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Actions of Neuropeptides and Neuroactive Agents on Membrane Potential and Ionic Currents in Cultured Mammalian Stellate Ganglion Neurons

by

Robert William Gilbert

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

at

Dalhousie University
Halifax, Nova Scotia

July 1998

DALHOUSIE UNIVERSITY

FACULTY OF GRADUATE STUDIES

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DEDICATION

This thesis is dedicated to my parents, to Debbie and to Bob, for their consistent moral support.

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ABSTRACT

Neuropeptides modulate the excitability of mammalian sympathetic neurons by their actions on various types of voltage- and ligand- gated ion channels. This thesis examines the actions of two neuropeptides, substance P (SP) and calcitonin gene-related peptide (CGRP) on dissociated adult guinea-pig and rat stellate ganglion (SG) neurons using whole-cell patch clamp recording methods. Voltage-gated (K^+ and Ca^{+2}) and ligand-gated (nicotinic acetylcholine receptor, (nAChR)) conductances have been identified and characterized in these experiments. Under current-clamp conditions SG neurons exhibited overshooting action potentials (AP) followed by afterhyperpolarizations (AHP). The K^+ channel blocker, tetraethylammonium (1 mM), the calcium channel blocker, Cd^{2+} (100-200 μM) and SP (500 nM) all depolarized SG neurons, reversibly decreased the AHP amplitude and increased the AP duration. In the presence of Cd^{2+}, the effect of SP on membrane potential and AHP of guinea-pig SG neurons was reduced. Under voltage-clamp, several different K^+ currents were observed including: a transient outward K^+ conductance, and a delayed rectifier outward K^+ current (I_K) consisting of Ca^{2+}-sensitive ($I_{K(Ca)}$) and Ca^{2+}-insensitive components. Substance P (500 nM) inhibited the delayed rectifier outward K^+ current. Pretreatment with Cd^{2+} (20-200 μM), or the high-voltage activated Ca^{2+} channel blocker ω-conotoxin (10 μM), blocked SP's inhibitory effects on I_K. This suggests that SP reduces the outward K^+ current primarily through the inhibition of $I_{K(Ca)}$, and that this may occur, in part, via a reduction of Ca^{2+} influx through voltage-dependent Ca^{2+} channels. Substance Ps actions on I_K were mediated by a pertussis toxin-insensitive G protein coupled to NK_1 tachykinin receptors. This mechanism did not appear to involve the actions of the cytosolic second messengers cyclic AMP, protein kinase A or C. A ω-conotoxin-, Cd^{+2}- sensitive inward Ba^{+2} current was also identified in guinea-pig SG neurons. Substance P (500 nM) and CGRP (500 nM) reduced inward Ba^{+2} current in guinea-pig SG neurons. Thus, the actions of SP on $I_{K(Ca)}$ may be due in part to a reduction in Ca^{2+} influx occurring via N-type Ca^{2+} channels.

To determine a potential modulatory role of SP on ganglionic neurotransmission the actions of SP on nicotinic AChR ligand gated ionic current were investigated in rat SG neurons. In current-clamp, application of the nicotinic acetylcholine receptor agonist, DMPP (100 μM) depolarized the membrane potential by 30 mV. When neurons were voltage-clamped at a holding potential of -60 mV, DMPP activated an inward current which desensitized in the continued presence of the agonist. The DMPP-induced inward current was reduced by the nicotinic acetylcholine receptor antagonist mecamylamine (10 μM). The DMPP-inward current was reduced in Na^+-free ringers indicating that the current was carried primarily by Na^+ ions. In the presence of 500 nM SP the amplitude of the DMPP induced current was reduced and the rate at which the current desensitized in the continued presence of DMPP was increased.

This study presents the first description of ionic currents in mammalian SG neurons and demonstrates that SP and CGRP may modulate excitability in SG neurons via actions on voltage-gated and / or ligand-gated channels.

ABBREVIATIONS USED

A-current	transient outward potassium current
ACh	acetylcholine
ANS	autonomic nervous system
AHP	afterhyperpolarization
4-AP	4-aminopyridine
AP	action potential
ATP	adenosine 5'-triphosphate
Ba^{+2}	barium
BK	calcium activated potassium current
Ca^{+2}	calcium
CCK	cholecystokinin
Cd^{+2}	cadmium
CGRP	calcitonin gene-related peptide
ChAT	choline acetyltransferase
ChAT-IR	choline acetyltransferase-immunoreactive
CNS	central nervous system
ω-CgTx	ω-conotoxin GVIA
cyclic AMP	cyclic adenosine monophosphate
DbcAMP	8-Bromoadenosine 3',5'-cyclic Monophosphate
DMEM	Dulbecco's Modified Eagle's Medium
DMPP	1,1-dimethyl-4-phenylpiperaznium iodide

DYN	dynorphin
EGTA	1 ethylene glycol-bis (β-aminoethyl ether) N,N,N',N'-tetraacetic acid
ENK	enkephalin
E_K	equilibrium potential
f-EPSP	fast excitatory postsynaptic potential
g	conductance
GΩ	gigaohm
GAL	galanin
GDP	guanosine diphosphate
GDPβS	guanosine 5'-O-(2-thiodiphosphate)
GF109203-X	2-[2-(3-dimethylaminopropyl)-1H-indo-3-Yl]-3-(1H-indol-3-Yl)-maleimide
G-protein	guanine nucleotide binding protein
GTP	guanosine 5'-triphosphate
HRP	horseradish peroxidase
HVA	high voltage-activated current
I_A	transient outward potassium current
I_{AHP}	afterhyperpolarizing current
I_{Ba}	barium current
IBMX	isobutylmethylxanthine
I_K	potassium current
I_{Kv}	delayed rectifier potassium current
$I_{K(Ca)}$	calcium-dependent potassium current

I_M	muscarine-sensitive potassium current
IML	intermedial lateral
IP_3	inositol triphosphate
IR	immunoreactive
I-V	current-voltage
K^+	potassium
LVA	low voltage-activated current
MC-SG	middle cervical-stellate ganglion
mV	millivolts
nA	nanoamps
Na^+	sodium
NA	noradrenaline
NA-IR	noradrenergic-immunoreactive
nAChR	nicotinic acetylcholine receptor
NCS	newborn calf serum
NGF	nerve growth factor
NK_1	neurokinin 1
NK_2	neurokinin 2
NK_3	neurokinin 3
NKA	neurokinin A
NKB	neurokinin B
nM	nanomolar
NOS	nitric oxide synthase

NPY	neuropeptide Y
NT	neurotensin
PBS	phosphate buffered saline
PKA	protein kinase A
PKC	protein kinase C
pA	picoamps
PNS	peripheral nervous system
PTX	pertussis toxin
PYY	peptide YY
RMP	resting membrane potential
s-EPSP	slow excitatory post synaptic potential
SCG	superior cervical ganglion
SG	stellate ganglion
SOM	somatostatin
SP	substance P
TEA	tetraethylammonium
TOH	tyrosine hydroxylase
TTX	tetrodotoxin
μM	micromolar
VIP	vasoactive intestinal peptide
V_h	holding potential
V_M	resting membrane potential

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PUBLICATIONS

Portions of this thesis have previously been published.

Papers

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Abstracts

1. Gilbert R, Ryan J, Horackova M, Smith F, Kelly MEM. (1996) Substance P modulation of K^+ and Ca^{+2} currents in guinea-pig stellate ganglion neurons. Biophys J **70**: A119.

2. Gilbert R, Smith F, Kelly MEM. (1995) Regulation of ionic currents in freshly isolated and cultured stellate ganglion neurons. Can J Physiol Pharmacol **73**: (suppl. 3) Aix-Ax.

3. Gilbert R, Smith F, Kelly MEM. (1994) Pharmacology of voltage-dependent K^+ currents in freshly dissociated and cultured rat stellate ganglion neurons. Can J Physiol Pharmacol **72**: (suppl. 1) 521.

INTRODUCTION

1. OVERVIEW

The mammalian heart receives innervation from both its parasympathetic and sympathetic limbs of the autonomic nervous system. Parasympathetic efferent neurons produce cardiodepression when activated while sympathetic efferent neurons augment cardiac function. It has long been known that sympathetic neurons in the stellate ganglion (SG) regulate cardiac output by actions on heart rate and contraction strength (Langley, 1893; Langley and Anderson, 1894), and that vasomotor, sudomotor and pilomotor effects are also mediated by this ganglion (Langley, 1893, 1891a,b). More recent investigation of sympathetic efferent projections in a number of different mammalian species, have revealed that sympathetic postganglionic neurons innervating the heart arise almost exclusively from the middle cervical ganglion and stellate ganglion (Janes et al., 1986; Pardini et al., 1990, 1989; Armour and Hopkins, 1984; Hopkins and Armour, 1984), with lesser numbers in the superior cervical ganglia (SCG) and mediastinal ganglia. While some aspects of the sympathetic control of the heart have been well documented (Randall, 1977; Horackova and Armour, 1995), there is a paucity of information on the electrophysiological properties of SG neurons supplying the heart. Since the SG is an important site for potential modulation of the sympathetic outflow to the heart, information on the types and properties of voltage- and ligand-gated ionic currents underlying excitability and action potential formation in these neurons would enhance our

understanding of the ways in which the autonomic nervous system regulates the heart via this pathway.

In addition to classical neurotransmitters such as acetylcholine (ACh) and noradrenaline (NA) a variety of neuropeptides are now known to be present in the nerve fiber networks of the mammalian SG. The functional role of peptidergic innervation of sympathetic ganglia is not yet clear, but it has become increasingly apparent that neuropeptides in other sympathetic ganglia modulate ganglionic neuro-transmission. The neurons in sympathetic ganglia respond to neuropeptides and neurotransmitters with changes in membrane potential and/or action potential firing patterns (Mo et al., 1994; Vanner et al., 1993). These changes are the result of neuropeptide-mediated alterations in the properties of various ligand- and voltage-gated ionic conductances of the cell membrane, whose activation is coupled to appropriate cell membrane receptors. In autonomic ganglia, nicotinic cholinergic receptors are responsible for the fast excitatory postsynaptic potentials (f-EPSP) which trigger the initiation of postsynaptic action potentials (AP) (Skok, 1983). The activation of neuropeptide receptors however, are most often associated with slow changes in membrane potential or action potential firing patterns which may play modulatory roles (Kobayashi and Tosaka, 1983; Vanner et al., 1993). It is now known that a variety of neuropeptides are present within the cell bodies and nerve fiber networks of a number of different mammalian autonomic ganglia, including both those of the enteric plexus (Furness and Costa, 1987; North, 1982) and the neurons of the SG *in situ* (Heym et al., 1993; Kummer and Heym, 1988; Morales et al., 1995). Immunohistochemical studies have indicated that nerve fibers in the guinea-pig and rat SG

may contain substance P (SP), calcitonin-gene related peptide (CGRP), vasoactive intesinal peptide (VIP), enkephalin (ENK) or combinations of the so mentioned (Heym et al., 1993; Morales et al., 1995; Horackova et al., 1993; Anderson et al., 1993). These peptide-containing fibers are believed to originate from either preganglionic sympathetic neurons of the spinal cord or from sensory neurons (Heym et al., 1993). In spite of the identification of these neuropeptides in the SG, their potential roles in the modulation of membrane excitability has received little attention.

Substance P, an undecapeptide, has been shown to act both as a neurotransmitter and as a neuromodulator in the central and peripheral nervous systems (North, 1982; Otsuka and Yanagisawa, 1987). The actions of SP in the mammalian peripheral nervous system are typically associated with slow depolarization (Nakajima et al., 1988; North, 1982; Shen and Suprenant, 1993; Tokimasa and Akasu, 1992; Vanner et al., 1993). In rat sympathetic SCG neurons, SP-coupled signaling pathways appear to be involved in the modulation of Ca^{2+} channels (Shapiro and Hille, 1993), whereas in guinea-pig submucosal and celiac neurons a decreased K^+ conductance (the M-current) is primarily responsible for the slow EPSP (s-EPSP) and depolarization produced by SP (Shen and Suprenant, 1993; Vanner et al., 1993). In the mammalian and avian sympathetic neurons, SP has been shown to modulate the actions of cholinergic agonists on nicotinic ACh receptor (nAChR) channels (Clapham and Neher, 1984; Role, 1984; Valenta et al., 1993; Simasko et al., 1985; Min and Weiland, 1992). Although the different ion channels underlying the modulation of AP threshold and excitability in mammalian SG neurons have not been determined, SP was found to depolarize ganglion neurons through a decrease in

membrane conductance, possibly that of a K^+ channel (Mo et al., 1994). In light of the neuromodulatory actions described for neuropeptides in neurons in other autonomic ganglia an understanding of the potential roles of peptides such as SP on SG neuron electrophysiology is important.

The aim of this thesis, therefore, was to examine the biophysical and pharmacological properties of the various voltage- and ligand-gated ionic conductances present in somatic membranes of cultured SG neurons, and tc determine whether these conductances are modified by SP and CGRP, two neuropeptides previously identified in the SG. The following sections summarize current information on the anatomy, neurochemistry and electrophysiology of the neurons of mammalian autonomic ganglia, with emphasis on the paraverebral ganglia of the sympathetic division including the SG.

2. The Autonomic Nervous System: Background

Anatomy and Function

The SG are a component of the sympathetic division of the mammalian autonomic nervous system. The autonomic nervous system (ANS) as defined by Langley (1921) is an efferent system which innervates and controls the vegetative functions of all of the organs and tissues of the body, with the exception of skeletal muscle fibers. The integrating actions of the ANS (also called the visceral, vegetative or involuntary nervous system) are of vital importance to the health of the organism through its actions in maintaining internal homeostasis (Claude Bernard, 1878,1879; Cannon, 1928,1932). Homeostatic functions

under control of the ANS are numerous and include cardiac output, blood flow to organs, thermoregulation, respiration, micturition and digestion (Elfin et al., 1993; Burnstock and Hoyle, 1992; Janig, 1988; Gilman et al., 1992).

The ANS is comprised of both central nervous system (CNS) and peripheral nervous systems (PNS) components. The peripheral components of the ANS includes all autonomic pathways lying outside the brain stem and spinal cord. En route to visceral targets, autonomic outflow from the CNS passes (via chemical synaptic or ganglionic transmission) through recognizable aggregates of macroscopic and microscopic peripheral autonomic neuronal cell bodies lying on the nerve trunks. These are referred to as autonomic ganglia. The autonomic ganglia are the sites of peripheral interneuronal synapses in the nervous pathway to the organs and tissues, with preganglionic axons arising from neurons within the brainstem and spinal cord forming connections with postganglionic neurons whose axons end at their targets.

Classically, autonomic ganglia were believed to serve the simple function of a relay station, distributing nerve signals from the CNS to the peripheral organs. However, with decades of research, neuroscientists have revealed the structure and function of autonomic ganglia to be more complex. Autonomic ganglia are now seen as serving the role of relaying, distributing, integrating and modifying signals originating in the CNS. Ultimately, a final pattern of action potential discharge resulting from these interactions is conducted along the post ganglionic axons to neuroeffector junctions in the periphery, where they determine various end organ responses. One example of the potential complexity of autonomic ganglion function is illustrated in the process of chemical and

electrical transmission of autonomic outflow through the ganglia at the level of the postganglionic neuron. For many years neuroscientists believed that chemical (synaptic or ganglionic) transmission through autonomic ganglia was mediated by a sole neurotransmitter ACh. Acetylcholine was released from presynaptic terminals in response to preganglionic nerve stimulation and activated excitatory postsynaptic receptors to relay the CNS autonomic outflow. However, over the last 30 years an overwhelming body of neurochemical data has revealed that, in addition to containing this classical neurotransmitter, nerve fiber networks within autonomic ganglia also contain a number of neuropeptides, and that these neuropeptides may modulate chemical and electrical aspects of classical ganglionic neurotransmission. The current view of the coexistence of various neurochemicals within ganglia confer an enormous potential for diversity of chemical signaling in the autonomic nervous system. It has therefore been proposed that autonomic neurons may utilize numerous neurochemicals in the process of synaptic transmission.

A further level of complexity is provided by recent investigations which show that autonomic ganglia can integrate and modify neural activity from sources and directions other than preganglionic nerves. In particular, signals may arise from sensory receptors within visceral targets and possibly from intraganglionic nerve fibers, and each of these nerve fibers may possess distinct neurochemical phenotypes which could contribute to the modulation of ganglionic transmission.

While it is still not clear which neurochemicals are neurotransmitters and which ones are neuromodulators, the role of a particular compound may be state-dependent and the distinction may therefore be arbitrary. If a compound is synthesized by a presynaptic

neuron, released by the terminals of that neuron in response to stimulation, and subsequently produces a direct receptor-mediated effect on a postsynaptic neuron then that compound can, for the purposes of this discussion, be considered a neurotransmitter. If a compound is synthesized by a presynaptic neuron, released by the neuron in response to a stimulation, and subsequently produces an indirect effect on the postsynaptic neuron, then it will be considered primarily a neuromodulator. In any case, the activity of postganglionic neurons is regulated by the process of synaptic transmission, which involves both neuromodulators and neurotransmitters. Thus the release of neuropeptides may play multiple roles in determining such variables as the neuronal membrane potential and patterns of action potential firing in response to presynaptic drive.

3. Anatomy and Function of the Sympathetic Nervous System

On the basis of distribution of peripheral autonomic ganglia, the projections of ganglion neurons, the pharmacological properties of these neurons, and taking into account Langley's (1921) definition, the ANS is traditionally separated into three divisions: parasympathetic (craniosacral), sympathetic (thoracolumbar) and enteric (see Figure 1-1). The SG is a component of the sympathetic division of the mammalian autonomic nervous system. The following section provides a brief review of the anatomical and functional relationship between the SG and the heart.

A. Function

FIGURE 1-1: Anatomical arrangement of the principal parts of the peripheral autonomic nervous system. SC, spinal cord: NE, norepinephrine; ACh, acetylcholine. Modified from Gilman (1992).

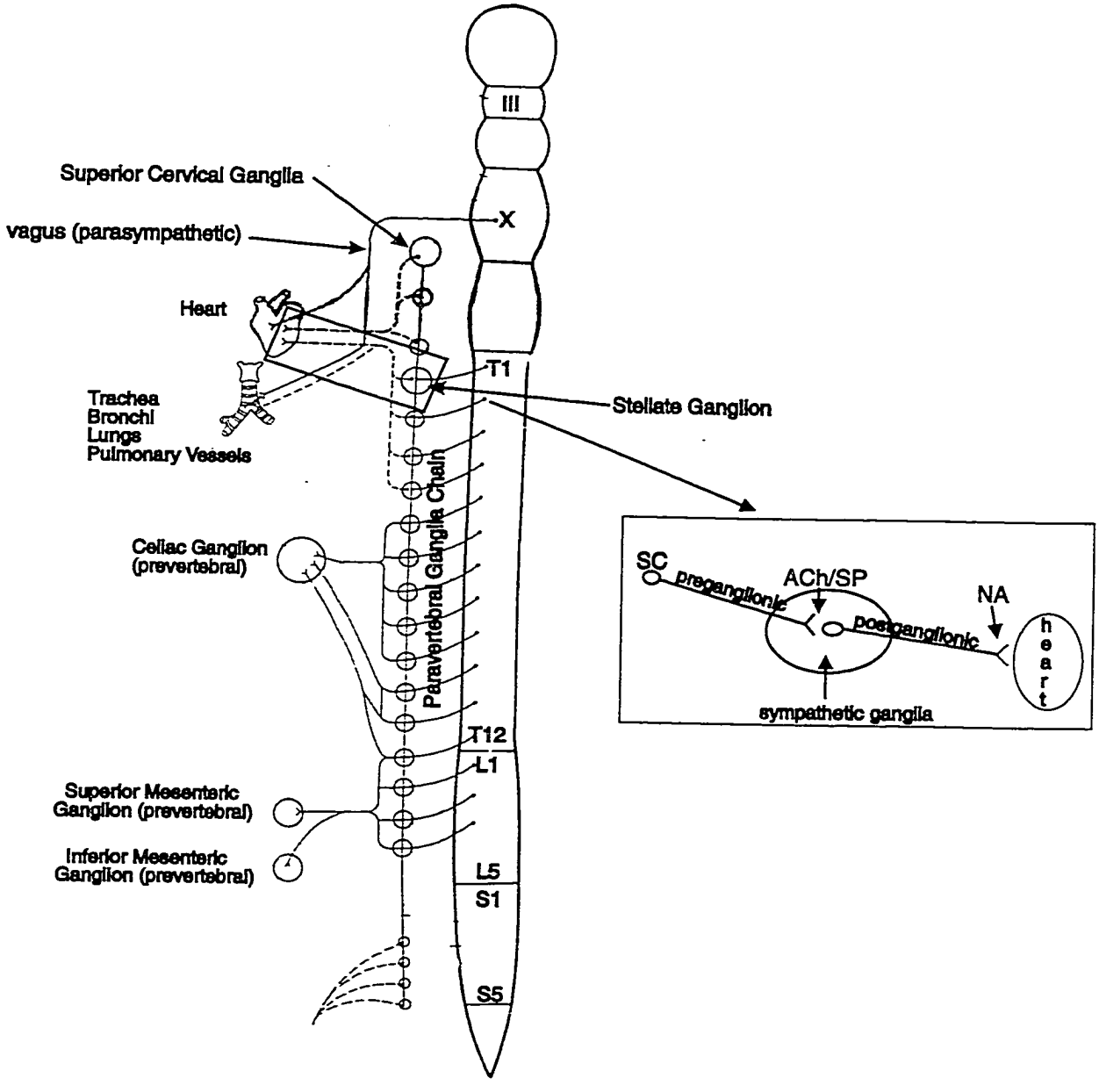

III
Superior Cervical Ganglia
vagus (parasympathetic)
X
Heart
T1
Trachea
Bronchi
Lungs
Pulmonary Vessels
Stellate Ganglion
Paravertebral Ganglia Chain
Celiac Ganglion
(prevertebral)
SC
preganglionic
ACh/SP
NA
postganglionic
heart
sympathetic gangila
T12
L1
Superior Mesenteric
Ganglion (prevertebral)
Inferior Mesenteric
Ganglion (prevertebral)
L5
S1
S5

FIGURE 1-1

The neurons of the SG have long been known to play an important role in the sympathetic control of the heart. For over 100 years it has been known that the sympathetic neurons of the SG augment heart rate and contraction strength (Langley, 1893; Langley and Anderson, 1894). Subsequently, the role played by the SG in the sympathetic control of the heart was investigated in numerous large and small mammalian species (Randall, 1977; Yasunaga and Nosaka, 1979). In the dog, stimulation of individual intrathoracic cardiac nerves containing sympathetic axons efferent to the heart, produces regional changes in mechanical and electrical cardiac indices (Armour and Randall, 1975; Randall et al., 1972). A number of reports have described the effects of stimulation, ablation or anesthetization of the SG and the possible clinical benefits of these procedures in heart disease (Gootman et al., 1992; Janse et al., 1985; Puddu et al., 1988; Schwartz and Stone, 1980; Schwartz et al., 1977; Yanowitz et al., 1966; Cinca et al., 1985; Cua et al., 1997; Drott, 1994), thus emphasizing the importance of this pathway to cardiac function. In addition to their role in control of the heart, neurons in the SG have also been shown to mediate pilomotor, vasomotor and sudomotor effects (Langley, 1893; Langley, 1891). In this respect some putative cardiac neurons of the SG have been recently shown (immunohistochemically and by retrograde neuroanatomical tracing methods) to also innervate pulmonary tissue (arteriole smooth muscle, bronchial smooth muscle and secretory cells). Non-cardiac neurons in the SG have been shown to innervate the skin and skeletal muscle vasculature, the brown adipose tissue, and sweat glands of the forepaw (Heym et al., 1993).

B. Anatomy

i. General Organization

The efferent sympathetic division of the ANS is organized as a two neuron pathway from the CNS to the peripheral target organs. Preganglionic neurons with their somata in the CNS project via rami communicants to postganglionic neurons in the sympathetic ganglia. The sympathetic ganglia are sub-categorized into those containing the paravertebral chain, the prevertebral ganglia and the terminal (or previsceral) ganglia (Simmons, 1985; Elfin et al., 1993; Smith, 1994). The SG are a pair of sympathetic ganglia located at the most rostral extent of the thoracic paravertebral chain and are attached to the dorsal body wall overlying the head of the first rib (De Lemos and Pick, 1966).

ii. Nerve Fibers Networks of the Paravertebral Sympathetic Ganglia

The major source of nerve fibers to the paravertebral ganglia are neurons in the intermedial lateral (IML) cell column of the lumbar and thoracic spinal cord. Preganglionic spinal fibers reach the paravertebral ganglia via white rami, and postganglionic axons leave the ganglia via gray rami to join the spinal nerves and distribute to the effector organs. Preganglionic neurons in nuclei more medial and lateral to the IML have also been shown to project to sympathetic ganglia. Retrograde tracing studies of connections to the adult rat SG have revealed that most preganglionic neurons projecting to this ganglion are located in the 8th cervical to the 9th thoracic spinal cord segments

(Pyner and Coote, 1994). The majority of these neurons are located in four distinct regions: 1) 70% of the nuclei reside in the nucleus intermediolateralis thoracolumbar par principalis, with the remainder in the 2) nucleus intermediolateral thoracolumbalis parfunicularis, 3) the nucleus intercalatapars paraependymatis or, 4) the central autonomic area (Pyner and Coote, 1994). Similar neuronal distribution patterns to those described for rat have been provided for the guinea-pig (Dalsgaard and Elfin, 1981).

In general, the number of postganglionic neurons within a ganglia greatly exceeds the number of preganglionic neurons which project to it. The number of post- to preganglionic neurons varies among ganglia, species, animal size and probably between different functional targets within a given ganglia. Studies in the rat SCG have shown that a total of approximately 1600 preganglionic neurons project to a population of between 25000 and 45000 postganglionic neurons (Rando et al., 1981; see Gabella, 1985). Arborization patterns of sympathetic preganglionic axons in the rat SG have indicated that axons bifurcate repeatedly and extend to lengths of 400-800 μM in the rostralcaudal direction and approximately 400 μM in the transverse direction (Asamoto et al., 1997). By implication, there therefore exists a divergence of preganglionic input in that preganglionic neurons branch repeatedly, each innervating a number of postganglionic neurons.

In addition to preganglionic inputs there is also evidence that synaptic contacts of axon collaterals from both visceral sensory fibers and from axons derived from postganglionic cells in the same or neighboring sympathetic ganglia, impinge on SG neurons. In the cat and the dog electrophysiological and intracellular labeling experiments

have provided evidence for peripheral afferent inputs on postganglionic neurons in the SG. The perikarya of these afferents may be located in sensory (dorsal root and vagal) ganglia, in the SG itself or in the mediastinum (Bosnjak and Kampine, 1982, 1985; Armour, 1987). In the guinea-pig SG a small population of intraganglionic axon terminals have been described. Several lines of evidence suggest that these fibers are of sensory origin (Quigg et al., 1988; Quigg et al., 1990; Heym et al., 1993). It is not yet clear whether these presumptive sensory axons form true synaptic contacts in the SG (as has been demonstrated in the prevertebral sympathetic ganglia) (Matthews and Cuello, 1982; Matthews et al., 1987) or whether they terminate freely in the neuropil. However, such sensory neurons may have a special relationship with ganglionic neurons and could represent an afferent input to peripheral autonomic neurons, independent of the CNS.

iii. Postganglionic Neurons of Paravertebral Sympathetic Ganglia

Postganglionic sympathetic neurons in mammals are multipolar with synapses from preganglionic neurons occurring predominantly on their dendrites, although axosomatic junctions have also been observed (Elfin et al., 1993). Neurons in mammalian sympathetic ganglia have a single axon and several dendrites. The perykarya of the guinea-pig SCG average 13 dendrites per cell (McLachlan, 1974). Within a sympathetic ganglion, each soma receives input from about seven preganglionic fibers and each preganglionic axon is extensively branched such that it may innervate up to 200 cell bodies (Smith, 1993). There is convincing evidence from electrophysiological studies that convergence of multiple preganglionic neurons onto postganglionic neurons exists, with

synapses being formed on postganglionic neurons by multiple varscosities arising from several convergent preganglionic nerve fibers (Purves and Lichtman, 1985a,b). Physiological studies showed that functionally distinct populations of neurons exist in sympathetic ganglia which are innervated by preganglionic fibers from approximately four separate segments of the spinal cord with the majority coming from a single segment. It is believed that postganglionic neurons supply only one type of target effector tissue and that those neurons which innervate a common target organ come from functionally distinct populations of neurons in the ganglia and from similar regions of the ganglion with common targets.

Neuroanatomical tracing studies in canines (Armour and Hopkins, 1981; Hopkins and Armour, 1984) and felines (Shin et al., 1985) and the rat (Pardini et al., 1989, 1990) have indicted that the cardiac sympathetic postganglionic somata originate primarily in the middle cervical and SG. In the rat up to 94% of the cardiac sympathetic postganglionic fibers originate bilaterally in the MC-SG complex, with the remainder occurring in the superior cervical and the 4th through 6th thoracic paravertebral ganglia (Pardini et al., 1989, 1990). The middle cervical and SG are located very close together in the rat and are often fused into a single mass (Pardini et al., 1989). In support of the neuroanatominical tracing studies, experiments examining the effects of bilateral middle cervical and stellate ganglionectomies (removing the somata of most cardiac sympathetic fibers) have produced a decrease in cardiac norepinephrine content of up to 94%. This lends further support to the concept that the SG-MC complex serves as the primary locus for postganglionic sympathetic neurons innervating the heart (Pardini et al., 1989, 1991).

Related results have been reported in both the cat (Hertting and Schiefthaler, 1964; Blaschke and Uvnas, 1979) and dog (Hikosaka, 1969). Properties of the postganglionic sympathetic neurons supplying the heart and arising in the SG and adjacent paravertebral ganglia of various mammalian species have been recently reviewed with respect to their location, morphology, synaptic input and membrane characteristics (Wallis et al., 1996).

4. Classical Neurotransmitters of the Autonomic Nervous System

Traditionally, peripheral autonomic nerves have been classified on the basis of the primary transmitter molecules which are released from their terminal boutons and varscosities: parasympathetic terminals release ACh, sympathetic terminals release norepinephrine (NE) (see Karczmar, 1986 for review). The terminals of autonomic neurons which synthesize ACh are referred to as cholinergic terminals. To date, these include all pre-ganglionic efferent autonomic fibers to the prevertebral and paravertebral ganglia, all parasympathetic pre- and post-ganglionic neurons, and a few postganglionic sympathetic nerve fibers.

Autonomic neurons which synthesize and act by releasing norepinephrine are called adrenergic fibers. The main transmitter in the sympathetic nervous system of mammals is NE (von Euler, 1946), and the majority of principal ganglion cells are noradrenergic, as demonstrated initially with histofluorescence techniques (Falck et al., 1962; Eranko and Harkonen, 1963; Norbeg and Hamberger, 1964) and later verified immunohistochemically with antisera against dopamine β hydroxylase (Geffen et al., 1969) the enzyme which convert dopamine to NE in the synthesis of catecholamines. In addition

to adrenergic neurons, a small population of cholinergic sympathetic postganglionic neurons was first demonstrated in the cat stellate and lumbosacral (L5-S2) ganglia by Holmstead and Sjoqvist (1959), (see also Sjoqvist, 1962), via studies which demonstrated the presence of the ACh metabolizing enzyme acetylcholinesterase (AChE) (Koell and Friedenwald, 1949). This was later confirmed immunohistochemically in the cat using antisera to the ACh synthesizing enzyme choline acetyltransferase (ChAT) (Buckley et al., 1967). Cholinergic sympathetic postganglionic neurons amount to about 10-15 % of the total cell population in stellate and lumbar sympathetic ganglia.

5. Neuropeptides in Mammalian Sympathetic Ganglia

Recent studies of autonomic ganglia have revealed that, in addition to the classical neurotransmitters, various monoamines such as histamine (Christian et al., 1989) and serotonin (Dun et al., 1980; Verhofstad et al., 1981), as well as a wide variety of neuroactive peptides (Lundberg and Hokfelt, 1983) are contained within the ganglia. For example, substances which are immunologically indistinguishable from CGRP, SP, neuropeptide Y (NPY), VIP, somatostatin (SOM), ENK and dynorphin (DYN) have been detected in autonomic ganglia (Hokfelt et al., 1977abc; Schultzberg et al., 1979, 1983; Furness et al., 1983; Heym, 1987; Debinski et al., 1987; Horn and Stofer, 1988, 1989). The functional roles of these substances, both within the ganglia and in their visceral targets, are not fully understood. One suggestion has been that patterns of multiple peptide content may distinguish functionally separate groups of neurons within a ganglion

and that peptide coding may be related to neuronal function and to the innervation patterns of peripheral targets (Costa et al., 1986; Gibbins et al., 1987; Jaing and McLachlan, 1992). In terms of function, peptides may act as either neurotransmitters, neuromodulators or both depending on the system (Kow and Pfaff, 1988). They may provide slow, long-lasting actions to supplement or modulate the more transient effects of the primary transmitter. They may also participate in feedback inhibition of the same or nearby nerve terminals.

A. Peptides Co-localized with NE

i.) Noradrenergic Immunoreactive Cell Bodies

In addition to the classical neurotransmitter NA, numerous neuropeptides have been identified in the perykarya of sympathetic ganglia. Adrenergic postganglionic sympathetic cell bodies which contain neuropeptides have been well characterized in a number of mammalian sympathetic ganglia. The first peptide determined to be colocalized with NA was SOM, which has been observed in SCG neurons of the guinea-pig, rat (Hokfelt et al., 1977) and human (Jarvi et al., 1987). Immunoreactivity for the opioid peptide ENK has been identified in rat and guinea pig SCG neurons (Schultzberg et al., 1979), and subsequent investigations in the rat lumbar paravertebral ganglia have revealed that as many as 55% of neurons contained immunoreactivity for met-ENK (Shimosegawa et al., 1985). Immunoreactivity to vasopressin has also been reported in the perikarya of rat and monkey SCG (Hanley et al., 1984) and a large population of adrenergic

paravertebral post ganglionic cell bodies containing NPY have been described in the lower lumbar ganglia and SCG of the guinea pig (McLachlan and Llewllyn-Smith 1986; Gibbins and Morris, 1987) and in the SG of the rat, guinea-pig and pig (Tyrrell and Landis, 1994; Happola et al., 1993; Heym et al., 1990). Galanin (GAL) has been identified in a large population of the NPY-positive neurons in the paravertebral ganglia of the cat (Kummer, 1987; Lindh et al., 1989), while a DYN like peptide has been observed in approximately 90% of the NPY containing neurons of the guinea pig SCG (Gibbins and Morris, 1987). CGRP has been identified in a population of human SGC and SG neurons (Schmitt et al., 1988). Finally, peptide YY(PYY) has been reported in 10-30% of rat SCG neurons where all PYY neurons expressed NPY (Happola et al., 1990).

ii.) Peptides in Non-adrenergic Cell Bodies

Whereas the peptides described above are mostly present in noradrenergic ganglion cell bodies, immunoreactivities to several other peptides including VIP and CGRP have been demonstrated in non-adrenergic postganglionic paravertebral perykarya of the guinea-pig (Heym et al., 1993; Kummer and Heym, 1991), rat (Landis and Fredieu, 1986; Morales et al., 1995), cat (Kummer and Heym, 1988; Lindh et al., 1987), and human (Schmitt et al., 1988) stellate ganglia. NOS like immunoreactivity was found in a population of TOH -immunoreactive negative neurons of the cat (Anderson et al., 1995) and guinea-pig (Hohler et al., 1995). ACh, VIP and CGRP have been shown to coexist in some postganglionic sympathetic neurons of the rat SCG (Morales et al., 1995).

