Sigma Receptors Modulation of Voltage-gated Ion Channels in Rat Autonomic Neurons

by

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ABSTRACT

Sigma receptors have been implicated in the regulation of the cardiovascular system. Some of the cardiovascular effects of sigma receptors may be through the modulation of autonomic neurons. Studies on the expression and cellular function of sigma receptors in autonomic neurons were conducted in neonatal rat intracardiac (ICG) and superior cervical ganglia (SCG).

Individual neurons from SCG and ICG were shown to express transcripts encoding the sigma-1 receptor. The effects of sigma receptors activation on high-voltage-activated Ca\(^{2+}\) channels was studied in isolated neurons of these ganglia. Bath application of sigma receptor agonists depressed peak calcium channel currents in a dose-dependent manner and the rank order potency of haloperidol > ibogaine > (+)-pentazocine > DTG is consistent with the effects being mediated by a sigma-2 receptor. Sigma receptor antagonist, metaphit, blocked DTG-mediated inhibition of Ca\(^{2+}\) current. Sigma ligands also altered the biophysical properties of these channels.
Activation of sigma receptors reversibly blocked delayed outwardly rectifying potassium channels, large conductance $\text{Ca}^{2+}$-sensitive $K^+$ channels, and the M-current with maximal inhibition >80%. The rank order potency of different sigma ligands suggests that the effect is mediated by sigma-1 receptor. While bath application of sigma ligands depolarized ICG neurons, the number of action potentials (AP) fired by the cells in response to depolarizing current pulses was decreased. Experiments on the signal transduction cascade mediated the inhibition of $K^+$ and $\text{Ca}^{2+}$ channels by sigma ligands showed that the signal transduction pathway does not involve a diffusible cytosolic second messenger or a G-protein.

Sigma ligands also modulate voltage-gated $\text{Na}^+$ channels (VGSC) in ICG neurons. Bath application of sigma ligands inhibited VGSC current with maximal inhibition >90% and altered the biophysical properties of VGSC. The latency of AP generation during depolarizing current ramp was increased by sigma ligands and this effect is through the inhibition of VGSC.

These data suggest that activation of sigma receptors on autonomic neurons modulates voltage-gated $\text{Ca}^{2+}$, $K^+$ and $\text{Na}^+$ channels and as a result, the generation of AP is inhibited in these neurons. Sigma receptors are likely altering the cell-to-cell signaling in autonomic ganglia and thus regulating cardiac function by the peripheral nervous system.
CHAPTER 1
BACKGROUND AND SIGNIFICANCE

SIGMA RECEPTORS

Sigma receptors were postulated in 1976 by Martin et al based upon the actions of the benzomorphan opiate SKF-10,047 and were originally thought to be a type of opiate receptor (Martin et al., 1976). Two subsequent findings convincingly demonstrated that sigma receptors are not members of the opiate receptor family. First, the enantioselectivity for the ligands is different: opiate receptors are selective for the (-) isomers of opium-derived narcotics, narcotic antagonists, and their congeners. Sigma receptors are enantioselective for the (+) isomers. Second, traditional opiate receptors antagonists such as naloxone or naltrexone are ineffective against both the in vivo and the in vitro effects of sigma ligands (Brady et al., 1982; Iwamoto, 1981; Katz et al., 1985; Slifer and Balster, 1983; vaupel, 1983). Therefore, the sites defined as sigma today are not opioid and represent a unique type of receptor.

Given that SKF10,047 and phencyclidine (PCP) produce many of the same psychotomimetic effects, some investigators proposed a common receptor for the two, called the sigma/PCP site (Zukin et al., 1984). Further analysis later
revealed that the two sites are separate and show different localization across brain area. The sigma site has high affinity for SKF10,047, lower affinity for PCP, and is also sensitive to low concentrations of haloperidol. In contrast, the PCP site has high affinity for PCP, lower affinity for SKF10,047, and is insensitive to low concentration of haloperidol. It is now well established that the sigma site represent a unique binding site in mammalian brain and peripheral organs, distinct from any other known transmitter receptors (Walker et al., 1990).

