was reduced on average by 75.9 $\pm$ 4.6% (mean $\pm$ S.E., n=14), and by about 75% in this cell.

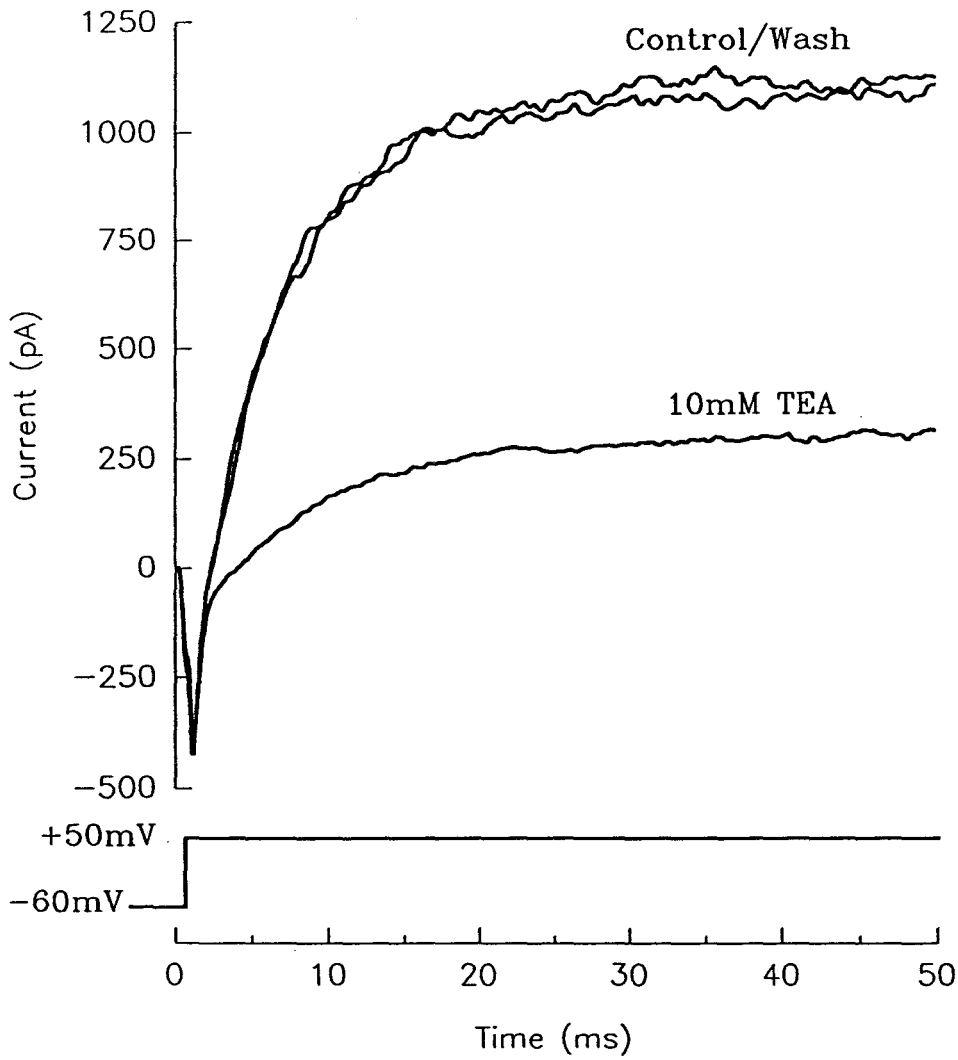

1250
1000
750
500
250
0
−250
−500
Current (pA)
Control/Wash
10mM TEA
+50mV
−60mV
0
10
20
30
40
50
Time (ms)

Figure 2.6B: I/V relationship for the experiment shown in Fig. 2.6A. Current was measured just prior to the end of the test pulses. Outward current was reduced at all test potentials with activation requiring an additional depolarization of approximately 30 mV beyond that to activate it in the absence of TEA.

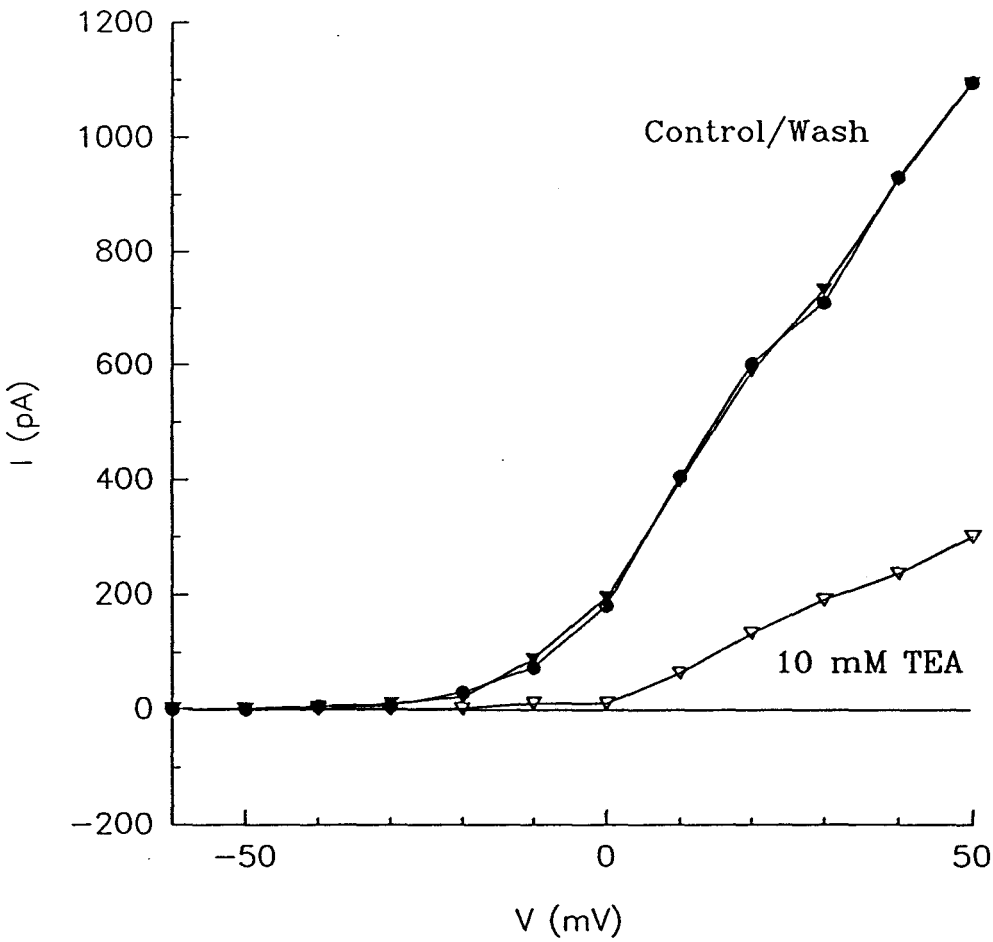

1200
1000
800
600
400
200
0
-200
I (pA)
-50
0
50
V (mV)
Control/Wash
10 mM TEA

Figure 2.6C: Effect of addition of 10 mM 4-AP to the bathing solution on the sustained outward current in SIF cells. The cell was held at -60 mV and stepped to +50 mV. Outward current was reduced on average by 48.5 $\pm$ 4.5% (mean $\pm$ S.E., n=13), and by about 55% in this cell.

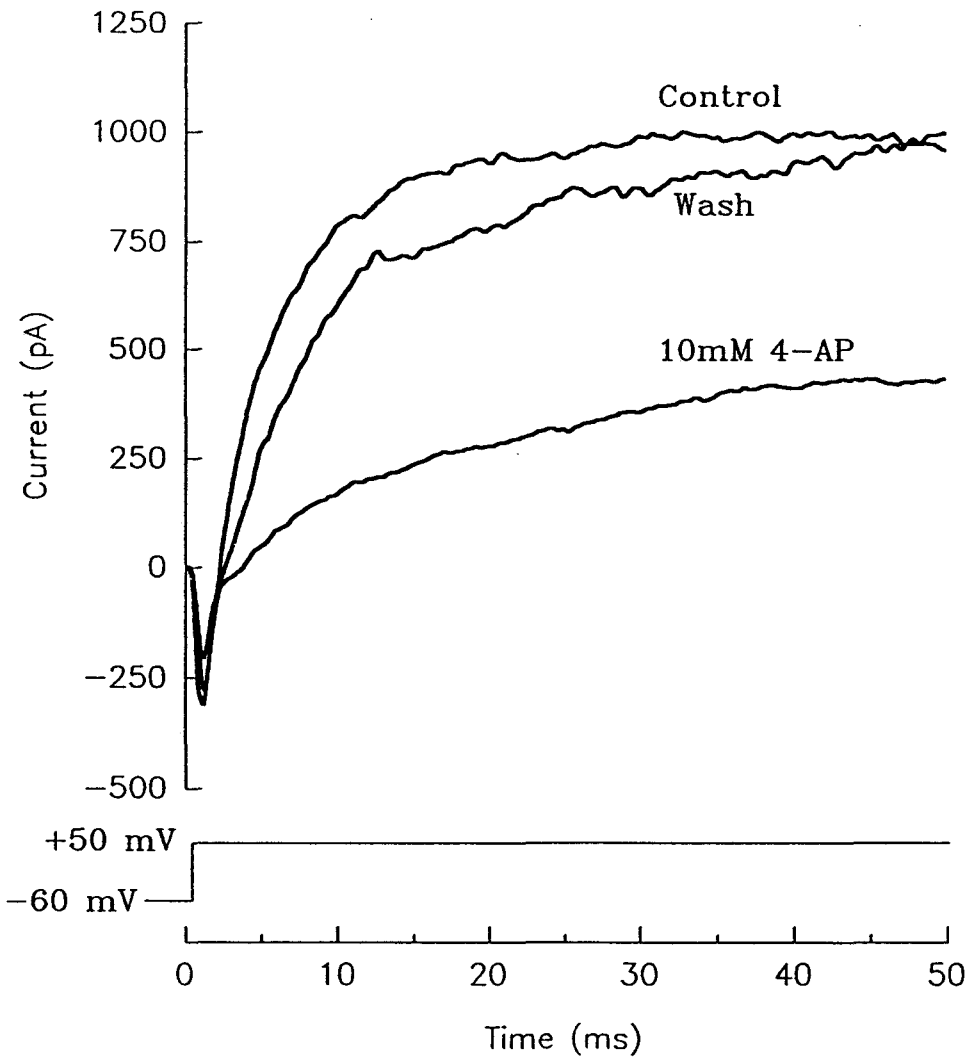
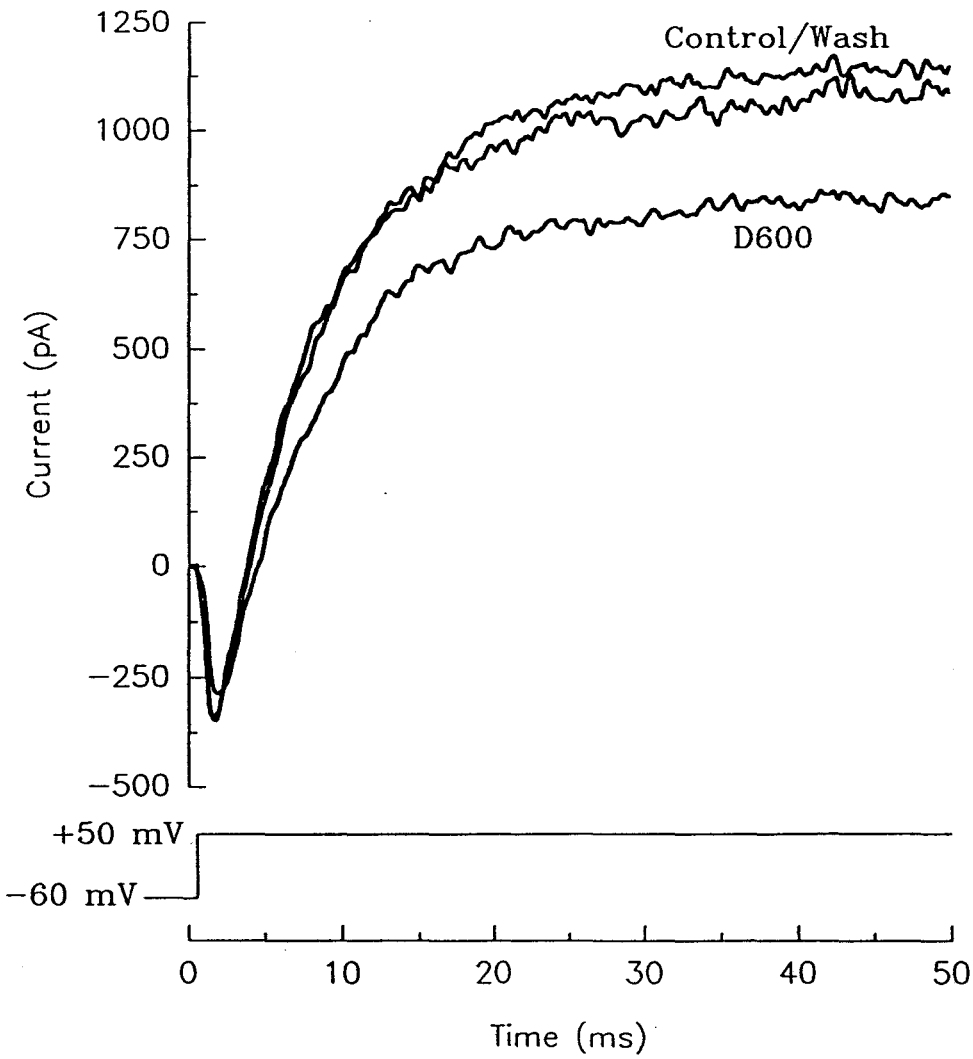

1250
1000
750
500
250
0
−250
−500
Current (pA)
Control
Wash
10mM 4−AP
+50 mV
−60 mV
0
10
20
30
40
50
Time (ms)

Figure 2.6D: Effect of addition of 50 μM D600 to the bathing solution on the sustained outward current in SIF cells. The cell was held at -60 mV and stepped to +50 mV. Outward current was reduced on average by 22.1 $\pm$ 4.7% (mean $\pm$ S.E., n=8), and by about 25% in this cell. This treatment also decreased the inward current peak as well, presumably via block of inward Ca^{2+} current (see later).