B. Peptidergic Nerve Fiber Networks in Sympathetic Ganglia

Numerous neuropeptides have been identified in nerve fiber networks of sympathetic ganglia. In paravertebral ganglia immunoreactivity to SP (Hokfelt et al., 1977c; Dalsgaard et al., 1983; Heym et al., 1991; Heym et al., 1993), VIP (Hokfelt et al., 1977b; Heym et al., 1993), cholecystokinin (CCK) (Larsson and Rehfeld, 1979), CGRP (Hokfelt et al., 1977; Lee et al., 1985; Gibbins et al., 1987; Lee et al., 1985; Heym et al., 1993), BOM/GRP (Schultzberg, 1983), GAL (Elfin et al., 1994), and ENK (Schultzberg et al., 1979; Lindh et al., 1986; Heym et al., 1993), have been described in the SCG and the SG of the rat and guinea-pig. DYN-immunoreactive fibers have been observed in the guinea-pig SCG (Gibbins et al., 1987). Guinea-pig lumbar ganglia have been shown to contain immunoreactivities for SP, CGRP, NPY, VIP, DYN, met-ENK-Arg-Phe and leu-ENK (Heym et al., 1990; Asmar and Kummer, 1993). SOM and ENK immunoreactivity have been demonstrated in the lumbosacral ganglia of the cat (Lindh, 1992b). SP and CGRP immunoreactivities have been described in nerve fibers of the cat stellate ganglion (Kummer and Heym, 1988; Lindh et al., 1989) and in the pig SP, CGRP, SOM, VIP and NPY immunoreactive nerve fibers and axonal varscosities were observed (Majewski et al., 1991; Happola et al., 1993). VIP, ENK, SP and NPY-immunoreactive processes have been identified in the middle cervical and SG of the dog (Darvesh et al., 1987). NT immunoreactive fibers have been identified in the paravertebral ganglia of the cat and guinea-pig (Morales et al., 1993; Maher et al., 1994; Lundberg et al., 1982b; Reinecke et al., 1983).

C. Projections and Connectivity of Peptidergic Nerve Fibers In Sympathetic Ganglia

In recent years it has become apparent that the integration of incoming signals to ganglia appear to involve not only peptidergic input from spinal preganglionic fibers, but also from neuropeptide containing axon collaterals of dorsal root primary afferents, and from fibers of visceral origin. The origins of ganglionic nerve fiber networks innervating the sympathetic ganglion have been investigated using a combination of axonal tracing and nerve lesion experiments, in combination with immunohistochemistry.

i) Motor Afferents

The major source of the cholinergic fibers in the paravertebral ganglia are the preganglionic neurons whose cell bodies reside in the IML cell column of the spinal cord. In the mouse SCG, cholinergic innervation of postganglionic perykarya has identified ChAT-IR fibers which are heterogeneously distributed in the ganglia, with more ChAT-IR stained fibers in the cranial portions of the ganglia (Kasa et al., 1991). Immunoreactivities to numerous peptides have also been shown in the cell bodies of the IML cell column of the spinal cord. In many cases these peptides occur colocalized in a nuclei and may represent a source for many of the neuropeptide immunoreactive nerve fibers of the ganglia. In the IML cell column of the cat, cell bodies containing SP, ENK and SOM (Kurkoff, 1987; Kurkoff et al., 1985) have been identified. NT immunoreactivity has been observed in the IML perikarya of the guinea-pig and the cat (

Reinecke and Forssmann 1983; Kurkoff, 1987; Kurkoff et al., 1985). SP and CGRP immunoreactive preganglionic sympathetic neurons have been identified in the cat (Kurkoff et al., 1985), and rat (Senba and Tohyama, 1988; Yamamoto et al., 1989). In the guinea-pig SG, the ultrastructural appearance of SP and CGRP immunoreactive profiles is consistent with a preganglionic sympathetic origin (Heym et al., 1993). CGRP (Yamamoto et al., 1989) and VIP (Baldwin et al., 1991) immunoreactivities have been demonstrated in the preganglionic nerve fibers projecting to the rat SCG. NT immunoreactivity has been observed in the IML perikarya of the guinea-pig and the cat (Reinecke and Forssmann, 1983; Kurkoff, 1987; Kurkoff et al., 1985). Indirect immunofluorescent techniques revealed CHAT-immunoreactive fibers in the SG and SCG of the rat, which showed co-immunoreactivity for NOS, ENK and CGRP, and which disappeared following cervical sympathetic trunk transection. These results suggested that some preganglionic fibers of the SG and SCG are cholinergic and may release nitric oxide, ENK and CGRP (Morales et al., 1995).

ii) Sensory Afferents

Electrophysiological experiments and intracellular labeling performed in the dog and cat provided evidence for peripheral afferent inputs on postganglionic neurons of the SG. The perikarya of these afferent neurons may be located in sensory (dorsal root and vagal) ganglia, in the SG itself and in the mediastinum (Bosnjak and Kampine, 1982, 1985; Armour, 1987). In a study examining the intracardiac distribution of sympathetic afferent fibers, retrogradely-labeled cell bodies were observed in sections of the guinea-pig

SG and in afferent sensory fibers following injection of HRP into the lower cervical and upper thoracic dorsal root ganglia (Quigg et al., 1988, 1990). These findings suggested that synaptic contacts occur between axon collaterals of the primary afferent fibers transversing the SG and the postganglion neurons. In the guinea-pig SG, double labeling immunohisochemistry has revealed a population of SP and CGRP immunoreactive intraganglionic axon terminals (Heym et al., 1993). Since the coexistence of immunoreactivity to SP and CGRP is common in guinea-pig sensory neurons (Gibbins, 1987) this small but persistent population of fibers may originate from primary afferent neurons.

iii) Intraganglionic Fibers

Nerve fibers have been identified in sympathetic ganglia which possess TOH- and DBH-immunoreactivity. It has been proposed that these fibers may originate, in part, from noradrenergic or cholinergic postganglionic neurons. Ultrastructural analysis after intracellular application of HRP, has demonstrated dendrodendritic and dendrosomatic interconnections between postganglionic neurons in rat SCG (Kiraly et al., 1989; Kondo et al., 1980), findings which confirmed results obtained previously by three-dimensional reconstruction of electron micrographs of ultrathin serial sections of the cat SCG (Elfin, 1963). These findings have led researchers to propose that some of the nerve fibers containing neuropeptides such as NPY-, DYN-, SOM-, and ENK-immunoreactivities, originate from the noradrenergic neurons of sympathetic ganglia, and further that the non-

adrenergic neuron population in the ganglia may be the source of some of the fibers containing SP, CGRP and VIP.

6. Electrophysiological Properties of Autonomic Ganglion Neurons

Autonomic ganglion neurons integrate and encode efferent signals in the form of action potentials to a variety of specific receptor cells. Ion fluxes via ionic channels found in autonomic ganglion neurons give rise to action potentials, as well as, membrane potential. These ion channels have been divided into functional groups based upon their ionic selectivity, pharmacological profiles and biophysical properties. The electrophysiological studies summarized in this section provides a brief description of the types of voltage-gated and ligand-gated ion channels present in the somatic membranes of autonomic ganglion neurons, with emphasis on those found within the sympathetic nervous system.

A. Voltage-Gated Ionic Currents

i.) Sodium Currents (I_{Na})

All autonomic neurons are capable of generating action potentials and the fast voltage-gated sodium current which underlies the rising phase of the action potential has been characterized in mammalian sympathetic (Belluzzi and Sacchi 1986, Schofield and Ikeda, 1988; Carrier and Ikeda, 1992) parasympathetic (Xu and Adams, 1992b; Aibara et

al., 1992) and enteric (Franklin and Willard, 1993) neurons. In mammalian autonomic neurons, action potentials are abolished in the presence of low doses of the specific Na^+ channel blocker tetrodotoxin (TTX), or by the removal of extracellular Na^+. The Na^+ current described in sympathetic neurons is completely blocked by the addition of 3 μM TTX (Belluzzi and Sacchi, 1986), with no evidence of the existence of a TTX-resistant component of the kind described in the rat dorsal root ganglia (Kostyuk et al., 1981a) or the bull frog sympathetic ganglion (Jones, 1987b).

ii.) Calcium Currents (I_{Ca})

Calcium influx through voltage-gated Ca^{2+} channels contribute to the electrical excitability of neurons but this may also mediate other functions, such as neurotransmitter release, regulation of gene expression, or the further control of membrane excitability via regulation of Ca^{2+}-activated ion channels (Hille, 1992 and see below section 5. B iii). The presence of a voltage-dependent Ca^{2+} current capable of influencing the action potential time course in mammalian sympathetic neurons was first proposed by McAfee and Yarowsky (1979). Subsequently, depolarization-activated ionic currents carried by Ba^{2+}, Ca^{2+} or Sr^{2+} through Ca^{2+} channels have been quantitatively described in mammalian autonomic neurons by way of voltage clamp (Galvan and Adams, 1982; Freschi, 1983; Belluzzi et al., 1985b; Belluzzi and Sacchi, 1989) and patch clamp studies (Marrion et al., 1987; Hirning et al., 1988; Ikeda et al., 1992; Weight 1986; Schofield and Ikeda 1988; Plummer et al., 1989; Carrier and Ikeda, 1992; Frank and Willard, 1993; Suprenant et al.,

1990). Based upon their thresholds for activation, Ca^{2+} currents have traditionally been separated into two groups: low voltage-activated (LVA) currents (Ca^{2+} currents which activate upon membrane depolarizations to potentials around -60 mV) and high voltage-activated (HVA) currents (currents requiring membrane depolarization's to -30 mV for activation) (Carbone and Lux, 1984; Nowycky et al., 1985; Fedulova et al., 1985). Four classes of Ca^{2+} channels, termed T-, N-, L-, and P-type, have been reported in a variety of mammalian central and peripheral neurons (for review see Bean, 1989; Hess, 1990; Bertolino and Llians, 1992; Tsien et al., 1988; Mintz et al., 1992). These channel types have been characterized by their thresholds for activation, voltage-dependent activation and inactivation parameters, and pharmacological profiles (Nowycky et al., 1985; Adams and Harper, 1995).

iii.) Potassium Currents

Various K^+ conductances are present in the cell membranes of mammalian autonomic neurons (Adams and Harper, 1995). At least seven distinct voltage-dependent K^+ conductances have been identified: (1) a delayed rectifier outward potassium current (I_{Kv}), (2) a transient outward potassium current (A-current, I_A), (3) three Ca^{2+}-dependent potassium currents (I_{KCa}, and two distinct I_{AHPs}) (4) a muscarine-sensitive outward potassium current (I_M) and (5) an inward rectifying potassium current (I_{Ki}).

The delayed rectifier in mammalian autonomic neurons has been proposed to contribute to the time courses of both repolarization of the AP and AHP (Marsh and Brown 1991). The kinetics of the delayed rectifier K^+ current (I_K) in mammalian neurons

resemble those of the classical delayed rectifier which was first identified in squid axon (Hodgkin and Huxley, 1952a,b). Presently, two criteria are employed in the identification of a membrane current as a delayed rectifier: 1) the macroscopic current must be similar in overall kinetic behavior and voltage-dependence to the delayed rectifier described by Hodgkin and Huxley, and 2) the current must not be activated by a rise in intracellular Ca^{2+} (Rudy, 1988). The kinetics and voltage dependence of I_{Kv} have been described in mammalian enteric neurons (Nishi and North, 1973) and sympathetic and parasympathetic neurons (Freschi, 1983; Galvan and Sedlmeir, 1984; Belluzzi et al., 1985b; Belluzzi and Sacchi, 1988, 1991; Schofield and Ikeda, 1989; Xu and Adams, 1992a). I_{Kv} in mammalian sympathetic neurons has been shown to possess a voltage dependence, slight or absent inactivation and TEA sensitivity similar to the Hodgkin-Huxley described delayed rectifier.

The A-current, or I_A, is a relatively fast, transient outward K^+ current. This current was originally described in molluscan neurons by Hagiwara et al. (1961) and was subsequently characterized by Connors and Stevens (1971a) and Neher (1971). The distinguishing properties of this current are: complete steady state inactivation close to the resting membrane potential; large removal of inactivation with modest membrane hyperpolarization; and activation with subsequent exponential inactivation following depolarization. Constanti and Brown (1981) and Galvan and Adams (1982) were among the first to report the presence of the I_A current in mammalian sympathetic neurons and gave preliminary descriptions of the I_A current. The properties of the A-current have subsequently been described in detail in voltage clamp (Kostyuk et al., 1981b; Freschi 1983; Galvan and Sedlmeir, 1984; Segal et al., 1984; Belluzzi et al. 1985a; Nerbonne and

Gurney, 1989; Green et al., 1990) and patch clamp studies (Cooper and Shrier, 1985,1989; Kasai et al., 1986; Schofield and Ikeda, 1989; Marrion et al., 1987) from a variety of mammalian neurons. In spite of the large number of studies devoted to the description of the A-current, there is still no unified concept for its functional role. A number of physiological mechanisms have been proposed for A-current in mammalian sympathetic neurons including: modulating synaptic potentials (Cassell and McLachlan, 1986); providing a major driving force for repolarization following an AP (Galvan and Sedlmeir, 1984; Belluzzi and Sacchi, 1991), an influence which is critically dependent upon the cells resting potential (Belluzzi and Sacchi, 1988); and in the regulation of low-frequency repetitive discharge in mammalian sympathetic neurons (Galvan, 1982; Freschi, 1983; Galvan and Sedlmeir, 1984; Belluzzi et al., 1985a; Cassell et al., 1986; Schofield and Ikeda, 1989; Rogers et al., 1990). One reason for this variety of proposed mechanisms may reflect differing modalities and substrates used in the determination of A-current time course and conductance.

The currents described above, "delayed rectifiers" and "A -type" are the result of ion movement through voltage-dependent channels, that is channels which are gated by membrane potential. However, in a third class of K^+ channels, the Ca^{2+}-dependent K^+ channels, channel opening and closing depends upon cytoplasmic Ca^{2+} activity, such that an increase in intracellular Ca^{2+} concentration leads to channel opening. Calcium-dependent K^+ conductances (Gardos, 1958; Meech and Standen, 1975) are consistently found in excitable cells (Blatz and Magleby, 1987) and single-channel studies have identified several types of Ca^{2+} -activated K^+ channels associated with neuronal

membranes. Calcium-dependent K^+ channels have been extensively analyzed in mammalian neurons (for a review, see Latorre et al., 1989; Sah, 1996) and two distinct families of K^+ channels (BK) (Marty, 1981) and (SK) (Blatz and Magleby, 1986) have been identified based upon their biophysical and pharmacological properties. BK channels are voltage-sensitive, possess a high unitary conductance, and are blocked by submillimolar concentrations of TEA (Marty, 1989; Blatz and Magleby, 1987). At membrane potentials near the RMP (-50 to -70 mV) these channels have been shown to require high concentrations of Ca^{+2} (1-10 μM) for activation (Sah, 1996). The macroscopic current corresponding to activation of BK channels has been identified in bullfrog ganglion neurons and has been named I_C (Adams et al., 1982; Pennefather et al., 1985). Functionally, this current contributes to action potential repolarization and the fast hyperpolarization (Adams et al., 1982; Lancaster and Nicoll, 1987; Sah and McLachlan, 1992; Storm, 1987). SK channels are voltage-insensitive, possess a lower single channel conductance, and are insensitive to TEA (Lang and Ritchie, 1990; Park, 1994). In neurons, SK channels are believed to underlie, at least in part, the AHP following Ca^{+2} influx during APs (Hille, 1992).

The M-current (I_M) is a muscarine-sensitive, slowly activating, non-inactivating K^+ current which was first characterized in bull-frog lumbar sympathetic neurons (Brown and Adams, 1980; Constanti and Brown, 1981; Adams et al., 1982ab) and then in the rat sympathetic neuron (Brown and Constanti, 1980). In mammalian neurons, the M-current exhibits sustained activation in the voltage range between the RMP and the threshold for AP firing (-70 mV to -30 mV). The M-current does not inactivate, therefore once

activated it functions to hyperpolarize the membrane back towards rest and thereby reduces membrane excitability (Adams et al., 1982a). As it is the only K^+ current tonically active at potentials near threshold it has a major impact on neuronal excitability. The M-current has been investigated in cultured sympathetic neurons of the rat (Brown et al., 1982; Freschi, 1983; Galvan and Sedlmeir, 1984; Brown and Selyanko, 1985, Rogers et al., 1990), rabbit (Hashiguchi et al., 1982) and in neurons of the inferior mesenteric ganglion in guinea-pig (Brown, 1988). Suppression of the M-current by muscarine provided the first clear example of neuromodulation of a K^+ current in vertebrate nerve cells (Brown and Adams, 1980). Suppression of the M-current depolarizes the membrane potential and increases the input resistance of the cell thereby increasing the likelihood that the neuron will fire an action potential. M-current can be inhibited by: ACh, other muscarinic receptor agonists including bethanechol, various neuropeptides and Ba^{2+} (Adams et al., 1982; Selyanko et al., 1990; Brown and Selyanko, 1985; Rogers et al., 1990; Cassell et al., 1986; Cassell and McLachlan, 1987b; Bley and Tsien, 1990). This current is insensitive to TEA, apamin and tubocurarine (a blocker of nicotinic AChRs of skeletal muscle). Synaptic regulation and synaptic suppression of M-current underlies a slow excitatory post synaptic potential (EPSP) described in both rat (Brown 1988) and frog (Adams and Brown, 1982) sympathetic neurons. M-channels have also been suggested to play a role in the pattern of action potential firing in autonomic ganglion neurons. Voltage clamp studies in guinea-pig sympathetic ganglion cells have revealed that the M-current is insignificant in neurons which fire tonically, but not in phasic neurons (Cassell et al., 1986). Furthermore, in numerous excitable cells phasic type neurons can be

induced to switch to tonic firing in the presence of muscarinic agonist, such as bethanechol or neuropeptides including Substance P (Vanner et al., 1993, and see Hille, 1992; Jones and Adams, 1987; Madison and Nichol, 1984; Tokimasa and Akasu, 1992).

Inward rectifying K^+ currents (I_{ki}) are anomalous in that their conductance increases under hyperpolarization and decreases under depolarization. These channels do permit a restricted outward K^+ current, and indeed this is their usual physiological function, since rarely does the membrane potential of the neuron become more negative than E_K (Hille, 1992). Through their conduction of outward current at voltages up to a few millivolts positive to E_K, these channels maintain resting membrane potentials near the E_K. Under voltage clamp conditions, inward rectifier K^+ currents have been described in a variety of autonomic neurons including mammalian sympathetic (Freschi, 1983; Cassell et al., 1986) and enteric neurons (Surprenant and North, 1988; Akasu and Tokimasa, 1989; Galligan et al., 1989).

B. Action Potentials

The work of Hodgins and Huxley (1952b) on the squid giant axon provided a quantitative two-channel model for the ionic mechanisms underlying the action potential and more generally for predicting membrane excitability. Subsequent studies have described the membrane potential change during an action potential spike in terms of time dependent change in the Na^+ and K^+ conductances. Since the initial work of Hodgkin and Huxley, investigations of the neuron cell body have shown that the complement of ion channels specific to any given neuron accounts for the properties of its somatic membrane,

including AP, afterhyperpolarization (AHP), repetitive firing, accommodation (adaptation) and pacemaker activity.

i.) AP Characteristics

Autonomic ganglion neurons display a variety of action potential and after-potential characteristics. Electrical stimulation of post- or preganglionic axons evoke antidromic or orthodromic action potentials, respectively, in the cell soma. Somatic action potentials can also be elicited by intracellular depolarizing current pulses. In mammalian autonomic neurons intrasomally-evoked action potentials have been characterized by their threshold potential, action potential amplitude and overshoot, duration, maximal rate of rise (dV/dt), amplitude and duration of the AHP of the action potential. The amplitude of the action potential mainly reflects primarily the equilibrium potential of the ion (Na^+) which carries the bulk of the inward, depolarizing current. Differences in action potential amplitude may be related to contributions from different minority charge carriers which accompany the main ion (ie. Ca^{2+}), although repolarizing conductances with fast onsets may also limit action potential amplitude (Surprenant 1984). Action potential duration is dependent upon the kinetics of activation and inactivation of the contributing ionic conductance's.

ii.) Properties of the AHP

Somatic action potentials in most mammalian autonomic neurons are followed by a phase of marked hyperpolarization, which can last up to several seconds. This hyperpolarizing potential has several phases, each resulting from the activation of different

types of K^+ currents. Immediately following the action potential, there is a fast hyperpolarization, which typically lasts 1-10 ms and is primarily due to the activation of voltage-gated K^+ currents. In some neurons a fast Ca^{2+}-activated K^+ current (I_C) contributes to this fast hyperpolarization (Sah, 1996, Marsh and Brown, 1991). Following the fast hyperpolarization, there may be a prolonged hyperpolarization phase, which can last from hundreds of milliseconds to several seconds. This phase is believed to be mediated by Ca^{2+}-dependent K^+ conductances which are secondary to Ca^{2+} entering the cell during the action potential, and has been termed the afterhyperpolarization (AHP). The reversal potential of the AHP is usually close to, or slightly more positive than the calculated equilibrium potential (E_K) for K^+ indicating that this phase is due to a prolonged increase in K^+ conductance (Holman and Hirst, 1977; McAfee and Yarowsky, 1979).

Voltage-clamp studies in autonomic neurons have revealed that the calcium-activated K^+ currents which underlie the AHP can be divided into two distinct types. One of these currents, termed I_{AHP} (also called medium AHP, $I_{K.Ca1}$, $I_{K.Cas}$) has been described in guinea-pig coelic ganglion neurons as possessing a relatively fast onset (<10 ms), attaining maximal amplitude immediately after the action potential and decaying exponentially with a time constant of about 130 ms (Cassell and McLachlan, 1987a). This I_{AHP} has been described in the sympathetic neurons of the rat (Gurney et al., 1987; Kawai and Watanabe, 1986; Belluzzi and Sacchi, 1990), the bullfrog (Pennefather et al., 1985; Tanaka and Kuba, 1987), and the guinea-pig (Jobling et al., 1993), where it has been shown to be voltage-insensitive and blocked by the bee venom, apamin. A second, slow-activating Ca^{+2}-activated K^+ current, (which is often not seen after single action potentials) has also been identified and is referred to as slow AHP or I_{KCa2}. This current has been

described as rising to a peak amplitude with a time constant of several hundred milliseconds and decaying with a time constant of 1-2 seconds (Sah and McLachlan, 1991; Cassell and McLachlan, 1987a). This current has been found in some sympathetic neurons (Cassell et al., 1986; Cassell and McLachlan, 1987a; Jobling et al., 1993) where it produces a long-duration afterhyperpolarization (LAH). This current is also voltage-insensitive but unlike I_{KCa1} it is not blocked by apamin. I_{KCa2} can be blocked by ryanodine and other agents which inhibit Ca^{2+} mobilization from intracellular stores. Physiologically the AHP has been suggested to play a role in limiting the firing frequency of neurons, and in generating spike frequency adaptation (Sah, 1996; but see Cassell et al., 1986).

iii.) Firing Patterns of Autonomic Neurons

One scheme for the differentiating among different physiological types of autonomic neurons is based upon the firing response of the somatic membrane to long intracellular depolarizing current pulses. Neurons are typically classified as being tonic (slowly adapting), phasic (rapidly adapting) or long afterhyperpolarizing (LAH). The term "adapting" refers to a decline in the frequency of action potentials during maintained depolarization (Adams and Harper, 1995). Neurons classified as LAH exhibit prolonged after-hyperpolarization and fired only a single action potential, at the start of the depolarizing current, even at very high intensity (Cassell and McLachlan, 1987a; McLachlan and Meckler, 1989). Mechanisms underlying the adaptation of discharge may be inherent in properties of the soma membrane or of the impulse initiation zone. Several membrane ionic currents have been suggested to play a role in the regulation of repetitive

activity and adaptation in autonomic ganglion neurons. These ionic currents have been characterized in autonomic neurons, and K^+ channels appear to be the main players. The transient voltage-dependent K^+ current (I_A), Ca^{2+} -dependent K^+ currents ($I_{K.Ca}$) and a muscarine-sensitive K^+ current, (I_M), have all been suggested to play a role in the firing patterns of autonomic neurons. The regulation of action potential discharge by I_A has been best evaluated in a study of the membrane currents underlying the firing behavior of sympathetic ganglion neurons of the caudal lumbar sympathetic chain (phasic firing neurons) and the mesenteric ganglia (tonic firing neurons) of the guinea-pig (Cassell et al., 1986). Suprathreshold current pulses of long duration evoked only a transient burst of APs in most lumbar sympathetic neurons (phasic) but produced repetitive discharge for the duration of the stimulus pulse in inferior mesenteric ganglion neurons (tonic). It was concluded that these properties defined two functionally discrete neurons. Examination of the kinetic and voltage-dependent properties of the I_A currents in tonic neurons revealed that the I_A current could be evoked from the resting membrane potential, while in phasic neurons activation of I_A required a conditioning hyperpolarization. An examination of the voltage dependence of inactivation for these two neuron types subsequently revealed that while the shape of the inactivation phase was essentially the same, the midpoint for inactivation was more depolarized in tonic neurons. This suggests that in the tonic neurons of the inferior mesenteric ganglion a substantial fraction of the I_A channels are available for activation at the resting membrane potential whereas in phasic neurons I_A channels are effectively inactivated. Examinations of isolated intact rat and cultured guinea-pig SG neurons have shown that the majority of neurons fire phasically, with a small proportion

firing tonically (Mo et al., 1994; Horackova et al., 1993). Similar trends have been observed in autonomic neurons from other parts of the body (Adams and Harper, 1995).

iv.) Mechanism of Action Potential Generation in Sympathetic Ganglion Neurons

Belluzzi and Sacchi (1991) have developed a quantitative model for the generation of the action potential in rat SCG neurons which incorporates the known properties of the ionic currents described above. A prominent feature of this model is that SCG neurons do not have a unique stereotyped mode of functioning but instead can adopt several alternative strategies of activity. These authors have shown that rat SCG neurons are outfitted with a number of conductances, from which they can, as a function of the actual membrane potential, make a weighted selection of the elements which will determine its electrical behavior. The salient features of this model, which predicts the shape of the action potential, are as follows: 1) the rising phase of the action potential is generated by the activation of I_{Na}; 2) during the latter part of the spike potential Ca^{2+} channels start to activate and Ca^{2+} load begins, the degree to which Ca^{2+} channels activate depending on the RMP and level of I_A deinactivation; 3) further Ca^{2+} influx, activation of I_{KCa}, I_A and possibly I_{Kv} (but see Marsh and Brown, 1991) leading to repolarization of the spike potential; 4) accumulation of cytoplasmic Ca^{2+} leading to activation of I_{AHP}; 5) the afterhyperpolarization abates as the I_{AHP} slowly turns off, with intracellular Ca^{2+} levels returning to normal. In summary, the model of Belluzzi and Sacchi (1991) suggests that different sets of conductances could be responsible for action potential generation. These would be determined by the RMP from which the AP is generated, or perhaps during

trains of action potentials when the channels would be susceptible to different potentials and intracellular Ca^{2+} concentrations.

C. Ligand-Gated Ionic Currents

i.) Nicotinic Postsynaptic Receptor Currents in Autonomic Ganglia

The nicotinic postsynaptic acetylcholine receptor (nAChR) is a protein complex integrating an ACh binding site and an ionic channel on one molecule (directly gated or ionotropic receptor). The role of nAChRs in ganglionic transmission was initially demonstrated by Langley et al., (Langley and Dickinson, 1889; Langley and Anderson, 1892). These authors demonstrated that autonomic ganglia were first activated and then blocked by nicotine, and that ACh is a transmitter in autonomic ganglia (Feldberg and Gaddum, 1934, see Skok, 1973). Currently it is known that nAChRs are present throughout the nervous system (e.g. on all autonomic ganglia studied so far, on the adrenal medulla and in the central nervous system), where they play crucial roles in brain and body function (reviewed in Changeux et al., 1992; Lucas and Bencherif, 1992; Sargent, 1993; Lukas, 1995). In mammalian sympathetic ganglia, stimulation of preganglionic fibers elicits a depolarization of the postganglionic membrane potential, termed the fast excitatory post-synaptic potential (f-EPSP). This f-EPSP has been shown to be generated by the interaction of endogenous ACh (released from preganglionic nerve terminals), with the nicotinic-sensitive nAChR located on the postsynaptic membrane. Underlying the f-EPSP is a fast excitatory postsynaptic cation-selective ionic current (f-EPSC), which flows through the channel of the nAChR complexs in response to

activation by ACh. The f-EPSC has been recorded under voltage-clamp conditions where it exhibits shorter growth and decay kinetics than the f-EPSP. When of sufficient strength the nicotinic f-EPSCs depolarizes the cell membrane (the f-EPSP) to a potential which exceeds the threshold for AP generation, resulting in AP discharge, which is the primary mechanism for the transfer of information through the autonomic ganglia. Typically, mammalian sympathetic ganglion cells receive multiple cholinergic synaptic inputs whose activation evokes multiple f-EPSP, which then trigger an AP. The majority of these neurons receive at least one synaptic input which is of sufficient strength to evoke an action potential, along with several weaker inputs which produce subthreshold f-EPSP's (Skok and Ivanov, 1983; Hirst and McLachlan, 1986; Selyanko et al., 1979). The characteristics of the nicotinic postsynaptic responses (f-EPSP and f-EPSC) in autonomic neurons have received much attention. Intracellular and voltage-clamp studies have clearly demonstrated that the activation of nAChRs in mammalian autonomic neurons induces the opening of an intrinsic ion channel. Most neuronal nAChR channels are cation-specific, but do not distinguish readily among cations (Fieber and Adams, 1991; Mulle and Changeux, 1990; Nutter and Adams, 1991). In mammalian sympathetic neurons, activation of nAChR's under physiological conditions leads to an influx of Na^+ and Ca^{2+} ions and an efflux of K^+ ions (the net result being an excitatory post synaptic current, EPSC) and membrane depolarization (or f-EPSP) (Derkach et al., 1983; Colquhoun et al., 1987; Marrion et al., 1987; Mathie et al., 1990; Selyanko, 1995; Sargent, 1993). Although the importance of the nAChR in ganglionic transmission has been recognized for well over 100 years, our current understanding of the ganglionic

nAChR channel is largely based upon data collected in the past 20 years as a result of the introduction of patch clamp recording techniques.

7. Neuromodulatory Role of Neuropeptides on Ganglionic Transmission

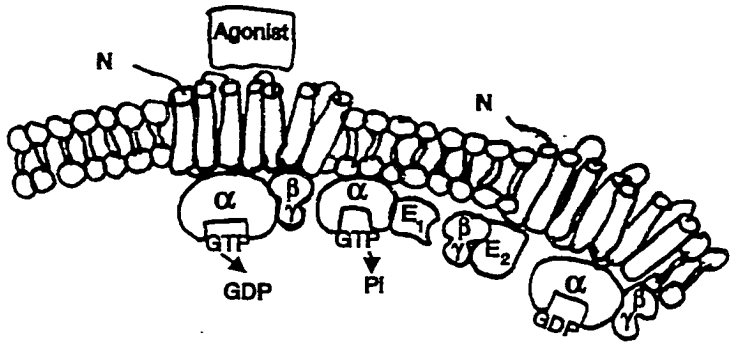
The excitability of peripheral neurons is regulated by both voltage- and ligand-gated ion channels. Neurotransmitters and neuropeptides control the tone of our autonomic nervous system by G-protein-dependent modulation of these channels. In mammals several neuropeptides, acting through G-protein coupled receptors, have been shown to be capable of regulating the electrical excitability of autonomic neurons by their modulations of voltage dependence, speed of gating, or the probability of opening of various ion channels. Furthermore, each neuron expresses a variety of cell surface receptors, and several intracellular signaling pathways are involved in modulating ion channel function.

A. Ion Channel Regulation by G Proteins

The major factors involved in the neuronal response to changes in their environment are receptors, coupling elements and effector proteins. In eukaryotic cells, heterotrimeric GTP-binding proteins (G proteins) directly couple receptors for hundreds of diverse ligands (see Wickman and Clapham, 1995) to intracellular effectors. Described in simple terms, a stimulus (change in the environment) activates a cell surface receptor protein to activate a G-protein (a coupling element), which in turn activates an effector protein (E). In the case of G-protein-coupled receptors, which modulate ion channels, the

effector protein may be the ion channel itself, or any of a variety of second enzymes including adenylate cyclase, phospholipase C, cyclic AMP, cyclic GMP, inositol triphosphate (IP_3), or diacylglycerol (DAG) the activation of which subsequently modifies ion channel function. A simplified description of a receptor mediated G-protein signal transduction system is illustrated in Figure 1-2 (for review see; Lamb and Pugh, 1992; Wickman and Clapham, 1995; Clapham, 1996; Hamm, 1998) . The G-protein-coupled receptors belong to a superfamily of rhodopsin-like transmembrane proteins for which more than 100 distinct plasma membrane receptors have been described (reviewed in Hedin et al., 1993; Iismaa and Shine, 1992; Kobilka, 1992; Speigel et al., 1992). In each case these receptors have a common three dimensional conformation with seven transmembrane spanning segments, an extracellular N-terminus and a intracellular C-terminus. Intracellular loops that connect the seven transmembrane spanning segments form the G-protein-binding domain (Hamm, 1998; Unger et al., 1997; Baldwin et al.,

FIGURE 1-2: An overview of G-protein signaling. α, α-subunit; β, β-subunit; γ, γ-subunit, N, N-terminus of receptor; E_1, E_1 effectors; E_2, E_2 effectors.