At least two subtypes of sigma receptor have been classified based on the findings from biochemical and radioligand binding experiments and have been designated as sigma-1 and sigma-2. The sigma-1 site was suggested for the guinea pig brain traditional sigma while sigma-2 was rat pheochromocytoma (PC12) cell sigma-like sites, respectively (Hellewell and Bowen, 1990; Quirion et al., 1992). Only sigma-1 receptor has been cloned and in human, sigma-1 gene is located on chromosome 9p13, a region associated with psychiatric disorders (Prasad et al., 1998). The structure of the sigma-1 receptors showed no homology with other known mammalian protein, but possessed some homology with fungal sterol isomerases (Hanner et al., 1996; Kekuda et al., 1996; Seth et al., 1998). The cDNA of sigma-1 receptor predicted a protein of 223 amino acids and the molecular weight is 25 KDa. Initial hydropathy analysis of the deduced amino acid sequence of sigma-1 receptor suggested a single putative transmembrane domain, which played a critical role in ligand binding of this receptor (Prasad., 1998; Yamamoto et al., 1999). Subsequently, when
expressed in Xenopus lavis oocytes, the domain accessibility experiments suggested that sigma-1 receptor has two membrane-spanning segments, with the NH₂ and COOH termini on the cytoplasmic side of the membrane (Aydar et al., 2002). In this model, the proposed ligand binding domain is located in the COOH-terminus intracellular portion.

In the oocyte, guinea pig sigma-1 receptor was found mainly in the plasma membrane (Aydar et al., 2002), however, it has been found that at N-terminus, there was an endoplasmic reticulum retention signal motif, suggests that sigma-1 receptor exists not only in the plasma membrane but also in intracellular membranes, which makes it different from conventional neurotransmitter receptors (Kekuda et al., 1996; Hanner et al., 1996).

The molecular nature of sigma-2 receptor is still unknown and it has been presumed that sigma-2 receptor gene encodes a protein of M_r 21,500. In both mice and humans, it has been suggested that the splicing variants of the sigma-1 receptor gene could display sigma-2 receptor binding activities (Monassier and Bousquet, 2002). However, in the sigma-1 receptor knockout mouse, sigma-2 receptor binding sites have no changes compared with the wild type mouse, suggesting that sigma-1 and sigma-2 are not two isoforms of the same protein (Langa et al., 2003). Similar to sigma-1 receptor, some studies also showed that sigma-2 receptors are intracellular receptors located in the membranes of the endoplasmic reticulum and mitochondria (Bowen 2000).
Sigma-1 and sigma-2 receptor exhibits a distinct pharmacological profile. Sigma-1 receptors display high affinity for (+)-pentazocine, (+)-SKF-10,047 and other (+)-benzomorphans, whereas sigma-2 receptors have low affinity for these compounds but have high affinity for ibogaine (Hellewell and Bowen, 1990; Hellewell et al., 1994; Bowen et al., 1995).

Since originally proposed, sigma receptors have been observed across many species and are present at high levels in CNS, immune system, liver and kidney. (Wolfe and De Souza, 1992; Hellewell et al., 1994) Although the function of these receptors is not well understood, sigma receptors have been implicated in the modulation of various biochemical, behavior and physiological processes (Walker et al., 1990). In the central nervous systems, sigma receptors are concentrated in brainstem areas that regulate motor function, limbic structures, some predominantly sensor areas and areas associated with endocrine function. Sigma receptors have been implicated not only in the regulation of posture and motor activity (Glodstein et al., 1989, Bowen et al., 1988), but also in the modulation of opioid analgesia (King et al., 1997), cocaine-induced toxicity (McCracken et al., 1999), learning and memory (Maurice et al., 1998), drug abuse (Maurice et al., 2003), neuroprotection in cerebral ischemia (Takahashi et al., 1996; Lockhart et al., 1995; Goyagi et al., 2001), as well as in regulation of neurotransmitter release and modulation of neurotransmitter receptor function (Matsuno et al., 1993; Couture and Debonnel, 1998; Patrik et al., 1993).
It was also found that both subtypes of sigma receptors are expressed in very high density in tumor cell lines derived from various tissues, which suggests important cellular functions of sigma receptors in cancer and potential diagnostic utility for tumor imaging agents which target sigma binding sites (Vilner et al., 1995; Brent and Pang 1995; Barbieri et al., 2003). Thus, it is likely that sigma receptors have important functions outside of the nervous system and may serve a more general role than a neurotransmitter receptor.