1250
1000
750
500
250
0
−250
−500
Current (pA)
Control/Wash
D600
+50 mV
−60 mV
0
10
20
30
40
50
Time (ms)

The outward currents in SIF cells appeared to contain a significant Ca^{2+}-activated K^+ component. This was revealed following blockade of inward Ca^{2+}-entry with the calcium channel blockers Co^{2+} or D600. The outward current was reduced at all test potentials above -20 mV, in the presence of either Ca^{2+} channel blocker. For example, the organic Ca^{2+}-channel blocker D600 (50μM) reduced the peak outward current by 22.1 $\pm$ 4.7% (mean $\pm$ S.E.; n=8) when the voltage was stepped from -60 to +50 mV; an example is shown in Fig 2.6D. In addition, Co^{2+} (2mM) had a more pronounced effect, reducing the peak outward current by 47.5 $\pm$ 7.6% (mean $\pm$ S.E.; n=10) for a comparable step (not shown).

Transient Outward K^+ Currents (I_A)

The possible presence of a fast inactivating K^+ current (I_A) in SIF cells was tested by stepping from more hyperpolarized potentials, between -80 and -100mV (Connor and Stevens, 1971a,b). As with the classic Na^+ channel, while a number of I_A channels are activated and available to open upon depolarization from a normal resting potential, hyperpolarized potentials are required for complete activation of the I_A channel population. The I_A current has been described in other cells of the sympathoadrenal lineage including the postganglionic sympathetic neurons from the same superior cervical ganglion (Galvan, 1982; Galvan and Sedlmeir, 1984; Belluzzi et. al., 1985a), and is thought to play an important role in the modulation of action potential frequency (Connor and Stevens, 1971c). In SIF cells obtained from one-day-old rats and cultured for several days, a transient I_A current was usually absent. This is indicated in Fig. 2.7A

Figure 2.7A: Absence of a fast inactivating outward I_A current in one-day-old SIF cells. This cell was in culture for 7 days. Upon holding the cell at -80 mV and stepping to the test potentials shown in the figure only a slowly activating sustained outward current was evoked by this protocol.

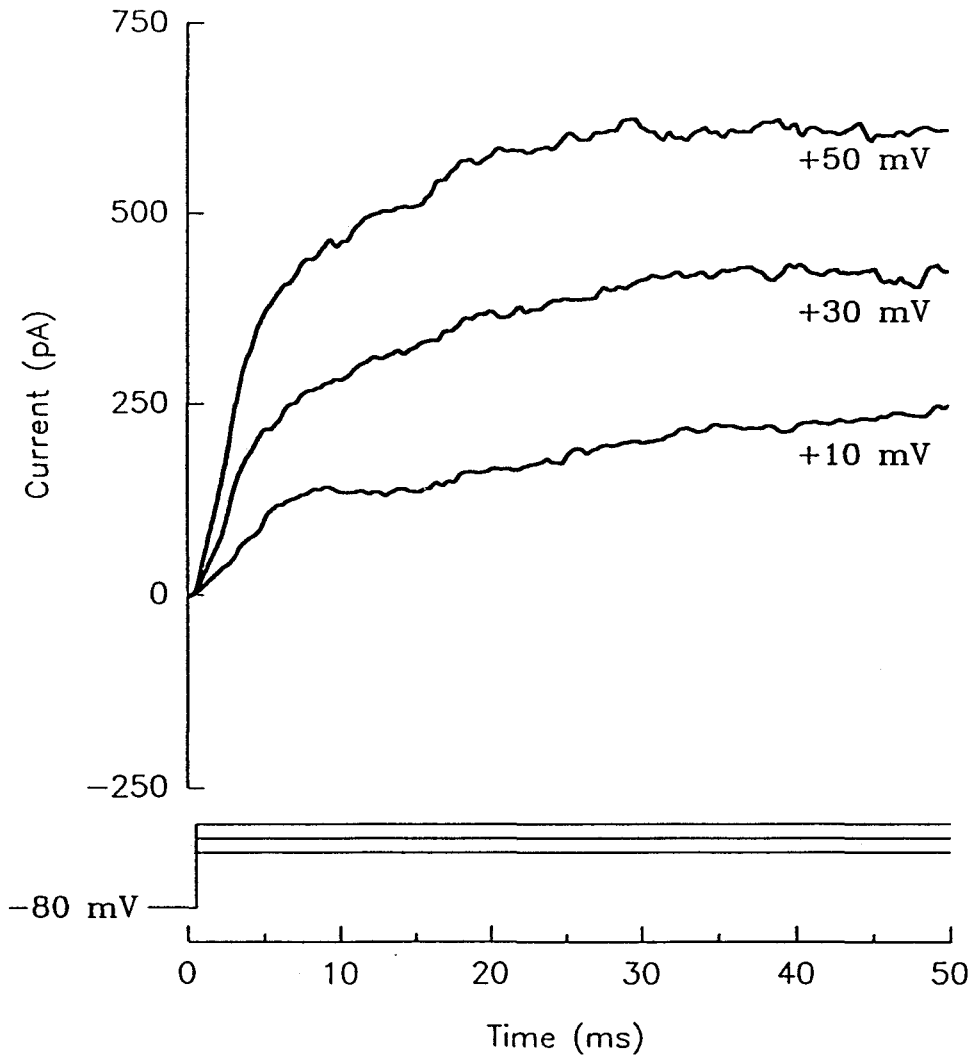

750
500
250
0
−250
Current (pA)
+50 mV
+30 mV
+10 mV
−80 mV
0
10
20
30
40
50
Time (ms)

Figure 2.7B: Presence of a fast inactivating outward I_A current in five-day-old SIF cells. This cell was in culture for 6 days. Upon holding the cell at -80 mV and stepping to the test potentials shown in the figure the outward current activated more quickly than in one-day-old cells (cf Fig. 2.8A) and contained a large quickly inactivating component.

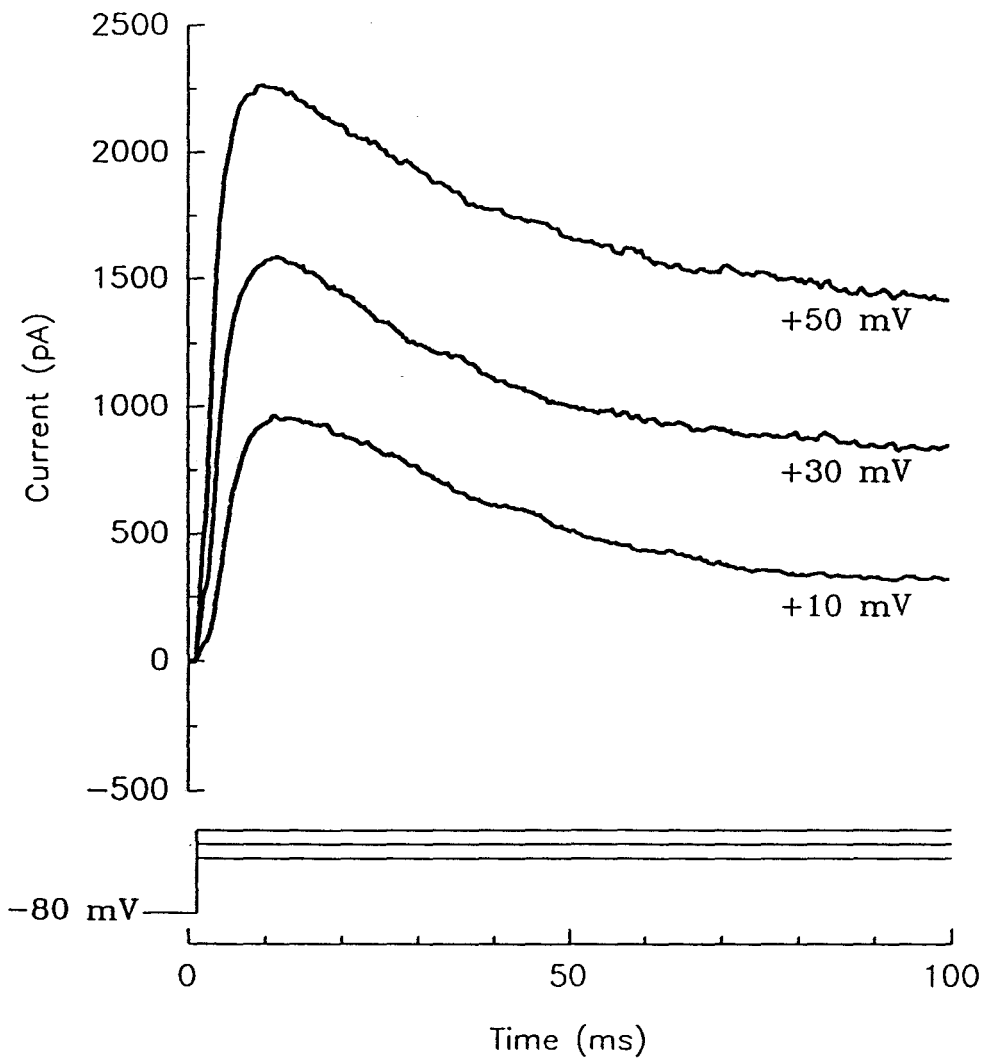

2500
2000
1500
1000
500
0
−500
Current (pA)
+50 mV
+30 mV
+10 mV
−80 mV
0
50
100
Time (ms)

Figure 2.7C: Effect of addition of 10 mM 4-AP to the bathing solution on the outward currents evoked from a holding potential of -80 mV in SIF cells possessing a fast I_A current. This cell was from a five-day-old rat pup in culture for 7 days and was stepped from a holding potential of -80 mV to a test potential of +20 mV. The 4-AP blocked the transient portion of the outward current while leaving the sustained component. This response was seen in 4 of 4 cells tested.