E_1 Effectors	E_2 Effectors
Adenylate cyclase	Adenlyate cyclase
Phospholipase C-β	Phospholipase C-β
Calcium channels	β-Adrenergic Receptor Kinase
cyclic GMP-PDE	

FIGURE 1-2

1997). Heterotrimeric G-proteins transduce ligand binding to these receptors to intracellular responses. G-proteins are made up of α, β and γ subunits and although there are many gene products encoding each subunit, four main classes of G-proteins can be distinguished. These are: G_s , which activates adenylate cyclase; G_i, which inhibits adenylate cyclase; G_q ,which activates phospholipase C; and G_{12} and G_{13} whose function remains unclear. To date, G proteins have been shown to link more than 100 distinct plasma membrane receptors to intracellular effectors. All of the "classical" G-proteins appear to function in a similar manner. G-protein α subunits (located on the cytoplasmic side of the plasma membrane) bind guanine nucleotides (GTP, GDP) with high affinity and hydrolyze bound GTP to GDP. In the absence of receptor activation, Gα-GDP and G$\beta\gamma$ coexist as a tight heterotrimeric complex, which is the preferential substrate for ligand bound receptors. G-proteins are activated when agonist-bound receptors promote a receptor-catalyzed guanine nucleotide exchange resulting in GTP binding to the α subunit. GTP binding leads to the dissociation of the heterotrimer into active Gα-GTP and G$\beta\gamma$ subunits which in turn activate of down-stream effectors (enzymes, regulatory proteins, and ion channels). G-protein deactivation is the rate-limiting step for inhibiting effector activity and occurs when the Gα subunit hydrolyzes GTP to GDP.

The delineation of components linking receptors, G-proteins, and ion channels has received much attention in the past 15 years. To date, two types of channel modulation have been described; 1) G-protein-dependent modulation of ion channels involving diffusable second messengers; and 2) membrane-delimited G-protein modulation of ion channels. In the first, activated G-proteins modulate a variety of effectors, including

those controlling the levels of cytosolic messengers such as Ca^{2+} and cyclic nucleotides. In many cases, Ca^{2+} and cyclic nucleotides have been shown to directly modulate ion channel activity (Hille, 1992; North, 1989). In other instances multiple messengers may be required for channel modification. For example, channel modulation by protein kinase mediated phosphorylation is fairly ubiquitous, and G-proteins modulate the major classes of serine/threonine protein kinases by controlling levels of Ca^{2+} (Ca^{2+}/ calmodulin-dependent kinases), cyclic nucleotides (cyclic AMP and cyclic GMP-dependent kinases), and diacylglycerol (PKC). Figure 1-2 illustrates modulation of selected second messengers by G proteins (for review see Wickman and Clapham, 1995). Second, several lines of evidence now suggest that the link between G-protein activation and modulation of some types of ion channels may also be a direct one, rather than acting through a downstream water soluble intracellular messenger (reviewed in Hille, 1992). This type of G-protein modulation of ion channels has been termed " membrane delimited" (Brown, 1990; Brown and Birnbaumer, 1988). Since G-proteins and ion channels are membrane-associated the simplest and most frequently used explanation for this phenomenon has been a direct association between the ion channel and a functional G-protein subunit ($G\alpha$-GTP or $G\beta\gamma$). This explanation has not gone unchallenged, however, as receptors, G-proteins and ion channels comprise only a small percentage of the total plasma membrane protein and the list of G-protein effectors is growing steadily (Clapham and Neher, 1993). For example, the finding that some ion channels are modulated by lipid soluble second messengers such as arachidonic acid emphasizes that membrane-delimited channel modulation does not prove a direct G-protein-ion channel interaction. A direct physical

and functional interaction between ion channels and G-proteins has not yet been demonstrated unequivocally.

B. Modulation of Ion Channels by G-protein-coupled Neuropeptide Receptors

i) Modulatory Effects of Neuropeptides on Voltage-Gated Ion Channels

A variety of neuropeptides have been shown to modulate neuronal excitability by affecting the function of voltage-gated ion channels. In particular, certain ion channels such as voltage-dependent Ca^{2+} and K^{+} channels are reciprocally modulated by excitatory and inhibitory neuropeptides. Through the activation of their respected receptors, neuropeptides have been shown to affect the voltage-dependence, the speed of gating and the probability of opening of various ion channels. Each neuron expresses many kinds of receptors and uses several intracellular signaling pathways to modulate channel function in different ways (Hille, 1994).

One example of a voltage-gated ion channel which has been shown to be modulated by neuropeptides is the ω-conotoxin sensitive N-type Ca^{2+} channel. N-type Ca^{+2} channels are the dominant type of Ca^{2+} channels in sympathetic neurons (Plummer et al., 1989; Regan et al., 1991; Schofield and Ikeda, 1988). The inhibition of N-type Ca^{2+} currents by neurotransmitters has received a great deal of attention in recent years due to their importance in the regulation of transmitter release and neuronal excitability. In rat sympathetic neurons a variety of agents including ACh (through muscarine sensitive receptors, Beech et al., 1992), angiotensin II (AII, Shapiro et al., 1994), SOM (Shapiro and Hille, 1993; Beech et al., 1992; Ikeda and Schofield, 1989) noradrenaline

FIGURE 1-3: Inhibitory modulatory pathways converging on N-type Ca^{+2} channels in rat SCG neurons. Abbreviations: AII, angiotensin II; SP, substance P; PP, Pancreatic polypeptide; $M_{1\&4}$, muscarinic receptor agonists; VIP, vasoactive intestinal peptide; SS, somatostatin; $alpha_2$, $alpha_2$ adrenoreceptors; G_{nonPTX}, PTX-insensitive G-protein. Adapted from Hille 1994.

FIGURE 1-3

(measured by whole-cell patch clamp) via interaction with NK_1 tachykinin receptors. Intracellular dialysis with 2 mM GDP-β-S attenuated inhibition of the Ca^{2+} channels by SP, implicating G-protein involvement in SPs modulatory actions. Pertussis toxin (PTX) which has been shown to inactivate G-proteins of the G_i, G_z, and G_o classes by catalyzing nicotinamide-adenine dinucleotide-dependent ADP ribosylation of the α subunit (Milligan 1988) did not block SP inhibition, suggesting that SPs actions were not mediated by these classes of G-proteins. In cell-attached patch configuration inhibition of Ca^{2+} current by SP was significantly reduced, suggesting the involvement of a membrane delimited pathway (Shapiro and Hille, 1993).

A second neuropeptide implicated in the regulation of N-type Ca^{2+} channels is CGRP. CGRP is a thirty-seven amino acid peptide which contains a 2,7-disulfide bridge and a carboxy terminal amide linkage. As described above CGRP has been shown to be distributed widely in the central and peripheral nervous systems and has been identified immunohistochemically in the preganglionic neurons innervating many mammalian paravertebral ganglia. In acutely dissociated rat nodose ganglion neurons CGRP increased HVA Ca^{+2} currents (most likely of the N-type) via a pertussis toxin-sensitive pathway (Wiley et al., 1992). In cultured rat neocortical neurons CGRP attenuated HVA Ca^{+2} currents possibly via a cyclic AMP second messenger system (Zona et al., 1992). In rat paracervical ganglion CGRP both increased and decreased the Ca^{+2} current in separate cell populations (Cohen et al., 1996). CGRP inhibited Ca^{+2} currents in rat neocortical neurons, a result which was similar to the inhibition of Ca^{+2} currents by cyclic AMP observed in rat sympathetic neurons (Horn and McAfee, 1980). It is clear that CGRP

affects cell function differently in different neuronal populations. This differential modulation of Ca^{+2} currents by second messenger systems may reflect differences in the biochemical neuronal machinery involved in channel protein regulation or in the phosphorylation sites of the channel proteins (Sumikawa and Miledi, 1989).

ii) Mechanisms Underlying the Modulatory Effects of Neuropeptides on the nAChR Ligand-Gated Ion Channel

Over the past 30 years, more than 40 neuropeptides have been identified in mammalian sympathetic nervous tissue. The further demonstration that many of these peptides coexist in neural elements with classical neurotransmitters underscores the enormous potential for diversity of chemical signaling in the nervous system, particularly since the possible modulatory mechanisms thus far elucidated for various peptides are quite diverse. Perhaps most interesting, however, in light of the potential neuropeptide-transmitter coexistence, are the modulatory actions of peptides on classical neurotransmission. For example, several neuropeptides have been shown to act on ligand-gated ion channels such as the nAChR of autonomic ganglia. During the past 15 years a variety of neuropeptides including; serotonin (Vijayaraghavan et al., 1993); NPY (Zheng et al., 1995); SP (Akasu et al., 1983; Role, 1984; Neher and Clapham, 1984; Simmons et al., 1990; Valenta et al., 1993; Lyford et al., 1990; Min and Weiland, 1992; Lukas and Eisenhour, 1996); and SOM (Inoue and Kuriyama, 1991) have been shown to be capable of influencing the cholinergic-nAChR responses of autonomic neurons.

Of all the neuropeptides shown to modulate the cholinergic-nAChR response, the tachykinins have received the most attention and several lines of evidence suggest that responses in a variety of tissues from different species are particularly sensitive to the effects of SP. The first description of the modulatory actions of SP on the nAChR was made at the Mauthner fiber-giant fiber synapse of the hatchet fish (Steinacker and Highstein, 1976). Since then, the inhibition of cholinergic-nAChR responses by SP have been reported in: neuronal tissue (Akasu et al., 1983; Belcher and Ryall, 1977; Boyd and Leeman, 1987; Clapham and Neher, 1984; Livett et al., 1979; Simasko et al., 1985; Stallcup and Patrick, 1980; Valenta et al., 1993); skeletal muscle (Simasko et al., 1985); and Torpedo electroplaque (Min and Weiland, 1992). In these reports, SP inhibited nAChR responses with low affinity (micromolar IC_{50} values) and it was proposed that SP acts as a desensitizing noncompetitive antagonist of ACh, interacting with the nAChR at a unique binding site whose structural specificity was distinct from that of the NK_1-type receptor (Boyd and Leeman, 1987; Simasko et al., 1985; Min and Weiland, 1992). The idea that a direct SP- nAChR interaction underlies the effects of SP on cholinergic nAChR responses has not gone unchallenged however. Other studies have indicated that SP can alter this response at picomolar or nanomolar concentrations (Hanley et al., 1980; Shinkai et al., 1993). Such studies suggests that nAChR responses may be altered indirectly following activation of intracellular signaling cascades and may be triggered by SP effects at tachykinin receptors (Hanley et al., 1980; Beaujouan et al., 1982; Torrens et al., 1989). This concept has been supported by studies which examined the effects of SP on single nAChR channel currents. Using the cell-attached patch configuration of patch-clamp,

micromolar concentrations of SP applied to the extra-patch membrane were shown to modulate nAChR single channel function (Simmons et al., 1990). These experiments demonstrated that SP may modulate neuronal AChR function by a second messenger mechanism.

Several laboratories have investigated potential mechanisms underlying the inhibition of nAChR responses by SP. Patch-clamp technology has demonstrated that SP promotes desensitization of some nAChR channel subtypes: bovine chromaffin cell, (Clapham and Neher, 1984); avian sympathetic neurons, (Downing et al., 1987; Role 1984; Simmons et al., 1990; Valenta et al., 1993); and PC12 cell ganglionic nAChR, (Boyd and Leeman, 1987; Simasko et al., 1985). In isolated bovine chromaffin cells, Clapham and Neher (1984) showed that when SP (20 μM) was applied simultaneously with ACh, it induced an apparent acceleration of the ACh-induced nAChR current desensitization. Single channel recordings in the same preparation demonstrated that SP acts to shorten the ACh-induced mean open time of single nAChR channels but did not alter single channel conductance. In the avian sympathetic neuron it has been demonstrated that SP accelerates the rate of desensitization of whole-cell ACh-induced nAChR currents (Role, 1984) while at the single channel level it decreases both ACh-induced channel opening frequency and mean open time kinetics (Simmons et al., 1990). Furthermore, as described above, SPs actions on nAChR function may be mediated by a second messenger mechanism, since SPs effects on single channel kinetics were observed in cell-attached patch mode with SP applied to the extra-patch membrane (Simmons et al., 1990). Additional studies, have demonstrated that nAChRs can be directly

phosphorylated by protein kinases A and C and by tyrosine kinases (Miles and Huganir, 1988) and that receptor phosphorylation accelerates the rate of nAChR desensitization (Huganir et al., 1988; Downing and Role, 1987).

Because SP has been shown to be present in, and released from avian sympathetic ganglia, and in light of the fact that SP inhibits synaptic activation and transmitter release from sympathetic neurons *in vitro*, it has been proposed that nAChR modulation by SP may regulate autonomic function *in vivo* (Valenta et al., 1993).

8. Rationale and Specific Objectives:

The neurons of the SG play an important role in the sympathetic control of both the heart and the vasomotor, pilomotor and sudomotor functions. In spite of the fact that the SG-MCG complex is the principal source of efferent sympathetic innervation of the heart little is known of the electrophysiological properties of SG neurons or the voltage- and ligand-gated ion channels which determine membrane potential and action potential firing patterns. A thorough analysis of these factors is essential to our understanding of the process of sympathetic transmission through these ganglia. Therefore, the first objective of this thesis was to identify and characterize the various types of ionic currents present in the postganglionic somata of cultured mammalian SG neurons.

A variety of neuropeptides have been shown to affect neuronal excitability by modulating the function of voltage- and ligand-gated ion channels as discussed above. Terminals in the mammalian SG have been shown to possess a variety of neuropeptides including SP and CGRP. In light of the presence of SP and CGRP in the SG, and the proposed roles played by these neuropeptides as neuromodulators of ion channel function, the second objective of this thesis was to investigate the potential neuromodulatory actions of SP and CGRP on the ionic currents and action potential properties of SG neurons.

MATERIALS AND METHODS

1. Materials

A. Materials for Stellate Ganglion Dissection and Neuronal Culture

Adult guinea-pigs (250-300 g) and adult Sprague-Dawley rats (250-300 g) were obtained from Charles River (St-Constant, Quebec, Canada). Halothane B.P. (Fluothane, Wyeth-Ayerst Canada Inc. (Montreal, Quebec), was used to induce anesthesia at a concentration of 1-2.5 % in air. For ganglion dissection, phosphate buffered saline (PBS) was prepared to the following specifications (in mM): NaCl 140, KCl 2.7, Na_2HPO_4 8.0, KH_2PO_4 1.5, at pH 7.4 and the solution was aerated with a gas mixture compound of 95% O_2 / 5% CO_2 . All chemicals used in preparing PBS were obtained from Sigma Chemical Co. and were dissolved in filtered water. For ganglion dissociation and cell culture, Dulbecco's Modified Eagle's Medium (DMEM), newborn calf serum (NCS), penicillin-streptomycin and Nunc tissue culture dishes were obtained from Canadian Life Technologies (Burlington, Ontario, Canada). Enzymes used for stellate ganglion dissociation (see below) were; trypsin (Sigma Chemical Co., St. Louis, Missouri, USA) and collagenase (type 2, 319 Units mg^{-1} , Worthington Biochemical Corporation, Lakewood, New Jersey, USA). Collagen (type 1, rat tail) was obtained from Boehringer Manneheim (La Vail, Quebec, Canada), and was used as a substrate for attaching neurons to glass coverslips. Nerve growth factor (NGF) was obtained from Collaborative Biomedical (Bedford MA, USA), and cytosine 1-β-D-arabinofuranoside from Sigma

Chemical Co. (St. Louis Mo, USA). Glass coverslips were obtained from Fisher Scientific Ltd. (Ottawa, Ontario, Canada).

B. Solutions and Chemicals for the Electrophysiological Recording Experiments

The standard external recording solution was composed of (in mM): 140 NaCl, 2 $CaCl_2$, 5 KCl, 20 Na^+-HEPES, 1 $MgCl_2$ and 10 glucose. In experiments examining Ca^{2+} currents, the external recording solution was composed of (in mM): 150 tetraethylammonium chloride (TEA), 5 $BaCl_2$, 10 HEPES, 0.8 $MgCl_2$, and 10 glucose. In experiments examining K^+ and Ca^{2+} currents, 1 μM tetrodotoxin (TTX) was present in the external solution, unless otherwise stated. The standard internal (patch pipette filling) solution used in whole-cell recordings was composed of (in mM): 140 KCl, 0.4 $CaCl_2$, 1 $MgCl_2$, 20 HEPES, 1 ethylene glycol -bis (β-aminoethyl ether) N,N,N',N'-tetreacetic acid (EGTA) , 5 ATP (Mg) and 0.3 GTP (Na). For recording Ca^{2+} currents, the patch pipette solution was composed of (in mM): 125 CsCl, 4.5 $MgCl_2$, 10 HEPES, 9 EGTA, 5 ATP (Mg), and 0.3 GTP (Tris). The pipette filling solution was filtered using a 0.22 μm filter (Millipore Products Division, Bedford, Massacheussetts, USA). All solutions were maintained at pH 7.3-7.4, and osmolarity of solutions was 324-329 mosM. The use of a slightly hyperosmotic external solution (relative to the internal solution), was found to be effective for eliminating any transient changes in ionic conductances, which may have occurred as a result of osmotic changes during the initial period of time following achievement of whole-cell recording configuration. Solution osmolarities were confirmed

by freezing point depression, using an osmometer (Osmette A, Fisher Scientific, Nepean, Ontario, Canada). Ca^{2+} concentration was calculated using a software program based on the algorithm of Goldstein (Goldstein 1979). Free Ca^{2+} in the standard pipette solution was estimated at 100 nM, and in the 125 mM CsCl solution it was < 20 nM.

Antagonists for potassium channels (TEA and 4 aminopyridine (4-AP)); calcium channels (cadmium chloride (CdCl) and ω-conotoxin-GVIA (ω-CgTX), and sodium channels (TTX) were dissolved in water and made into concentrated stocks. Other drugs including 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP); mecamylamine, pertussis toxin (PTX); neurokinin A (NKA); neurokinin B (NKB); forskolin; GDP-β-S; CGP49823, were dissolved in water, made into concentrated stocks, and frozen in aliquots (-10 ^{0}C). Stock solutions were then freshly diluted with the standard external solution to desired concentrations on the day of the experiment. Substance P was reconstituted by first dissolving in a nitrogen bubbled solution containing 1mg/ml bovine serum albumin (as per, Sigma), which was then made into a concentrated stock solution with water, and frozen in aliquots. GF109203X , an protein kinase C inhibitor, was dissolved in Dimethyl sulfoxide (DMSO) to make a concentrated stock solution with water, and then diluted to desired concentrations with standard external recording solution before use. The final concentration of DMSO in the experimental solutions used was less than 0.05% (vol/vol). DMSO itself at this concentration does not significantly affect the electrophysiological properties of cells (Joseph and Miller 1992).

All drugs and chemicals were obtained from Sigma Chemical Co. (Mississauga, Ontario, Canada), except for PTX, NKA, NKB and GF109203X which were obtained

from Rose Scientific (Edmonton, Alberta, Canada) and Calbiochem (San Diego, California, USA) and CGP49823 which was a kind gift from CIBA Geigy (Alberta, Canada).

2. Methods

A. Cell Dissociation and Culture

Adult Sprague-Dawley rats (250-300 g) or adult guinea-pigs were used for the preparation of isolated SG neurons in culture. All procedures conformed to guidelines of the Canadian Council on Animal Care. A simplified schematic diagram of the SG dissociation procedure is shown in figure 2-1. Animals were anesthetized with halothane and decapitated. Using aseptic conditions the thorax was opened and the right and left SG were removed and placed in aerated (95% O_2 / 5% CO_2) sterile PBS solution. Excess connective tissue was removed and ganglia were placed into a dish containing a solution composed of 5 mg/ml collagenase and 1 mg/ml trypsin, dissolved in warmed (37 ^{0}C) DMEM for dissociation. This preparation was then placed in an incubator (37 ^{0}C, atmosphere composed of 95% air and 5% CO_2) for 1 hour and trituration was performed with a fire polished Pasteur pipette at 15 minute intervals. Trypsin action was then terminated by the addition of 1 ml of NCS. Cells were then centrifuged (1000 g) for 4 minutes, resuspended in 2 ml of fresh DMEM, and gently triturated. Cells were

FIGURE 2-1: Simplified schematic diagram of rat (or guinea-pig) stellate ganglion neuron dissociation and cell culture. Diagram of lower cervical and thoracic sympathetic trunks was modified and reproduced from De Lemos and Pick 1966)

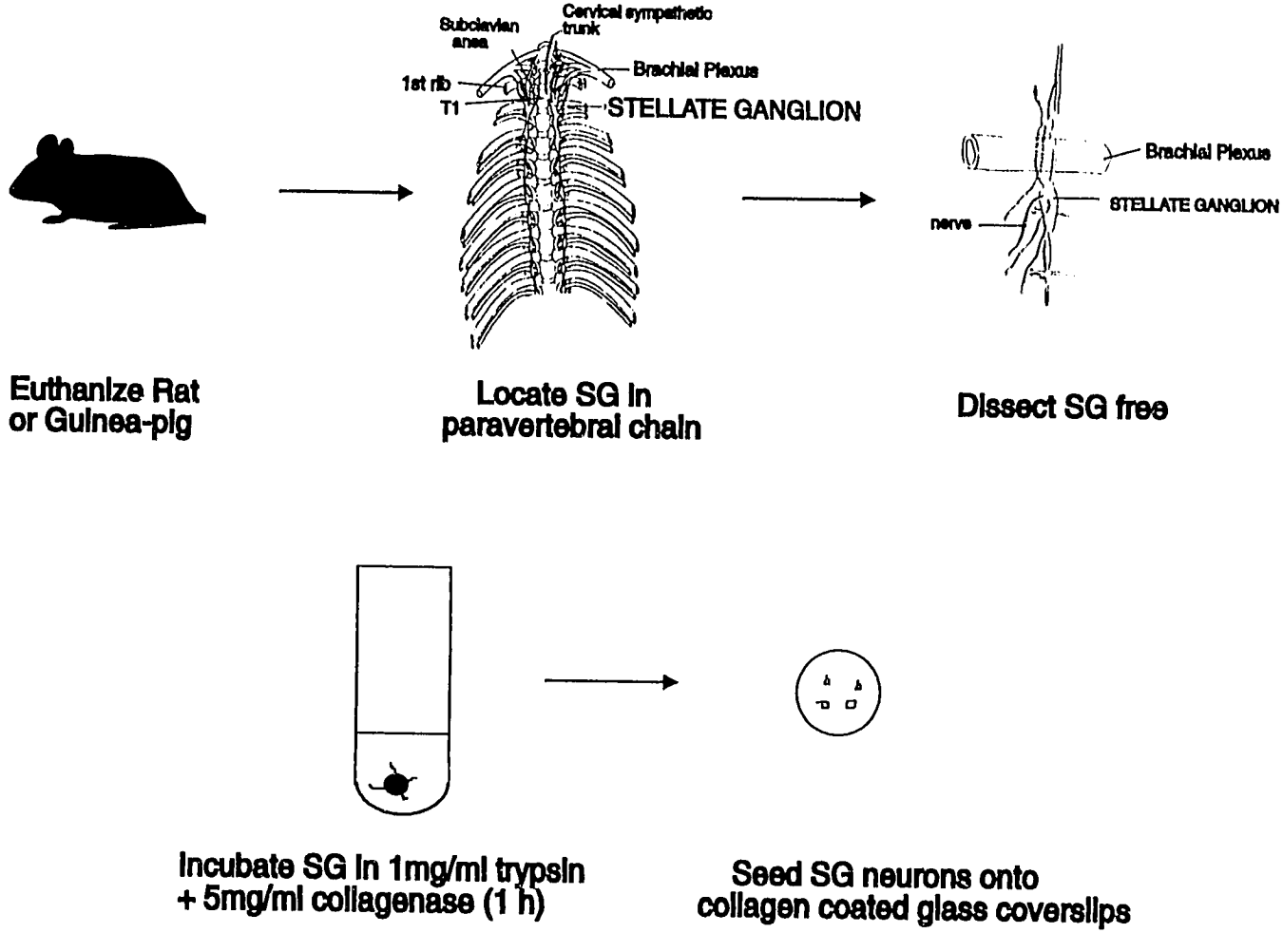

Subclavian area
Cervical sympathetic trunk
Brachial Plexus
1st rib
T1
STELLATE GANGLION
Brachial Plexus
STELLATE GANGLION
nerve
Euthanize Rat or Guinea-pig
Locate SG in paravertebral chain
Dissect SG free
Incubate SG in 1mg/ml trypsin + 5mg/ml collagenase (1 h)
Seed SG neurons onto collagen coated glass coverslips

FIGURE 2-1

centrifuged once more and resuspended in the culture media, which was composed of DMEM plus 15% NCS, 0.1 % penicillin-streptomycin, 10 ng ml^{-1} NGF and 5 $\mu g\ ml^{-1}$ cytosine 1-β-D-arabinofuranoside, used to inhibit the growth of non-neuronal cells (Horackova et al., 1996). Cells were seeded onto collagen-coated glass coverslips (12 mm diameter), placed in 1 ml wells of tissue culture dishes and kept at 37 ^{0}C in a humidified atmosphere composed of 5% CO_2 and 95% air. Under these conditions cells settled and became attached to coverslips within 12 hours.

For experiments in which the role of G protein subtypes were examined, pretreatment with PTX was accomplished by the addition of PTX (500 ng/ml) to the culture medium at the time of neuron plating. SG neurons were exposed to PTX for at least 24 hr before electrophysiological recordings were made. Control SG neurons were plated from the same ganglion culture as the PTX-treated cells, but were not exposed to PTX. Recordings from control and PTX-treated neurons were obtained on the same day to ensure consistent experimental conditions.

B. Immunohistochemistry of Cultured SG Neurons

Stellate ganglion neurons were examined for immunoreactivity to tyrosine hydroxylase following the procedure of Horackova et al. (Horackova et al., 1996). Cultured neurons were washed three times in cold (4^{0}C) 0.2 M PBS (pH 7.4) and then fixed in a 4% paraformaldehyde in 0.1 M PBS (pH 7.4) for 10 min. After fixation the cultures were washed three more times (5 minute periods each) in cold PBS and were then

transferred into blocking sera to block non-specific immunoglobulin binding sites (1% normal goat serum (NGS, (Bio/Can Scientific, Mississauga, Ontario, Canada) in PBS) at room temperature. Fixed cells were then incubated for 1 hour at 37°C with primary antisera to tyrosine hydroxylase (TOH) raised in rabbit (EugeneTech International, Ridgefield Park, New Jersey, USA) and diluted 1:2000 in PBS containing 1% NGS and 1% Triton X-100. The cells were washed four more times with PBS and incubated for a further 1 h at room temperature with a fluorescently tagged CY3 antirabbit secondary antibody. The cultures were washed with PBS inverted on glass microscope slides in 50% PBS/glycerol and viewed under epifluorescence illumination (Nikon Diaphot). Control experiments were performed with no primary antiserum.

C. Superfusion System for Electrophysiological Recording

SG neurons, attached to glass coverslips, were placed in a shallow recording chamber (1 ml total volume) which was positioned on the stage of a Nikon inverted microscope (Nikon Canada Instruments Inc., Mississauga, Ontario, Canada). Cells were superfused (at 1-2 ml/min) with external recording solutions. All external solutions were equilibrated with 95% air / 5% CO_2, and were gravity-fed into the recording chamber from elevated reservoirs which could be individually selected by a series of valves. In experiments in which pharmacological modulation of ionic currents was studied, test solutions were applied in one of two ways: 1) by superfusion or, 2) by pneumatic pressure ejection from micropipettes (tip diameter 1-2 μm). For test solutions applied by

pneumatic pressure ejection, micropipettes were positioned 50-100 μm from the cell and approximately 2-4 lb/in^2 pressure was applied to the back of the micropipette using a Picospritzer II (General Valve Corp., Fairfield, New Jersey, USA). Test solutions delivered via the perfusate were applied for a minimum of five complete (1 ml) bath exchanges. In experiments in which the reversal potential for whole-cell K^+ current was examined, the external K^+ was increased from 5 mM to 20 or 50 mM $[K^+]_o$ by equimolar replacement of NaCl with KCl. In those experiments investigating the ionic selectivity of the nicotinic acetylcholine receptor currents (nAChR), NaCl was substituted with Tris-Cl. In experiments investigating the effects of low Ca^{2+} on K^+ currents, external Ca^{2+} was replaced with 4 times equimolar Mg^{2+}.

D. Fabrication of Recording Electrodes

Micropipettes were prepared from borosilicate glass with a 1.5 mm outside diameter and a 1.1 mm inside diameter (Sutter Instruments, Novato, California, USA), using a two-stage vertical microelectrode puller (model PP83, Narishige, Tokyo, Japan). The tips of the electrodes were coated with beeswax, a hydrophobic substance, to within about 50 - 100 μm of the tip, leaving the tip uncoated. This procedure was employed to minimize the background noise by preventing the creep of bath fluid up the pipette shaft and to reduce the pipette-to-bath capacitance (Rae and Levis 1984).

E. Electrophysiological Recording Techniques

Neurons selected for recording were first identified by viewing the preparation with phase contrast microscopy on a Nikon inverted microscope. To ensure adequate space-clamp most recordings were made from small neurons with spherical cell bodies (20 - 30 μm diameter) and neurites that extended for less than 1 cell diameter from the soma. Membrane potential (V_m) and currents were recorded using standard whole-cell patch clamp recording procedures (Hamill et al 1981). Figure 2-2 summarizes the procedure leading to the whole-cell patch clamp recording configuration. Briefly, by pressing the tip of a patch pipette against the membrane of a cell and applying gentle suction to the back of the pipette a gigaohm seal (GΩ) is thereby formed over a small patch of the cell membrane (this is referred to as "cell attached mode"). A subsequent brief application of stronger suction then ruptured the patch of membrane leaving the seal intact (this is referred to as "whole-cell configuration"). In this configuration, the patch pipette interior is continuous with the interior of the cell, permitting experimental control over the cytoplasmic constituents (which can be exchanged with the pipette internal solution). In this configuration both membrane current and potential can be recorded.

An Axopatch 1-D amplifier (Axon Instruments, Foster City, California, USA) was used to record whole-cell current and voltage. Figure 2-3 shows the arrangement of a whole-cell patch-clamp experiment. The bath solution was connected to the reference electrode by a silver-silver chloride electrode. Liquid junction potentials (LJP) between the bath and patch pipette (electrode) were measured experimentally before obtaining a seal on the cell; the LJP was taken as the potential between the bath solution and the

FIGURE 2-2: Schematic representation of the procedures leading to the whole-cell patch clamp recording configuration. The upper left configuration shows a patch pipette in simple mechanical contact (MΩ seal resistance) with the membrane of a cell. Upon slight suction the seal between the cell membrane and the patch pipette increases in resistance between 2 to 3 orders of magnitude, forming a cell-attached patch (GΩ resistance seal). From this cell-attached mode, a brief application of stronger suction can rupture the patch of membrane leaving the giga-seal intact. In this configuration the cell can then be current- or voltage-clamped to measure membrane voltage or current changes, respectively. Diagram modified from Hamill et al., 1981.

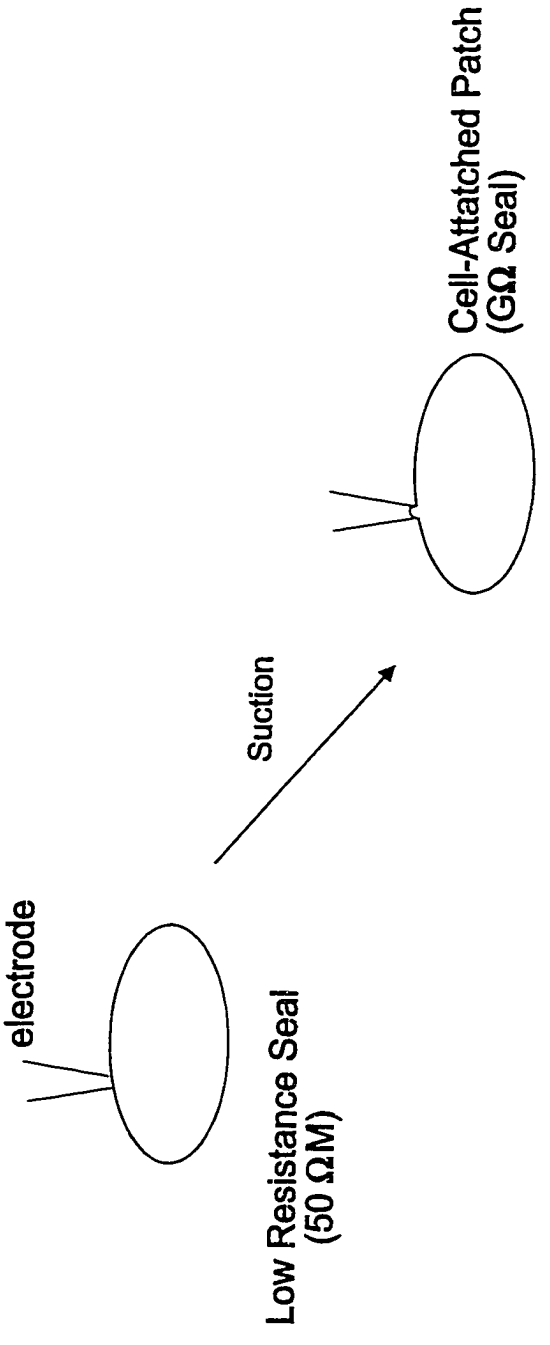

electrode
Low Resistance Seal
(50 ΩM)
Suction
Cell-Attatched Patch
(GΩ Seal)
Pulse of Suction
or Voltage
Whole-cell Recording Patch

FIGURE 2-2

FIGURE 2-3: Schematic diagram for whole-cell patch clamp recording. The patch pipette forms a tight seal with the cell membrane and the cytoplasm is electrically continuous with the intracellular recording solution in the pipette. Ionic currents flow through open ion channels, located in the cell membrane, then flow out of the pipette. The pipette is connected to the negative input of the amplifier. The amplifier serves two functions: it is a variable clamp-battery and it is an ammeter which measures the ionic currents in the electrode. The clamping current flows into the cell through the pipette and is used to impose a voltage on the cell membrane. The bath is grounded by a reference electrode. Diagram was modified and reproduced from Armstrong and Gilly 1992. Abbreviations: R_s: series resistance; R_f: feedback resistor; R: cell input resistance; V_{clamp}: clamp-battery voltage; V_{out}: output voltage; I: current flowing; C: cell capacitance.