Although there is evidence for the existence of sigma receptor binding sites in brain and tissue extracts, no endogenous functional ligand for sigma receptors has been conclusively identified. Various candidates have been suggested, such as, progesterone (Su et al., 1988), Neuropeptide Y (NPY), peptide YY (Roman et al., 1989) and zinc (Connor and Chavkin, 1992). These data suggest that sigma-binding sites constitute a true receptor that binds to endogenous ligands released in response to neurostimulation.

SIGMA RECEPTORS IN CARDIOVASCULAR SYSTEM

Much of the initial interest in sigma receptors was driven by their potential role in the actions of antipsychotic drug. This was due mainly to their high affinity for most of the typical neuroleptics, such as haloperidol and their shared affinity for compounds known to cause psychotomimetic activity such as N-allylnormetazocine (SKF10,047) (Su, 1982; Tam and Cook, 1984). Sigma receptors are also found in high density in many tissues outside of the central
nervous system, such as, endocrine, immune, and gastric system (see Walker et al., 1990). Sigma binding sites have been detected in cardiac myocytes from neonatal and adult rats (Ela et al., 1994; Novakova et al., 1995). In neonatal rat cardiac myocytes, sigma receptor ligands, at nanomolar concentrations, exert a complex effect on the amplitudes and frequencies of contraction, $\text{Ca}^{2+}$ fluxes and $[\text{Ca}^{2+}]_i$ transients (Ela et al., 1994). Pretreatment of these cells with sigma ligands caused the desensitization of the receptor (Ela et al., 1996). However, sigma ligands, at concentrations of 10 nM and higher, markedly increased the amplitude of systolic cell contraction and the amplitudes of $[\text{Ca}^{2+}]_i$ transients in adult rat cardiac myocytes (Novakova et al., 1995). These effects were shown to be mediated by elevated production of inositol 1,4,5-trisphosphate (IP$_3$) and potentiation of the systolic release of Ca$^{2+}$ from sarcoplasmic reticulum-Ca$^{2+}$ stores.

It has been shown that sigma receptor antagonist DuP 734 increase the heart tolerance to ischemic and reperfusion arrhythmias both in vivo and in vitro (Lishmanov et al., 2000a). Conversely, the sigma receptor agonists (+)-SKF-10,047, 1,3-di-O-tolyguanidin (DTG), and (+)-3-[3-hydroxyphenyl]-N-(1-propyl)piperidine have been shown to produce tachycardia by activating central and peripheral sigma receptors (Wu and Martin, 1989; Lishmanov et al., 2000b). Clinical study has also been shown that some psychiatric patients during therapy with haloperidol, which is deemed as sigma-1 receptor antagonist and sigma-2 receptor agonist, tend to have cardiac side effects such as arrhythmia,
tachycardia and sudden cardiac death (Settle and Ayd, 1983; Mehta, 1979; Turbott et al., 1984). While the effects of sigma ligands in cardiac muscle may be involved in the cardiovascular effects of these agents, very little is known about sigma receptors in autonomic neurons that innervate the heart and regulate cardiovascular function.

SIGMA RECEPTORS IN AUTONOMIC NEURONS

Some evidence does exist that suggests that sigma receptors play an important role in the function of peripheral neurons. For example, sigma receptors have been shown to block contractions of longitudinal muscle elicited by both electrical stimulus or by exogenous serotonin in the guinea pig ileum/myenteric plexus preparation via inhibition of acetylcholine release from myenteric neurons (Campbell et al., 1989). Conversely, sigma receptors potentiate neurogenic twitch contraction in the mouse vas deferens by inhibiting $K^+$ channels in sympathetic neurons for the hypogastric ganglion, which increase norepinephrine release from these cells (Campbell et al., 1987). Both sigma-1 and sigma-2 receptors have been detected in rat pheochromocytoma (PC12) which exhibit a phenotype similar to sympathetic neurons and sigma-2 receptors were shown to enhance amphetamine-stimulated dopamine release from these cells (Hellewell and Bowen, 1990; Sagi et al., 1996; Weatherspoon and Werling, 1999).
SIGMA RECEPTORS MODULATION ON VOLTAGE-GATED ION CHANNELS