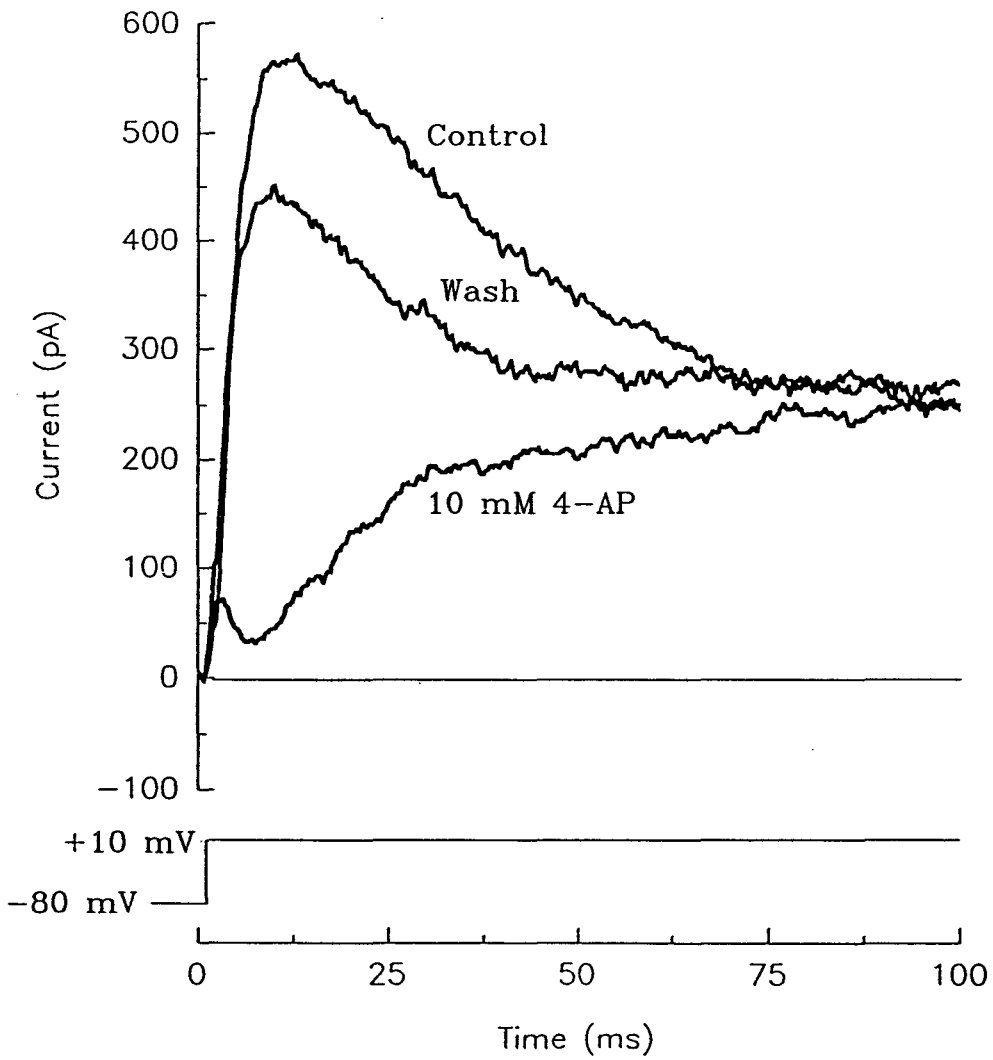
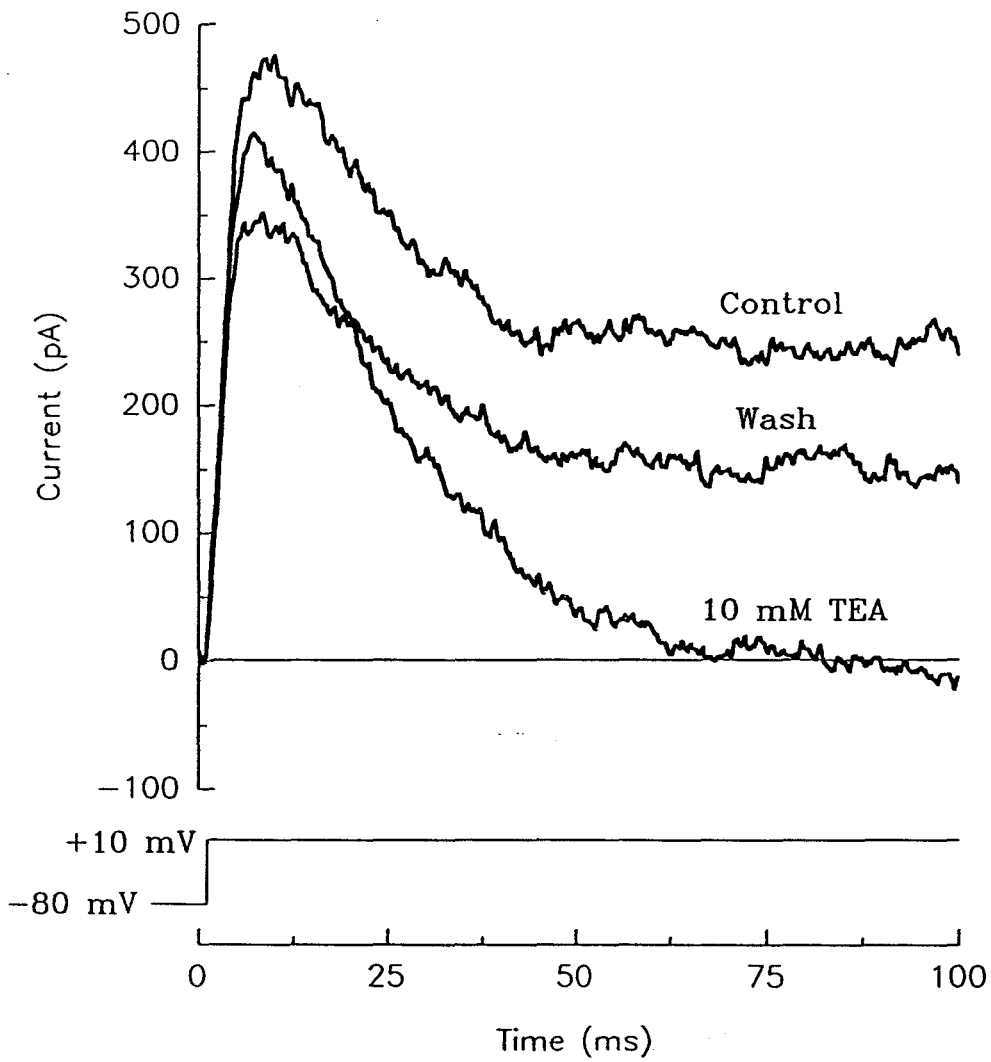

600
500
400
300
200
100
0
−100
Current (pA)
Control
Wash
10 mM 4−AP
+10 mV
−80 mV
0
25
50
75
100
Time (ms)

Figure 2.7D: Effect of addition of 10 mM TEA to the bathing solution on the outward currents evoked from a holding potential of -80 mV in SIF cells possessing a fast I_A current. This cell was from a five-day-old rat pup in culture for 7 days and was stepped from a holding potential of -80 mV to a test potential of +20 mV. The TEA reduced the peak of the transient portion of the trace only slightly, but the peak was reduced further with washing out of the TEA. This is probably a result of deterioration of the preparation resulting in loss of the transient peak with time as opposed to treatment. The sustained component was completely eliminated leaving a small inward current presumably due to Ca^{2+} influx (cf Fig. 2.7A, B and D).


500
400
300
200
100
0
−100
Current (pA)
Control
Wash
10 mM TEA
+10 mV
−80 mV
0
25
50
75
100
Time (ms)

where steps from more hyperpolarized holding potentials (e.g. -80 mV) produced only sustained outward currents that did not inactivate at least over 50-100 msec. In only one out of 40 such cases was a rapid transient component in the outward current (I_A) detected (see below). This may indicate that a fast I_A-current is not normally present in SIF cells at one day postnatal age, or that this property is lost in culture. To test for the possibility that the I_A-current develops with postnatal age, and is retained in culture, SIF cells from three-day-old, five-day-old, and seven-day-old animals were cultured for several days. Interestingly, in 3 of 6 cases SIF cells from 3 day-old animals contained a prominent fast I_A-current after 5-10 days of culture. Further, in 10 of 10 cases for 5 day-old pups, and 6 of 6 cases for 7 day-old pups, SIF cells contained a fast I_A after a similar period in culture. An example of the fast I_A current in a SIF cell is shown in Fig. 2.7B where, in contrast to Fig. 2.7A, a rapid inactivating component in outward current is evident as early as 20 msec after voltage steps from a large negative holding potential (-80 mV) to more positive potentials. To confirm the presence of an I_A current additional pharmacological tests were used. In 4 out of 4 cases, when the perfusate contained 4-AP (10 mM) which is known to block the I_A in other preparations (Thompson, 1977; Galvan, 1982; Rogawski, 1985), the transient portion of the outward current was reversibly abolished, while the sustained component remained virtually unaffected (Fig. 2.7C). In contrast, application of TEA (10 mM) in 2 of 2 cases hardly affected the transient outward K^+ current I_A, whereas the sustained component of K^+ current was substantially reduced (Fig. 2.7D). At this concentration of TEA (10 mM)

the delayed rectifier K^+ current should be virtually completely blocked with the Ca^{2+}-activated K^+ current highly suppressed (see Fig 2.6A above); as well, the I_A current has inactivated by the end of the 100 ms test pulse. The negative, inward current seen at 75 msec is presumably due to an inward Ca^{2+} current, which will be described in the next section, masking any outward Ca^{2+}-activated K^+ current that remained after TEA blockade. When SIF cells from one-day-old postnatal pups were cultured for longer periods, such that the total postnatal age of the cells (age of animal plus days in culture) was approximately equivalent for the 3 groups, the fast I_A was again not detected in 5 of 5 cases. Taken together these data suggest that the fast transient I_A current develops rapidly in SIF cells *in vivo* during the first week of postnatal life. As has been shown in other preparations (*Aplysia*: Connor and Stevens, 1971b), the development of this current may help modulate the frequency of multiple spiking shown to be present in SIF cells of bullfrog sympathetic ganglia (Dunn and Marshall, 1985), and of rat superior cervical ganglia (Fig. 2.3; this chapter).

Inward Ca^{2+} Currents

Following blockade of all outward currents in K^+-free solutions (see methods) a sustained inward current was unmasked. Simultaneous elimination of Na^+ currents by choline substitution and/or application of TTX allowed this sustained inward Ca^{2+}-current to be studied in isolation (Fig. 2.8A). Since conventional whole-cell recording resulted in a rapid run-down of these Ca^{2+}-currents, the perforated-patch technique was also used since it preserves cytoplasmic integrity and permits long-term stable recordings of Ca^{2+}-

Figure 2.8A: Ca^{2+} currents in SIF cells. Na^{+} and K^{+} were eliminated from the intracellular and extracellular solutions to view Ca^{2+} currents in isolation. This cell was isolated from a one-day-old rat pup and cultured for 5 days before being stepped from a holding potential of -60 mV to the test potentials shown. Upon a step to -10 mV only a sustained component of Ca^{2+} current was evoked. At 0 mV a small amount of inactivating Ca^{2+} current was also seen. The sudden change in current level seen in this trace at 40-50 mV is discussed in the text. Upon depolarization to +10 mV the trace showed a large transient component. The average peak Ca^{2+} current, with 10 mM external Ca^{2+}, was 127 $\pm$ 17 pA (mean $\pm$ S.E.) when stepping to +10 mV from -60 mV.

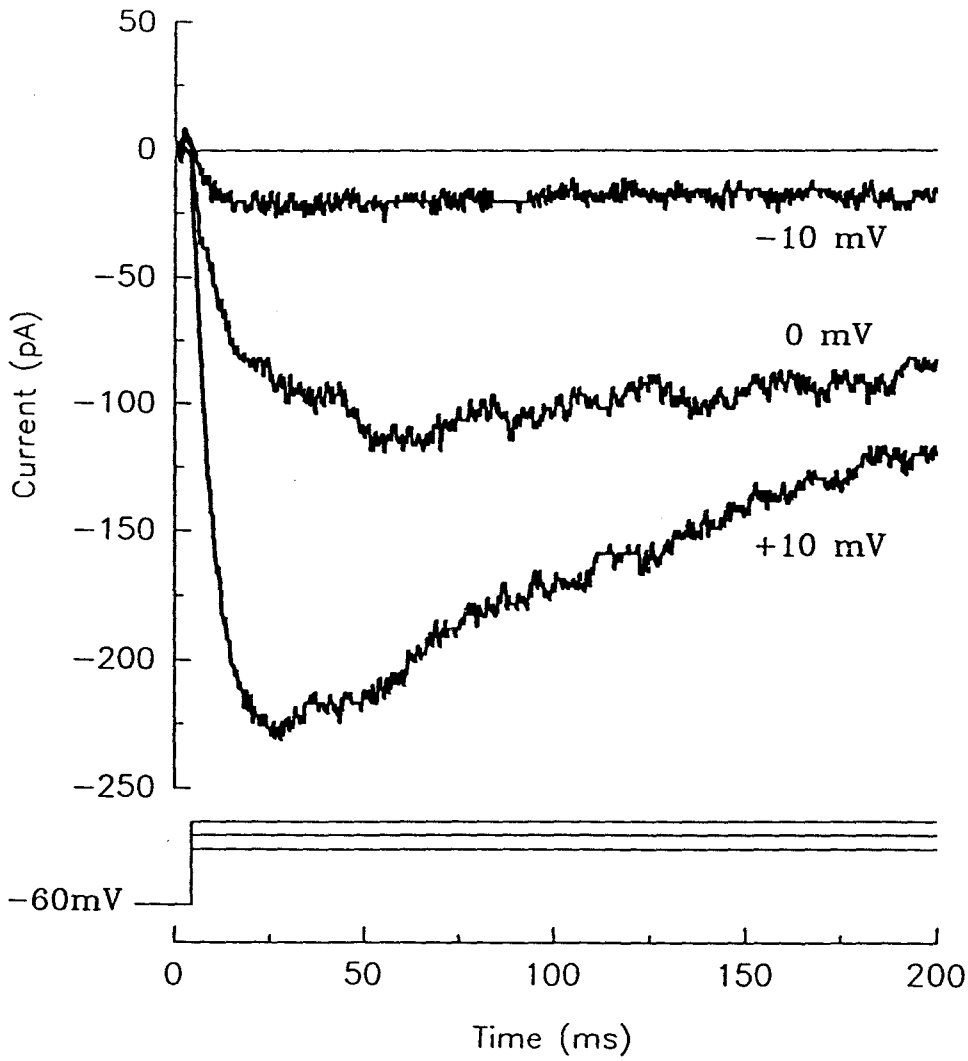

50
0
−50
−100
−150
−200
−250
Current (pA)
−10 mV
0 mV
+10 mV
−60mV
0
50
100
150
200
Time (ms)

Figure 2.8B: Dependence of Ca^{2+} currents in SIF cells on holding potential. This cell was isolated from a one-day-old rat pup and cultured for 6 days before it was stepped from the holding potentials shown to the test potential of +10 mV in Na^+/K^+-free solutions. The transient component was completely inactivated between a holding potential of -40 and -20 mV, while the sustained component required a holding potential of between 0 and 20 mV for virtually complete inactivation. A similar result was seen in 5 cells.