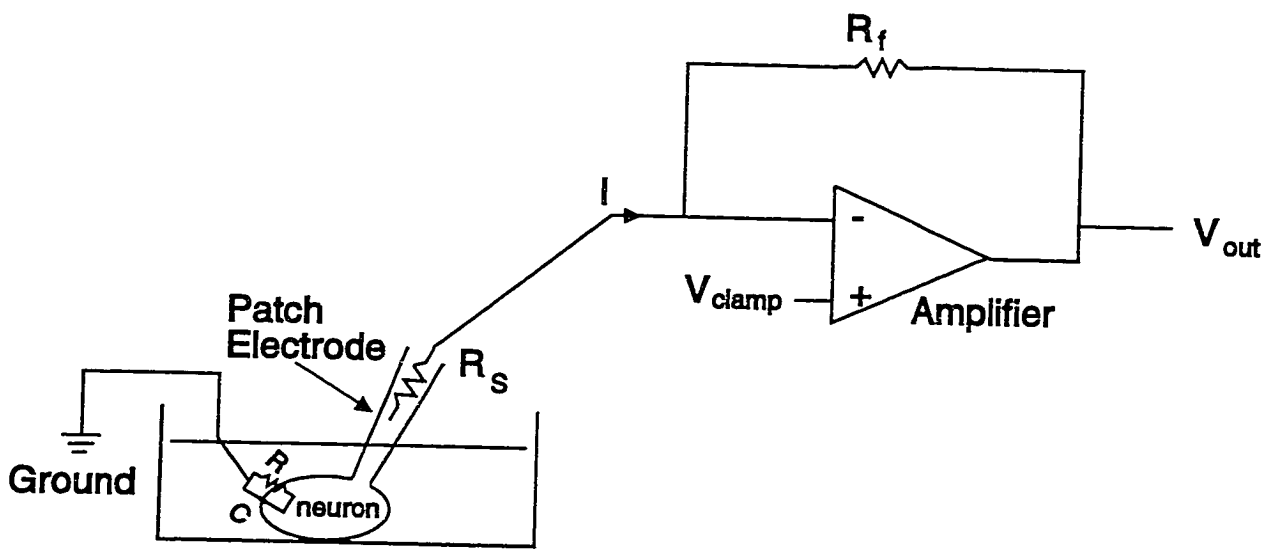

R_f
I
-
+
V_{out}
V_{clamp}
Amplifier
Patch
Electrode
R_s
Ground
R_m
C_m
neuron

FIGURE 2-3

pipette solution (Barry and Lynch 1991). For analyzing whole-cell recording data , the V_m was calculated according to equation 1,

$$V_m = V_p - LJP \quad (1)$$

where V_m is corrected membrane potential and V_p is pipette potential. The LJP was 3 mV for the standard internal and external recording solutions used to record K^+ currents, and was not accounted for in the data shown. For solutions used to record Ca^{2+} currents, the LJP was 11 mV, and V_m was corrected in the current-voltage plots shown.

Patch pipettes had tip resistance's of 2 to 4 MΩ when filled with the standard internal recording solution and placed in the standard external solution. Access (series) resistance was obtained directly from the amplifier (series resistance dial), and was always less than 10 MΩ. Series resistance compensation (90%) was routinely employed. Cell capacitances were measured from the capacitance compensation circuitry on the amplifier. Headstage current was filtered with a four pole low pass Bessel filter (3 dB at 1 kHz), to eliminate signals and noise above the frequency bandwidth of interest. Current and voltage data were digitized at a sampling frequency of 5 kHz using pClamp 6 software (Axon Instruments Inc.), displayed on a Gould TA240 chart recorder for monitoring during the experiment, and stored on computer disk as well as on videotape with the aid of a digital data converter (Medical Systems, Greenvale, New Jersey, USA). Except where indicated, current-voltage relationships were corrected for linear leak current, measured from hyperpolarizing command pulses. Neurons with membrane potentials more positive than -40 mV, and with attenuated action potentials which did not overshoot 0 mV were eliminated from these studies. All experiments were conducted at room temperature (20-

22^{0}C). At this temperature, the effect on channel conductance would be expected to be negligible, however, the channel kinetics may be slowed as compared to those observed at 37^{0}C (Hille 1975).

F. Data Analysis

Mathematical functions were fitted to sets of data points in either CLAMPFIT (Axon pClamp Version 6 software program) or Origin, a graphing software program (MicroCal Software Inc., Northampton, Ma, USA). The relationship between the reversal potential of the whole-cell tail currents and $[K^+]_0$ was determined by linear regression analysis and was performed using the Origin program. The Origin program was used to fit data with a Boltzman equation. Fitting functions are described in the results, where appropriate. Graphing of experimental data was accomplished using Origin and final figures were constructed using Coreldraw (Version 3.0 Rev B, Corel Corp., Ontario, Canada). Numerical data in this thesis are presented as mean ± standard error (SE).

RESULTS

1. ESTABLISHMENT OF DISSOCIATED MAMMALIAN STELLATE GANGLION SOMATA CULTURES SUITABLE FOR WHOLE-CELL PATCH CLAMP RECORDING, FROM RAT AND GUINEA-PIG

A. Morphological and Histochemical Properties of Cultured Guinea-Pig and Rat Stellate Ganglion Neurons Grown in Culture

Immediately after dissociation all SG neurons were almost spherical in shape. During the first 12 hours in culture they adhered to the coverslips and by 24 hours began to develop neurites, but the shape of the soma remained spherical. The mean diameter of the neurons was 21 $\pm$ 3 μm (n = 20) at 24 hours in culture. Photomicrographs of guinea-pig SG neurons at times 12 and 48 hours in culture are shown in Figure 3-1. *In vivo* studies have indicated that the SG of the rat (Landis, 1988; LeBlanch and Landis, 1986; Landis and Fredieu, 1986) and guinea-pig (Kummer and Heym, 1991) possess two populations of neurons distinguished on the basis of their immunoreactivity to TOH. Tyrosine hydroxylase is a rate limiting enzyme in the biosynthesis of catecholamines (Gilman et al., 1990) and neurons immunoreactive for TOH were considered to be catecholaminergic while those staining negative were considered to be of non-catecholaminergic phenotype. Therefore, we examined SG neurons at 24 hrs post dissociation for the presence of TOH. Labeling for TOH was observed in more

FIGURE 3-1: Photomicrographs of cultured guinea-pig SG neurons. A & B: Stellate ganglion neurons at 12 hrs and 72 hrs in culture respectively. C & E: Phase view of SG neurons cultured for 72 hrs. D & E: Immunohistochemical staining for TOH, same neurons as shown in C & E, respectively. Scale bars in panels A-D are 35 μm. Scale bars in panels E & F are 15 μm.

than 90% of neurons at 24 hrs in culture. Figure 3-1 shows photographs of SG neurons displaying immunoreactivity for TOH.

B. Suitability of the Stellate Ganglion Culture Model for Patch-Clamp Analysis

All electrophysiological experiments described in this study were carried out on phase- dense neurons, in culture for a minimum of 12 hrs and a maximum of 48 hrs. This time range was used because: 1) after 12 hrs, SG neurons had surfaces suitable for obtaining GΩ seals with the patch-clamp technique; 2) neurons in culture for less than 48 hrs averaged approximately 20 μm in diameter, were devoid of synapses and dendrites, and had minimal neurite outgrowth thereby allowing for good space-clamp; 3) the RMP's of SG neurons were similar to those of neurons in intact mammalian SG ganglia (Mo et al., 1995); 4) neurons cultured within this time frame had high input resistances (>200 MΩ) and overshooting (> 0 mV) action potential spikes, both signs of healthy cells; 5) the neurons clearly exhibited robust ionic currents similar to currents previously reported in other mammalian sympathetic ganglion neurons both in culture and *in situ*; 6) the typical voltage- and ligand-gated ionic currents found in mammalian sympathetic neurons (i.e. Na^+, Ca^{2+}, K^+, nAChR) were present and in sufficient density to allow convenient study and 7) the neurons were shown to possess viable receptors for SP, ACh analogues and CGRP.

2. PROPERTIES OF THE RESTING CELL MEMBRANE OF CULTURED MAMMALIAN STELLATE GANGLION NEURONS IN CULTURE

The properties of the resting cell membrane were characterized largely in guinea-pig SG neurons, with some results presented from rat SG neurons for comparison. Figures presented in this, and the following three sections (sections 2-5), were obtained from recordings in the guinea-pig. Where comparisons were made in the rat, this data is included in either Table 3-1 or in the text.

A. Passive Electrical Properties of Rat and Guinea-pig Stellate Ganglion Neurons

i) Resting Membrane Potential

Data on the mean RMP of cultured guinea-pig and rat SG neurons determined under current-clamp conditions are presented in Table 3-1. Briefly, for guinea-pig SG neurons the mean RMP was -49 ± 2 mV (n= 40), cell capacitance averaged 42 ± 2 pF, (n=114). The input resistance for guinea-pig SG neurons was 577 ± 212 MΩ (n=5, range 52-1350 MΩ). For rat SG neurons the mean RMP was -46 ± 2 mV (n = 37), and whole-cell capacitance was 47 ± 4 pF (n = 37). These values are similar to mean values described for dissociated rat SCG neurons (Schofield and Ikeda, 1988) and rabbit coeliac ganglion neurons (Gola and Neil, 1993) recorded from with whole-cell patch-clamp.

Electrophysiological Properties of Dissociated Mammalian Stellate Ganglion Neurons Grown in Culture

	Rat	Guinea-pig
Resting Membrane Potential (mV)	-46 ±2.0 (n=37)	-49 ± 2.0 (n=40)
Whole-cell Capacitance (pF)	47 ± 4.0 (n=37)	42 ±2.0 (n=114)
Current to Generate an AP (pA)	58 ±10 (n=6)	20 ±2.0 (n=22)
AP Amplitude (mV)	+50 ±6.0 (n=6)	+79 ± 6.0 (n=38)
AHP Amplitude (mV)	-10 ± 2.0 (n=5)	-14 ± 1.0 (n=34)
AP Duration at 0mV (msec)	3.0 ± 2.0 (n=6)	5 ± 0.0 (n=36)
AHP Time to 1/2 Inactivation (msec)	6.4 ± 1.0 (n=5)	42 ± 8.0 (n=27)

TABLE 3-1

B. Electrical Behavior of Guinea-Pig and Rat Stellate Ganglion Neurons

i) Action Potential Characteristics

Somatic action potentials were elicited in guinea-pig and rat SG neurons by injecting depolarizing current pulse through the electrode. Injection of depolarizing current during current-clamp typically elicited fast overshooting AP in both guinea-pig and rat SG neurons, which showed spike frequency adaptation (a slow decrease in frequency of AP elicited by maintained depolarization) (Figure 3-2A). Previous investigations of neurons from isolated intact rat SG (Mo et al., 1994), and from cultured guinea-pig SG neurons (Horackova et al., 1993) have demonstrated that, in response to sustained depolarization, the majority of neurons fire phasically, while a smaller proportion fire tonically. While we did not investigate firing properties in detail in isolated cultured guinea-pig SG neurons, we found that approximately 80% of neurons (32 of 40) from which APs were recorded could best be described as phasic (Figure 3-2A), whereas the remainder (20%, 8 of 40) exhibited tonic firing (Figure 3-2B). In the cultured rat SG neuron, all cells examined (n = 6) exhibited phasic firing properties. Similar results have been reported in guinea-pig lumbar sympathetic chain (Cassell et al., 1986); coeliac (McLachlan and Meckler, 1989) neurons and in SCG neurons of the rat (Yarowsky and Weinreich, 1985).

Characteristics of the action potentials recorded from in these neurons have been summarized in Table 3-1. Briefly, in the guinea-pig , the minimum current required to

FIGURE 3-2: Responses of cultured SG neurons to depolarizing currents. **Panel A),** phasic neuron; **Panel B),** tonic neuron. **Panel A)** Sustained depolarizing current injection induced a SG neuron to fire a single action potential. Action potentials evoked by a brief (100 ms) pulse of depolarizing current of (*a*), twice threshold and (*b*) three time threshold intensity. **Panel B)** Sustained depolarizing current injection induces a SG neuron to fire a series of action potentials. Action potentials evoked by a brief (100 ms) pulse of depolarizing current of (*a*) just threshold and (*b*) three time threshold intensity.

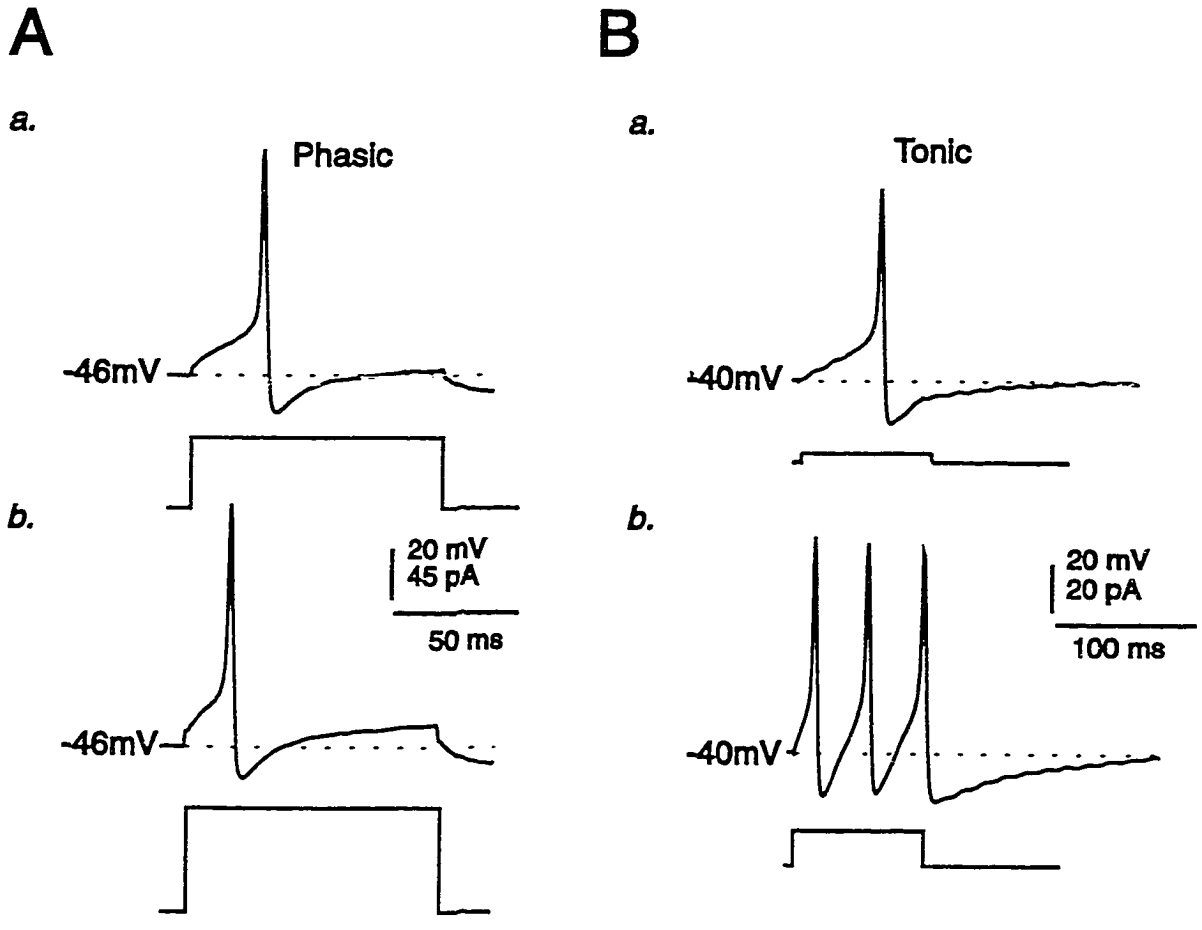

A
a.
Phasic
-46mV
b.
20 mV
45 pA
50 ms
-46mV
B
a.
Tonic
-40mV
b.
20 mV
20 pA
100 ms
-40mV

FIGURE 3-2

generate an AP in 22 neurons examined was 20 ± 2 pA. The average AP amplitude, measured from the RMP to the peak of the AP was 79 ± 6 mV (n=38); the peak AHP amplitude was -14 ± 1 mV (n=34); the AHP duration (measured at half maximal AHP amplitude) was 42 ± 8 ms (n=27), and the AP duration measured at RMP and at 0 mV was 20 ± 3 ms (n=24) and 5 ± 0.3 ms (n=36), respectively. The values reported in Table 3-1 are comparable to those reported for neurons in *in vitro* rat SG and SCG preparations (Mo et al., 1994; Perri et al., 1970). The amplitude of the APs generated by depolarizing current injection was diminished by exposure to 1μM TTX. Figure 3-3A demonstrates the action of 1μM TTX on the action potential of a guinea-pig SG neuron. For the cell shown in Figure 3-3B, when 1 mM tetraethylammonium chloride (TEA) was added to the superfusate, the RMP depolarized by 2 mV, the AP duration increased by 13 %, and the AHP amplitude was reduced by 41 %, respectively. Similar effects were observed in 2 other cells exposed to TEA in the guinea-pig and for 2 neurons in the rat. The data are consistent with block of voltage-dependent K^+ current. Exposure to the Ca^{2+} channel blocker Cd^{2+} (200 μM) depolarized the membrane potential (11.0 ± 5.0 mV) in 4 of 7 guinea-pig neurons sampled, while in the remaining 3 neurons, Cd^{2+} produced either no effect or a slight hyperpolarization (1-5 mV). However, in all neurons examined Cd^{2+} consistently decreased the AHP amplitude and duration by 43 ± 13 % and 25 ± 2 % (n=7), respectively (Fig. 3-3C,), suggesting the contribution of a Ca^{2+}-activated K^+ current to AP repolarization.

FIGURE 3-3: Action potential generation and firing properties of cultured SG neurons. Action potentials were recorded in control recording solution and after addition of 1 μM TTX (**Panel A**), 1 mM TEA (**Panel B**), or 0.2 mM Cd^{+2} (**Panel C**). Action potential traces have been superimposed for comparison. Resting membrane potential is indicated at the left of each trace and by dashed line (*, presence of drug).

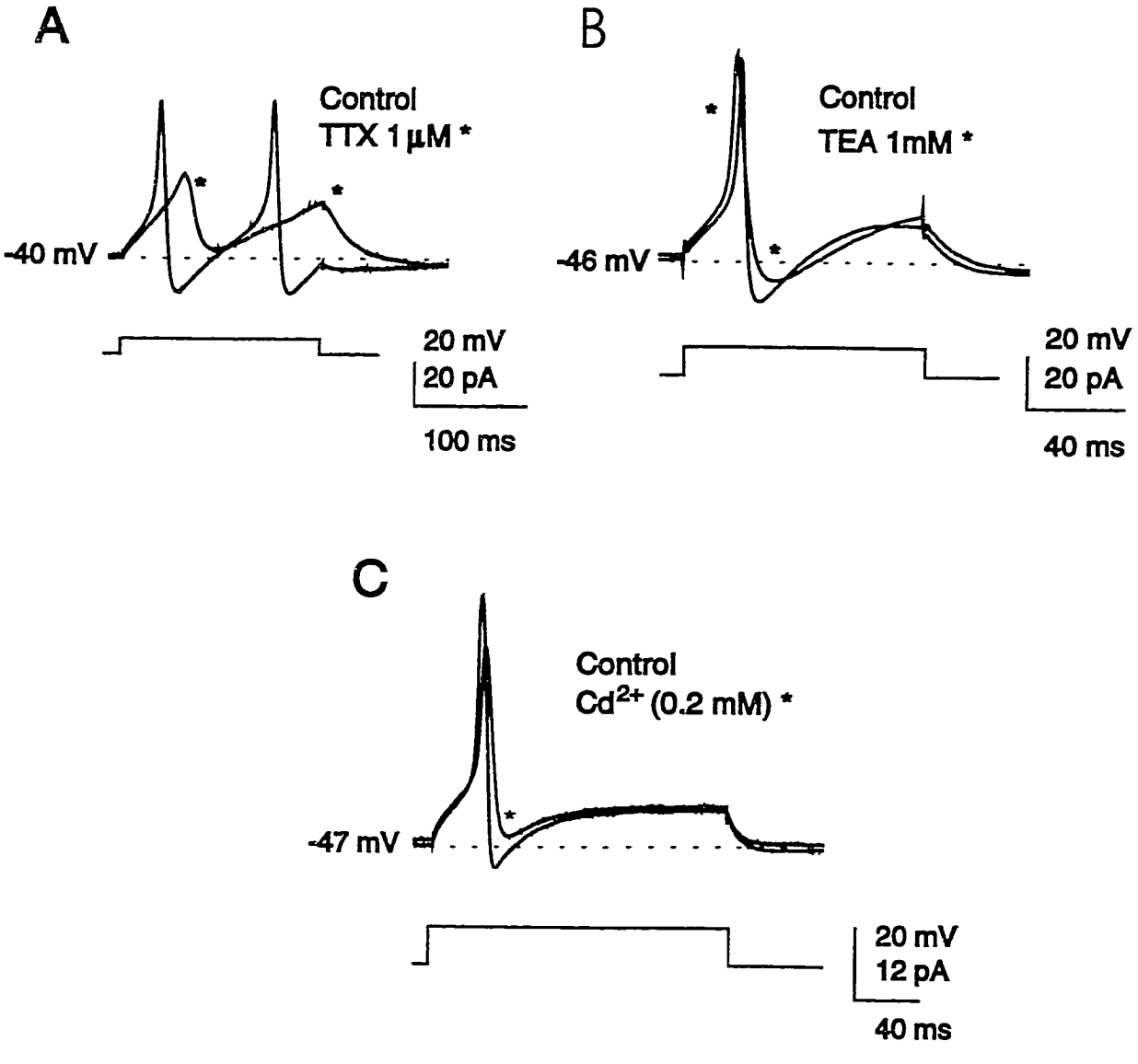

A
Control
TTX 1 µM *
-40 mV
20 mV
20 pA
100 ms
B
Control
TEA 1mM *
-46 mV
20 mV
20 pA
40 ms
C
Control
Cd2+ (0.2 mM) *
-47 mV
20 mV
12 pA
40 ms

FIGURE 3-3

3. VOLTAGE-GATED WHOLE-CELL POTASSIUM CURRENTS IN CULTURED MAMMALIAN STELLATE GANGLION NEURONS

A. A Transient Outward Potassium Current in Rat and Guinea-Pig Stellate Ganglion Neurons

Figure 3-4 illustrates representative whole-cell currents recorded in guinea-pig SG neurons using standard recording solutions. The protocol shown in the figure consisted of 500 ms depolarizations from a holding potential (V_H) of either -100 mV (a) or -35 mV (b), followed by a series of test potential steps ranging from -40 to +60 mV in 20 mV increments. In this example a SG neuron is depolarized from a V_H of -100 mV, evoking a transient outward current followed by a sustained component (Figure 3-4A*a*). Depolarization of V_H to -35 mV inactivated the transient outward current component , leaving the sustained component intact (Fig. 3-4A*b*). Figure 3-4B shows the current-voltage (I-V) relationship for the peak outward currents recorded at a V_H of -100 and -35 mV.

The K^+ selectivity of the outward current was confirmed by examination of current tails, obtained by stepping the membrane potential to values negative and positive to the holding potential following step depolarizations to +10 mV. In guinea-pig SG neurons outward current tails reversed at a membrane potential (V_M) of -71.0 ± 5.0 mV (n=14, calculated E_K = -84 mV) in standard recording solutions. The reversal potential of the tails shifted positively to -48.0 ± 7.0 mV (n=3, calculated E_K = -41.0 mV) and -24.0 ±

FIGURE 3-4: Whole-cell K^+ currents in a cultured SG neuron. **Panel A)** Representative currents elicited by a series of step depolarizations from -40 mV to +60 mV from a holding potential (V_h) of -100 mV (*a*) or -35 mV (*b*). **Panel B)** Current-voltage (I-V) relationship showing peak outward currents for the neuron shown in panel A, over a range voltage potentials from -100 mV to +60 mV, applied in 10 mV increments and from a V_h = -100 mV (■) or -35 mV (●). **Panel C)** Conductance (g)-voltage curve for transient outward current. Conductance was obtained by subtracting currents measured from V_h = -35 mV from currents measured from V_h = -100 mV and calculated according to equation 3-1. Conductance was expressed as a percentage of maximal conductance (g_{max}) measured at 0 mV. Inset: representative subtracted currents measured at -20, 0 and +20 mV. **Panel D)** Outward currents are carried by K^+ ions. Tail currents recorded at various potentials in standard 5 mM K^+ recording solution following a preceeding pulse to +20 mV to activate outward current. The voltage-clamp protocol is shown above the traces. Tail currents reversed near -75 mV.

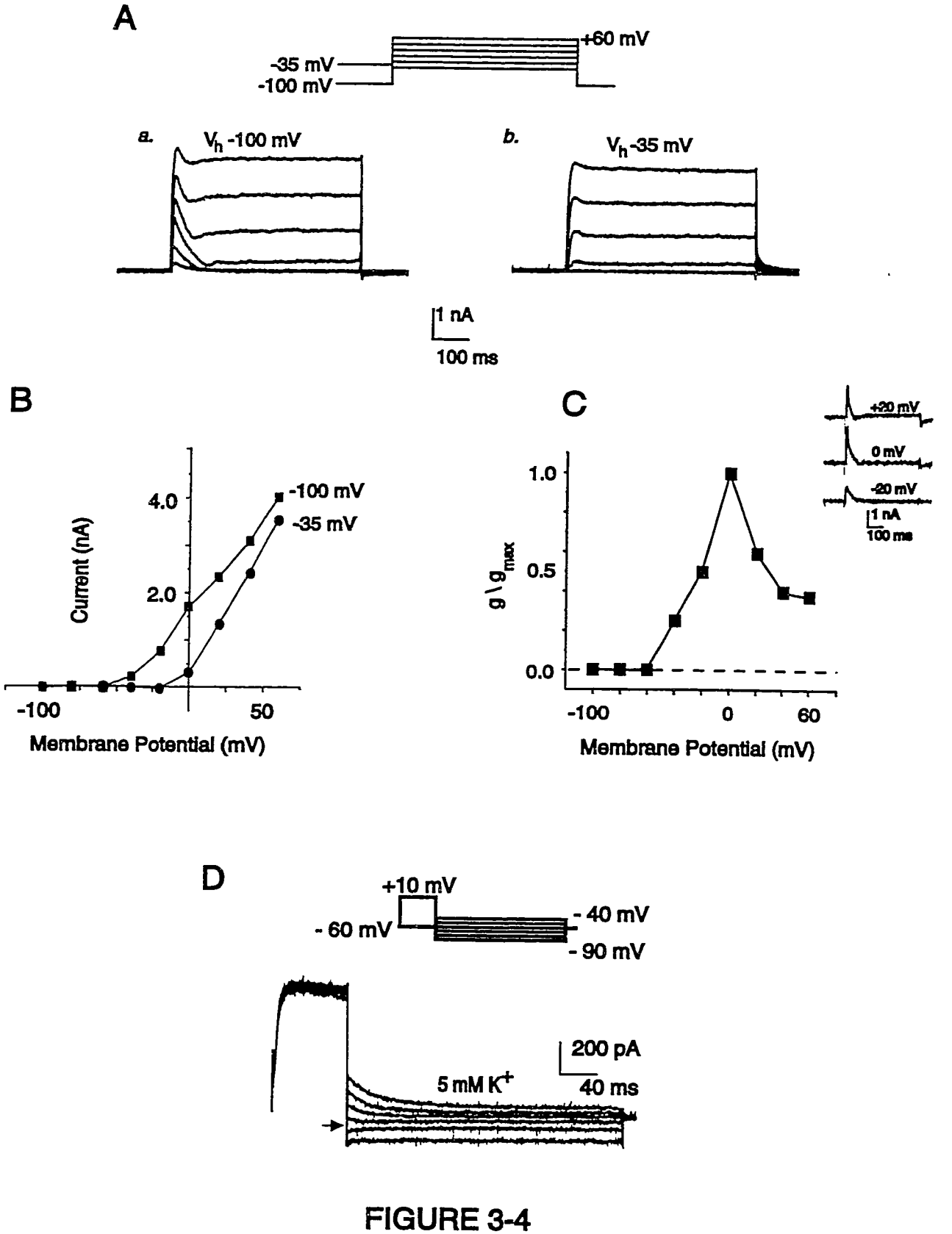

A
+60 mV
-35 mV
-100 mV
a.
V_h -100 mV
b.
V_h -35 mV
1 nA
100 ms
B
-100 mV
-35 mV
4.0
2.0
Current (nA)
-100
50
Membrane Potential (mV)
C
+20 mV
0 mV
-20 mV
1 nA
100 ms
1.0
0.5
0.0
$g \setminus g_{max}$
-100
0
60
Membrane Potential (mV)
D
+10 mV
- 60 mV
- 40 mV
- 90 mV
200 pA
40 ms
5 mM K^+

FIGURE 3-4

1.0 mV (n=4, calculated E_K = -26.0 mV) when the external K^+ concentration was increased to 20 mM and 50 mM, respectively. This shift of 47 mV per ten fold change in $[K^+]_{out}$ approaches the theoretical value of 58 mV predicted by the Nernst equation. Similar results were obtained in the rat SG where outward tails reversed at a membrane potential of -70 ± 1 mV (n=8) in standard recording solutions.

Figure 3-4C shows the conductance-voltage plot for the peak transient outward current, obtained by subtracting the whole cell currents activated by step potentials of -100 to +60 mV in 20 mV increments from V_H of -35 mV, from currents activated from V_H of -100 mV. Figure 3-3C (insert) shows representative subtracted current traces elicited by step depolarizations to -20, 0 and +20 mV. Conductance was calculated from the equation:

$$g=(I_{p(-100)} - I_{p(-35)})/(V-E_K) \qquad (3\text{-}1)$$

Where g is the transient outward K^+ conductance, $I_{P(-100)}$ is the peak outward current measured from a V_H of -100 mV, $I_{P(-35)}$ is the peak outward current measured from a holding potential of -35 mV, V is the membrane potential and E_K is the equilibrium potential for outward K^+ currents measured in SG neurons (as above). The transient K^+ conductance became activated at a membrane potential of -50 mV, and the conductance increased with incremental depolarization to 0 mV, and decreased at more depolarized levels. The decrease in conductance at potentials positive to 0 mV primarily reflects inactivation of the transient current (see insert, figure 3-4C) but may also reflect a small reduction in $I_{K(ca)}$, resulting from Ca^{2+} channel inactivation at the depolarized V_H of -35 mV (Cox and Dunlap, 1992; Plummer et al., 1989; Regan et al., 1991). The transient

outward K^+ current was observed in 60% (25/42) and 72% (67/92) of rat and guinea-pig SG neurons examined, respectively, when neurons were depolarized from a holding potential of -60 mV.

In mammalian sympathetic neurons, the voltage-gated K^+ channels which mediate transient outward current (I_A) exhibit greater sensitivity to 4-AP than do other K^+ currents (Belluzzi et al., 1985). Figure 3-5A shows representative current recordings in an SG neuron. The neuron was held at a V_H of -60 mV and 500 ms duration steps from potentials of -100 to +20 mV were applied in 20 mV increments. The outward current evoked by depolarization from a V_H of -60 mV consisted of both an initial transient component and a sustained current component. Both components of the outward current were reduced by 4-AP. However, 4-AP was more selective for the transient current in guinea pig SG neurons and at a concentration of 2 mM, 4-AP essentially eliminated the transient outward current component over the V_M range of -50 mV to -20 mV (this is within the range of potentials where the transient current is activated separately from the sustained current). The I-V relationship shown graphically in Figure 3-5B was constructed from the peak current traces shown in Figure 3-5A, and demonstrates the reduction in transient K^+ current in the presence of 2 mM 4-AP.

B. Delayed Outward Rectifying Potassium Currents in Rat and Guinea-Pig Stellate Ganglion Neurons

Delayed rectifier K^+ currents (I_{KV}) have been identified in all mammalian

FIGURE 3-5: Pharmacological block of outward K^+ currents in cultured SG neurons. **Panel A)** Whole-cell currents elicited by a series of step depolarizations to +20 mV from V_h = -100 mV recorded in the absence (control) and presence of 2 mM 4-aminopyridine (4-AP). **Panel B)** Current-voltage relationship for the peak component of outward K^+ current (*) measured from current traces shown in Panel A in the absence (control, ■) and presence of 4-AP (●). **Panel C)** Whole-cell currents elicited by a series of step depolarizations to +140 mV from V_h = -100 mV in the absence (control) and presence of 0.2 mM Cd^{+2}. **Panel D)** Current-voltage relationships for sustained outward current component measured from traces shown in panel C (*) in absence (control, ■) and presence of 0.2 mM Cd^{+2} (▲). Cadmium-sensitive current (●) was obtained by subtracting current records measured in presence of Cd^{+2} from control currents.