The effects of sigma receptor ligands on voltage-gated ion channels have been investigated in several types of cells, and K⁺ channels are the most extensively studied. In frog pituitary melanotrophs, for example, sigma receptors activation increased electrical activity by inhibiting K⁺ currents via activation of a cholera toxin-sensitive G protein; even though sigma receptors share no homology with classic G-protein coupled receptors (Soriani et al., 1998; Soriani et al., 1999a). Conversely, in rat neurohypophysial terminals, neither G-protein nor any soluble cytoplasmic factor were involved in the modulation of K⁺ channels by pentazocine or (+)SKF10047 (Lupardus et al., 2000). Aydar et al reconstituted sigma-1 receptor modulation of Kv1.4 and Kv1.5 in oocytes by heterologous expression of the K⁺ channels with sigma-1 receptors. It was found that (+)SKF10047 depressed K⁺ current only in the oocytes that injected with both sigma-1 receptors and K⁺ channel subunits and it was also showed that the sigma-1 receptor alters the kinetic properties of Kv1.4 channel in the absence of sigma ligands (Aydar et al., 2002). These findings together with the observation that sigma-1 receptor forms an immunoprecipitating complex with K⁺ channels both in rat neurohypophysis and when coexpressed in oocytes, led investigators to propose that the sigma receptor functions as a ligand-regulated auxiliary K⁺ channel subunit (Aydar et al., 2002). These studies have made advances in deducing the mechanisms of the signal transduction pathway of the ion channel modulation.
Ion channels are expressed in several types of tumor cells and play an important role in metastasis, an integral aspect of which is the control of cell growth and proliferation. For example, down-regulation of K\(^+\) channel amplitude has been associated with the metastatic phenotype in human breast and prostate cancer (Wonderlin and Strobl, 1996; Fraser et al., 2003) and voltage-gated Na\(^+\) channels are also involved in the metastatic cascade in prostate cancer (Fraser et al., 2003). In DMS-114 cells (a tumor cell line derived from human small cell lung carcinoma), sigma receptors are highly expressed and sigma ligands have been shown to inhibit voltage-gated K\(^+\) channel currents (Wilke et al., 1999). Because both subtypes of sigma receptors are expressed in very high density in tumor cell lines (Vilner et al., 1995; Brent and Pang., 1995; Barbieri et al., 2003) and sigma-1 receptors functions as a auxiliary K\(^+\) channel subunit, it raises the possibility that sigma receptor ligands could be useful as novel anti-cancer agents (Adyar et al., 2002).

**BIOMEDICAL SIGNIFICANCE**

It has been suggested that sigma receptors may regulate the cardiovascular system (Ela et al., 1994) and direct effects of sigma ligands on cardiac muscle have been documented, very little is known about sigma receptors in autonomic neurons that innervate the heart. The presence of putative endogenous ligands of sigma receptors, including neuropeptide Y (Roman et al., 1989) and substance P (Larson and Sun 1993), in these ganglia
(Hassall and Burnstock 1984; Karhula 1995; Kessler and Black 1982; Papka et al., 1981) suggests that sigma receptors may be activated under physiological conditions, affecting cell-to-cell signaling in the ganglia, and ultimately the regulation of cardiac functions by the autonomic nervous system. It is also known that drugs of abuse, such as cocaine and PCP, have high binding affinity with sigma receptors, and their cardiovascular side effects may be due to the activation of sigma receptors in autonomic neurons.

Current studies on the modulation of voltage-gated ion channels by sigma receptors of autonomic neurons will not only serve to increase our understanding of the functions of these receptors in the heart but also on the cellular functions of these poorly understood receptors.
CHAPTER 2
SIGMA RECEPTORS MODULATION ON HIGH-VOLTAGE-ACTIVATED CALCIUM CHANNELS IN RAT SYMPATHETIC AND PARASYMPATHETIC NEURONS

INTRODUCTION

Sigma receptors are widely distributed in mammalian brain and peripheral systems and organs. These receptors have been pharmacologically defined into two subclasses of receptors, sigma-1 and sigma-2 (Hellewell and Bowen 1990; Quirion et al., 1992), with a major difference being the higher affinity of sigma-1 receptors for (+) pentazocine (Quirion et al., 1992) and the greater affinity of sigma-2 receptors for ibogaine (Bowen et al., 1995). While only the sigma-1 receptor has been cloned, studies using photolabeling techniques with sigma ligands on guinea pig brain and PC12 cell membranes suggest that distinct molecular entities exist that correspond to the two sigma receptor subtypes (Hellewell and Bowen 1990). The function of these receptors is not well understood; however, sigma receptors have been implicated in the modulation of
various biochemical, behavioral, and physiological processes (Walker et al., 1990).