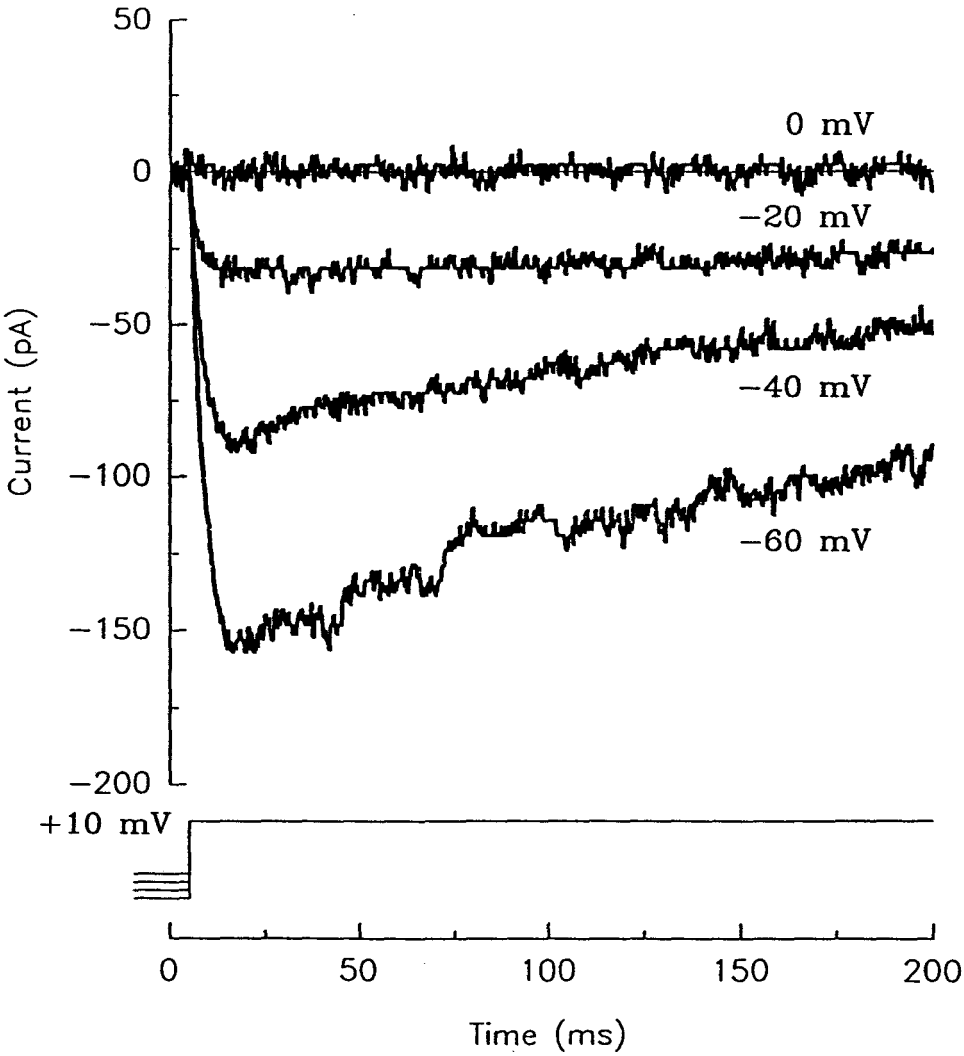

50
0
−50
−100
−150
−200
Current (pA)
0 mV
−20 mV
−40 mV
−60 mV
+10 mV
0
50
100
150
200
Time (ms)

Figure 2.8C: Effect of substitution of Ca^{2+} by Co^{2+} on Ca^{2+} currents in SIF cells. This cell was isolated from a one-day-old rat pup and cultured for 6 days before it was stepped from -60 mV to 0 mV. The Ca^{2+} current was reversibly eliminated by substitution of the Ca^{2+} by the Ca^{2+} channel blocker Co^{2+} in Na^{+}/K^{+}-free solution. This result was seen in 5 experiments.

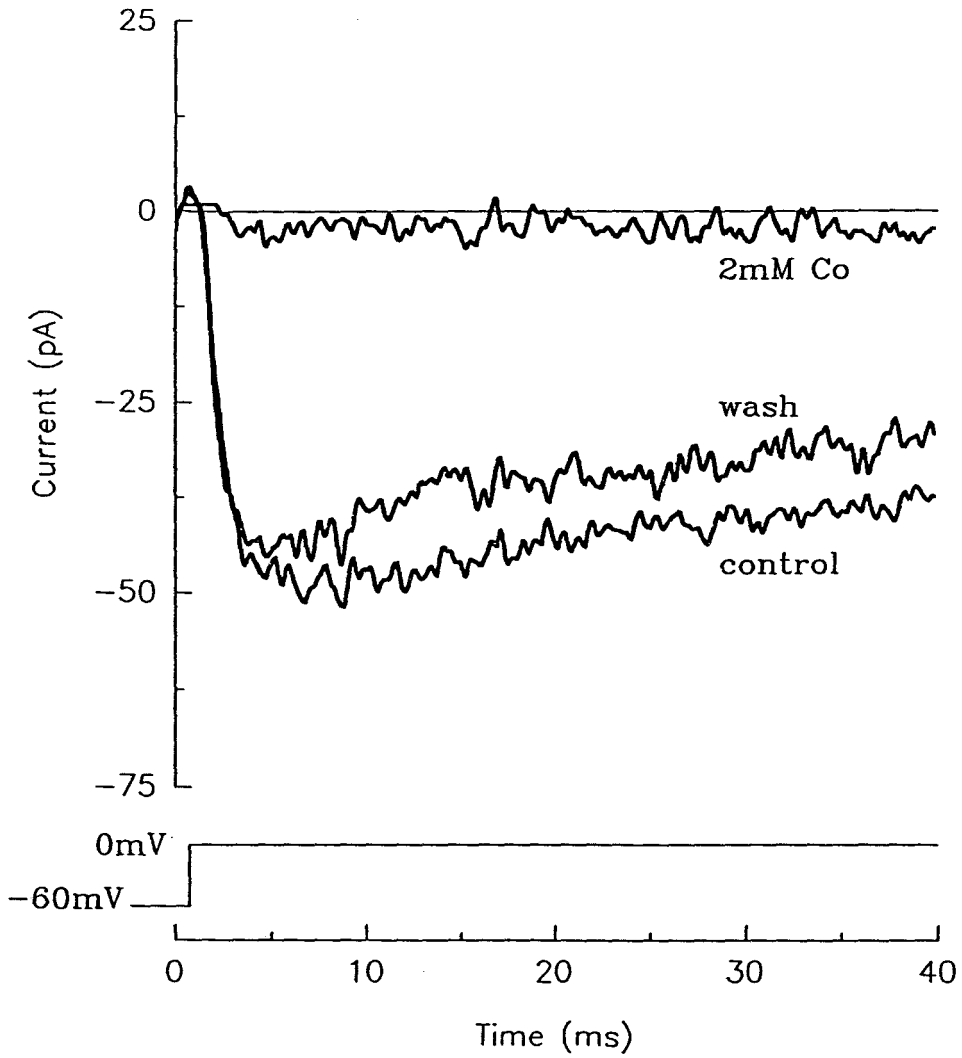
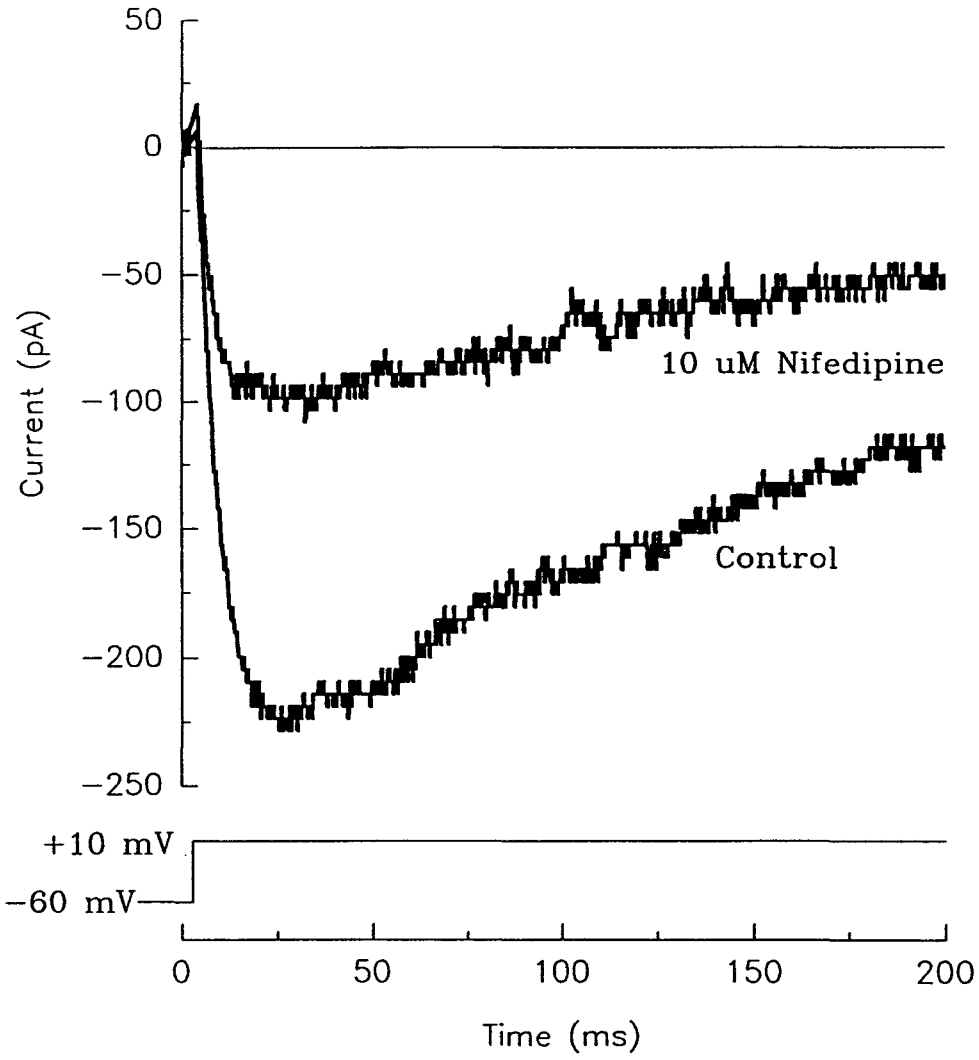

25
0
−25
−50
−75
Current (pA)
2mM Co
wash
control
0mV
−60mV
0
10
20
30
40
Time (ms)

Figure 2.8D: Effect of 10 μM nifedipine on Ca^{2+} currents in SIF cells. This cell was isolated from a one-day-old rat pup and cultured for 6 days before it was held at -60 mV and stepped to a test potential of +10 mV. The nifedipine appeared to eliminate most of the sustained component and part of the transient component as well. This experiment was only performed in 2 cells, and reversibility following wash out of the drug was not demonstrated.


50
0
−50
−100
−150
−200
−250
Current (pA)
10 uM Nifedipine
Control
+10 mV
−60 mV
0
50
100
150
200
Time (ms)

currents (Falke, Gillis, Pressel and Misler, 1989). The Ca^{2+}-currents recorded immediately after break-through using the whole-cell configuration resembled the more stable recordings obtained with the perforated-patch technique. The latter method, however, permitted these Ca^{2+} currents to be analyzed. The peak value of the overall Ca^{2+} current occurred during voltage steps to between 0 to +10 mV from a holding potential of -60 mV (Fig. 2.8A). As indicated in Fig. 2.8A, there appears to be both a transient and sustained component of the Ca^{2+} current, especially evident during the step to +10 mV. The values for the peak Ca^{2+} current were 71.9 $\pm$ 5.1 pA (mean $\pm$ S.E., n=14) for whole cell, vs 65.8 $\pm$ 6.6 pA (mean $\pm$ S.E., n=9) for perforated-patch; 2 mM extracellular Ca^{2+} present in both cases.