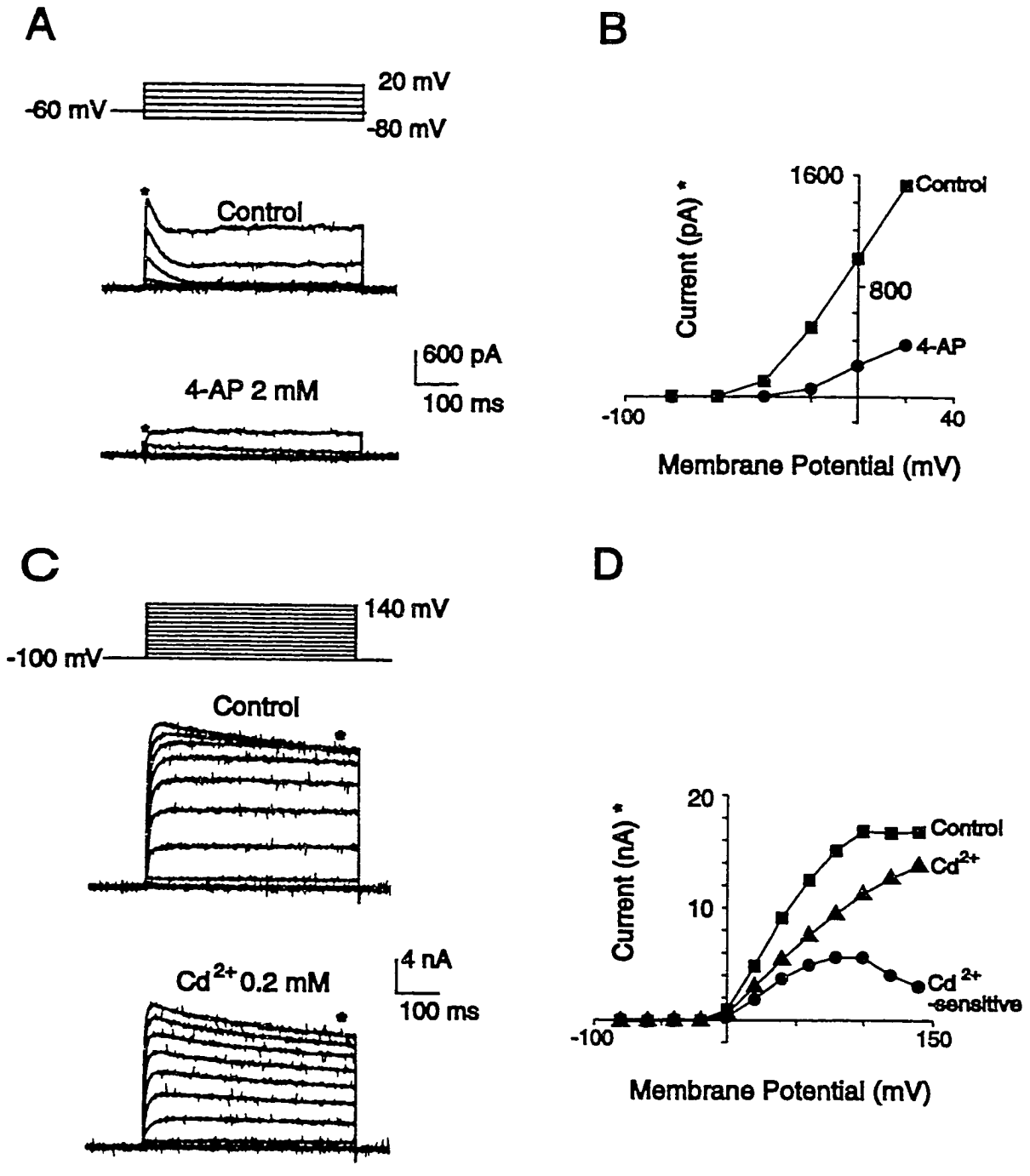

A
20 mV
-60 mV
-80 mV
Control
600 pA
4-AP 2 mM
100 ms
B
1600
Control
Current (pA) *
800
4-AP
-100
40
Membrane Potential (mV)
C
140 mV
-100 mV
Control
4 nA
Cd2+ 0.2 mM
100 ms
D
20
Control
Current (nA) *
Cd2+
10
Cd2+
-sensitive
-100
150
Membrane Potential (mV)

FIGURE 3-5

sympathetic neurons examined. In order to isolate I_{Kv} from other current contributions, cells are typically bathed in a solution containing Cd^{2+} (to suppress both calcium and calcium-dependent K^+ currents) and TTX, to block sodium inflow. Figure 3-5C shows current recordings obtained from a representative guinea-pig SG neuron in the presence and absence of the Ca^{2+} channel blocker Cd^{2+}. The neuron was stepped, for 500 ms, from a V_H of -100 to a maximum potential of +140 mV, in 20 mV increments. Depolarization of membrane potential in SG neurons elicited sustained outward K^+ current, the amplitude of which showed some decline at potentials positive to +80 mV. The I-V relation (Fig. 3-5D) obtained for the current traces in Figure 3-5C demonstrates that in the presence of 200 μM Cd^{2+} the sustained outward current was reduced at all membrane potentials positive to -30 mV. This Cd^{2+}-insensitive component of outward current was activated around -20 mV and increased with increasing depolarization. This current, which resembles the delayed rectifier (I_{Kv}) current described in both rat SCG (Belluzzi and Sacchi, 1988) and guinea-pig celiac ganglion neurons (Vanner et al., 1993), will be referred to as I_{Kv} in this study. The Cd^{2+}-difference current was obtained by subtracting currents recorded in the presence of Cd^{2+} from control currents. The Cd^{2+}-sensitive current activated around -30 mV and increased with depolarization to +75 mV, which approaches the calculated E_{Ca} (+125 mV) for these neurons under the recording conditions used, following which the current declined. In 21 neurons tested, 200 μM Cd^{2+} reduced the outward current by 35 ± 6% and 46 ± 5% at 0 and +60 mV, respectively. In 4 other guinea-pig neurons exposed to nominally Ca^{2+}-free extracellular solution, the outward K^+ current was reduced by 41 ± 11%, thus indicating that approximately 30-50%

of I_K activated at depolarized potentials in SG neurons may be due to activation of a calcium-dependent K^+ current ($I_{K(Ca)}$).

C. An M-Type Potassium Current in Guinea-Pig Stellate Ganglion Neurons

A non-inactivating voltage- and time-dependent component of K^+ current termed the M-current (I_M) has been described in mammalian sympathetic neurons (Mo and Wallis, 1994; Marrion et al., 1989; Constanti and Brown, 1981; Vanner et al., 1993). I_M is typically measured from the amplitude of current decay (relaxation) during hyperpolarized voltage steps from potentials where the I_M is pre-activated. Current relaxations similar to those described in other mammalian sympathetic ganglia were recorded in 29% of SG neurons tested (n=15) (Fig. 3-6.). The protocol (shown in Fig. 3-6) consisted of step hyperpolarizations, from a V_H of -30 mV to a potential of -80 mV, in 10 mV increments. A slow current relaxation evident during the step hyperpolarization represents the slow deactivation of that component of M-like current which had been activated at -30 mV (Fig. 3-6A). The time constant (τ) of the slow deactivation measured at - 60 mV and determined with the fitting program of the pClamp 6 software was 101 ms (for the cell shown in Figure 3-6A). The relaxations reversed direction at potentials negative to -70 mV. An outward current developed upon depolarizations back to V_H. The outward current consisted of a fast transient and sustained currents. The range of membrane potentials over which the M-like current is activated is shown in Fig. 3-6B. This figure demonstrates membrane current changes for the current records shown

FIGURE 3-6: M-type K^+ current in cultured SG neurons. **Panel A)** Membrane currents induced by 500 ms step hyperpolarizations from a constant V_h = -30 mV to the potentials indicated. Slow current relaxations after the initial current step during the hyperpolarization show the slow decay (deactivation) of that component of M-type current which had been activated at V_h = -30 mV. This deactivation relaxation reverses direction at command potentials negative to -70 mV. **Panel B)** Membrane current changes in panel A (over a full range of voltage commands), measured as deflections from the V_h current (ordinate), plotted against step potentials (abscissa): (■) 'instantaneous' current at the beginning of the voltage step; (●) 'steady state' current at the end of the 500 ms voltage step (as shown in the insert).

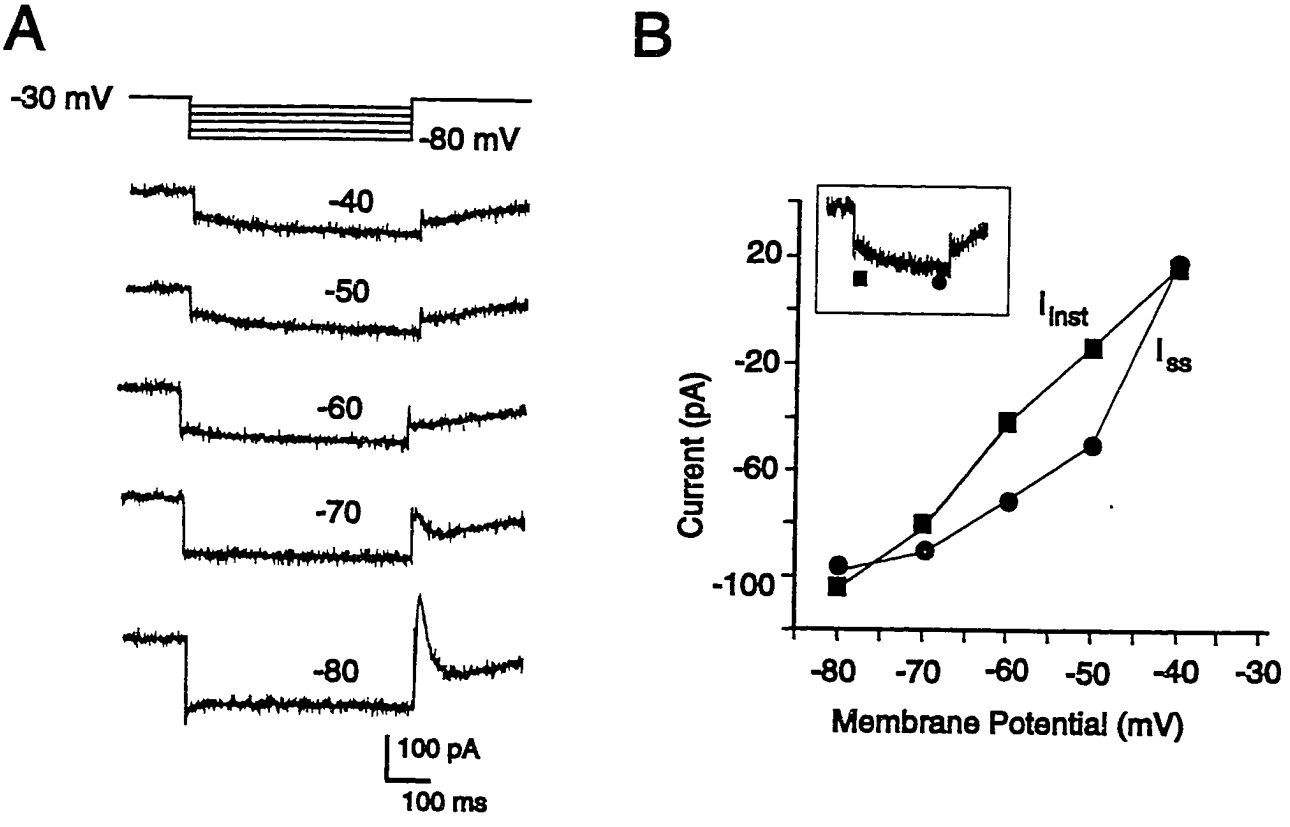

A
-30 mV
-80 mV
-40
-50
-60
-70
-80
100 pA
100 ms
B
20
-20
-60
-100
Current (pA)
I_{inst}
I_{ss}
-80
-70
-60
-50
-40
-30
Membrane Potential (mV)

FIGURE 3-6

in Fig. 3-6A, measured as deflections from the holding current, plotted against the command potential (see insert Figure 3-6B). M-like current activation is manifest by the appearance of outward rectification at potentials positive to -70 mV. The amplitude of the M-like current in SG neurons was very small (10 ± 5.6 pA, n=15, range 5 - 44 pA), as compared to values reported in other mammalian sympathetic neurons recorded using sharp microelectrodes (Marrion et al., 1989; Vanner et al., 1993). Attempts to optimize conditions for increasing the incidence and/or amplitude of M-current (Horn and Marty, 1988; Vanner et al., 1993) by use of an ATP regenerating systems, failed to increase the number of cells in which I_M was observed. This finding in SG neurons is consistent with reports indicating that recording of M-current is difficult in the whole-cell patch-configuration (Vanner et al., 1993). For example, in the guinea-pig celiac ganglion neurons a prominent M-current could be recorded using intracellular microelectrodes, but similar current was not apparent with whole-cell patch (Vanner et al., 1993). Furthermore, attempts to record whole-cell M-current in rat SCG have determined the M-current of the acutely isolated neuron (8 hours post dissociation) to be very small (Constanti and Brown, 1981; Shapiro and Hille, 1994; Beech et al., 1991).

4. VOLTAGE-GATED WHOLE-CELL CALCIUM CURRENTS IN GUINEA-PIG STELLATE GANGLION NEURONS

Ion substitutions and pharmacological agents used to isolate Ca^{2+} channel currents are as described in the METHODS. In K^+- and Na^+- free solutions, with external 5 mM

Ba^{2+} as the current carrier, all K^+ and Na^+ currents were blocked and on membrane depolarization an inward current (I_{Ba}) was observed. Ba^{+2} currents in SG neurons typically activated upon step depolarization positive to -30 mV, and exhibited I-V relationships with a single peak at approximately -10 to 0 mV. Figure 3-7A shows inward I_{Ba} recorded from a guinea-pig SG neuron following step depolarization to +10 mV from a V_H of -70 mV. The inward current in this neuron was reduced by >88 % by 0.2 mM Cd^{2+}. Similar results were observed in all cells examined with a mean reduction in I_{Ba} amplitude measured at 0 mV by Cd^{2+} of 96.0 ± 6.0% (n = 5). Figure 3-7B shows I_{Ba} recorded following a step depolarization to 0 mV with and without 10 μM ω-CgTx. In the presence of ω-CgTx, > 95% (97 ± 1.5, n=9) of the inward current at 0 mV was inhibited and showed no recovery by 15 minutes after application. In 5 other cells, I_{Ba} at 0 mV was reduced by 96 ± 3.0% following a 15 min exposure to ω-CgTx. Subsequent application of 0.2 mM Cd^{2+} to these cells resulted in no further reduction in I_{Ba} in 4 cells and an 11% reduction in 1 cell, suggesting that current through ω-CgTx-sensitive Ca^{2+} channels accounts for almost all the Ca^{2+} channel current in SG neurons under the conditions used in this study. Figure 3-7C shows the I-V plot for the ω-CgTx-sensitive current. Inward current, from a V_H of -90 mV, activates at around -30 mV with peak inward current occurring around a potential of -10 mV. However, voltage steps from -90 mV to membrane potentials positive to +30 mV elicited outward ω-CgTx-sensitive currents, which may represent cesium ions moving out of the cell via ω-CgTx-sensitive calcium channels (Cox and Dunlap, 1992; Fenwick et al., 1982). Nifedipine applied at a

FIGURE 3-7: Pharmacological block of voltage-dependent Ba^{+2} currents (I_{Ba}) in cultured SG neurons. **Panel A)** I_{Ba} elicited with a step depolarization to +10 mV from V_h = -70 mV in absence (control) and presence of 0.2 mM Cd^{+2}. **Panel B)** Superimposed I_{Ba} elicited with a step depolarization to 0 mV from V_h = -90 mV in the absence (control) and the presence of 10 μM ω-CgTx. **Panel C)** Current-voltage relationship for ω-CgTx-sensitive current obtained by subtracting currents recorded in presence of ω-CgTx from control currents (same neuron as in panel B). **Panel D)** Steady-state inactivation curve for I_{Ba}. The steady-state inactivation curve was determined by measuring the peak inward I_{Ba} upon step deopolarization to 0 mV from a variety of test pulses. V_H are indicated on the x-axis and normalized to the maximum I_{Ba}. The data were fit by the Boltzman equation as described in the text.

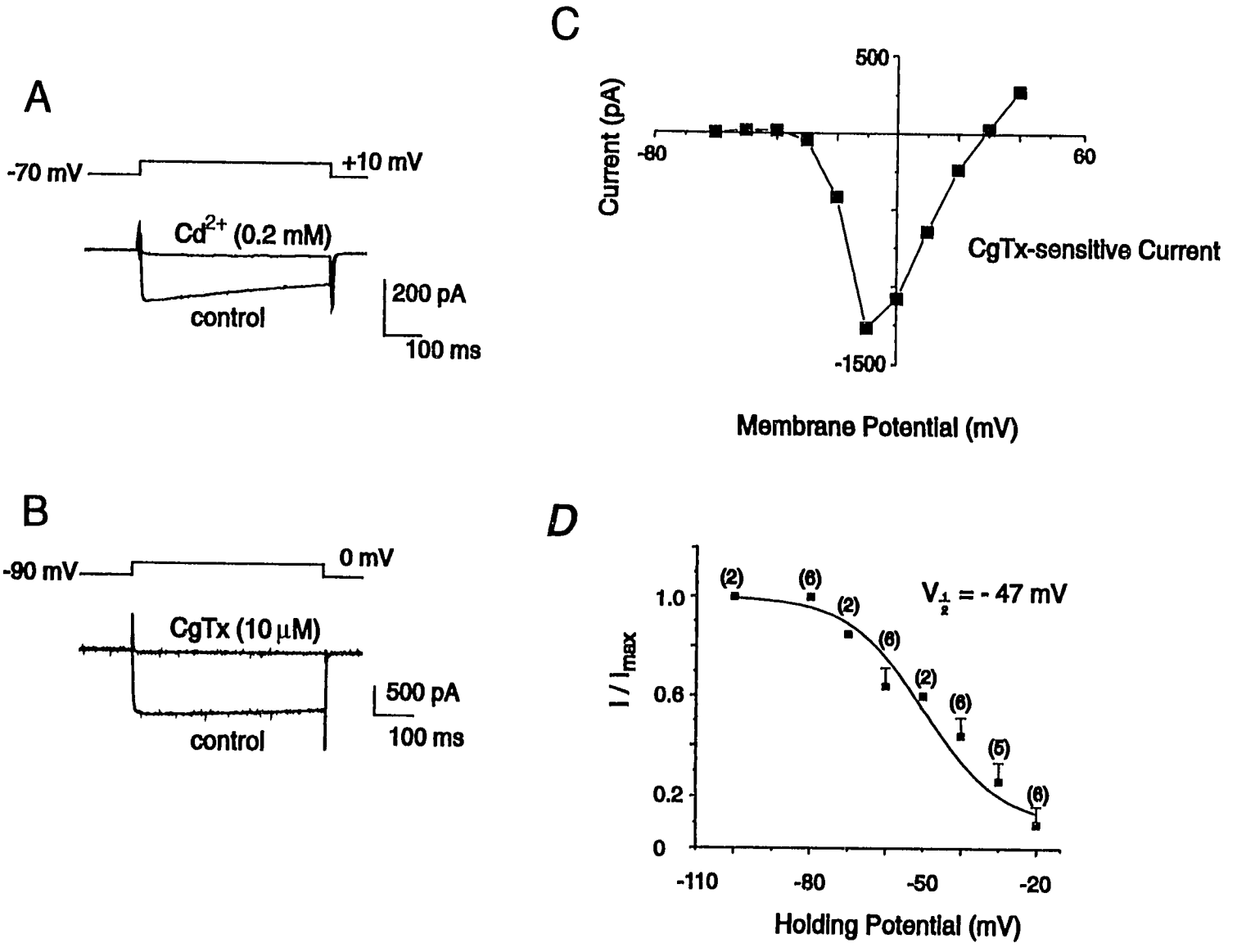

A
-70 mV
+10 mV
Cd2+ (0.2 mM)
control
200 pA
100 ms
B
-90 mV
0 mV
CgTx (10 μM)
control
500 pA
100 ms
C
Current (pA)
500
-80
60
-1500
CgTx-sensitive Current
Membrane Potential (mV)
D
I / Imax
1.0
0.6
0.2
0
(2)
(6)
(2)
(6)
(2)
(6)
(5)
(6)
V½ = - 47 mV
-110
-80
-50
-20
Holding Potential (mV)

FIGURE 3-7

concentration (3 μM) known to block L-type Ca^{2+} currents (Bean 1992) was without significant effect on the Ca^{2+} currents. A steady state inactivation curve for the Ca^{2+} current recorded in guinea-pig SG neurons is shown in Figure 3-7D. In this figure, the peak I_{Ba} normalized relative to maximum current recorded from a holding potential of -100 mV, and plotted as a function of the holding potential, is well fitted by a Boltzman function:

$$I/I_{max} = \{1+\exp[(V-V_n)/k_n]\}^{-1} \qquad \text{eq. 3-2}$$

The $V_{1/2}$ (the potential at which half the current was inactivated) was approximately -47 mV and resembled that reported for N-type Ca^{+2} channels in chick dorsal root ganglion neurons (Fox et al., 1987).

5. PEPTIDERGIC MODULATION OF MEMBRANE POTENTIAL AND IONIC CURRENTS IN CULTURED MAMMALIAN STELLATE GANGLION NEURONS

Voltage-gated ion channels are phosphoproteins (Catterall, 1988; Rossie and Catterall, 1988) that can be modulated by second messenger or membrane delimited responses to G-protein activation (Hille, 1994). Among the various extracellular messengers, such as hormones and neurotransmitters, the neuropeptides SP and CGRP have been shown to modulate K^+ and Ca^{+2} channel function in numerous autonomic ganglia neurons (Hille, 1994; Rudy, 1988; Wiley et al., 1993). Since CGRP and SP have

been identified in the nerve fiber networks of the SG we have investigated whether these neuropeptides affect the function of the voltage-gated ion channels identified in this study.

A. Actions of Substance P on Membrane Potential and Voltage-Gated Ionic Currents in Rat and Guinea-Pig Stellate Ganglion Neurons

i) Actions of Substance P on Membrane Potential and Afterhyperpolarization in Rat and Guinea-Pig Stellate Ganglion Neurons

Substance P (500 nM) or standard external solution (control) were applied to individual SG neurons by a single pressure ejection from a glass pipette with its tip positioned approximately 50 μm from the cell. Pressure application of standard external solution alone did not evoke a response in SG neurons. Application of SP (500 nM) produced a small depolarization (7 ± 3 mV) of the membrane potential in 7 of 7 neurons tested, in which the mean RMP was -50 ± 2 mV (Fig. 3-8A*a*). However, when neurons were current-clamped to a more depolarized potential of -30 mV, a second SP application of the same duration produced a repeatable depolarization of 16 ± 5 mV (n=7) (Figures 3-8A*b* and 3-8A*c*), which was associated with a 27 ± 8 % (n=7) decrease in membrane conductance. In 4 neurons an initial pressure application of SP at -30 mV evoked a 25 ± 5 mV depolarization and was associated with an 42 ± 8 % decrease in membrane conductance. When these same 4 neurons were superfused with 200 μM Cd^{2+}, the SP-evoked depolarization and conductance decrease was significantly less than

FIGURE 3-8: Effects of substance P (SP) and Cd^{+2} on membrane properties of cultured SG neurons. **Panel A)** Membrane depolarization measured at -50 mV (a) and -30 mV (b) and (c) in same neuron in response to sequential 10 sec. pressure application of 500 nM SP; in the presence of 0.2 mM Cd^{+2}, depolarizing effects of 500 nM SP are reduced (d). **Panels B) and C)** Decrease in AP afterhyperpolarization (AHP) induced by 500 nM SP in another neuron in which membrane potential was continuously clamped at resting membrane potential (panel B); in the presence of 0.2 mM Cd^{+2}, the effects of 500 nM SP on AHP are attenuated (panel C). Resting membrane potential is indicated at left of each trace and by dashed line.

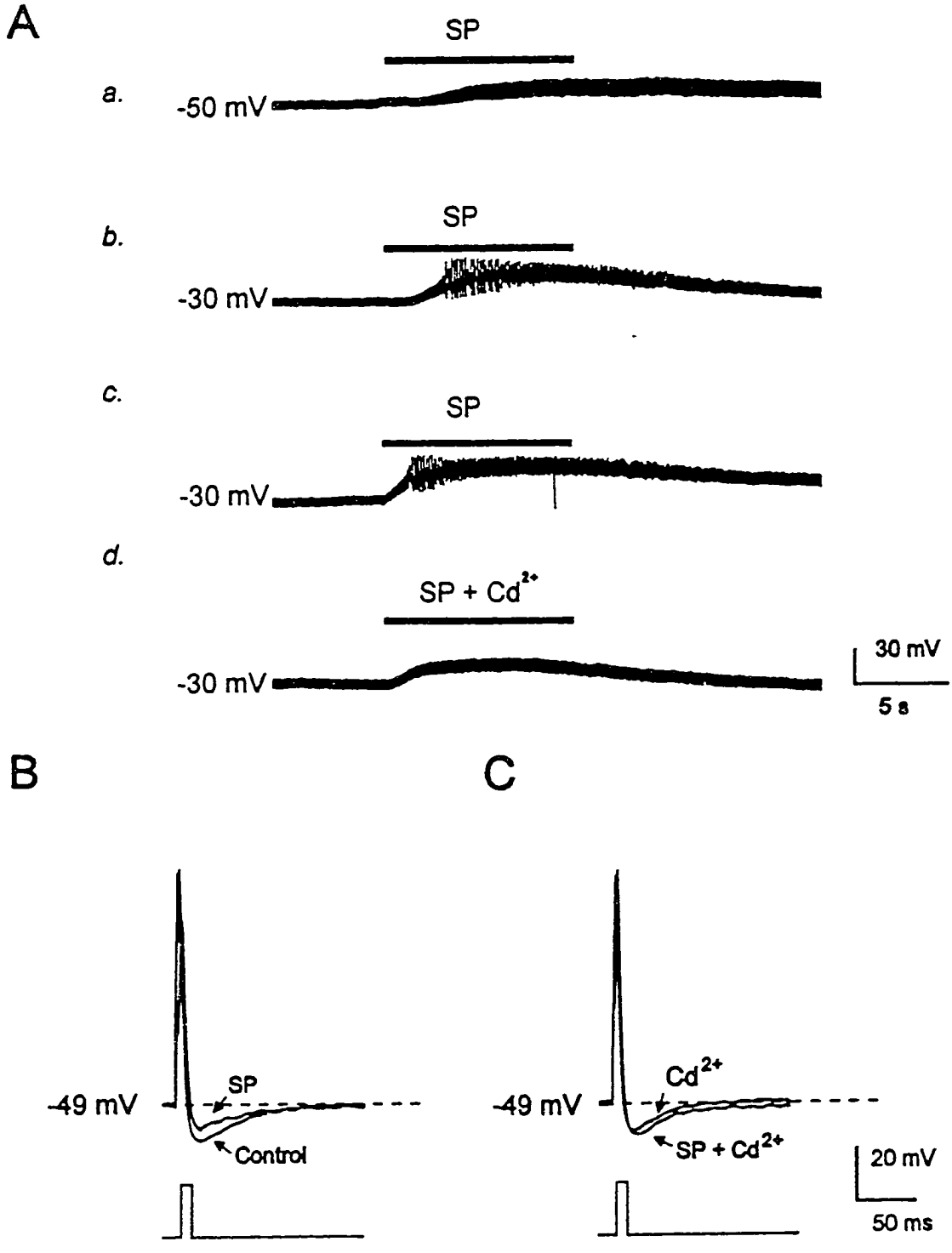

A
SP
a.
-50 mV
SP
b.
-30 mV
SP
c.
-30 mV
d.
SP + Cd2+
-30 mV
30 mV
5 s
B
C
-49 mV
SP
Control
-49 mV
Cd2+
SP + Cd2+
20 mV
50 ms

FIGURE 3-8

that observed with SP alone ($p<0.01$, Student's paired t-test); in these cells repeat application of SP produced a mean depolarization of 18 ± 5 mV accompanied by a 19 ± 9 % decrease in conductance (see Fig. 3-8Ad).

The effect of SP application on depolarization-elicited AP's was examined (Fig. 3-8B). Pressure application of SP (500 nM) decreased the AP hyperpolarization amplitude from 14.5 to 9 mV and decreased the AHP duration from 35 to 28 ms, with an increase in the AP duration from 11 to 14 ms. In 6 other neurons examined, SP produced a mean decrease in AP hyperpolarization amplitude of $54 \pm 15\%$ and a reduction in the time to one half AHP inactivation by $59 \pm 13\%$ (n=6). Subsequent application of SP (500 nM) in the presence of Cd^{2+} in the neuron shown in Figure 3-8C and in 3 other neurons tested, failed to significantly affect AHP. Thus, the depolarizing actions of SP on membrane potential, which are associated with a decrease in the total membrane conductance as well as the inhibition of the AHP in SG neurons, are consistent with inhibition of K^+ conductance. Since the actions of SP on membrane potential and AHP were reduced in the presence of Cd^{2+}, which blocks voltage-dependent Ca^{2+} influx, this further suggests that the actions of SP on SG neurons may include in part inhibition of $I_{K(Ca)}$.

In 2 of 9 neurons tested, SP-induced depolarization caused a "switch" in the neuronal firing pattern from phasic to tonic (Figure 3-9), thus increasing their excitability. A similar observation has been made in numerous other excitable cells, including guinea-pig celiac ganglia neurons (Vanner et al., 1993).

FIGURE 3-9: Action potential discharge induced by depolarizing current injection in cultured SG neurons. Action potentials were recorded in control and in the presence of 500 nM SP, were evoked by a current pulse of threshold intensity and were recorded at the same membrane potential in each case. The resting membrane potential for this neuron was -65 mV. The neuron was clamped at -65 mV throughout the experiment.

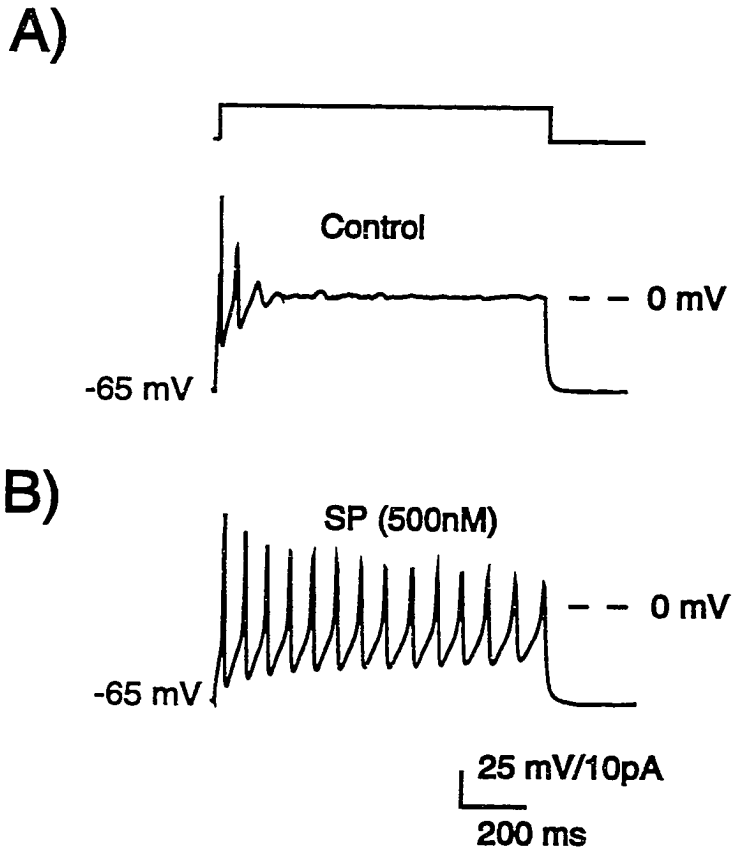


FIGURE 3-9

ii) Actions of Substance P on Whole-Cell Outward Potassium Currents in Rat and Guinea-Pig Stellate Ganglion Neurons

Figure 3-10 shows current recordings made from a guinea-pig SG neuron before and during a 30 s pressure application of 500 nM SP, measured at two different holding potentials. The current records in Figure 3-10A*a* demonstrate the lack of sensitivity of the transient outward current to SP. Current records were obtained from a guinea-pig neuron at a V_H of -100 mV and the membrane potential was stepped in 20 mV increments from -100 to +60 mV. Control recordings indicate that both transient and sustained outward K^+ currents are activated by depolarization in this neuron (Figure 3-10A*a*., upper traces). Application of 500 nM SP reduced the sustained component of the outward K^+ current but did not appear to affect the transient component of outward conductance (Figure 3-10*a*., lower traces). The I-V relationship for the peak outward currents recorded in both the presence and absence of 500 nM SP is shown in Figure 3-10B. At membrane potentials where the transient component of the outward current was activated in relative isolation from the sustained component (-50 mV to -30 mV) SP had little effect. Depolarizing V_H to -30 mV in the same neuron produced a voltage-dependent inactivation of the transient component of the outward current, yet left the sustained outward K^+ current (I_K) relatively unaffected (Figure 3-10A*b*, top traces). Application of 500 nM SP reduced I_K (Figure 3-10A*b*, bottom traces). The I-V relation

FIGURE 3-10: Inhibition of outward K^+ current (I_K) by SP. **Panel A)** I_K elicited by a series of step depolarizations to +60 mV from V_h = -100 mV (a) or -30 mV (b) recorded in absence (control) and presence of 500 nM SP. **Panels B) and C)** Current-voltage relationships for current records shown in A measured for peak (panel a; *) and sustained (panel b; #) components of I_K in the absence (■) and presence (●) of 500 nM SP.

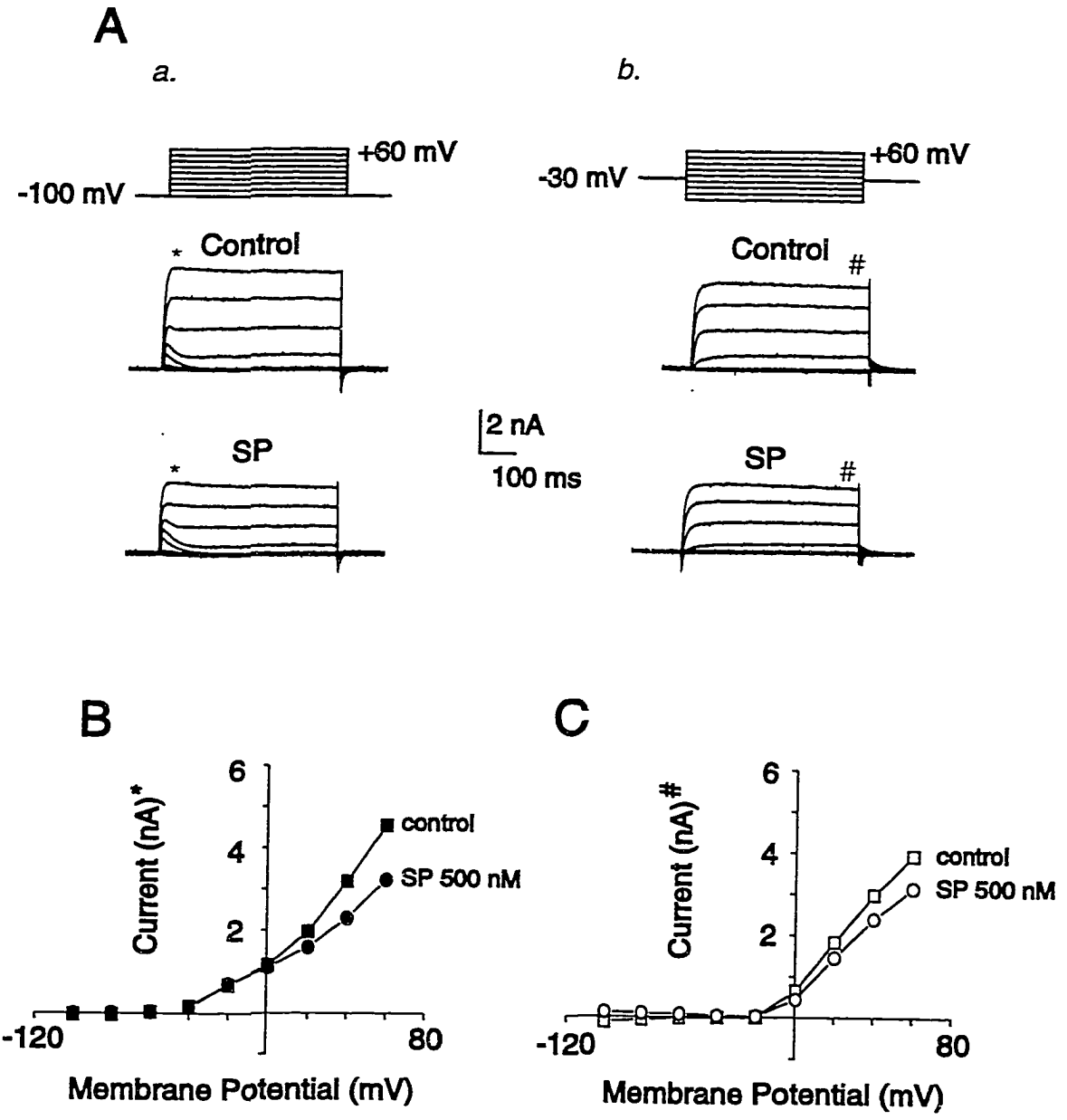

A
a.
b.
+60 mV
-100 mV
-30 mV
+60 mV
Control
Control
SP
SP
2 nA
100 ms
B
C
Current (nA)*
Current (nA)#
6
4
2
control
SP 500 nM
-120
80
Membrane Potential (mV)
Membrane Potential (mV)

FIGURE 3-10

shown in Figure 3-10C for current measured at the end of the voltage pulse demonstrates that SP now reduced the outward current over the range of potentials (-30 to +60 mV) at which sustained I_K would be activated. For the cell shown in Figure 3-10, the SP-mediated inhibition of outward current, measured at +60 mV, was greater at V_H = -100 mV (31%) than the inhibition observed when V_H was -30 mV (20%). This may reflect some inactivation of both Ca^{2+} and K^+ channels at depolarized potentials (Adams and Harper, 1995; Plummer et al., 1989; Xu and Adams, 1992a,b). Similar findings were seen in rat SG neurons in which SP (500 nM) reduced I_K measured at +60 mV by 23% ± 5 (n = 5).