As expected, increasing extracellular Ca^{2+} to 10 mM resulted in a larger average peak current of 127 $\pm$ 17 pA (mean $\pm$ S.E., n=31) for the same voltage step (determined with perforated-patch method). In 26 of these 31 cases with 10 mM extracellular Ca^{2+} (eg. Fig. 2.8A) an inactivating component of Ca^{2+}-current appeared to be present with the sustained, non-inactivating component when the cells were stepped from a holding potential of -60 mV. The remaining 5 cases possessed no definite inactivating component. The sudden increase in Ca^{2+} current, between 40 and 50 ms, seen in the step to 0 mV in figure 2.8A, may be due to the activation of a separate channel type, but its origin is presently unclear. It was seen in a number of different traces of Ca^{2+} current, but the time during the voltage step at which it occurred is quite variable. At low test potentials between -10 to -20 mV there appeared to be no obvious

transient component of Ca^{2+} current over a 200 ms test pulse; only a slow sustained component appeared to be present. The inactivating component of Ca^{2+}-current was detected at depolarizations to > 0 mV, and this was consistent with the activation of L-type Ca^{2+} channels for the sustained component, and N-type Ca^{2+} channels for the transient component of Ca^{2+} current (Nowycky, Fox and Tsien, 1985). When the SIF cells were held at more positive potentials a reduction in both the sustained and transient components occurred following steps to the same test potential (Fig. 2.8B). The transient component was absent during steps from a holding potential of -20 mV to a test potential of +10 mV (n=5), while the sustained component was inactivated at holding potentials > 0 mV (n=5). The different dependence on holding potential for the two current components is also consistent with there being both L- and N-type Ca^{2+} channels present (Nowycky, Fox and Tsien, 1985).

In order to confirm that these currents (e.g. Fig. 2.8) were carried via Ca^{2+} channels, the effect of known Ca^{2+} channel blockers was tested. As expected, the addition of the Ca^{2+} channel blocker Co^{2+} to a nominal Ca^{2+}-free bathing solution abolished reversibly both the transient and sustained components of inward current in 5 experiments (e.g. Fig. 2.8C), where Na^{+} contributions were eliminated. In addition, in 2 successful experiments, the dihydropyridine antagonist nifedipine (50 μM), a known blocker of the sustained L-type currents appeared to reduce the sustained component, though a transient component was still detectable (Fig. 2.8D). In these preliminary results with nifedipine reversibility of the response could not be demonstrated before

termination of the experiment; the effects of this drug therefore requires further study.

Taken together, these results suggest two possibilities for the Ca^{2+} channel type, or types present. Nowycky et. al., (1985) described 3 separate Ca^{2+} channel types in chick dorsal root ganglion (DRG) cells; (i) a sustained, high-voltage activated L-type Ca^{2+} channel; (ii) a transient, high-voltage activated N-type Ca^{2+} channel that is completely inactivated at holding potentials of about -20 mV; and (iii) a transient, low-voltage activated T-type Ca^{2+} channel. Plummer et. al., (1989) described a Ca^{2+} current in rat sympathetic neurons that appeared to consist of both N-type and L-type Ca^{2+} currents together, however single channel recording revealed that the current was governed by a single channel that had a transient and a sustained conductance state. It is possible that at least two separate Ca^{2+}-channel types are present in SIF cells, probably the N-type Ca^{2+}-channels (the inactivating component) and L-type Ca^{2+}-channels (the sustained component) according to the classification of Nowycky et. al., (1985). Alternatively, there is only one type of Ca^{2+}-channel present, the N-type Ca^{2+}-channel of Plummer et. al., (1989), that possesses different conductance states with both an inactivating and a sustained component present in one channel. Clear distinction between these two possibilities is best resolved with single channel studies.

Discussion

These studies provide the first complete characterization of the ionic currents present in small intensely fluorescent (SIF) cells from neonatal rat sympathetic ganglia. Since these currents are likely to be modulated by various agents, including nuerotransmitters or neuromodulators released during activity of the autonomic nervous system, their characterization is essential for understanding the physiology of SIF cells in ganglionic transmission or in other possible endocrine functions. The studies were greatly facilitated by the availability of methods for growing isolated SIF cells in culture (Doupe, Patterson and Landis, 1985). This was important since it meant that these small cells (<20 μm diam.), which are difficult to study with conventional intracellular microelectrodes, could be conveniently studied with the high resolution patch-clamp/whole-cell recording technique (Hamill et. al., 1981), which is ideal for studying the electrophysiology of small cells under current or voltage clamp. The small size, combined with the fact that these cells are sparsely distributed contributed to the paucity of information available on the electrophysiology of SIF cells. In fact there was only one previous report on intracellular recording of action potentials in a few SIF cells from bullfrog autonomic ganglia (Dunn and Marshall, 1985).

The superior cervical ganglion (SCG) of neonatal rats was used in this study since it provides an excellent source of SIF cells and the culture techniques for SIF cells were first described for this preparation (Doupe, Patterson and Landis, 1985). These ganglia are the largest and most easily accessible of the sympathetic chain ganglia and

contain hundreds of SIF cells (Björklund, Cegreli, Falck, Ritzén and Rosengren, 1970; Davies, 1978; Eränkö and Soinila, 1981; Smölen, Wright and Cunningham, 1983). Selectivity for SIF cell survival over ganglionic principal neurons was achieved by adding glucocorticoid and omitting nerve growth factor (NGF) from the culture medium (Doupe, Patterson and Landis, 1985). SIF cells in culture have an absolute requirement for corticosteroids in the growth media (e.g. the synthetic analog, dexamethasone) whereas the principal neurons die if NGF is omitted. The ganglia also contains other cell types including support glial cells and endothelial cells from blood vessels which may survive and proliferate in culture. To suppress this proliferation the antimitotic agent cytosine arabinoside (ara-C; 10^{-5} M) was added to the culture medium. Since the majority of SIF cells from neonatal animals are postmitotic, this treatment did not obviously affect SIF cell survival nor their ability to express a variety of voltage-gated ion channels, or characteristic enzyme markers e.g. tyrosine hydroxylase immunoreactivity.

SIF cells from the rat SCG have been shown to exhibit two distinct morphologies (Chiba and Williams, 1975; Williams et. al., 1975; Williams et. al., 1976). Type I SIF cells were solitary and had processes directed towards neighbouring ganglionic neurons, while type II SIF cells were clustered near fenestrated capillaries. Doupe, Patterson and Landis (1985) found that by varying the concentration of the synthetic corticosteroid dexamethasone (DEX) they could select for either type I or type II SIF cells; 10^{-8} M DEX selected for type I cells whereas 10^{-6} M DEX resulted in a bias towards type II SIF cells. Though the concentration of DEX used in this study (10^{-6} M)

might be expected to select for type II SIF cells, the situation was somewhat complicated since a variable number of ganglionic non-neuronal cells was still present in the cultures even after treatment with Ara-C. In contrast Doupe, Patterson and Landis (1985) used relatively pure SIF cell cultures and found that factors in medium conditioned by non-neuronal cells favoured type I SIF morphology even in the presence of high glucocorticoid levels. However, since type I SIF cells bear neurites or short processes, and such cells were excluded from the present electrophysiological recordings (so as to minimize space clamp errors during voltage clamp experiments) it is likely that the majority of SIF cells studied was type II. Clamp errors arise in situations where the membrane potential is non-uniform over the region studied; regional differences in membrane potential produce "extraneous" currents which complicate voltage clamp data. Measurements of the passive properties of SIF cells indicated they had relatively large input resistances (mean $\approx$ 0.92 GΩ) and estimates of their size based on capacitance measurements were similar to that seen under the microscope (ca. 18 μm diameter) in this and previous studies (Doupe, Patterson and Landis, 1985).

Spiking Behaviour in SIF Cells

Dunn and Marshall (1985) recorded a series of action potentials in SIF cells from bullfrog sympathetic ganglia during intracellular recording as a result of ACh application. The closely related sympathetic principal neuron (Belluzzi et. al., 1985a), adrenal medullary chromaffin cell (Fenwick et. al., 1982a), and type I, or glomus cell of the carotid body (Duchen et. al., 1988) also exhibit action potentials during

depolarization. In this thesis, SIF cells from the rat SCG were shown to respond to a steady depolarizing current pulse with multiple spikes, similar to that seen in bullfrog sympathetic ganglion SIF cells (Dunn and Marshall, 1985). This property, i.e. multiple spiking, is known to be controlled in excitable cells by a variety of K^+ channels (Hille, 1984) including the delayed rectifier (I_{DR}) (Hodgkin and Huxley, 1952a), Ca^{2+}-activated K^+ (Meech and Standen, 1975; Eckert and Lux, 1976; Smith, 1978; Gorman and Thomas, 1978; Gorman, Hermann and Thomas, 1981), and the fast transient K^+ (or I_A) (Connor and Stevens, 1971c). The delayed rectifier K^+ current acts simply to keep action potentials short by opening upon depolarization and allowing flow of repolarizing current (Hille, 1984). The Ca^{2+}-activated K^+ current creates long hyperpolarizing pauses between trains of action potentials (Meech and Standen, 1975; Eckert and Lux, 1976; Smith, 1978; Gorman and Thomas, 1978; Gorman et. al., 1981), and is sensitive to both voltage, and Ca^{2+} bound near the vicinity of the channel on the cytoplasmic face. Each spike in a train elicits Ca^{2+} entry into the cell faster than the cell can remove or sequester it, thus decreasing the threshold for Ca^{2+}-activated K^+ channel opening, and allowing more repolarizing K^+ current. After a number of spikes the $Ca^{2+}{}_i$ builds up to the point that even upon repolarization the Ca^{2+}-activated K^+ channels stay open and the K^+ current flowing through them prevents further depolarization resulting in a long hyperpolarizing pause. With no spiking, and therefore limited Ca^{2+} entry, the cell can reestablish normal low $Ca^{2+}{}_i$ levels whereupon the Ca^{2+}-activated K^+ channel closes and a new train of action potentials can occur. The fast transient I_A current functions to

increase time between repetitive spikes (Connor and Stevens, 1971c). The afterhyperpolarization following an action potential removes inactivation of both Na^+ and fast I_A channels coupled with closing of the delayed rectifier channels. The cell can then start to depolarize again by opening of Na^+ channels; however, the fast I_A channels also open and the accompanying K^+ efflux counteracts the depolarization. The cell's membrane potential is controlled by these two opposing currents until the I_A starts to inactivate; this enhances depolarization and the probability of eliciting another action potential. It was found in this thesis that all 3 of these channel types contribute to the overall K^+ current in SIF cells, though interestingly the fast transient I_A appears to develop rapidly during the first postnatal week of life in the rat. The development of a fast I_A suggests that the firing frequency of action potentials is modulated during this period. Since this frequency controls Ca^{2+} entry, it should help regulate neurotransmitter release (e.g. dopamine) from SIF cells. Thus the endocrine, or secretory functions of these cells may well depend in part on factors that modify firing frequency.

Active Membrane Currents

Under these culture conditions at least five different time- and voltage-dependent transmembrane currents were found. (i) a TTX-sensitive, rapidly inactivating Na^+ current; (ii) a delayed rectifier K^+ current; (iii) a Ca^{2+}-dependent K^+ current; (iv) a rapidly inactivating, transient K^+ current or I_A (depending on postnatal age), and (v) a calcium current mediated by both sustained L-type and inactivating N-type Ca^{2+} channels (Fox et. al., 1985), or a single Ca^{2+} channel type, with both inactivating and

sustained components (Plummer et. al., 1989).

Comparison of SIF Cell Currents to Other Cells of the Sympathoadrenal Lineage.

With respect to the expression of voltage-activated currents, SIF cells share several common features, at least qualitatively, with other members of the sympathoadrenal lineage, i.e. the principal neurons, adrenal chromaffin cells and glomus, or type I cells of the carotid body. For example, they all possess the TTX-sensitive inward Na^+ current (rat SCG: Belluzzi et. al., 1985a; bovine chromaffin: Fenwick et. al., 1982b; rat glomus: Duchen et. al., 1988), the delayed rectifier K^+ current (rat SCG: Galvan and Sedlmeir, 1984; rabbit and rat glomus cells: Duchen et. al. , 1988; Stea and Nurse, 1989a, 1991; rat and bovine chromaffin cells: Kidikoro and Ritchie, 1980; Fenwick et. al., 1982a), as well as Ca^{2+}-dependent K^+ current (rat SCG neurons: Galvan and Sedlmeir, 1984; rabbit and rat glomus cells Duchen et. al., 1988; Hescheler et. al., 1989; Stea et. al., in press; bovine chromaffin cells: Marty, 1981). The fast I_A current, which rapidly develops postnatally in SIF cells (this thesis) has been found in principal neurons (Galvan, 1982; Galvan and Sedlmeir, 1984; Belluzzi et. al., 1985a), but has not been reported in either chromaffin or glomus cells. The Ca^{2+} current profile consisted of both L-type and N-type Ca^{2+} currents in bovine chromaffin cells (Bossu et. al., 1991a,b), and in rat sympathetic neurons (Hirning et. al., 1988; Plummer et. al., 1988) though in glomus cells it appears to be exclusively L-type (Duchen et. al., 1988; Hescheler et. al., 1989; Ureña et. al., 1989). The evidence present in this thesis is

consistent with the presence of both L- and N-type currents in SIF cells. In this laboratory, an O_2-sensitive K^+ current described in rat glomus cells was not found in SIF cells (Stea and Nurse, 1991) suggesting the SIF cells investigated in these cultures probably lack chemosensory function.

Inward Na^+ Currents

The fast transient inward current seen with depolarizing voltage steps in SIF cells was the classical TTX-sensitive, time- and voltage-dependent Na^+ current. Since Na^+-free solutions and TTX (500 nM) virtually abolished the fast transient inward current, there appeared to be a negligible TTX-insensitive Na^+ component. The small amount of inward current remaining after these treatments was due to the inward Ca^{2+} current that was activated by similar depolarizing steps (see below). The threshold for activation of the Na^+ current in SIF cells was about -40 mV, a value similar to that for principal neurons ($\approx$-30 mV, Belluzzi and Sacchi, 1986), and chromaffin cells ($\approx$ -40 mV, Fenwick et. al., 1982b). Further, the steady state half-inactivation potential for SIF cells was around -55 mV, similar to the principal neurons ($\approx$-56 mV; Galvan and Sedlmeir, 1984), glomus cells ($\approx$-50 mV; Stea and Nurse, 1991), while chromaffin cells had a more positive half-inactivation potential (-35 mV; Fenwick et. al., 1982b). Thus the Na^+ channels in these related cell types appear similar, or identical.

Outward K^+ Currents

A significant portion of outward K^+ current in SIF cells was dependent on Ca^{2+} influx. Similar Ca^{2+}-activated K^+ currents are found in many other species and cell

types including rat sympathetic neurones (Galvan and Sedlmeir, 1984; Belluzzi et. al., 1985b; Smart, 1987), glomus cells (Hescheler et. al., 1989; Stea et. al., in press) and chromaffin cells (Marty, 1981). The presence of this current was demonstrated by exchanging the normal bathing solution with one that had Co^{2+} substituted for Ca^{2+}. The Ca^{2+}-activated K^+ current has been shown to be activated by a combination of intracellular Ca^{2+} and depolarization (rat muscle: Barrett, Magleby and Pallotta, 1982; rat sympathetic neuron: Smart, 1987). In this study it was found that during depolarizations to +50 mV a large portion of the total outward current ($\approx$48%) is composed of this Ca^{2+}-dependent K^+ current. The rest of the outward current resembled mainly the ubiquitous delayed rectifier K^+ currents in the majority of cells examined in this study. This current was elicited by depolarizations to between -30 and -20 mV as in other cell types (e.g. rat SCG principal neurons: Belluzzi et. al., 1985b; and rabbit glomus cells; Duchen et. al., 1988). The SIF cells did not display slow time-dependent inactivation ($\geq$ 200 ms test pulses) of the delayed rectifier current that was reported for rat SCG principal neurons (Belluzzi et. al., 1985b), and shown, but not discussed in rabbit glomus cells (Ureña et. al., 1989). This slow decrease in outward K^+ current was studied by McFarlane and Cooper (1991) in rat sympathetic neurons, and was determined to be a result of inactivation of a separate channel type from the delayed rectifier, that they named the slow I_A channel. This channel had two components to inactivation, the first, and one previously seen (Belluzzi et. al., 1985b; Ureña et. al., 1989), inactivated over 150-300 msec, and the second over 1-3 sec. The glomus cells from rat, like the

SIF cells, failed to show slow time-dependent inactivation of the total outward current, at least over 100 ms (A. Stea, personal communication).

I_A Current

Along with the slow I_A current reported in sympathetic neurons (McFarlane and Cooper, 1991) there is also a fast I_A current (Constanti and Brown, 1981; Galvan and Adams, 1982; Galvan 1982; Belluzzi et. al., 1985a,b; McFarlane and Cooper, 1991). The fast I_A current has not been reported in either the glomus cell or the chromaffin cell. In this study, almost all of the SIF cells isolated from one day old rat pups, and cultured for 5-10 days, did not display a fast I_A current. In contrast, a fast I_A was commonly found in SIF cells obtained from older animals (3-7 days old) and cultured for a similar period. When present, the fast I_A current resembled that found in sympathetic principal neurons (Galvan, 1982; Galvan and Sedlmeir, 1984; Belluzzi et. al., 1985a). Though it was seen during depolarizing voltage steps from a holding potential of -60 mV, its full development required more hyperpolarized holding potentials, between -90 and -100 mV. The I_A current was fully inactivated at holding potentials of -40 mV in all cases. Failure to detect the I_A in SIF cells isolated from 1 day-old rat pups, and cultured for even longer periods, suggested that this property develops rapidly in early postnatal life and requires *in vivo* conditions that are not present in the cultures (e.g. presence of pre-synaptic input). The late development of the fast I_A relative to the other K^+ currents in SIF cells appears similar to the situation in rat sympathetic neurons where the fast I_A was also the last of the K^+ currents to develop (Nerbonne, Gurney and Rayburn, 1986; Nerbonne and

Gurney, 1989).

Pharmacology of the K^+ Currents in SIF Cells

Tetraethylammonium (TEA) ions at mM concentrations are known to block the Ca^{2+}-activated K^+ current (Adams, Constanti, Brown and Clark, 1982; Latorre, Vergara and Hidalgo, 1982; Latorre and Miller, 1983; Blatz and Magleby, 1984; Smart, 1987; Blatz and Magleby, 1987), as well as the delayed rectifier K^+ current (Lorente de No, 1949) and reduce the fast transient outward I_A current (Thompson, 1977; Rogawski, 1985; Rogawski, 1987). In this dose range TEA blocks approximately 75% of the total outward current in SIF cells. In contrast, aminopyridines (e.g. 4-AP) in mM concentrations block only the delayed rectifier and fast I_A channels in other studies (Connor and Stevens, 1971b; Thompson, 1977). In SIF cells cultured from one-day-old animals 4-AP blocks approximately 50% of the outward current, and presumably the sensitive component is mainly the delayed rectifier since the fast I_A is small or absent at this age. In SIF cells from older animals (5-7 days old) the fast I_A is prominent and in 4 out of 4 cases, this transient current was blocked by 10 mM 4-AP, whereas the sustained component was left mostly intact. This is in agreement with the situation in molluscan neurones where TEA (10 mM) had little effect on the transient K^+ current but markedly suppressed the sustained current (Thompson, 1977)

Taken together, these results suggest that both the delayed rectifier K^+ channel and the fast I_A channel in SIF cells are virtually completely blocked by 10 mM 4-AP, whereas the Ca^{2+}-activated K^+ channel is unaffected. Further, 10 mM TEA blocks all

or most of the Ca^{2+}-activated K^+ channel and the delayed rectifier K^+ current, whereas the fast I_A current is hardly affected.

Inward Ca^{2+} Currents

It has long been known that Ca^{2+} currents are labile during whole-cell recording presumably because of loss of cytoplasmic integrity (Rorsman and Trube, 1986). The perforated-patch technique of Horn and Marty (1988) permits much more stable recordings of Ca^{2+} currents (Falke et. al., 1989), since the presence of the pore-forming antibiotic nystatin in the patch pipette limits diffusive exchange to small monovalent ions and the cytoplasm remains largely intact.

Following blockade of all Na^+ (by choline substitution and TTX addition) and K^+ (by Cs^+ substitution) currents, the Ca^{2+} currents in SIF cells were studied in isolation. Confirmation that Ca^{2+} ions carried the residual currents was obtained since increasing external Ca^{2+} from 2 to 10 mM increased the size of the current, whereas addition of the Ca^{2+} channel blocker Co^{2+} (2 mM) abolished this current. Following a sufficiently large depolarizing step it was clear that the Ca^{2+} current had 2 components, a transient or inactivating component and a slower sustained one. These Ca^{2+} currents resemble those seen in bovine chromaffin cells (Bossu et. al., 1991a; see however Fenwick et. al., 1982b) though in some cell types either the transient or sustained component may occur without the presence of the other. For example, in rat sympathetic neurons the Ca^{2+} current inactivates completely (Belluzzi and Sacchi, 1989), whereas in rabbit type I cells the current does not inactivate over long times (Duchen et. al., 1988).

In principal sympathetic neurons, glomus cells and chromaffin cells the Ca^{2+} current activates between -40 and -30 mV, compared to SIF cells where activation occurs between -20 and -10 mV, with test potentials of $\geq$ 0 mV required for significant activation of the transient portion. The inactivation of the transient component also differs from the sustained component; the former is completely inactivated at holding potentials of $\approx$-30 mV, compared to $\approx$-10 mV for the latter. Preliminary results using the Ca^{2+} channel blocker nifedipine (50 μM) suggest that the sustained component is blocked by this treatment whereas the transient component is hardly affected.

The specific Ca^{2+} channel types present in the cells of the sympathoadrenal lineage show similarities and differences. When compared to the Ca^{2+} channel types of Nowycky et. al., (1985) the glomus cells of the carotid body had only sustained, L-type channels (Duchen et. al., 1988), the adrenal medullary chromaffin cell had both the L-type and the transient, N-type channels (Bossu et. al., 1991), and sympathetic principal neurons had both types (Hirning et. al., 1988). Plummer et. al., (1989) described an 'N-like' channel in the rat SCG principal neuron, that was responsible for both a transient and sustained component, on the basis of single channel recordings, and considered the N-type to be specific for neuronal cells. SIF cell Ca^{2+} channels appeared to consist of both the L-type and N-type channels, and as in the case of the others no transient low-threshold T-type channels were present. The sustained component of SIF cell Ca^{2+} current observed in this study is presumed to be due to the L-type current, the only non-inactivating current of the three types. The different dependence on holding potential,

and sensitivity to nifedipine, of the transient and sustained components of SIF cell Ca^{2+} current also suggest that the sustained portion is due to a separate channel type from the N-type channel. The susceptibility of Ca^{2+}-activated K^+ current to the organic Ca^{2+} channel blocker D600 is consistent with this interpretation, since in adult bovine chromaffin cells D600 is reported to block the slow, sustained L-type Ca^{2+} current. (Bossu et. al., 1991a). Further, the dihydropyridine antagonist nifedipine blocks L-type channels while it appears to have no effect on N-type channels (Hirning et. al., 1988; Plummer et. al., 1989). The N-type channel requires low holding potentials of $\approx$-80 mV for full activation, while the L-type channel is fully active at potentials as high as -20 mV. Taken together these results suggest that SIF cells possess both L- and N-type channels, though the presence of the N-type channel with a sustained component (Plummer et. al., 1989) can not yet be excluded. Single channel recording is required to resolve this issue satisfactorily. Since these channel types may differentially control neurotransmitter release (see Hirning et. al., 1988), their distribution in SIF cells may yet be important during secretion.

Chapter 3

The Effect of Intracellular Acidification on Membrane Currents in Cultured SIF Cells.

Introduction

The preceding chapter of this thesis explained methods for, and results of identification and characterization of the whole-cell currents present in cultured small intensely fluorescent (SIF) cells isolated from rat superior cervical ganglion (SCG). This chapter utilizes the results reported in the previous one as a basis for the study of one aspect of a possible functional role for the SIF cell in chemosensation: the effect of intracellular acidification of SIF cell currents.

The SIF cell bears a considerable ultrastructural resemblance to the chemosensory glomus cell from the carotid body (Kobayashi, 1971; McDonald and Blewett, 1981; Doupe, Patterson and Landis, 1985), and has been shown in some cases to possess a similar innervation pattern indicating that the predominant direction of information flow is from the SIF cell to the pre-ganglionic neuron (Kondo, 1976, 1977b, 1980; McDonald and Mitchell, 1975). SIF cells and glomus cells are also both found with regions of their membranes that are devoid of satellite cover apposed to fenestrated capillaries (Eränkö and Härkönen, 1965; Siegrist et. al., 1968; Matthews and Raisman, 1969; Biscoe, 1971; Eränkö and Eränkö, 1971; Chiba et. al., 1976; Williams et. al., 1976; Chiba, 1980; Hélen et. al., 1980; Jew, 1980). These morphological studies agree

well with the SIF cell being chemosensory, but functional studies have yet to prove this role. In fact, it has recently been shown that in response to hypoxia the SIF cell does not respond with a reduction of outward current as occurs in the glomus cell (Stea and Nurse, 1991), while it does, however, respond similarly to the glomus cell with an increase in number and intensity of TH staining, and a decrease in cGMP activity in rat SCG (Wang et. al., 1991). Rat SCG SIF cells were also shown to respond to long-term normobaric hypoxia with an increase in dopamine turnover and TH-reactivity that was independent of both pre- and post-ganglionic input (Dalmaz et. al., 1991).