Figure 3-11A shows a concentration-response curve for inhibition of the I_K at +60 mV by SP. I_K was measured at the end of a 500 ms voltage pulse to +60 mV in cells held at V_H = -60 mV. SP had little effect on I_K at doses of 5 nM, with maximal inhibition obtained at 500 nM SP. The reduction of I_K by 500 nM SP at 0 and +60 mV was 29 ± 5% and 33 ± 4% (n=31), respectively.

iii) Substance P Inhibits Outward Potassium Current in Guinea-Pig SG neurons through an NK_1 Tachykinin Receptor

The receptors for SP belong to the tachykinin receptor family and include receptors classified as NK_1, NK_2 and NK_3. The preferred endogenous ligands for these receptor types are SP, NKA (Substance K) and NKB, respectively (Lavielle et al., 1990, Maggi and Schwartz 1997). We investigated which receptor type might be involved in SP-mediated inhibition of I_K in

FIGURE 3-11: Substance P inhibits I_K via interaction with NK_1 tachykinin receptors. **Panel A)** Concentration-response curves for inhibition of I_K by SP. [SP], SP concentration. Each point is mean ± SE; numbers in parentheses is the number of cells tested. **Panel B)** SP-induced inhibition of I_K is attenuated in the presence of NK_1 selective antagonist CGP-49823 (1 μM). Bars represent mean ± SE inhibition of I_K by SP in neurons incubated in absence and presence of 1.0 and 0.25 μM CGP-49823. **Panel C)** Mean inhibition of I_K by SP, neurokinin A (NKA) and neurokinin B (NKB); error bars, SE. NKA and NKB produced significantly less inhibition of I_K than SP (*$P < 0.05$, **$P < 0.01$). Current in panels B and C was measured at the end of a 500 msec step depolarization to +60 mV from V_h = -60 mV.

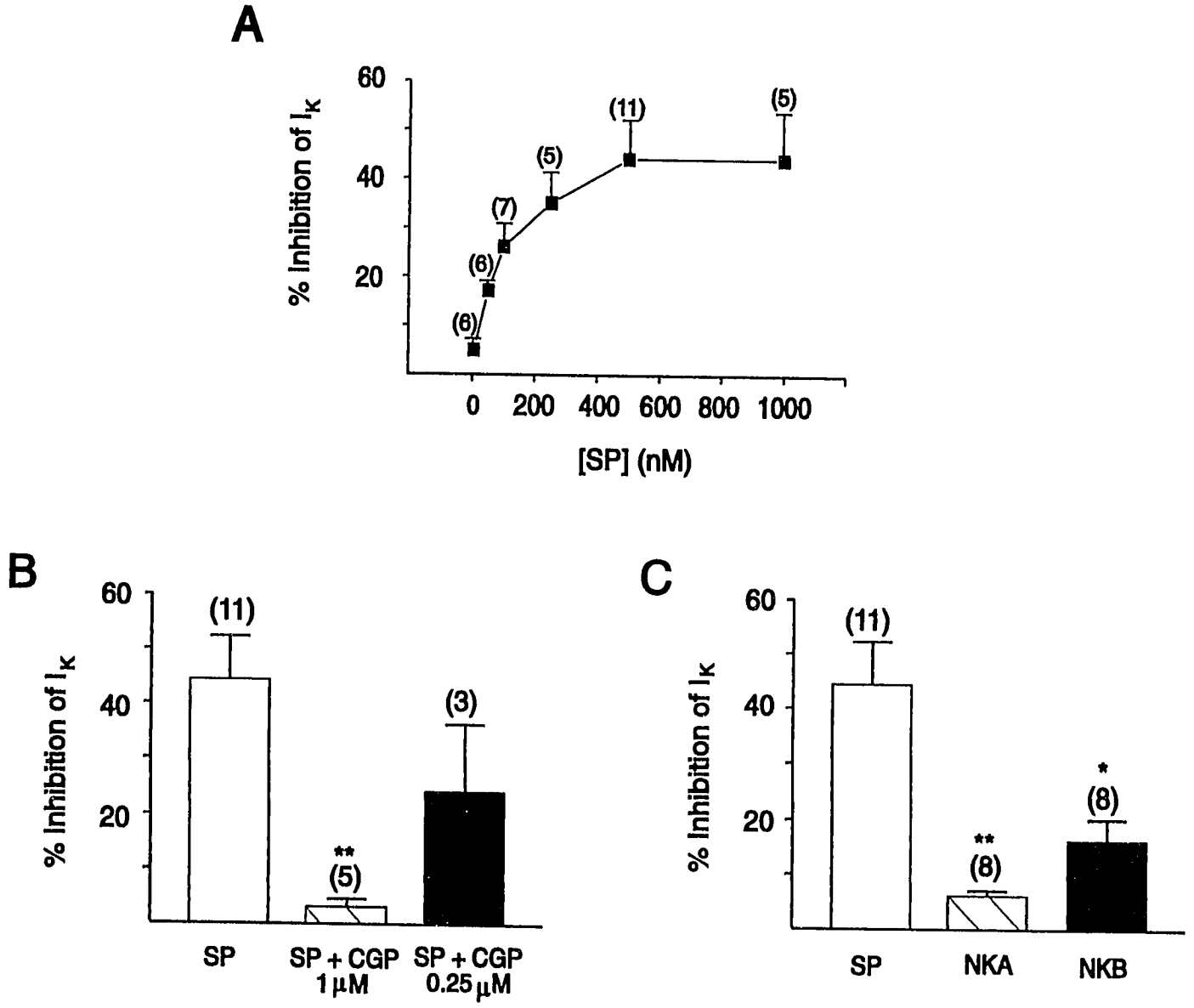

A
% Inhibition of I_K
60
40
20
(6)
(6)
(7)
(5)
(11)
(5)
0
200
400
600
800
1000
[SP] (nM)
B
% Inhibition of I_K
60
40
20
(11)
**
(5)
(3)
SP
SP + CGP 1 μM
SP + CGP 0.25 μM
C
% Inhibition of I_K
60
40
20
(11)
**
(8)
*
(8)
SP
NKA
NKB

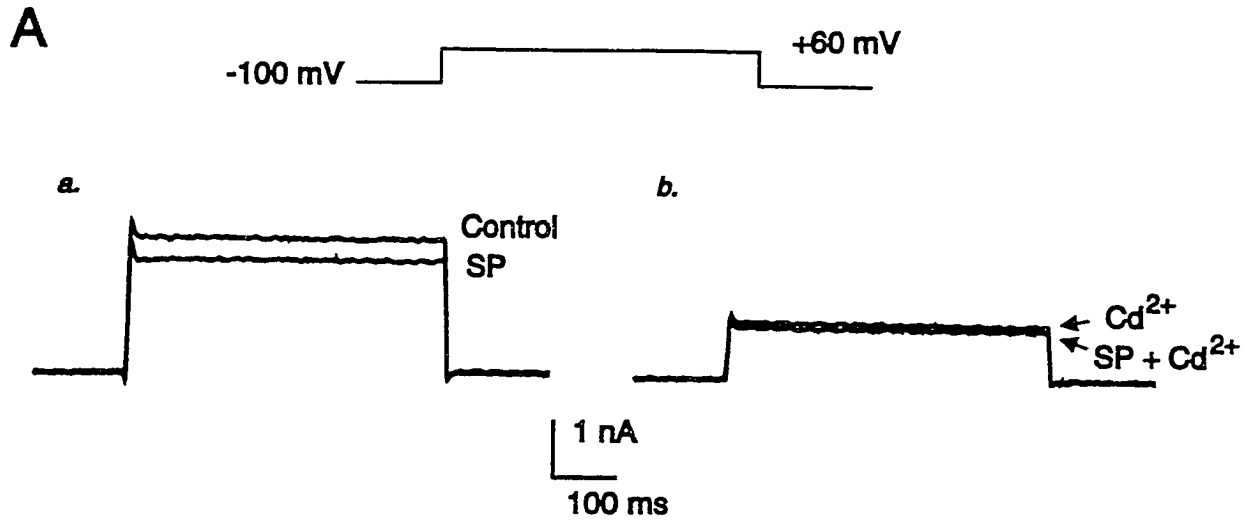
FIGURE 3-11

SG neurons by using the non-peptide NK_1 selective antagonist CGP49823 (Saleh et al., 1996). Figure 3-11B summarizes the effects of CGP49823 (0.25 and 1μM) on the response to 500 nM SP. In the absence of the antagonist, SP reduced I_K by 44 ± 8% (n=11, see also Figure 3-10A). However, when neurons were exposed to 1 μM CGP49823, the inhibition of I_K by SP was reduced to 3 ± 1.5% (n=5, $P < 0.01$). At a concentration of 0.25 μM, CGP49823 did not significantly affect SP's ability to inhibit I_K (24% ± 12, n = 3, $p>0.05$).

We also compared the effects of the agonists SP, NKA and NKB on the inhibition of I_K in guinea-pig SG neurons. The potency order has been reported to be: SP>> NKA> NKB for the NK_1 receptor; NKA>NKB>>SP for the NK_2 receptor and NKB> NKA> SP for the NK_3 receptor (Lavielle et al., 1990; Shapiro et al., 1994). In the present study, Figure 3-11C shows the mean inhibition of I_K produced by 500 nM of either SP, NKA or NKB in 27 separate cells stepped to +60 mV for 500 ms from a V_H=-60 mV. Whereas inhibition by SP was 44 ± 8.0% (n=11), NKA and NKB reduced I_K only by 6.0 ± 1.0% (n=8) and 16.0 ± 4.0% (n=8), respectively ($p<0.01$ and $p< 0.05$, with respect to control). These data indicate that SP effects on I_K are mediated via NK_1 receptors, since the affinity order for the inhibition of I_K was SP>>NKA ≥ NKB.

iv) Actions of Substance P on Calcium-Activated Potassium Current in Guinea-Pig Stellate Ganglion Neurons

FIGURE 3-12: The effects of SP on $I_{K(Ca)}$. **Panel A)** Outward current traces elicited by a 500 ms depolarizing pulse to +60 mV from a holding potential of -100 mV, in an representative SG neuron before (control) and after a 30 s pressure application of 500 nM SP (panel A*a*). In the same neuron superfused with Cd^{2+} (0.2 mM), SP has no effect (panel Ab). **Panel B)** Bars on the histogram represent mean (± SE) inhibition of I_K by SP (500 nM) in the absence and the presence of 0.2 mM Cd^{+2}, 0.02 mM Cd^{+2}, and 10 μM ω-CgTx in the external recording solution. Outward K^+ current was measured at the end of a 500 ms voltage step to 0 mV (open bars) and +60 mV (hatched bars), from V_H= -60 mV. *$p<0.05$, **$p < 0.01$ compared to inhibition with SP in absence of Cd^{2+} or ω-CgTx.


A
-100 mV
+60 mV
a.
b.
Control
SP
Cd2+
SP + Cd2+
1 nA
100 ms

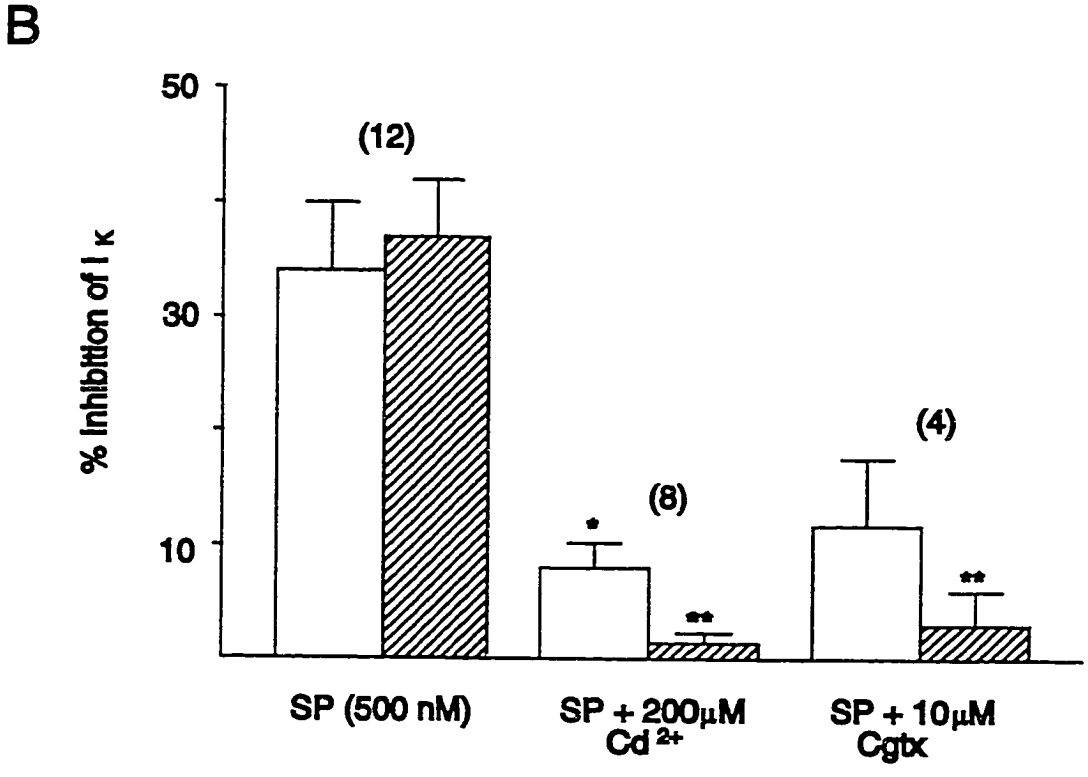

B
% Inhibition of I K
50
30
10
(12)
(8)
(4)
*
**
**
SP (500 nM)
SP + 200µM Cd 2+
SP + 10µM Cgtx

FIGURE 3-12

The inhibition of I_K by SP was significantly diminished in neurons exposed to Cd^{2+}. Figure 3-12A shows current traces recorded before and after SP application in a neuron superfused with standard extracellular recording solution and subsequently with solution containing 0.2 mM Cd^{2+}. Depolarizing voltage steps to +60 mV were applied from a V_H of -100 mV for 500 ms. In the presence of Cd^{2+}, the inhibitory actions of SP on the outward current were reduced, suggesting that the SP may be inhibiting a Ca^{+2}-dependent K^+ conductance ($I_{K(Ca)}$). Figure 3-12B represents the mean inhibition of I_K by SP (500 nM) in the presence of 0.2 mM Cd^{2+} or 10 μM ω-CgTx. Membrane potential of SG neurons was held at a V_H of -60 or -100 mV and stepped to 0 and +60 mV for 500 ms and I_K was measured at the end of the voltage pulses. Under control conditions, SP reduced I_K by 34 ± 6 % at 0 mV, and by 37 ± 5 % at +60 mV (n= 15). However, when the neurons were superfused with 0.2 mM Cd^{2+}, I_K measured at 0 mV and +60 mV was only reduced by only 8 ± 2 % and 1.4 ± 1 % (n=8), respectively ($p<0.01$ and $p< 0.05$ with respect to control). Similarly, in the presence of ω-CgTx, inhibition of the I_K by SP at 0 and +60 mV was only 12 ± 6 % and 3.0 ± 3.0 % (n=4).

v) Actions of Substance P on I_K are Mediated by a PTX-Insensitive G-Protein

The involvement of G-proteins in the signaling pathway of SP are well established (Hille, 1994). To confirm that SP modulation of I_K is mediated through a G protein-coupled pathway in SG neurons, we compared cells dialyzed with a GTP-free pipette solution containing 2 mM GDPβS, which is an antagonist of G protein activation,

FIGURE 3-13: Inhibition of I_K by SP is mediated by a PTX-insensitive G-protein. **Panel A)** Inclusion of GDPβS in the pipette attenuated the SP response. Bars on the histogram are mean (± SE) inhibition of I_K by 500 nM SP with the standard pipette solution (0.3 mM GTP), or with inclusion of 2 mM GDPβS (0 GTP). In all experiments cell were dialyzed with internal solution for 10 minutes before recording to allow for sufficient guanine nucleotide dialysis. **Panel B)** Inhibition of I_K by SP is PTX insensitive. Bars are mean (± SE) inhibition of the steady state I_K at +60 mV by SP. Cells were cultured overnight, as described in the methods with 500 ng/ml PTX. Current inhibition in control neurons was recorded with standard recording solutions. $p < 0.05$ *.

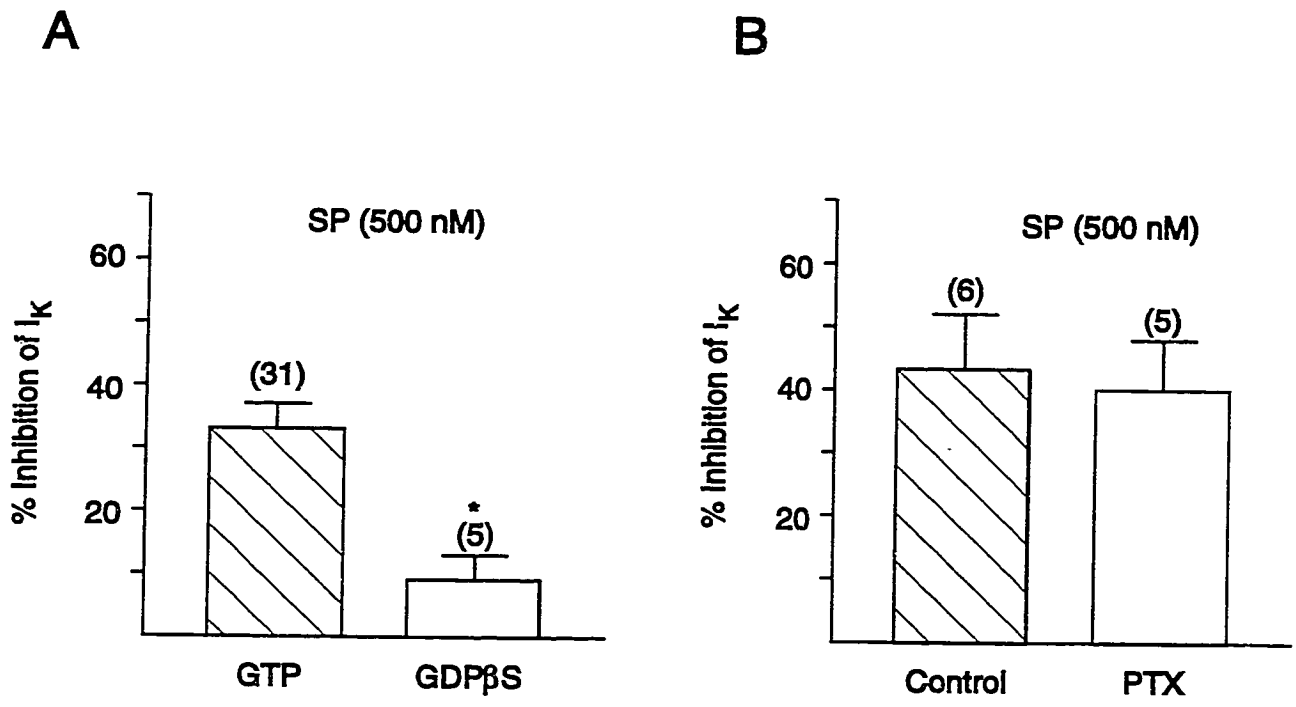

A
SP (500 nM)
% Inhibition of I_K
60
40
20
(31)
(5)
*
GTP
GDPβS
B
SP (500 nM)
% Inhibition of I_K
60
40
20
(6)
(5)
Control
PTX

FIGURE 3-13

with results obtained using standard pipette solution (0.3 mM GTP) (Holz et al., 1986; Shapiro et al., 1994). Neurons were dialyzed in the whole-cell configuration for at least 10 minutes before pressure application of SP (500 nM). Figure 3-13A summarizes the effects of GDPβS dialysis on the responsiveness of SG neurons to SP. SP inhibited I_K (measured at the end of a 500 ms voltage-step to +60 mV from V_H=-60 mV) by only 9.0 ± 4.0%, (n=5) in neurons dialyzed with 2 mM GDPβS as compared to the 33 ± 4.0% (n =31) observed in control cells which were dialyzed with GTP (Fig. 3-13A). These data confirm that the SP mediated inhibition of I_K is via a G protein-coupled pathway. To identify the type of G protein involved in SP mediated inhibition of I_K, we incubated SG neurons with PTX. Pertussis toxin inactivates G proteins of the G_i, G_Z or G_o class by catalyzing nicotinamide-adenine dinucleotide-dependent ADP ribosylation of the α subunit (Milligan, 1988). Incubation for 24 hr with PTX (500 ng/ml) was ineffective in blocking SP inhibition of I_K (40% ± 8, n=5) (Figure 3-13B), compared to control untreated cells (43% ± 9, n=6). These results suggest that PTX-sensitive G-proteins are not involved in SP modulation of I_K in SG neurons.

vi) SP-mediated I_K Inhibition is Independent of Cyclic AMP, Protein Kinase C or Protein Kinase A.

It is well established that the behavior of ion channels can be modulated by a host of intracellular mediators and by phosphorylation (Hille, 1994; Leviaton, 1988; Schultz et al., 1990). Experiments were carried out to determine whether kinase-dependent

FIGURE 3-14: Inhibition of I_K by SP is not mediated by cyclic AMP, protein kinase A or C. Bars on the histogram are mean (± SE) % inhibition of I_K by SP, measured at +60 mV in the absence (control) or presence of forskolin, dibromo cyclic AMP, and the PKC inhibitor GF109203-X.

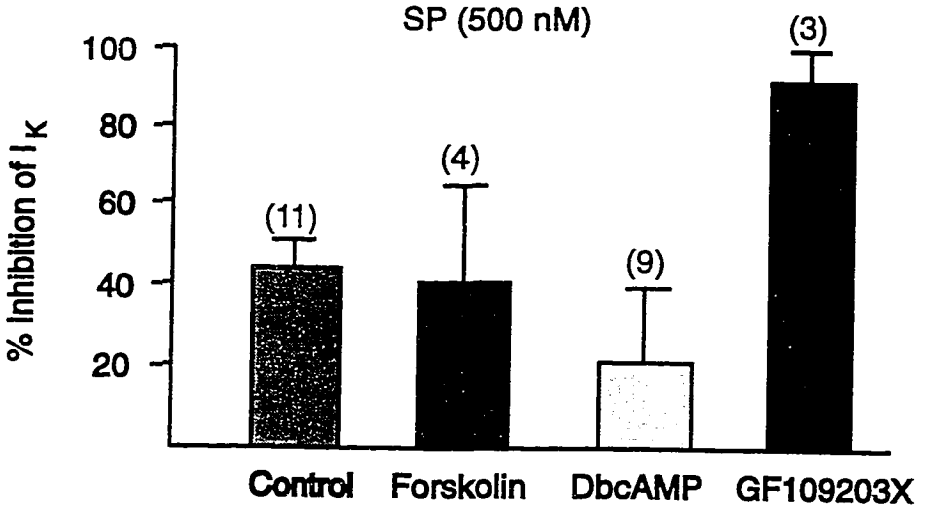

SP (500 nM)
% Inhibition of I_K
100
80
60
40
20
(11)
(4)
(9)
(3)
Control
Forskolin
DbcAMP
GF109203X

FIGURE 3-14

phosphorylation was involved in SP-mediated inhibition of $I_{K(Ca)}$ in cultured SG neurons (figure 3-14). To examine a potential role of protein kinase A, the effects of SP on $I_{K(Ca)}$ were examined when cyclic AMP levels were augmented by inclusion of dibromo cyclic AMP (1 mM) plus IBMX (0.1 mM, an inhibitor of phosphodiesterase) in the whole-cell path pipette, or by superperfused with the adenylate cyclase activator forskolin (10 μM) in the external recording solution. Neither inclusion of dibromo cyclic AMP / IBMX in the pipette, nor bath perfusion with forskolin significantly reduced the SP-mediated inhibition of $I_{K(Ca)}$. To determine a potential role of protein kinase C (PKC) in mediating the actions of SP on $I_{K(Ca)}$ SG neurons were superfused with 3 μM of the selective PKC inhibitor bisindomalemide (GF109203-X) (Bonev and Nelson, 1993; Kubokawa et al., 1995; Toullec et al., 1991). GF109203-X treatment did not reduce the SP-mediated inhibition of $I_{K(Ca)}$ but rather enhanced the SP-induced inhibition of the sustained whole-cell K^+ conductance in these neurons. GF109203-X, dibromo cyclic AMP and forskolin applied by themselves were without effect on I_K. These findings do not support a role for PKA or PKC in the SP-induced inhibition of $I_{K(Ca)}$ in SG neurons.

B. Actions of Substance P and Calcitonin Gene Related Peptide on Calcium Currents in Guinea-Pig Stellate Ganglion Neurons

i) Substance P inhibits an N-type Calcium Current in Guinea-Pig Stellate Ganglion Neurons

The finding that SP inhibition of I_K is reduced in SG neurons pretreated with either Cd^{2+}, or the high voltage-activated (HVA) N-type Ca^{2+} channel blocker ω-CgTx (Tsien et al., 1988), suggests that SP may act to inhibit $I_{K(Ca)}$ in SG neurons via the reduction of voltage-dependent Ca^{2+} influx. SP has been reported to act on NK_1 receptors inhibiting N-type Ca^{2+} channels in dissociated rat superior cervical ganglion neurons (Shapiro et al., 1994). Therefore, we investigated the effects of SP on Ca^{2+} channel currents in SG neurons. Ion substitutions used to isolate Ca^{+2} channel current are described in the METHODS. Using 5 mM Ba^{+2} as the charge carrier, in solutions designed to eliminate all K^+ and Na^+ currents, membrane depolarization elicited an inward current (I_{Ba}). Figure 3-15A shows I_{Ba} recorded at 0 mV in an SG neuron before and during application of 500 nM SP. In this cell, SP decreased the amplitude of I_{Ba} by 33%. Similar results were observed in 8 cells examined; in these cells the mean inhibition of I_{Ba} by SP was 49% ± 8. The I-V relationship in Figure 3-15B represents the SP-sensitive inward current measured from peak inward currents recorded in the absence (squares) and presence (circles) of 500 nM SP. As shown for the ω-CgTx-sensitive current in Figure 3-7C, the inward SP-sensitive current activates at -30 mV with peak current around 0 mV. These data indicate that SP inhibition of N-type Ca^{2+} current may account in part for the observed SP-induced decrease in $I_{K(Ca)}$.

ii) Calcitonin-Gene Related Peptide Inhibits N-Type Calcium Currents in Guinea-Pig Stellate Ganglion Neurons

FIGURE 3-15: Substance P reduces voltage-dependent Ba^{2+} currents in cultured SG neurons. **Panel A)** Superimposed I_{Ba} elicited with a step depolarization to 0 mV from a holding potential of -90 mV in both the absence (control) and presence of 500 nM SP. **Panel B)** The I-V relationship for the SP-sensitive currents, was obtained by subtracting currents recorded in the presence of SP from control currents (same neuron as in A).

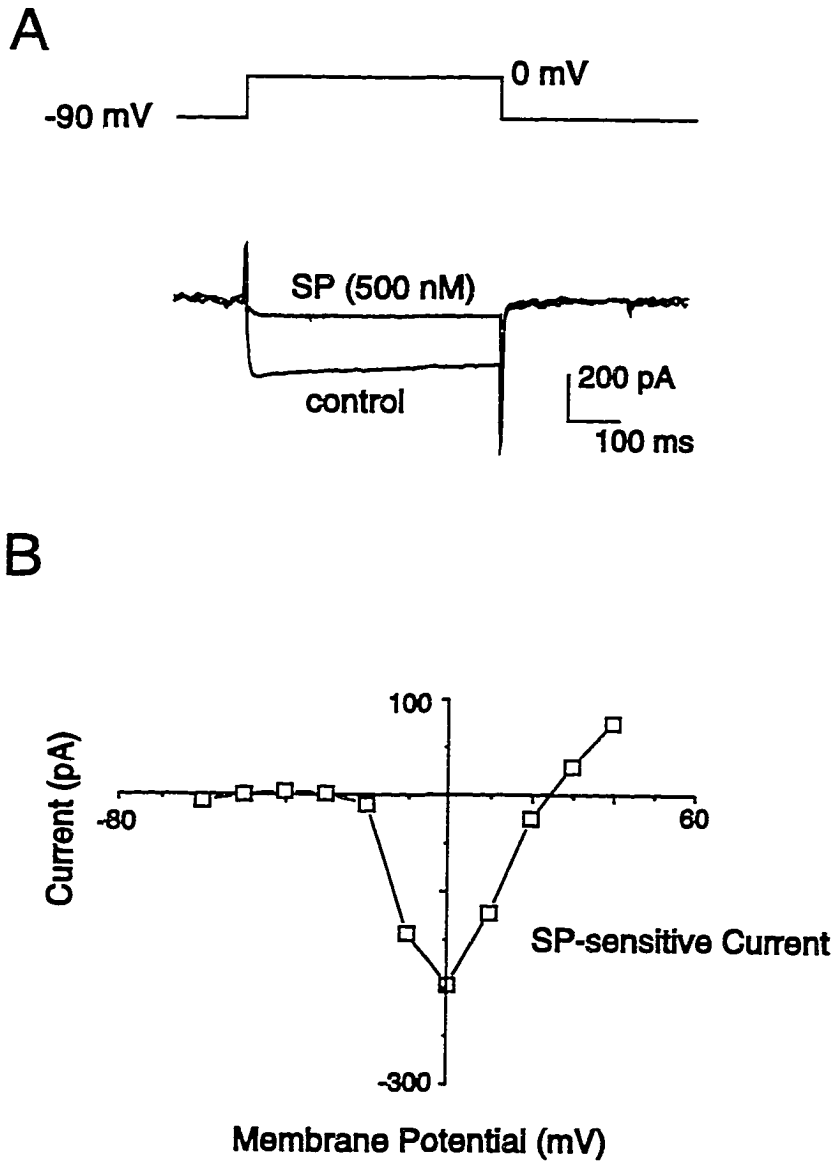

A
0 mV
-90 mV
SP (500 nM)
control
200 pA
100 ms
B
100
-80
60
-300
Current (pA)
SP-sensitive Current
Membrane Potential (mV)

FIGURE 3-15

CGRP has been reported to inhibit HVA type Ca^{2+} channels in cultured rat neocortical neurons (Zona et al., 1991), while in rat dorsal root ganglion neurons (Ryu et al., 1988; Wiley et al., 1992) it enhanced Ca^{2+} current. Therefore, we investigated the effects of CGRP on Ca^{2+} currents in guinea-pig SG neurons. Figure 3-16A shows I_{Ba} recorded at 0 mV in a SG neuron before and during application of 500nM CGRP. In this cell CGRP decreased the amplitude of I_{Ba} by 19 %. Similar results were observed in 5 other cells examined. In these cells the mean inhibition of I_{Ba} by CGRP was 16% ± 7. The I-V plot in figure 3-16B shows the CGRP -sensitive inward current measured from peak inward currents recorded in the presence of 500 nM CGRP in a representative SG neuron. The inward CGRP-sensitive current activates at -30 mV, with peak current around 0 mV. Figure 3-16C shows the dose-response curve for inhibition of the I_K at +60 mV by CGRP. I_{Ba} was measured at the end of a 500 ms voltage pulse to +60 mV in cells held at V_H =-60 mV. CGRP had little effect on I_{Ba} at doses of 5 nM, with maximal inhibition obtained at 500 nM CGRP.

6. LIGAND-GATED WHOLE-CELL NICOTINIC ACETYLCHOLINE RECEPTOR CURRENT IN CULTURED GUINEA-PIG AND RAT STELLATE GANGLION NEURONS

Activation of nAChRs in mammalian sympathetic neurons induces the opening of a cation-selective channel (Derkach et al., 1983; Skok, 1983; Colquhoun et al., 1987; Mathie et al., 1990). Under physiological conditions this leads to an influx of Na^+ and

FIGURE 3-16: Effects of CGRP on voltage-dependent Ba^{+2} current in cultured SG neurons. **Panel A)** Superimposed I_{Ba} elicited with a step depolarization to 0 mV from a holding potential of -90 mV, in the absence (control) and presence of 500 nM CGRP. **Panel B)** The I-V relationship for the CGRP-sensitive current for the neuron in panel A, was obtained by subtracting currents recorded in the presence of CGRP from control currents. **Panel C)** Concentration-response curves for inhibition of I_{Ba} by CGRP. [CGRP], CGRP concentration. Each bar represents the mean inhibition if I_{Ba}; error bars, SE; numbers in parentheses indicates the number of cells tested.