Aside from hypoxia, low pH is another stimulus known to effect carotid body glomus cells (Peers, 1990; Stea et. al., in press). The effect of intracellular acidification on SIF cell whole-cell membrane currents is examined in this chapter. The perforated-patch whole-cell method is employed for recording currents as it does not result in dialysis of cellular contents and concomitant loss of the cellular pH homeostatic mechanisms. Acidification of the cell interior was effected by application of the K^+/H^+ ionophore nigericin. As with the perforated-patch technique, the intracellular contents are not dialysed out; as well, intracellular pH can be set by the intracellular and extracellular solutions chosen.

Materials and Methods

Culture Conditions

The procedures for growing small intensely fluorescent (SIF) cells in culture were identical to those in Chapter 2.

Perforated Patch Recording

Only perforated-patch recordings were employed when investigating the effects of internal pH (pH_i) on SIF cell whole cell currents. The same methods for perforated-patch recordings were utilized as in Chapter 2.

Data Acquisition & Analysis

Whole-cell currents were recorded and analyzed essentially as described in Chapter 2. The only differences were that the amplifier used was the Dagan 3900 patch clamp amplifier, and only PCLAMP version 5.5 software (Axon Instruments) was used for data acquisition and analysis.

Solutions

The standard extracellular, and nystatin-containing pipette solutions from Chapter 2 were used in these experiments. Acidification of the cell interior was achieved by perfusion of extracellular solution containing the Na^+/H^+ antiporter nigericin (Sigma). To prepare this, a stock solution of 5 mg/ml nigericin in distilled water was diluted in normal extracellular solution to a final concentration of 3.0 μg/ml ($\approx$4.0 μM).

Results

Appearance of Cultures

The SIF cell cultures appeared essentially the same as those described in chapter 2 of this thesis.

Passive Membrane Properties

The passive membrane properties of the cells in this study agreed with those reported in chapter 2. The input resistance as determined from the slope of the I/V curve generated by a voltage-clamp protocol of hyperpolarizing pulses, in the same manner as in chapter 2, was 0.88 $\pm$ 0.11 GΩ (mean $\pm$ S.E.; n=21). Input capacitance, also determined as in chapter 2, by integration of the capacity transient elicited under a hyperpolarizing voltage step, was 9.5 $\pm$ 1.0 pF (mean $\pm$ S.E.; n=21). This corresponds to a cell diameter of approximately 17 μm when assuming a specific cell capacitance of 1μF/cm^2. This is a valid assumption for these cells as shown by the agreement of diameters calculated in this manner versus those measured using phase contrast microscopy (see Chapter 2). The cell size calculated for this study coincides with values reported earlier (Chapter 2; Doupe, Patterson and Landis, 1985; Stea and Nurse, 1991; Stea et. al., in press).

Active Membrane Currents

The active current profile was the same as that seen in Chapter 2, with the same time- and voltage-dependence (not shown). The active current magnitudes recorded were similar to those previously reported (Chapter 2; Stea and Nurse, 1991; Stea et. al.,

in press). The inward Na^+ current recorded after a voltage step from a holding potential of -60 mV to a test potential of 0 mV was 676.1 ± 76.6 pA (mean ± S.E.; n=21). Normalizing to capacitance as an indicator of cell size gave a value of 71.5 pA/pF. The average outward current elicited upon stepping from -60 mV to +50 mV was 748.0 ± 73.5 pA (mean ± S.E.; n=21), which when normalized yielded a value of 78.7 pA/pF.

Effect of Intracellular Acidification on

Active Membrane Currents in SIF Cells

The addition of 3 μM nigericin to the bath solution resulted in rapid reduction of both the total inward and outward current peaks (FIG 3.1A,B,C). Within approximately 2 minutes of application of nigericin the resultant cellular acidification effected a reduction in the inward Na^+ current of 39.1 ± 8.7 % (mean ± S.E.; n=11) upon a step from -60 mV to 0 mV (FIG. 3.1A). Reduction of the K^+ current by 3 μM nigericin was slightly less at 28.7 ± 6.1 % (mean ± S.E.; n=9) when stepping from -60 mV to +50 mV (Fig. 3.1B, a step from -60 mV to +40 mV is shown). These reductions in current were readily reversible. Figure 3.1C shows an I/V curve for the reductions in both inward and outward current in response to 3 μM nigericin. The cell was held at -60 mV and currents were measured at peaks for inward, and just before the end of the voltage step for outward, for each voltage step shown. At voltage steps to ≥ -20 mV nigericin reduced inward current while steps to ≥ -10 mV resulted in reduction of outward current.

Figure 3.1A: Effect of Intracellular acidification on the fast transient inward Na^+ current in SIF cells. The pH_i was changed to 6.0 from 7.2 via bath application of nigericin (see text). The inward current was rapidly and reversibly decreased.

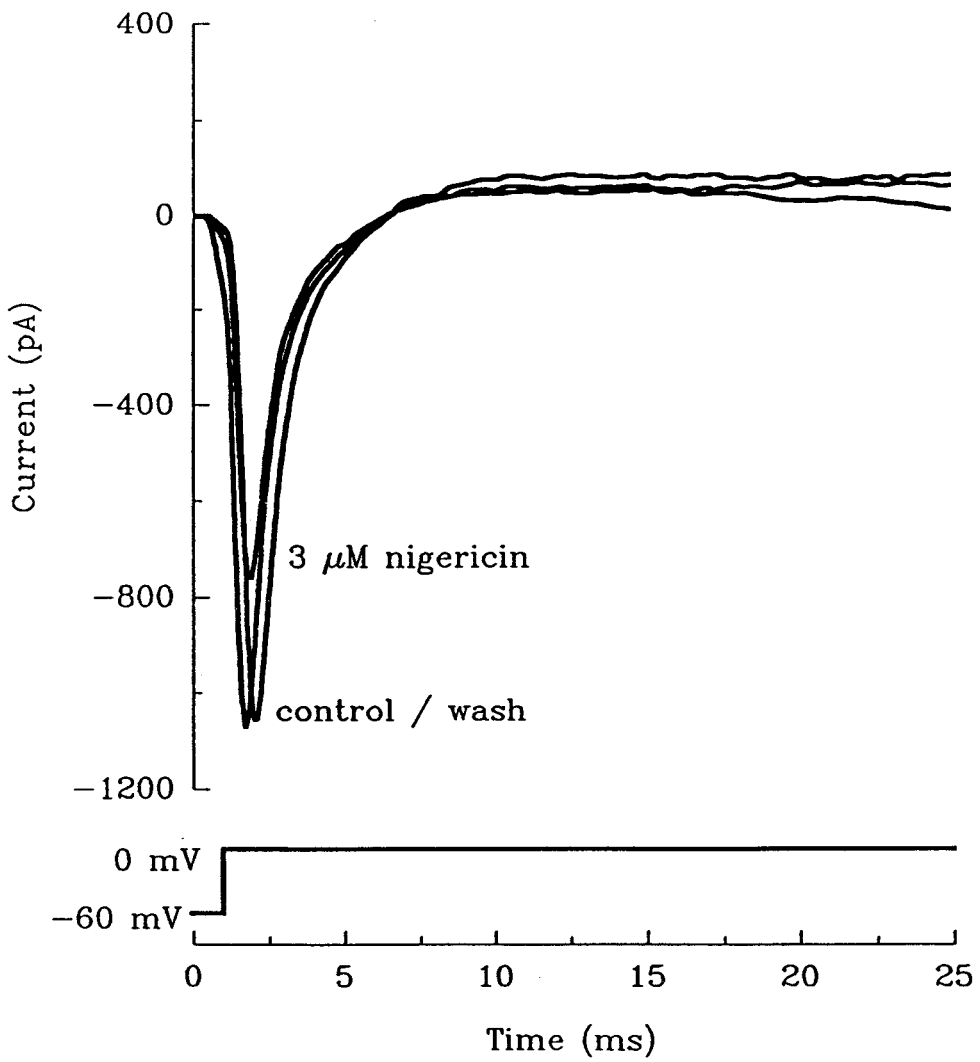

400
0
−400
−800
−1200
Current (pA)
3 μM nigericin
control / wash
0 mV
−60 mV
0
5
10
15
20
25
Time (ms)

Figure 3.1B: Effect of intracellular acidification on the outward K^+ current in SIF cells. The pH_i was changed to 6.0 from 7.2 via bath application of nigericin (see text). The outward current was rapidly and reversibly decreased.

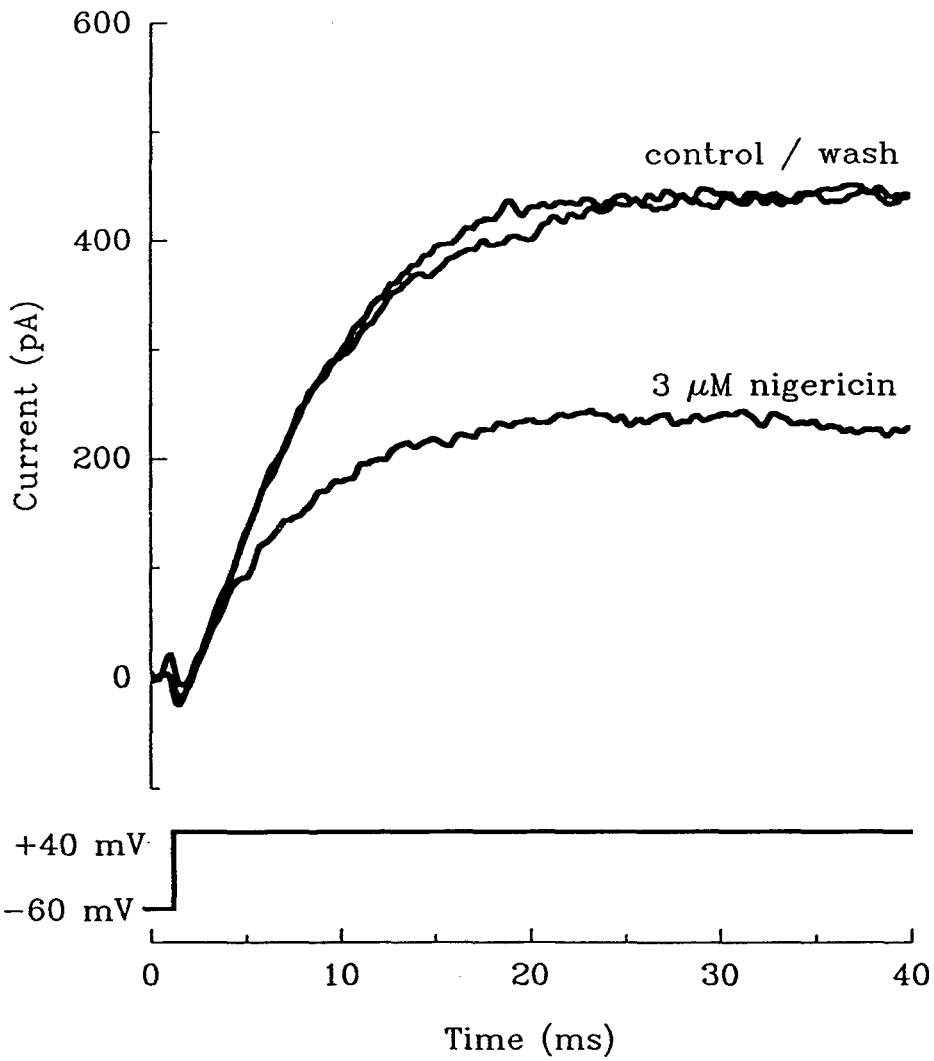
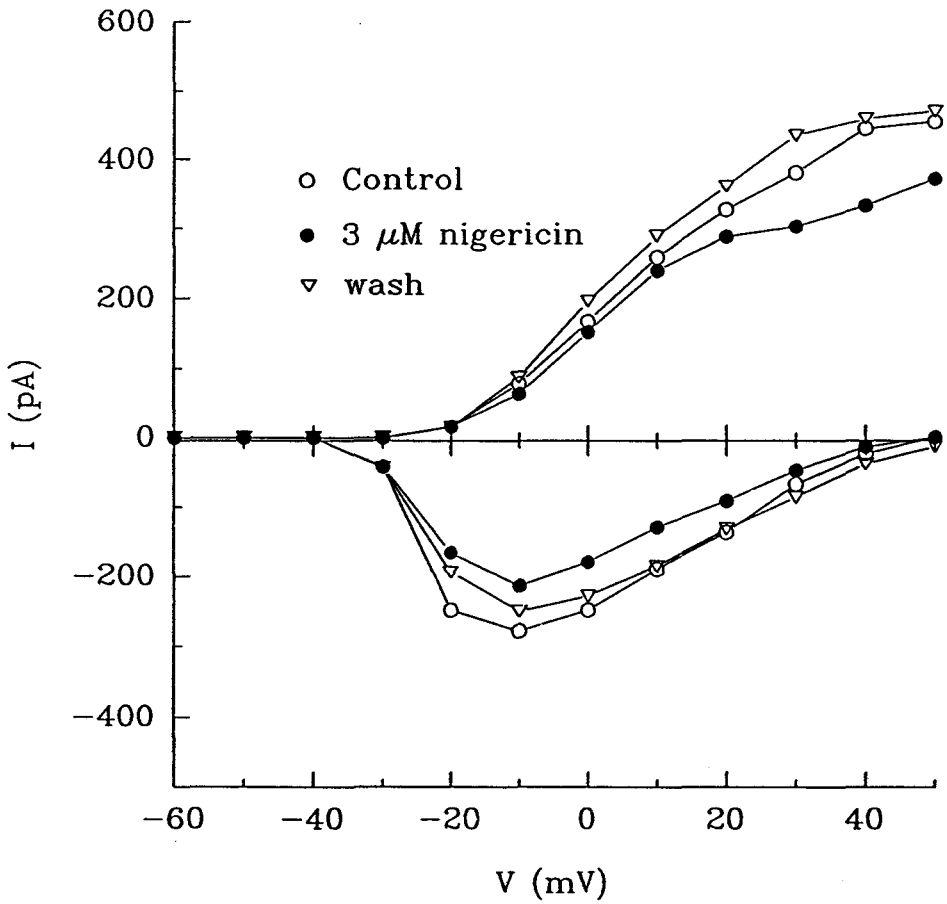