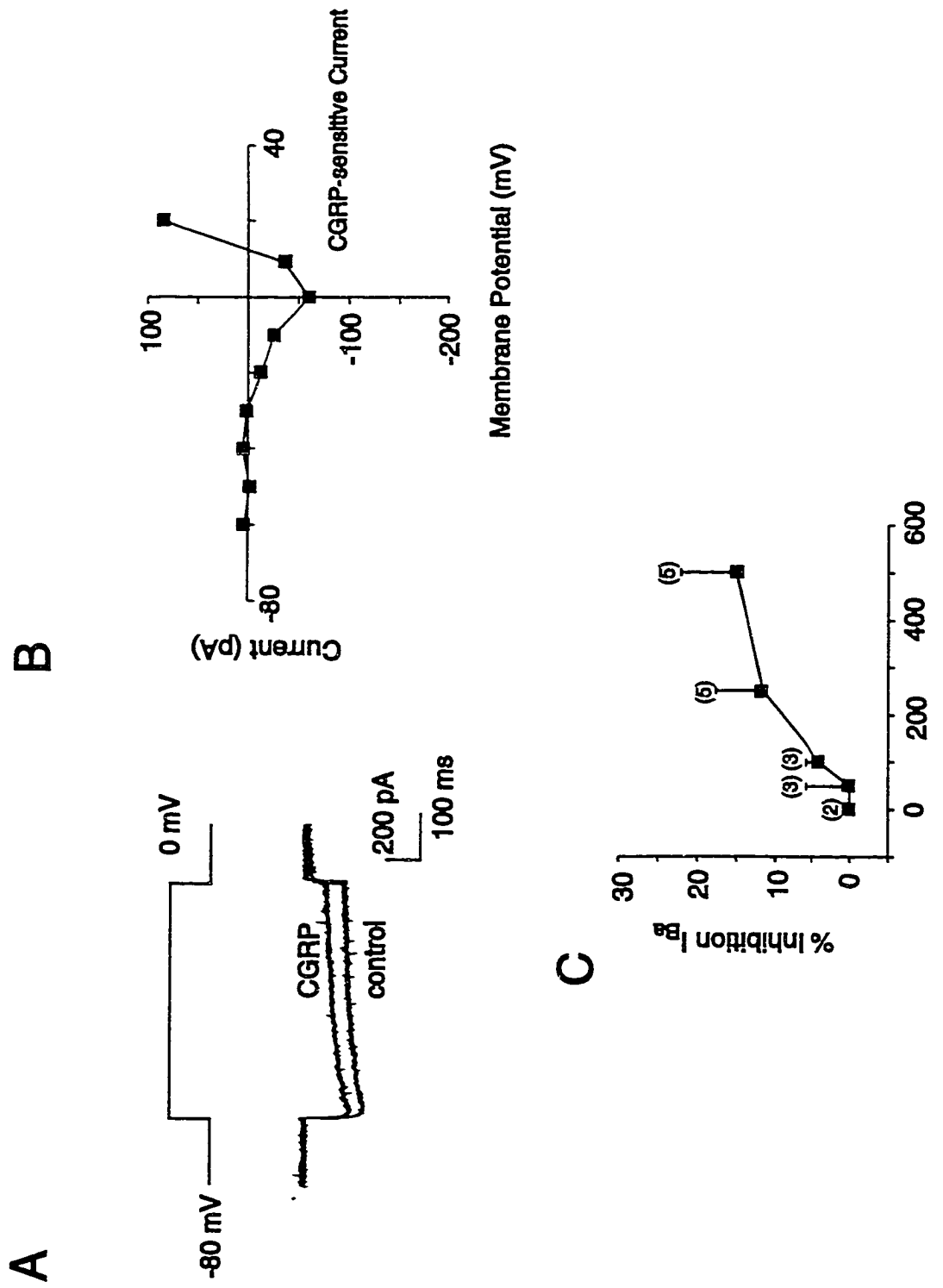

A
-80 mV
0 mV
CGRP
control
200 pA
100 ms
B
Current (pA)
100
-80
-100
-200
40
CGRP-sensitive Current
Membrane Potential (mV)
C
% Inhibition I_Ba
30
20
10
0
0
200
400
600
[CGRP] (nM)
(2)
(3)
(3)
(5)
(5)

FIGURE 3-16

membrane depolarization. Substance P is known to modify the actions of cholinergic agonists on nAChRs in neuronal tissue, skeletal muscle and electroplaque (Livett and Zhou 1991). Since SP has been identified in the nerve fiber networks of the SG we have investigated whether this neuropeptide affects the function of nAChR ion channels in isolated SG neurons.

A. Whole-Cell Currents Evoked by Nicotinic Agonists

Guinea-pig and rat SG neurons responded to prolonged (> 5 sec) application of ACh analogues with an initial brisk depolarization (activation of inward current) that decayed with continued exposure to these analogues. Under current-clamp mode, pressure application of the nicotinic AChR analogues 1,1-dimethyl-4-phenylpiperaznium iodide DMPP (100 μM) and carbachol (1mM) to rat SG neurons induced a mean rapid depolarization from the RMP of 10 mV ± 1.4 (n=3) and 20 mV (n=2), respectively. Associated with membrane depolarization was a increase in membrane conductance. Figures 3-17A and 3-18A illustrate typical responses of current-clamped rat SG neurons to prolonged (> 5 sec) application of 100 μM DMPP or 1 mM carbachol. Under voltage-clamp conditions, from a V_H of -60 mV, pressure application of 100 μM DMPP or 1 mM carbachol induced an inward current. Figures 3-17B and 3-18B illustrates the typical responses of voltage-clamped rat SG neurons to a 10 sec application of 100 μM DMPP or 1 mM carbachol. In the continued presence of DMPP inward currents increased rapidly to an initial peak value (I_{peak}) and then relaxed back towards baseline. This decay in the

FIGURE 3-17: DMPP-induced depolarization and whole-cell inward current in cultured rat SG neurons. In this and subsequent and in figures, DMPP was applied by pressure application at times indicated by the bar. **Panel A)** Under current-clamp application of 100 μM DMPP induced a membrane depolarization which was associated with an decrease in membrane resistance (measured as changes in response to repetitive 0 pA current injections) . **Panel B)** Whole-cell current responses induced by DMPP from a holding potential of -60 mV, and measured at successive 3 minute intervals.

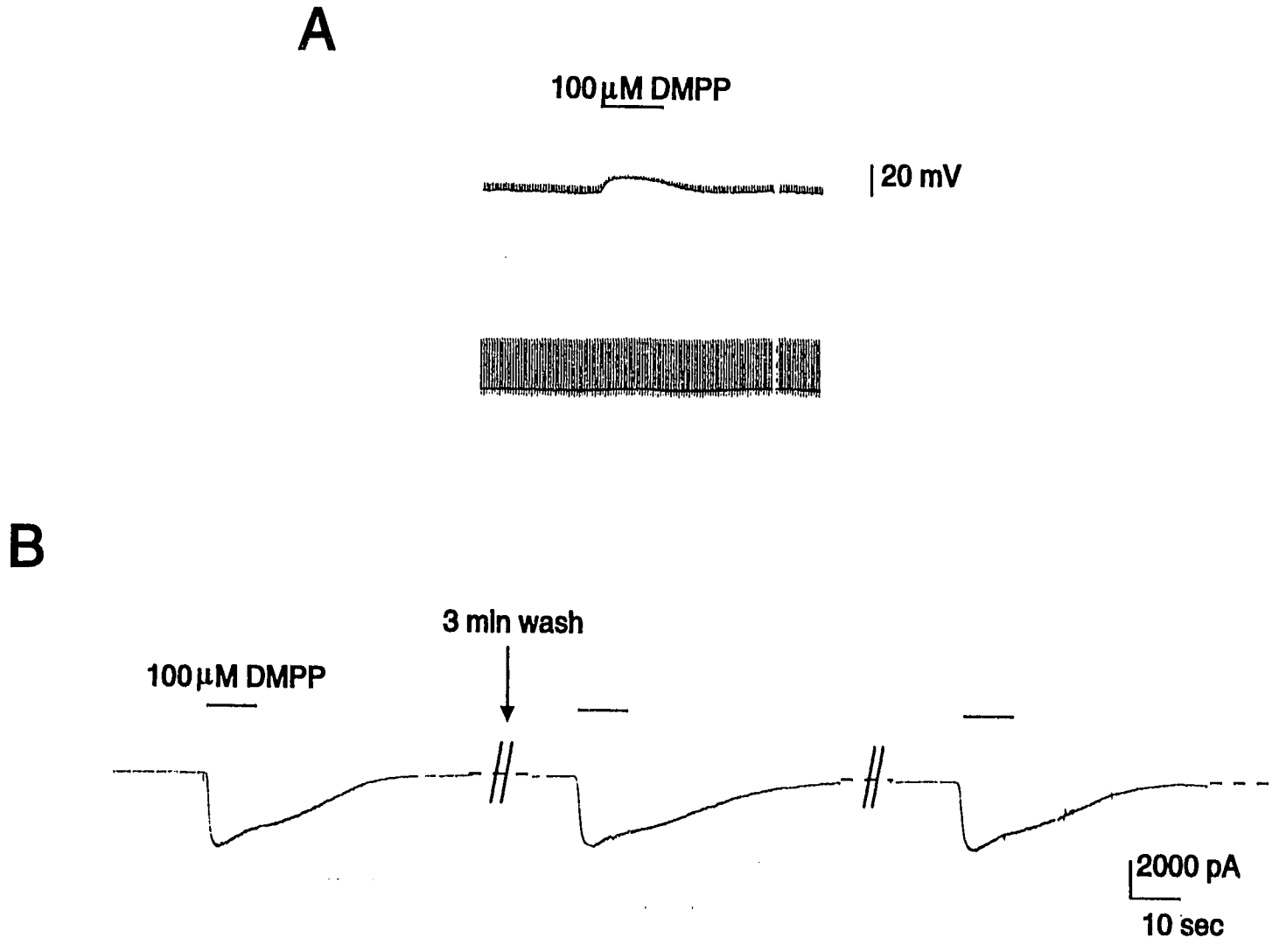


FIGURE 3-17

FIGURE 3-18: Carbachol-induced membrane depolarization and whole-cell inward current in cultured rat SG neurons. **Panel A)** Under current-clamp pressure application of carbachol (1 mM) induced a membrane depolarization which is associated with a decrease in membrane resistance. **Panel B)** Whole-cell current responses induced by carbachol at a holding potential of -60 mV.

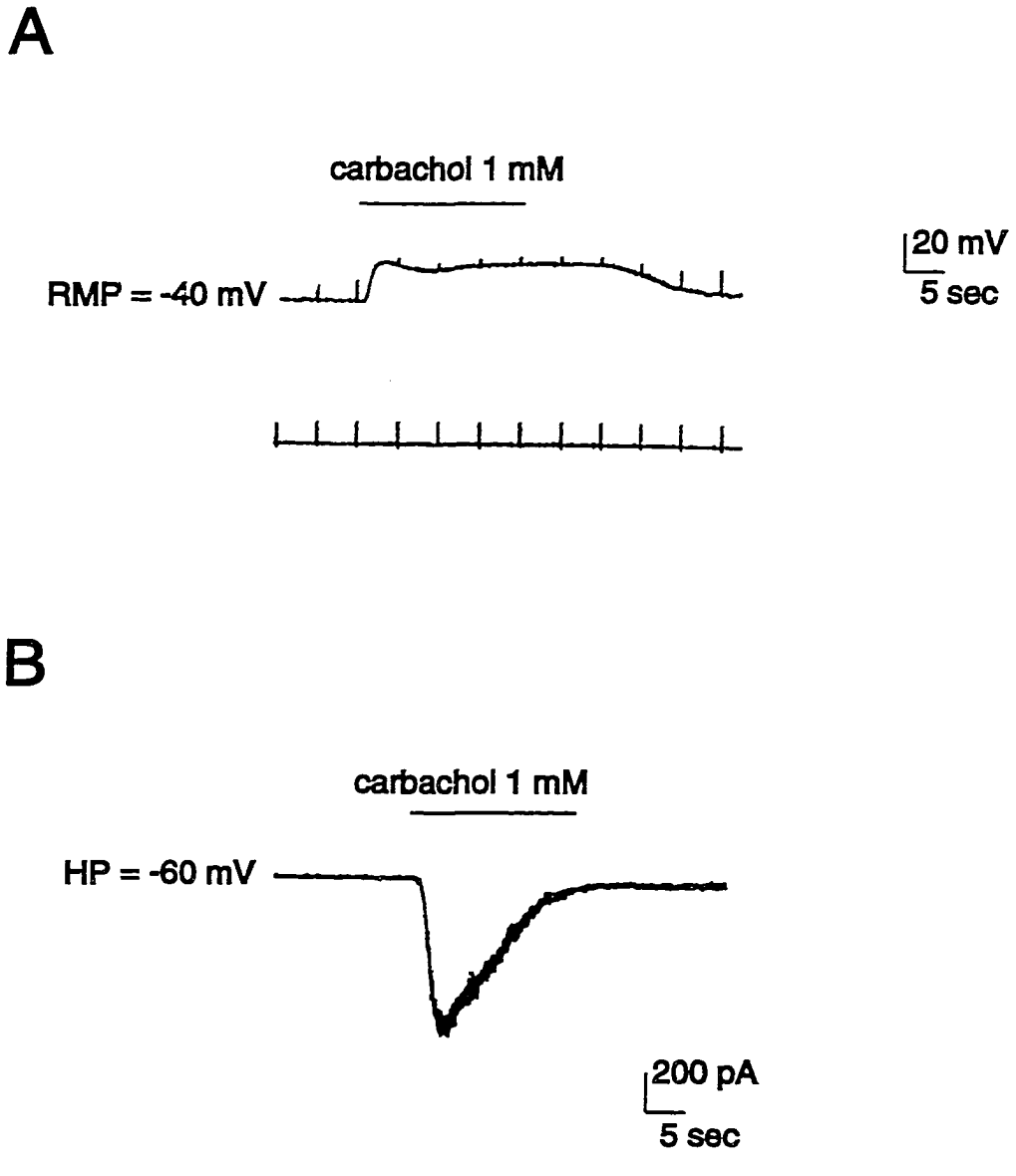

A
carbachol 1 mM
RMP = -40 mV
20 mV
5 sec
B
carbachol 1 mM
HP = -60 mV
200 pA
5 sec

FIGURE 3-18

response resulted in an inward current level which when measured 5 sec after the onset of DMPP application $I_{(5)}$ was 30 ± .05 % (n = 12) lower than I_{peak}. The rate of relaxation of inward current in the continued presence of DMPP can be measured by the I_5/I_{peak} ratio and is probably analogous to the agonist-dependent desensitization described in sympathetic neurons of the chicken (Role, 1984) and rat (Mathie et al., 1990). Whole-cell inward currents recorded in response to DMPP (10-100 μM) or carbachol 1 mM normally desensitized and recovered rapidly in both the rat and guinea-pig. An example of this recovery from desensitization is illustrated in figure 3-17B, for 100 μM DMPP. In this neuron the whole-cell responses were fully restored by a 3 min. wash between successive DMPP applications. Figure 3-19 shows the dose-response curve constructed for the DMPP-induced inward current in rat SG neurons. Peak inward current was measured in response to DMPP application in cells held at V_H = -60 mV. DMPP did not activate inward current at doses ≤ 1 μM, with maximal inward current obtained at 100 μM DMPP. The mean normalized peak inward current produced by 100 μM DMPP was 47.8 ± 5 pA/pF (n=13). In all rat and guinea-pig neurons examined application of DMPP (100 μM) in standard extracellular solution evoked inward currents of several hundred pA at a holding potential of -60 mV(n = 13 and n = 3, respectively). When extracellular NaCl was replaced with equimolar choline chloride the response to 100 μM DMPP application was reduced by 98 % ± 2 (n = 3, Figure 3-20). In the presence of the nicotinic antagonist mecamylamine (10 μM), the DMPP-induced inward current was inhibited by 81% ± 9.6 (n = 5) in rat SG neurons. Similar results

FIGURE 3-19: Concentration-response curves for peak nAChR current (pA/pF) induced by DMPP in cultured rat SG neurons. [DMPP], DMPP concentration. Each point represents the mean ± SE; numbers in parentheses indicate the number of cells tested.

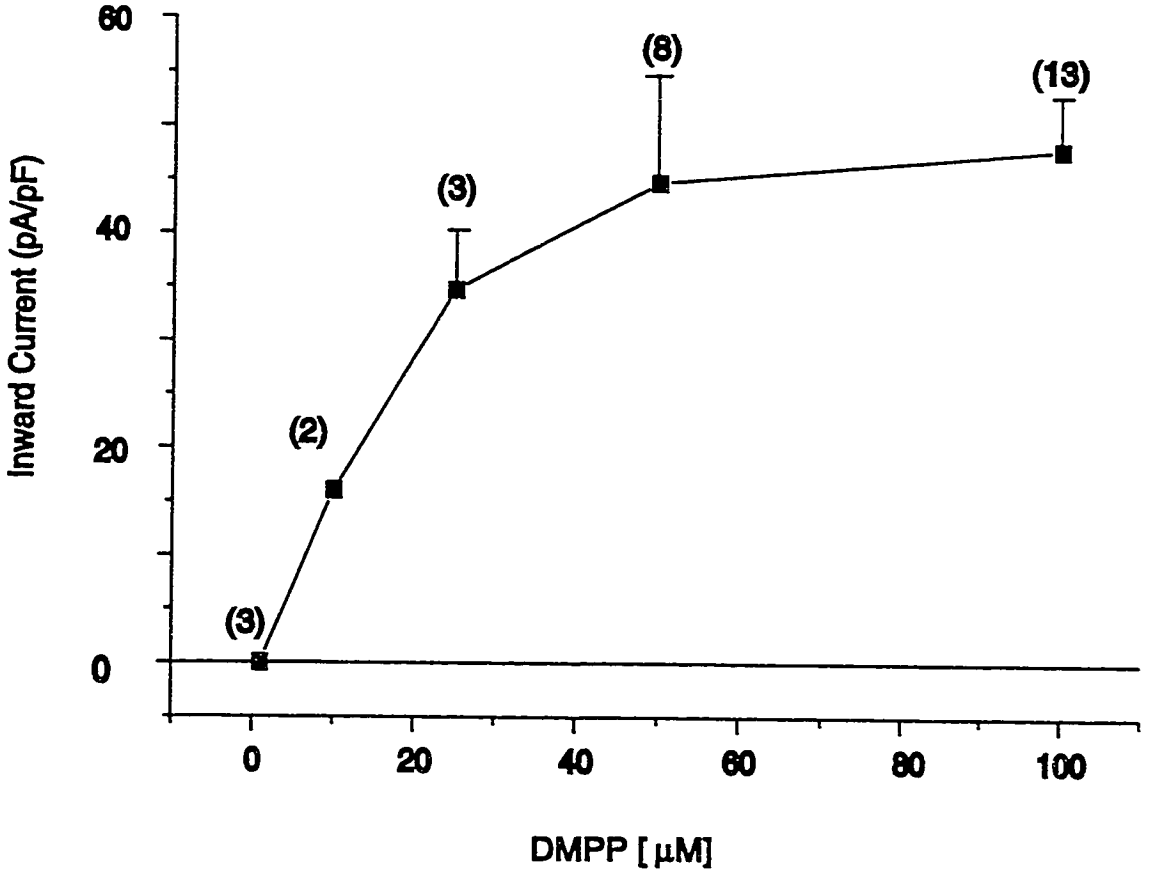


FIGURE 3-19

FIGURE 3-20: DMPP-induced inward current is reduced in the absence of extracellular Na^+. Whole-cell currents were recorded following pressure application of 100 μM DMPP in a single cultured SG neuron voltage-clamped at -60 mV. In this figure, DMPP was applied by pressure application at times indicated by the bar. Currents were recorded in standard extracellular recording solution (A), and in extracellular recording solution where the extracellular NaCl was replaced by an equimolar concentration of choline chloride (B).

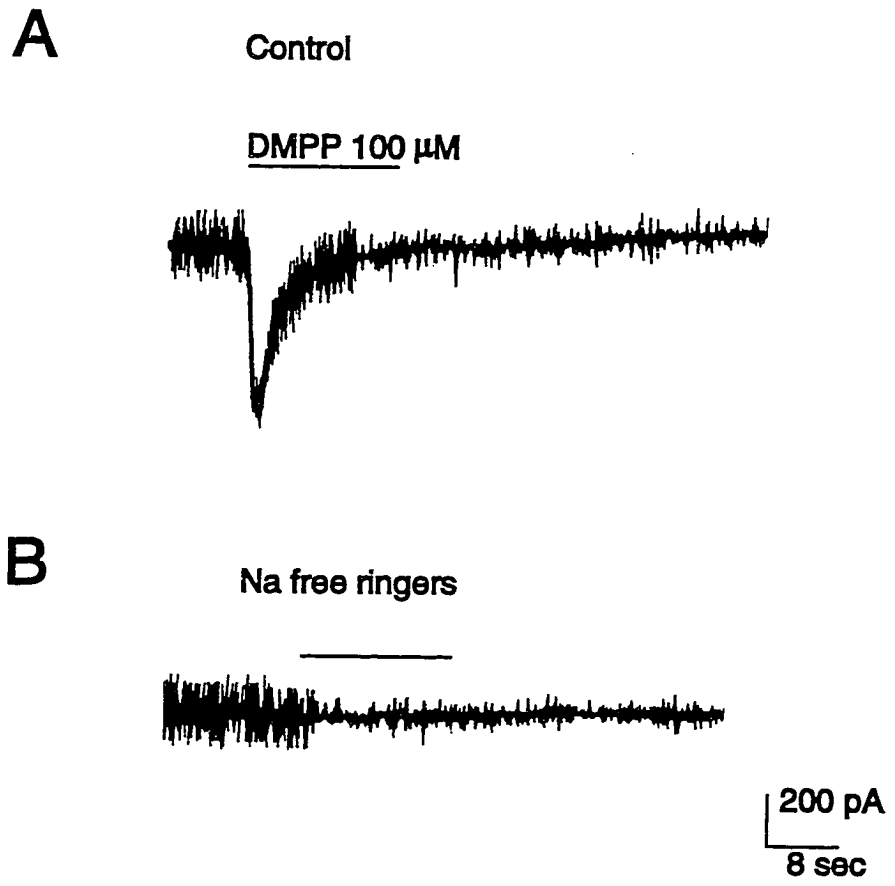


FIGURE 3-20

were observed in the guinea-pig SG neuron (90 %, n = 2). The effects of mecamylamine on a representative cultured SG neuron is shown in figure 3-21.

Peak whole-cell currents induced by ACh, DMPP and carbachol in rat sympathetic neurons have been shown to exhibit current-voltage relationships which are reasonably linear at negative membrane potentials, but rectify sharply with little current apparent at positive potentials (Mathie et al., 1987; Selyanko et al., 1988; Mathie et al., 1990). Figure 3-22A demonstrates that the DMPP-evoked current in rat SG neurons possess similar characteristics to those described in other sympathetic neurons. In this example, 100 μM DMPP was applied for the duration of the bar while the cell was held at -60 mV and four successive ramps of 2 sec duration between -120 mV and +50 mV were applied. At a membrane potential of -60 mV, DMPP application in this neuron produced an inward current of approximately -9000 pA . The current-voltage relationship for the DMPP-induced inward current was reasonably linear at negative potentials while at positive ramp potentials the inward current showed rectification and there was no outward current. Figure 3-22B shows the DMPP-sensitive current-voltage relationship for the neuron shown in panel A.

B. Substance P Modulation of DMPP-Induced Currents in Rat Stellate Ganglion Neurons

Since *in vivo* evidence has indicated that SG neurons receive peptidergic as well as cholinergic input, we examined the potential for modulation by SP of the DMPP-induced response in isolated SG neurons. As previously described, application of DMPP

FIGURE 3-21: Effects of mecamylamine on DMPP-induced current in cultured SG neurons. A representative neuron was voltage-clamped at -60 mV, and inward current was measured in response to pressure application of: (panel A) DMPP (100 μM), or (panel B) DMPP (100 μM) plus 10 μM mecamylamine (bath applied).

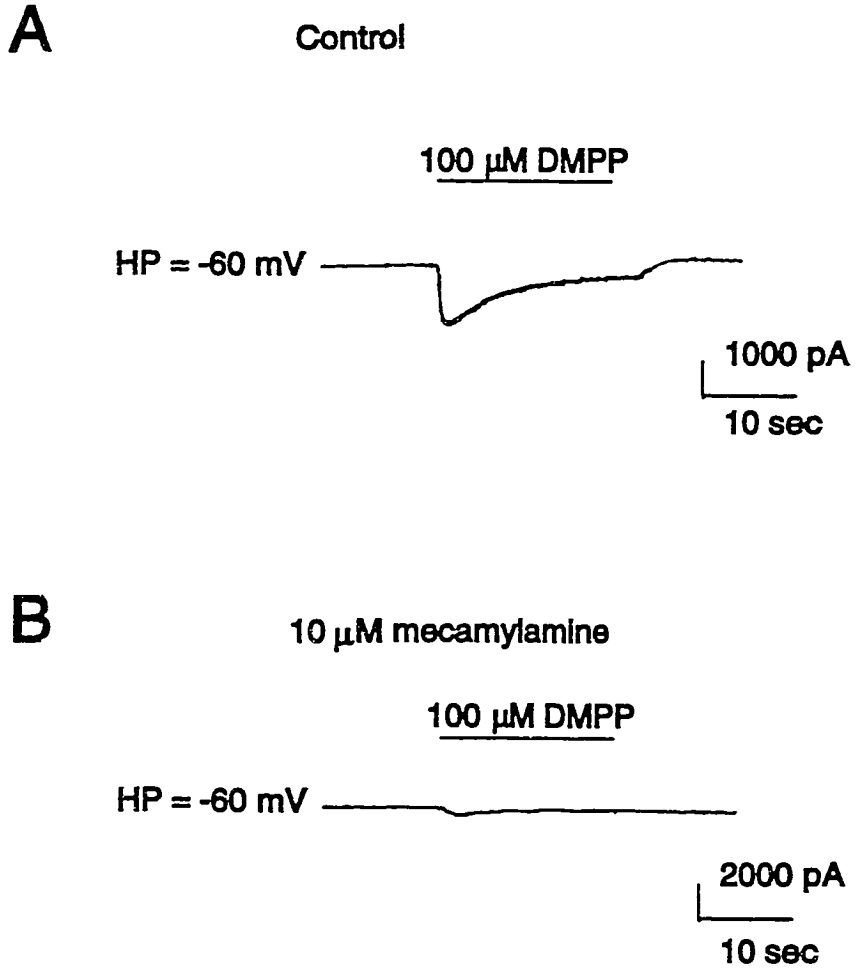

A
Control
100 µM DMPP
HP = -60 mV
1000 pA
10 sec
B
10 µM mecamylamine
100 µM DMPP
HP = -60 mV
2000 pA
10 sec

FIGURE 3-21

FIGURE 3-22: Whole-cell current responses induced by pressure application of DMPP (100 μM). **Panel A)** The cell was held at -60 mV and ramps (between -120 and +50 mV) of 2 sec. duration were applied in groups of 4 with 6 sec. intervals between ramps within a group. **Panel B)** Current-voltage relationship for DMPP-difference current from same neuron as shown in panel A. Ramps recorded in the absence of DMPP (*) are subtracted from those obtained in the presence DMPP (**).

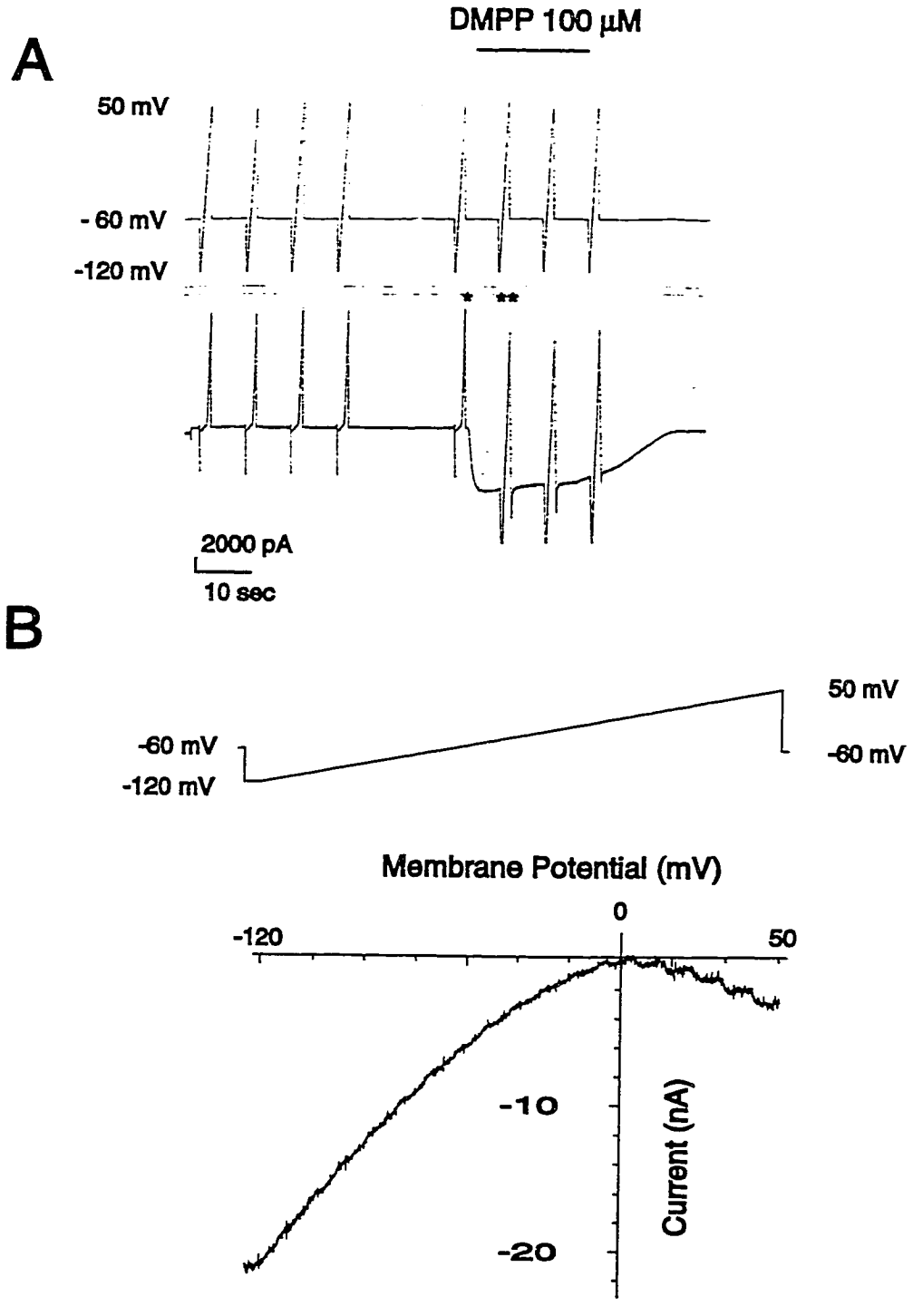

A
DMPP 100 µM
50 mV
- 60 mV
-120 mV
2000 pA
10 sec
B
50 mV
-60 mV
-60 mV
-120 mV
Membrane Potential (mV)
0
-120
50
-10
-20
Current (nA)

FIGURE 3-22

(100 μM) alone caused a rapid depolarization of the membrane potential associated with induction of an inward current. Concurrent application of DMPP and SP (500nM) however, produced a very different response. Substance P reduced the amplitude of the DMPP-induced peak inward current and altered the rate at which the current decayed. During prolonged application of DMPP, SP (500 nM) increased the rate of decay of the inward current, Figure 3-23). In this representative example, the I_5 / I_{peak} in the presence of DMPP alone was 0.54 while the I_5 / I_{peak} in the presence of SP was 0.37. The peak current amplitude was also decreased by 17%. The effect of SP on the rate of decay of the DMPP-induced inward current was observed in all rat SG neurons tested (n = 6). Mean I_5 / I_{peak} for the DMPP-induced inward current ($0.66 \pm .07$) was significantly different from that recorded in the presence of SP ($0.43 \pm .09$) ($P \leq 0.05$).

FIGURE 3-23: Effects of SP on DMPP-induced inward current in cultured rat SG neurons. Inward current induced by pressure application of DMPP (100 μM) was measured at -60 mV in the absence or presence of 500 nM bath-perfused SP.

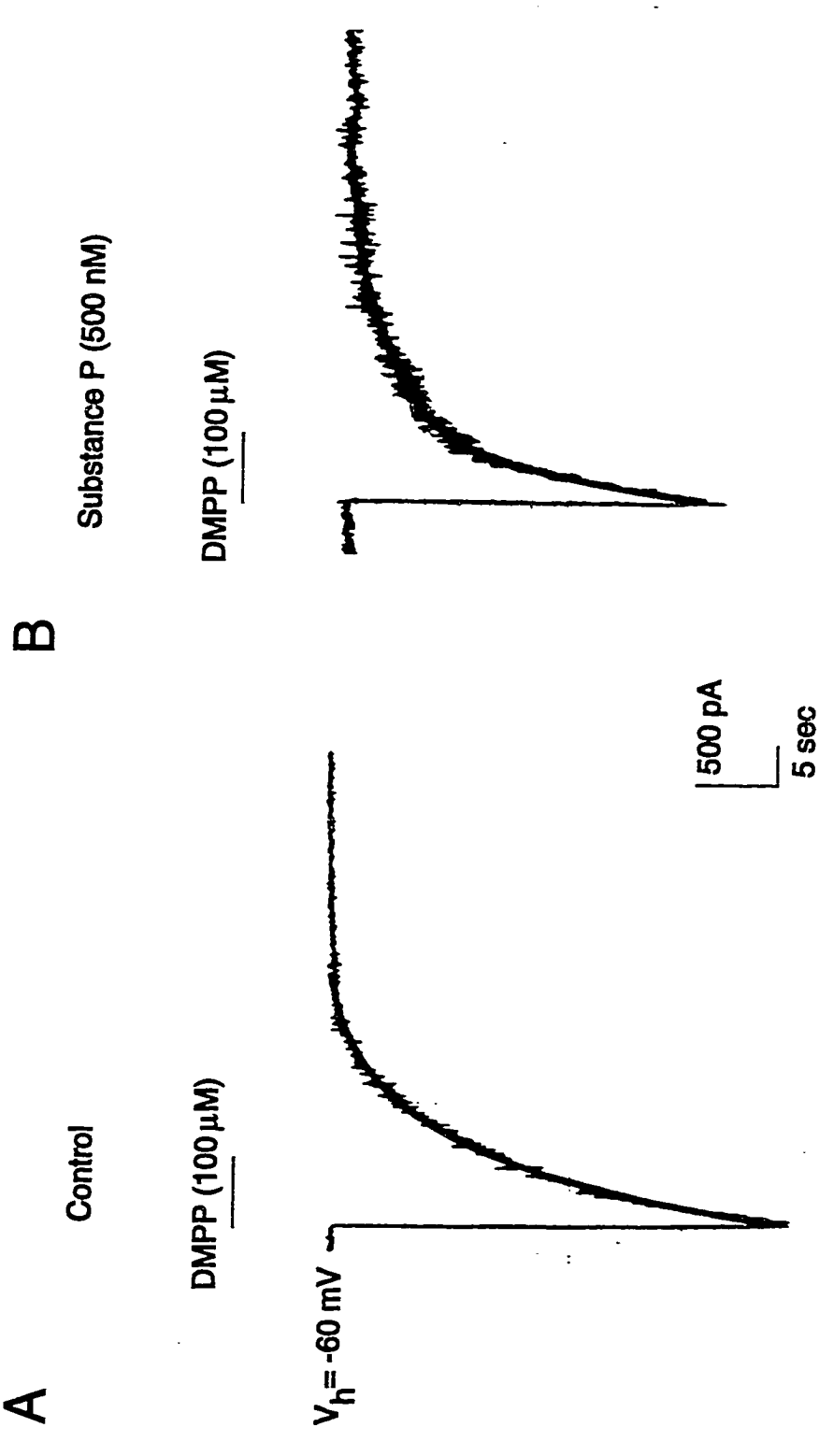

A
Control
DMPP (100 µM)
V_h= -60 mV
500 pA
5 sec
B
Substance P (500 nM)
DMPP (100 µM)

FIGURE 3-23

DISCUSSION

1. Overview

Neuropeptides, in particular SP and CGRP, are present within neuronal elements in peripheral autonomic ganglia. In the sympathetic ganglia these neuropeptides are believed to modulate the excitability and the responses to synaptic input of principal ganglion neurons. The research presented in this thesis characterizes the cellular mechanisms by which the neuropeptides SP and CGRP modulate specific ionic conductances that underlie the RMP and AP generation in SG neurons. In this study, the technique of whole-cell patch-clamp recording was used to investigate the membrane properties and voltage- (K^+, Ca^{2+}), and ligand- (nAChR agonist-induced) gated ionic currents in guinea-pig and rat SG neurons grown in culture. Details of K^+, Ca^{2+}, and nAChR ionic currents recorded in mammalian SG neurons are discussed below and compared to ionic conductances described for sympathetic neurons of other vertebrate autonomic ganglia and species.

This study also demonstrated that SP depolarized guinea-pig SG neurons, decreased membrane conductance at potentials positive to RMP and inhibited the action potential hyperpolarization. These actions of SP were reduced by the Ca^{2+} channel blocker, Cd^{2+} and are consistent with inhibition of a Ca^{2+}-activated K^+ conductance. Voltage-clamp analysis of currents in SG neurons revealed that SP inhibited at least two ionic conductances: a Ca^{2+}-sensitive K^+ conductance and an inward Ca^{2+} conductance (I_{Ba}). The inhibitory actions of CGRP on this inward Ca^{+2} conductance are also described. Evidence for the modulation of these currents by SP, and the results of an investigation of the molecular signaling pathways underlying the action of SP on $I_{K(Ca)}$ are discussed. Finally, I report that SP reduces the agonist-induced activation, and increases the rate of desensitization of nAChR current in rat SG neurons.

2. Voltage-dependent K^+ and Ca^{2+} Currents in Mammalian SG Neurons: Comparison with other Autonomic Neurons.

The outward current in guinea-pig and rat SG neurons was selective for K^+ and consisted of several distinct voltage-dependent K^+ conductances. These conductances were identified on the basis of voltage-dependence and pharmacological blockade and consisted of: a transient outward K^+ conductance, which resembled the A-type (I_A) conductance described in mammalian autonomic neurons (Adams and Harper, 1995; Belluzzi et al., 1985) and a delayed rectifier outward K^+ current which consisted of Ca^{2+}-sensitive and a Ca^{2+}-insensitive component.

The A-current is an operationally distinct K^+ current. In rat sympathetic neurons A-current has been separated from the delayed rectifier K^+ current and the Ca^{+2}-activated K^+ current on the basis of its gating kinetics and its time- and voltage-dependence (Belluzzi et al., 1985; Schofield and Ikeda, 1989). The A-current activates quickly, when compared with other K^+ currents, and it also inactivates quickly. In intact rat SCG, A-current has been shown to closely mirror the fast voltage-dependent Na^+ current and the time course of activation of these currents has been described with equations having similar form to those used for the I_{Na} in the squid axon (Belluzzi et al., 1985). The threshold for A-current activation has been reported to be lower than that of other K^+ currents, and it operates in a range of membrane potentials which are subthreshold of neuronal excitation.

In mammalian SG neurons the transient outward K^+ current demonstrated voltage-dependent activation, and was significantly inactivated at potentials near the RMP. This current typically activated within 1 ms of step depolarization to membrane potentials positive to -50 mV, and reached a maximum amplitude within 10 ms. Peak conductance was followed by a rapid phase of current decay. The transient outward conductance was maximal after hyperpolarization, and decreased sharply when the membrane voltage prior to the depolarizing steps was made less negative. These features of the transient K^+ current in guinea-pig and rat SG neurons resembled those of the transient outward K^+ current (I_A) described in other mammalian sympathetic neurons (Adams and Harper, 1995; Belluzzi et al., 1985; Cassell et al., 1986; Marrion et al., 1987). Another property of the A-current, often used as a criterion for its presence, is it's sensitivity to 4-AP and its derivatives. In rat sympathetic neurons, external application of 1-2 mM 4-AP

reversibly reduced I_A (Marrion et al., 1987; Galvan, 1982; Galvan and Sedlmeir, 1984; Belluzzi et al., 1985). Consistent with the pharmacological properties observed for I_A in neurons of other mammalian sympathetic ganglia, the transient K^+ current in mammalian SG neurons in the present study was also sensitive to 4-AP. While the sensitivity to 4-AP is often used as a criteria to identify a current as "A-type", caution should be exercised in making such interpretations, particularly in the absence of dose-response studies. This need for caution is reinforced by the fact that other K^+ currents may also be sensitive to 4-AP (Marsh and Brown, 1991).

In spite of the large number of studies devoted to the description of the A-current, there is as yet no unified concept for its functional role. However, a number of physiological roles have been proposed for A-current in mammalian sympathetic neurons, including: clamping the membrane potential at a hyperpolarized level (Adams and Harper 1995); modulating synaptic potentials (Cassell and McLachlan, 1986); providing a major driving force for repolarization following an action potential (Galvan and Sedlmeir, 1984; Belluzzi and Sacchi, 1991), an influence which is critically dependent upon the cells resting potential (Marsh and Brown, 1991; Belluzzi and Sacchi, 1988); the regulation of low-frequency repetitive discharge in mammalian sympathetic neurons (Galvan, 1982; Freshi, 1983; Galvan and Sedlmeir, 1984; Belluzzi et al., 1985; Cassell et al., 1986; Schofield and Ikeda, 1989; Rogers et al., 1990).

The predominant current expressed in all guinea-pig SG neurons studied was a sustained outward K^+ current. This sustained outward current activated with a brief delay following the onset of membrane depolarization to potentials positive to -30 mV, and persisted while the depolarization was maintained. The voltage-dependence and kinetics of the sustained K^+ current in guinea-pig SG neurons closely resembled delayed rectifier K^+ currents (I_{Kv}) described in neurons from several different sympathetic ganglia including the rat SCG (Belluzzi et al., 1985), and guinea-pig enteric ganglia (Vanner et al., 1993) where I_{KV} is reported to activate with a delay at membrane potentials more positive than -40 mV and to increase in amplitude with increasing depolarization. The delayed rectifier outward current in mammalian SG neurons was also reversibly decreased by the external application of 4-AP and abolished by replacement of internal

K^+ with Cs^+. This is consistent with the pharmacological properties described for delayed rectifier K^+ currents in neurons of other mammalian autonomic ganglia (Adams and Harper, 1995; Marsh and Brown, 1991; Vanner et al., 1993; Xu and Adams, 1992). The role of this current in ganglion function is not yet clear but the delayed rectifier in mammalian autonomic neurons has been proposed to contribute to both the repolarization and the early afterhyperpolarization following the action potential (Marsh and Brown, 1991).

The currents described above, "A-type" and "delayed rectifiers" currents are voltage-dependent channels, that is, they are gated by the membrane potential. For a third class of K^+ channels, the Ca^{2+}-activated K^+ channels, channel opening and closing depends Ca^{2+} influx and on cytosolic Ca^{2+} activity; an increase in intracellular Ca^{+2} concentration leads to channel opening. Ca^{+2}-dependent K^+ conductances (Gardos, 1958; Meech and Standen, 1975) are virtually a constant feature of excitable cells (Blatz and Magleby, 1987). Voltage-clamp studies of mammalian sympathetic neurons (Belluzzi and Sacchi, 1990; Jobling et al., 1993), together with data from other neuronal types (Lancaster and Nicoll, 1987; Xu and Adams, 1992), have provided support for the existence of several classes of Ca^{2+} dependent K^+ currents, identified on the basis of their kinetic and pharmacological properties. In rat SCG neurons a Ca^{2+}-dependent K^+ current (I_C) which is activated by elevated cytoplasmic free Ca^{+2} concentrations, and which is also voltage-sensitive has been described (Belluzzi et al., 1990). This conductance, which activates rapidly upon depolarization ($\tau_f = 2$ msec), has been suggested to function primarily in the AP repolarization and fast hyperpolarization (Belluzzi and Sacchi., 1990; see also Storm, 1987), and is believed to be mediated via large conductance (BK) Ca^{+2}-dependent K^+ channels (Smart, 1987; Gola et al., 1992). BK channels are not believed to contribute significantly to the AHP in sympathetic neurons. This is not surprising considering the high concentrations of intracellular Ca^{2+} required for BK channel activation at negative membrane potentials (Sah 1996). Two additional Ca^{2+}-dependent K^+ conductances which are believed to be involved in the generation and shape of the AHP have also been described in guinea-pig sympathetic neurons and are termed I_{KCa1} and $I_{K,Ca2}$ (Cassell and McLachlan, 1987a; Cassell et al., 1986; Jobling et al., 1993). I_{KCa1} is a

Ca^{2+}-dependent, voltage-insensitive K^+ conductance, which in guinea-pig celiac neurons is maximal immediately after the action potential and decays exponentially with a time constant of about 130 msec (Cassell and McLachlan, 1987). $I_{K,Ca2}$ is a Ca^{2+}-dependent, voltage-insensitive K^+ conductance believed to underlie the long afterhyperpolarization (LAH) observed in some sympathetic neurons (Cassell et al., 1987; Cassell and McLachlan, 1986; Sah, 1996). In the present study, I observed a reduction of a portion of the sustained outward K^+ current following superfusion with Ca^{2+} channel blockers or nominally Ca^{2+}-free solution. Our data indicate that approximately 35-50% of the delayed rectifier K^+ current in guinea-pig SG neurons can be attributed to the activation of Ca^{2+}-activated K^+ ($I_{K(Ca)}$) channels. While we did not attempt to classify $I_{K(Ca)}$ in detail in this study, this conductance may be mediated, at least in part by an I_C. This statement is supported by the finding that the application of Cd^{2+} induced an increase in AP duration and a decrease in AP hyperpolarization in these studies. Similar changes in AP duration and hyperpolarization amplitude have been reported by others, following blockade of I_C with TEA or charybdotoxin (a blocker of BK channels) (Sah and McLachlan, 1992; Lancaster and Nicoll, 1987; Adams et al., 1982; Storm, 1987). As discussed above, through its contribution to the repolarizing phase of the AP, I_C has been shown to regulate the shape of the AP. The LAH which has been found in some sympathetic neurons (Jobling et al., 1993), was not apparent in the SG neurons in this study. This may be an artifact of the whole-cell technique, reflecting washout of the cytosolic constituents which are required for the maintenance of the currents which underlie the AHP in these neurons (Hamill et al., 1981), as has been described by others (Vanner et al., 1993).

In addition to the $I_{K(Ca)}$, a small non-inactivating outward current was occaisonally recorded in guinea-pig SG neurons which exhibited voltage- and time-dependent properties similar to that of the M-current (I_M), described in several other types of sympathetic neurons, including SCG neuron (Brown and Selyanko, 1985; Marrion et al., 1989), and guinea pig inferior mesenteric ganglia (Galligan et al., 1989). In mammalian sympathetic neurons the M-current is activated in a voltage-range between -70 mV and -30 mV, thereby contributing a background K^+

current to the normal resting membrane current, and affecting the general level of neuronal excitability. In the present study, because of the infrequent occurrence (<15% of neurons) and the small amplitude of current relaxations (10 ± 5.6 pA, n=15 with voltage steps from V_H = -30 mV to -70 mV) of M-like current in cultured guinea-pig SG neurons, this current is unlikely to make a significant contribution to the actions of SP. In support of this, the difficulty in studying M-current in sympathetic neurons has been reported previously in neurons where this current was evident using intracellular recording techniques (Vanner et al., 1993), but was not observed in the same cell type by the same workers using whole-cell patch-clamp recording techniques.

Calcium influx through voltage-gated Ca^{2+} channels contributes to the electrical excitability of neurons but may also mediate other functions, such as neurotransmitter release or the indirect control of membrane excitability via regulation of Ca^{2+}-activated ion channels (Hille, 1992). The presence of a voltage-dependent Ca^{+2} current capable of influencing the time course of the action potential in mammalian sympathetic neurons was first proposed by McAfee and Yarowsky (1979), and has subsequently been described in numerous mammalian autonomic neurons through the use of voltage-clamp and patch-clamp techniques. Based upon their thresholds for activation, Ca^{2+} currents have traditionally been separated into two groups: low-voltage activated (LVA) currents (Ca^{2+} currents which activate upon membrane depolarizations to potentials around -60 mV) and high voltage-activated (HVA) currents (currents requiring membrane depolarizations to -30 mV for activation) (Carbone and Lux, 1984; Nowycky et al., 1985; Fedulova et al., 1985). Four classes of Ca^{+2} channels, coined T (an LVA type) and three HVA types called N, L and P have been reported in a variety of mammalian peripheral neurons (for review see Bean, 1989; Hess, 1990; Bertolino and Llians, 1992; Tsein et al., 1988; Mintz et al., 1992). These channels have been characterized by their threshold for activation, voltage-dependent activation and inactivation parameters, and pharmacological profiles (Nowycky et al., 1985).

In mammalian sympathetic neurons the major component (85-90%) of whole cell inward Ca^{2+} current has been shown to be carried by dihydropyridine-insensitive, high-threshold N-type

Ca^{2+} channels (Plummer et al., 1989; Regan et al., 1991; Schofield and Ikeda, 1988). N-type Ca^{2+} channels are believed to be expressed specifically in neurons (Hess, 1990; Plummer et al., 1989; Usowicz et al., 1990) and play a dominant role in the control of transmitter release from sympathetic neurons (Hirning et al., 1988, Lipscombe et al., 1989; Swandulla et al., 1985). N-type Ca^{+2} channels are blocked by the peptide toxin ω-CgTx-GVIA (Regan et al., 1991; Cox and Dunlap, 1994) which is isolated from the venom of the marine snail *Conus geographus* (Olivera et al. 1985). This toxin has been reported to be selective for N-type Ca^{+2} channels and does not block Ca^{+2} channels of the L- or P-type (Aosaki and Kasai, 1989; Cox and Dunlap, 1992; Plummer et al., 1989; Regan et al., 1991). The remaining conductance present in mammalian sympathetic neurons has been attributed, in part, to current carried through dihydropyridine sensitive L-type Ca^{2+} channels (Plummer et al., 1989; Regan et al., 1991; Boland et al., 1994).

In cultured mammalian SG neurons I have identified an inward Ca^{2+} current (I_{Ba}). Guinea-pig SG neurons exhibited a voltage-dependent Ca^{2+} current which activated upon step depolarization to voltages more positive than -30 mV, and was abolished by 0.2 mM Cd^{2+} or 10 μM ω-CgTx. This current resembles the descriptions of HVA, dihydropyridine insensitive, ω-conotoxin GVIA-sensitive Ca^{2+} currents reported in a number of mammalian autonomic neurons, including rat SCG neurons (Plummer et al., 1989; Plummer and Hess, 1992; Regan et al., 1991; Schofield and Ikeda, 1988; Tsien et al., 1988), the rat myenteric and guinea-pig submucosal neurons (Hirning et al., 1990; Shen and Suprenant, 1993). The block of > 95 % of the Ca^{2+} current by ω-CgTx-GVIA in guinea-pig SG neurons and the voltage-dependence of the ω-CgTx-GVIA-sensitive current indicates that the whole-cell Ca^{2+} current observed in these neurons is due primarily to the activation of a HVA N-type Ca^{2+} conductance. The lack of dihydropyridine (nifedipine 3 μM) sensitivity in SG neurons, in the present study, suggests that unlike descriptions in other mammalian sympathetic neurons, L-type Ca^{+2} currents do not contribute to the I_{Ba}. The apparent absence of L-type Ca^{+2} currents in SG neurons may be the result of the culture conditions, and/or the time in culture during which the electrophysiologcial experiments were performed. This finding is supported by studies in chick dorsal root ganglion neurons where Ca^{+2}

channels identified at times < 1 day *in vitro* were exclusively of the N-type with L-type Ca^{+2} currents only developing at later times in culture (Cox and Dunlap, 1992).

3. Ligand-Gated Ionic Currents in Mammalian Stellate Ganglion Neurons: Comparison with Other Autonomic Neurons

In this study whole-cell nAChR current was characterized in cultured rat and guinea-pig SG neurons. The inward rectification of the whole-cell current evoked by the selective nicotinic agonist DMPP and by carbachol was examined in guinea-pig and rat SG neurons. In current-clamp, SG neurons responded to prolonged (20 sec) pipette application of DMPP [100 μM] and carbachol [1 mM] with an initial depolarization that decayed with continued exposure to the agonist. Under voltage-clamp conditions, at a V_H = -60 mV, application of these agonists induced a rapidly-activating inward current that desensitized in the continued presence of the agonist. The DMPP-induced current recovered from desensitization rapidly following the removal of the agonist and was reduced by the nAChR channel antagonist mecamylamine. The current-voltage relationship of the whole-cell response induced by DMPP was linear in the negative voltage range: however there was no detectable outward current when the voltage approached and passed 0 mV. The voltage-dependence, pharmacology and kinetics of the nAChR current in SG neurons resembles the nAChR current described in other mammalian and avian sympathetic ganglion neurons (Clapham and Neher, 1984; Trouslard et al., 1993; Marrion et al., 1987; Mathie et al., 1987; Mathie et al., 1990; Role, 1984). In cultured rat SCG neurons, the addition of DMPP induced a large inward (depolarizing) current, associated with an increase in membrane conductance. This DMPP-induced current desensitized in the continued presence of the DMPP and was abolished by mecamylamine (Marrion et al., 1987). In rat sympathetic neurons the current-voltage relationship of the DMPP-induced whole cell response was shown to be linear in negative voltage ranges, and there was no detectable outward current at membrane potentials near or positive to 0 mV (Mathie et al., 1990).

4. Actions of Substance P and Calcitonin Gene-Related Peptide on Voltage-Dependent Ionic Currents in Mammalian Stellate Ganglion Neurons

Substance P and CGRP are among a variety of peptides which modulate neuronal ionic conductances (Bley and Tsien, 1990; Ehrlich and Elmslie, 1995; Hille, 1994; Hirning et al., 1990; Nakajima et al., 1988; Sah, 1990; Shapiro and Hille, 1993; Shapiro et al., 1994; Shen and Suprenant, 1993; Vanner et al., 1993; Zona et al., 1991). Many of these neuropeptides have been identified in the nerve fibers of the rat and guinea-pig SG (Anderson et al., 1993; Heym et al., 1993; Horackova et al., 1993; Morales et al., 1995). These peptide-containing fibers are believed to originate from either preganglionic sympathetic neurons of the spinal cord or from sensory neurons (Heym et al., 1993). Because of the prevelance of SP and CGRP in the nerve fiber networks of the SG the actions these peptides were examined.

Under our recording conditions, SP reversibly depolarized V_M and decreased membrane conductance. These actions are consistent with inhibition of an outward K^+ conductance by SP in SG neurons. In the presence of the Ca^{+2} channel blocker Cd^{2+}, a reduction in SPs ability to elicit membrane depolarization and a conductance decrease were observed, suggesting that SP may exert its effects, in part, via inhibition of $I_{K(Ca)}$. Our current clamp studies revealed a residual depolarization and small conductance decrease still elicited by SP in the presence of 0.2 mM Cd^{+2}, which may reflect an incomplete block of $I_{K(Ca)}$ by Cd^{+2} in those neurons tested and/or actions on K^+ currents distinct from the $I_{K(Ca)}$. For example, SP inhibition of a background (leak) K^+ conductance has been described in other autonomic neurons, including guinea-pig celiac and submucosal neurons (Shen and Suprenant, 1993; Tokimasa and Akasu, 1992; Vanner et al., 1993). The effects of SP application on depolarization-elicited AP's were also examined. Substance P decreased action potential hyperpolarization amplitude, increased AP duration and reduced the time to one-half AHP inactivation. Although I did not attempt to characterize the various classes of Ca^{+2}-activated K^+ currents in SG neurons, the actions of SP on AP duration and

hyperpolarization amplitude in these neurons may reflect a reduction in an I_C, which has previously been described to play a role in AP repolarization and fast hyperpolarization (Belluzzi et al., 1990; Gola et al., 1992; Sah, 1996; Smart, 1987; Storm, 1987).

Subsequent voltage-clamp studies focused on $I_{K(Ca)}$. The actions of SP on outward K^+ current were primarily mediated by inhibition of $I_{K(Ca)}$, since SP's inhibitory actions measured at 0 and +60 mV were blocked in the presence of 0.2 mM Cd^{2+} or the N-type Ca^{2+} channel blocker ω-conotoxin-GVIA (10 μM). There was a slightly greater reduction in SPs inhibitory actions on $I_{K(Ca)}$ at more positive potentials in the presence of Ca^{2+} channel inhibition, which may reflect alterations in the driving force for Ca^{2+} and decreased Ca^{2+} availability at these potentials (Sah, 1996). The Ca^{2+}-insensitive component of the sustained outward K^+ current, evident in the presence of Cd^{2+}, was not significantly affected by SP application.

The identity of the receptor believed to mediate SP induced modulation of $I_{K(Ca)}$ in guinea-pig SG neurons was investigated. Three tachykinins; SP, NKA and NKB, each possess a defined affinity for the three known mammalian tachykinin receptors (NK_1 , NK_2 , and NK_3 (Lavielle et al., 1990)). The rank order of affinity for inhibition of I_K in this study was SP>NKA≥ NKB which demonstrates an NK_1 subtype profile for SP inhibition of $I_{K(Ca)}$. Shapiro et al. (1993) have also reported a clear NK_1 subtype profile in rat SCG neurons for the ability of SP, NKA and NKB to inhibit Ca^{2+} channels.

The effects of the neuropeptides SP and CGRP were then examined on the Ca^{2+} channel current in guinea-pig SG neurons. Pipette application of SP produced an inhibition of I_{Ca} similar to that described in frog and rat sympathetic neurons (Bley and Tsien, 1990; Shapiro and Hille, 1993). This study indicated that SP inhibited approximately 50% of I_{Ca} in SG neurons, in which the majority of inward I_{Ca} represents activation of ω-CgTx-sensitive HVA Ca^{2+} channels. Because less than 5% of the whole-cell I_{Ba} in SG neurons is likely to be carried via L-type Ca^{2+} channels, no attempt was made to determine the effectiveness of SP on L-type currents in isolation. Although these findings suggest that the effects of SP on $I_{K(Ca)}$ may represent a

secondary consequence of the inhibition of Ca^{2+} influx, this does not exclude any additional direct effects of SP on K^+ currents.

Our data also indicate that CGRP inhibits approximately 16% of I_{Ca} in SG neurons. This inhibition of I_{Ca} by CGRP is dose-dependent and resembled inhibition of a HVA I_{Ca} reported in cultured rat neocortical neurons (Zona et al., 1991). In neocortical neurons, the CGRP effect could be mimicked by external application of forskolin, and by loading neurons with cyclic AMP, effects which had previously been reported in rat sympathetic neurons (Horn and McAfee 1980). Findings reported here in the SG neurons are in contrast however, with other reports of CGRP-induced enhancement of Ca^{+2} current. In rat dorsal root ganglion neurons CGRP was shown to enhance an N-type Ca^{+2} currents via a PTX sensitive pathway (Ryu et al., 1988; Wiley et al., 1992). It is possible that this differential regulation of Ca^{2+} currents by CGRP and second messenger systems reflects differences in the phosphorylation sites of the channel proteins and/or in the biochemical machinery involved in channel protein regulation (Sumikawa and Miledi 1989).

5. Transduction Mechanisms Underlying Substance P Induced $I_{K(Ca)}$ Inhibition in Stellate Ganglion Neurons

A series of experiments were designed to further examine the potential signaling pathway underlying SPs actions on I_K in guinea-pig SG neurons. To confirm G-protein modulation of I_K by SP, I used GDPβS and PTX . In the presence of GDPβS, an antagonist of G-protein activation (Holz et al., 1986), SP's ability to inhibit I_K was significantly diminished, thereby indicating G protein involvement. I found no effect of PTX pretreatment on the ability of SP to inhibit I_K in SG neurons, indicating that the receptors responding to SP are not coupled to G proteins of the G_i, G_o, G_z family (Milligan, 1988; Moss and Vaughan, 1988; West et al., 1985). These findings are consistent with other reports of receptor-mediated SP actions which are PTX-insensitive. These include the inhibition of N-type Ca^{2+} channels in rat SCG neurons (Shapiro and Hille, 1993) and frog sympathetic neurons (Bley and Tsien, 1990) and the inhibition of inwardly

rectifying K^+ channels in the nucleus basalis of the rat forebrain (Nakajima et al., 1988), both of which occur through interaction with an NK_1 tachykinin receptor.

It is well established that the behavior of ion channels can be regulated by a host of intracellular mediators and by phosphorylation (Hille, 1994; Leviatan, 1988; Schultz et al., 1990). In mammalian sympathetic neurons, voltage-dependent ion channels are modulated by several neurotransmitters and neuropeptides, and the coupling of receptors to ion channels is known to involve G proteins (Hille, 1994). Numerous studies have indicated that the SP-induced modulation of ion channels in sympathetic neurons involves the activation of a PTX-insensitive-G-protein, but does not require the actions of protein kinases or diffusible second messengers. (Bley and Tsien, 1990; Shapiro and Hille, 1993). The inhibition of N-type Ca^{+2} current by G proteins in sympathetic neurons has been described as membrane-delimited (Hille, 1994). It is not clear yet, however, which subunits of the G-protein heterotrimer actually produce these effects, or to which part of N-type Ca^{+2} channels they bind (Wickman and Clapham, 1996).

A series of experiments were thus designed to test for the involvement of selected G protein effectors as possible links between the SP activated G protein(s) and I_K inhibition in SG neurons. External application of forskolin (an activator of adenylate cyclase) or intracellular dialysis with dibromo cyclic AMP, both of which lead to increases in intracellular cyclic AMP levels and promote the activation of cyclic AMP-dependent protein kinase A, failed to either mimic or to alter the actions of SP on the I_K. This suggests that SPs actions on I_K are not mediated by cyclic AMP-dependent protein kinase A. Furthermore, bath perfusion with GF109203-X (a selective inhibitor of protein kinase C) enhanced rather than inhibited the reduction of I_K by SP in SG neurons. While the mechanisms underlying this action of GF109203-X on the SP response have not been investigated, this increase in I_K inhibition may reflect the removal of an endogenous protein kinase C mediated modulation of the Ca^{+2} - insensitive I_K, thereby making this current component suspectible to the actions of SP and or actions on other K^+ currents such as the delayed rectifier K^+ current. Taken together these data suggest that SP does not reduce I_K in guinea-pig SG neurons via cyclic AMP-dependent protein kinase A or protein

kinase C pathways. The possibility that SP inhibits $I_{K(Ca)}$ in these SG neurons by a membrane-delimited or unidentified second messenger mechanism awaits further confirmation.

6. Actions of Substance P on DMPP-Induced nAChR Current in Stellate Ganglion Neurons

In the mammalian sympathetic nervous system immunohistochemical techniques have revealed the presence of a variety of neuropeptides, including SP within the nerve fiber networks of the SG. This finding, combined with the demonstrations that SP can alter the responses of autonomic neurons to applied nAChR agonists, suggests a possible physiological role for the peptide in modulation of synaptic function. At a number of sites in the nervous system, SP modifies the actions of acetylcholine on the nAChR (Livett and Zhou, 1991). The inhibitory influences of SP on nicotinic cholinergic-induced responses has been observed in various preparations, such as bovine chromaffin cells (Livett et al. 1979, Clapham ad Neher, 1984; Lyford et al., 1990); avian sympathetic neurons (Valenta et al., 1993) and the clonal nerve cell line, PC12 (Stallcup and Patrick, 1980). Considerable controversy exists regarding the site and mode of action of SP. A number of studies have suggested that SP acts directly on nAChRs to increase agonist-induced desensitization (Stallcup and Patrick, 1980; Clapham and Neher, 1984; Boyd and Leeman, 1987). On the other hand, cell-attached patch studies on avian sympathetic neurons have concluded that the actions of SP on nAChRs involve diffusible second messengers (Simmons et al., 1990).

The inhibitory actions of SP were examined on the nAChR current in rat SG neurons. Results from these experiments showed that nanomolar concentrations of SP, applied simultaneously with DMPP, enhanced the rate of desensitization, and reduced the amplitude of the DMPP-induced inward current. Similar actions of SP on ACh induced currents have been reported in bovine chromaffin cells (Clapham and Neher, 1984) and in avian sympathetic neurons (Role, 1984; Valenta et al., 1993), although in these experiments much higher concentrations

(μM) of SP were required to achieve current modulation. Findings reported in this thesis indicate SP modulation of nAChR currents at nanomolar concentrations lends support to previous studies demonstrating attenuation of the nicotinic cholinergic response by SP at nanomolar concentrations (Hanley et al., 1990; Shinkai et al., 1993). It may therefore be suggested that nicotinic responses are altered indirectly following activation of intracellular signaling cascades, and may also be triggered by SP effects at tachykinin receptors (Hanley et al. 1990; Beaujourn et al., 1982; Torrens et al., 1989). This concept has been supported by studies in avian sympathetic neurons where cell-attached patch recordings demonstrated SP modulation of nAChR function by a second messenger (protein kinase C associated) mechanism (Downing and Role, 1987; Simmons et al., 1990).

Potential physiological roles for SP in the modulation of autonomic ganglion function have been proposed. In the bullfrog parasympathetic ganglia where ganglionic neurons were activated synaptically by high frequency presynaptic nerve stimulation, exogeneously applied SP was shown to enhance the rate of repolarization of the resulting EPSPs (Bowers et al., 1986). In the avian sympathetic neurons SP has been demonstrated to enhance the rate of decay of both synaptic currents and currents evoked by cholinergic agonists (Ramirez and Chiapinelli, 1987; Simmons et al., 1990; Role, 1984). The consequences of nAChR modulation by SP are evident in a reduction of neurotransmitter release from the sympathetic neurons, an effect which is directly related to the extent of SP modulation of nAChR desensitization (Valenta et al., 1993). Since SP is present in and released from avian sympathetic ganglia and the neuropeptide inhibits synaptic activation and transmitter release *in vitro*, it has been proposed that the modulation of nAChR by SP may regulate autonomic function *in vivo*. Because SP has been identified in the neuronal elements of mammalian SG, and in light of the demonstration of modulatory effects of this peptide on nAChR currents in these studies, it is reasonable to propose that SP regulates autonomic function in the SG *in vivo*. Such peptide-mediated regulatory actions will modulate sympathetic outflow to visceral targets innervated by the postganglionic neurons of the SG, such as the heart.

Intraganglionic release of SP may be the result of either preganglionic or peripheral afferent activities.

7. Conclusion

The discovery of numerous neuropeptide substances in central and peripheral neurons has stimulated many studies of their possible roles in synaptic transmission. Neuropeptides may be linked to a variety of modulatory roles including: altering membrane conductances and therefore electrical excitability; or altering the postsynaptic action of neurotransmitters. The neuropeptides, SP and CGRP, are widely distributed in the mammalian ANS. The presence of these neuropeptides in the ANS implies a functional role for these neuropeptides in the regulation of autonomic transmission and ultimately cardiac function. The work in this thesis supports the suggestion of many other workers that neuropeptides may effect a variety of physiological mechanisms in autonomic ganglia. Substance P can directly depolarize sympathetic neurons, apparently by inhibiting potassium conductances (Adams et al., 1983; Vanner et al., 1993), and has been shown capable of switching the firing patterns of neurons from phasic to tonic type (Vanner et al., 1993). Furthermore, SP can modulate the time course of cholinergic responses in sympathetic neurons (Role 1984, Valenta et al., 1993), perhaps by enhancing nAChR desensitization (Boyd and Leeman, 1987; Clapham and Neher, 1984; Simmons et al., 1990). Even so, in contrast to the relatively well documented cellular modulatory actions of SP and CGRP, the overall functional significance of these modulatory mechanisms are not all clear. In this thesis I have demonstrated that mammalian SG neurons respond by changes in their membrane potential and action potential properties to the actions of SP. These changes may reflect changes in K^+ and Ca^{2+} ionic permeabilities. The SP-induced membrane depolarization increases the level of SG neuron excitability and reflects, at least in part, an inhibition of the $I_{K(Ca)}$. Similarly, inhibition of a $I_{K(Ca)}$ in SG neurons underlie the reported increases in AP duration and decreases in AP hyperpolarization amplitude. Such actions may be presumed to lead to a

modulation of the sympathetic output to the heart and associated changes in cardiac indicies, such as the force and /or rate of contraction. I have also demonstrated that SP may modulate cholinergic responses in SG neurons via receptor mediated action. This modulation contrasts in part with the actions of SP on the membrane potential and AP firing patterns in SG neurons in that a SP mediated decrease in cholinergic responsiveness might be proposed to reduce the amplitude of EPSP's in these cells. Such actions may lead to reduced AP generation and decreased sympathetic outflow to the heart and associated end organs.

In conclusion, I propose three major modualtory mechanisms in the SG: 1) SP-induced membrane depolarization may facilitate postsynaptic responses to endogenous neurotransmitter release; 2) the modulation of AP characteristics could underlie changes in AP firing patterns leading to increased excitability; and 3) SP increases the rate of nAChR desensitization leading to increased postsynaptic frequency responses to sustained synaptic inputs. However, this enhanced desensitization is accompanied by a decrease in f-EPSC amplitude which may have a balancing inhibitory effect. At this point the ultimate actions of SP on SG neurons may only be speculated upon, and further experiments are required to determine which of the modulatory effects of SP predominate in the physiological setting. It may be proposed the the various modualtory actions of SP possess distinct concentration dependencies and that physiological concentrations of endogenous SP released may determine physiological response.

8. Future Studies

Future studies will extend the observations on acutely isolated and cultured SG neurons and examine the actions of these peptides in the intact ganglia. These will include experiments to:

1) To further characterize receptors and signaling pathways associated with the actions of CGRP on SG neurons including actions on I_{Ca} and other voltage- and ligand-gated ionic conductances.

2) Examine the effects of exogeneously applied and synaptically released SP and CGRP on the membrane properties, firing patterns and synaptic currents in the intact SG, using intracellular recording techniques.

In vivo studies coupled with *in vitro* information on the receptor types and signaling pathways underlying actions of these peptides will provide a clearer understanding of the function of these peptides in regulating sympathetic outflow through the SG. Since the SG is the primary source of sympathetic postganglionic neurons innervating the heart, an understanding of the role of peptides and their receptors in regulation of cardiac function may lead to development of novel agents to treat cardiac dysfunction.

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IMAGE EVALUATION TEST TARGET (QA-3)

150mm

6″

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