600
400
200
0
Current (pA)
control / wash
3 μM nigericin
+40 mV
−60 mV
0
10
20
30
40
Time (ms)

Figure 3.1C: I/V relationship showing changes in current for the cell pictured in figures 3.1A and 3.1B. Nigericin reduced both inward and outward current at each voltage step.


600
400
200
0
−200
−400
I (pA)
Control
3 μM nigericin
wash
−60
−40
−20
0
20
40
V (mV)

Discussion

The perforated-patch method was employed in this study to observe the effects of a decrease in pH_i on the SIF cell membrane currents. Once forming a cell-attached patch, this method permeabilizes the membrane patch, delineated by the patch microelectrode, thus creating electrical continuity between the pipette and cell interior, without leading to loss, or 'wash-out', of cytoplasmic compounds necessary for the cell's natural pH homeostatic mechanism. This provides a situation closer to *in vivo* conditions than would be obtained by replacing the cellular mechanisms with the artificial pH buffer HEPES, as occurs in traditional whole-cell methods. The greater stability of the patch over those formed by whole-cell methods also provides for longer times in which to perform manipulations of pH_i by application of nigericin. The K^+/H^+ ionophore nigericin applied in the bath inserts into the SIF cell membrane and affects the pH_i by antiporting H^+ ions into the cell in response to K^+ ions moving down their electrochemical gradient out of the cell (Thomas et. al., 1979), ($E_K \approx$ -83 mV, E_M held at -60 mV). The pH_e is unchanged by this procedure due to the large relative volume of the bathing solution, while the pH_i is affected in the ratio $[K^+]_i/[K^+]_e = [H^+]_i/[H^+]_e$ (Thomas et. al., 1979) which, under the conditions employed (see methods), theoretically results in a decrease to a $pH_i \approx 6.0$ once steady state is reached. This method for manipulation of pH_i is preferable to other previously utilized procedures. Moody (1984) describes a method of internal dialysis of cells with solutions of various pH. This creates a large hole in the cell membrane through which the solutions may pass, and may perturb

normal intracellular pH regulatory mechanisms. With the nigericin method, as with the perforated-patch technique, the small pores formed in the cell membrane allow only specific small ions to pass while retaining the large proteins. Addition of another ionophore, FCCP, to the bathing solution has also been performed (Deutsch and Lee, 1989). Nigericin enjoys an advantage over the use of the proton ionophore FCCP in that with nigericin the pH_e can be set, and then according to the K^+ and external H^+ concentrations employed the pH_i can then be manipulated to obtain a number of different values. With FCCP, protons readily cross the cell membrane and the concentration gradient runs down resulting in only one possible situation where the intracellular and extracellular H^+ concentrations are equal, and therefore $pH_i = pH_e$. A dose-response curve of different pH_i values should be performed on SIF cells in order to determine threshold and maximum levels of stimmulation, however limitations of time prevented this.

Acidification of the SIF cell interior by perfusion with 3 μM nigericin had a noticeable effect on both peak inward Na^+ and outward K^+ currents. The maximum currents recorded were reduced by about 40% and 30% respectively and were completely reversible upon removal of the nigericin. The decrease in outward current was considerably less than that reported in the related glomus cell (>50% reduction, Stea et. al., in press), while a comparison to the decrease in inward current in glomus cells is more difficult as glomus cells possess very little inward current under normal conditions (mean of $\approx$27 pA, Stea et. al., in press). These comparisons are important as the

glomus cell is generally accepted as possessing the chemosensory role in the carotid body (Biscoe and Duchen, 1989, 1990; Hescheler et. al., 1989; López-López et. al., 1989, Stea and Nurse, 1991), while a possible chemosensory role has been postulated for the SIF cell (Kondo, 1977b, 1980). SIF cells bear remarkable ultrastructural resemblance to the glomus cell; as well, they are both found in clusters near fenestrated capillaries and have incomplete Schwann cell coverings apposed to the fenestrations (rat SIF: Eränkö and Härkönen, 1965; Siegrist et. al., 1968; Matthews and Raisman, 1969; Eränkö and Eränkö, 1971; monkey SIF: Chiba and Williams, 1975; galago SIF: Williams et. al., 1976; human SIF: Chiba, 1980; Hélen et. al., 1980; rabbit and guinea pig SIF: Jew, 1980; rat glomus: Biscoe, 1971; innervation: Kondo, 1977, 1980), and may possess short processes that enter the perivascular space (SIF: Chiba, 1980; glomus: Biscoe, 1971). SIF cells from rat SCG were found to possess both afferent and efferent synapses with the same axon, with efferent synapses predominating indicating that the primary direction of transmission is from SIF cell to neuron (Kondo, 1977b, 1980). Since severing the pre-ganglionic trunk resulted in loss of most of the SIF cell contacts it was apparent that the axon making contact with the SIF cell was indeed pre-ganglionic. This innervation pattern was first seen in the rat carotid body, a known chemosensory organ, where sensory axons form a number of afferent and many efferent synapses with glomus cells, again indicating transmission to the axon predominates (McDonald and Mitchell, 1975; Kondo, 1977b, 1980). Kondo (1977b) also found this innervation pattern for SIF cells in the nodose ganglion, a chemosensory ganglion (Borison and Fairbanks,

1952; Chai and Wang, 1966; Jacobs and Comroe, 1971). The morphological results indicate a chemosensory role is possible for SIF cells, however, it is still unclear if functionally, SIF cells do possess a chemosensory role.

It has recently been shown that the SIF cell does not respond to a hypoxic stimulus that elicits a decrease in outward current in glomus cells (Stea and Nurse, 1991), while they do respond to a decrease in pH_i with a decrease in both inward and outward current as the glomus cell does (Stea et. al., in press). The hypoxic stimulus employed (which consisted of a $Po_2 \approx 20$ torr for a few minutes while doing whole-cell recording), and the parameters recorded may not be appropriate to discern a response. Dalmaz et. al., (1991) demonstrated an increase in dopamine (DA) turnover and an increase in TH-positive SIF cells in rat SCG, *in vivo*, in response to long-term normobaric hypoxia (10% O_2 for 2 weeks) that was not dependent on either of pre- or post-ganglionic input. As well, Wang et. al., (1991) found in rat SCG SIF cells and carotid body, after exposure to hypoxia, increases in number and intensity of TH staining, and a reduction in cGMP content, while post-ganglionic principal neurons were not affected. These SIF cell responses may be part of a long-term change in properties as opposed to short-term changes in current seen in the glomus cell.

It has been suggested that the fast response of glomus cells to acidity, hypercapnia and hypoxia may all be mediated or modulated by changes in pH_i (Stea et. al., in press). This pathway is viable for the glomus cell as it has been shown to possess intracellular carbonic anhydrase, (Nurse, 1990), the enzyme that catalyzess the hydration

of CO_2 and therefore the acidification of pH_i. Since SIF cells do not possess carbonic anhydrase (Nurse, 1990), the pH_i would not acidify as rapidly in CO_2 medium. It is apparent that while the SIF cell can mimic some responses present in the carotid body glomus cells (Wang et. al., 1991; Stea et. al., in press) it does not function in entirely the same manner.

CHAPTER 4

General Discussion

This thesis describes a method adapted from Doupe, Patterson and Landis (1985) for the long-term maintenance of small intensely fluorescent (SIF) cells in primary culture for the purpose of electrophysiological experiments. It is the first study to perform a characterization of the membrane ionic currents present in SIF cells. Identification of the individual current components present in cultured SIF cells isolated from postnatal day one rat superior cervical ganglia (SCG) revealed: (i) a fast transient TTX-sensitive inward Na^+ current; (ii) a slowly activating, non-inactivating outward delayed rectifier K^+ current; (iii) an outward non-inactivating Ca^{2+}-activated K^+ current; (iv) an inactivating, inward, Ca^{2+} current likely to be due to an N-type Ca^{2+} channel analogous to that of Nowycky et. al., (1985); and (v) a non-inactivating, inward, Ca^{2+} current probably due to a channel related to the L-type channel of Nowycky et. al., (1985). It was also found that a fast transient, outward K^+, or I_A current, seen in only 1 of 40 SIF cells from one-day-old rat SCG, became fully developed by postnatal day 5 *in vivo*, but did not develop when 1 day old SIF cells were cultured *in vitro*. The perforated-patch whole-cell recording method was used for long-term recording of current responses in SIF cells, and was especially useful for recording Ca^{2+} currents which tend to wash-out with conventional whole cell recording. As well, intracellular acidification of SIF cells was determined to result in a decrease in both inward and

outward current levels that was readily reversible upon removal of the stimulus.

The results of this thesis raise further questions concerning the SIF cell, its function, and development. With only whole-cell records of Ca^{2+} currents it can not unequivocally be determined whether there are both L- and N-type Ca^{2+} channels present (Nowycky et. al., 1985), or if the transient and sustained components of the total Ca^{2+} current represent two conductance states of one channel (Plummer et. al., 1989). Single-channel records are the only means of absolutely identifying the Ca^{2+} channel type, or types, responsible for the different components of Ca^{2+} current in SIF cells. Development of the SIF cell Ca^{2+} current may also occur around the time of birth as was found for an I_A current in these cells. It was found that not all SIF cells cultured from one-day-old rat pups exhibited the inactivating portion of Ca^{2+} current. It may be that the transient Ca^{2+} current develops during the late embryonic stage and is present in most cells at birth. Recording from cells isolated from late embryonic pups should answer this question. As well, the requirements for development of any currents, such as the I_A could be investigated. The fast I_A current was shown to develop *in vivo*, but not with the *in vitro* conditions used. Possible stimuli for I_A development, such as innervation and growth factors, could be introduced into the culture dishes and investigated with the whole-cell recording methods.

Studies on specific functional aspects of SIF cells can be answered with this preparation as well. The application of specific stimuli such as neurotransmitters, neuromodulators or known chemosensory stimuli can be easily performed, and the

response of the currents can be recorded. As well, metabolism of dopamine and other intracellular markers can be followed under these stimulatory conditions. Changes in SIF cells have been shown to occur in response to hypoxic stimuli *in vivo* that require neither pre- nor post-ganglionic input (Dalmaz, Borghini, Pequignot and Peyrin, 1991), it would be interesting to follow these responses in the relative isolation of cell culture to see if these changes are a result of direct stimulation of SIF cells.

Certain aspects of the development of the sympathoadrenal lineage may also be investigated using the methods and results of this thesis. Conversion of adrenal chromaffin cells into sympathetic adrenergic neurons has been shown (Doupe, Landis and Patterson, 1985), where the chromaffin cell passes through an intermediate stage resembling the SIF cell. The SIF cell was also shown to take on neuronal characteristics upon removal of the corticosteroids necessary for SIF cell survival, and addition of nerve growth factor (NGF) required for neuron survival (Doupe, Landis and Patterson, 1985). Changes in current profiles during this developmental progression can be followed and compared to the literature on currents present under normal conditions.

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