It has been suggested that sigma receptors may regulate the cardiovascular system (Ela et al., 1994). Sigma ligand binding sites have been detected in cardiac myocytes, and sigma ligands, including (+)-pentazocine and haloperidol, have been shown to alter contractility, Ca$^{2+}$ influx, and contraction rate in cultured cardiac myocytes (Ela et al., 1994; Novakova et al., 1995). However, while direct effects of sigma ligands on cardiac muscle have been documented, very little is known about sigma receptors in autonomic neurons, and in particular sympathetic or parasympathetic neurons that innervate the heart.

A process frequently targeted by sigma receptor modulation is intracellular calcium homeostasis. In the human neuroblastoma cell line, SK-N-SH, sigma-2 receptors have been shown to evoke release of Ca$^{2+}$ from intracellular stores (Vilner and Bowen, 2000). Studies have also suggested that sigma ligands may block Ca$^{2+}$ channels in hippocampal neurons and vascular smooth muscle (Church and Fletcher, 1995; Flaim et al., 1985), although these effects were attributed to direct modulation of Ca$^{2+}$ channels by the sigma ligands. The effect of sigma receptor activation on calcium channels, and in particular calcium channels of autonomic neurons, remains to be elucidated. Regulation of calcium channel function is a means by which various neurotransmitters exert their effects on autonomic neurons (Jeong et al., 1999). For example, both neuropeptide Y
and norepinephrine depress calcium channel currents in rat ICG neurons (Jeong et al., 1999; Xu and Adams 1993). This inhibition of calcium channels is believed to be a mechanism by which the sympathetic nervous system modulates the activity of the parasympathetic nervous system. Similarly, acetylcholine, acting via M_4 muscarinic receptors, blocks calcium channel currents in intrinsic cardiac neurons (Cuevas and Adams 1997). This phenomenon is likely to represent a feedback mechanism in cholinergic parasympathetic neurons.

Experiments were undertaken to determine whether sigma receptors are present in autonomic neurons of the sympathetic superior cervical ganglion (SCG) and the parasympathetic intracardiac ganglion (ICG), and whether activation of these receptors modulates the biophysical properties of calcium channels in these cells. Results indicate that sigma-1 receptor transcripts are expressed by individual autonomic neurons. Furthermore, sigma receptors were shown to depress peak Ca^{2+} channel currents, increase the rate of Ca^{2+} channel inactivation, and shift the voltage dependence of both steady-state inactivation and activation toward more negative potentials. Pharmacological experiments suggest that sigma-2 receptors modulate Ca^{2+} channels in these cells and that these receptors couple to Ca^{2+} channels via a signal transduction cascade that involves neither a diffusible cytosolic second messenger nor a G protein.
METHODS

Preparation and Electrical Recording

Modulation of depolarization-activated Ca\(^{2+}\) channels by sigma receptor activation was studied in isolated neurons of neonatal rat ICG and SCG. The preparation of cultured neurons of neonatal rats (3-10 day old) and the electrophysiological recording methods used here have been previously described (Cuevas and Adams, 1994; Cuevas et al., 2000). For the SCG preparation, neonatal rats were killed by inhalation of carbon dioxide; whereas for isolation of ICG neurons, rats were killed by decapitation. All procedures were done in accordance with the regulations of the Institutional Animal Care and Use Committee.

Membrane currents in autonomic neurons, cultured for 24-72 h, were studied under voltage-clamp mode using the whole cell recording configuration of the patch-clamp technique (Hamill et al., 1981). Electrical access was achieved through the use of the amphotericin B perforated-patch method (Rae et al., 1991) to preserve the intracellular integrity of the neurons and prevent calcium current rundown (Xu and Adams, 1992). For perforated-patch experiments, a stock solution of amphotericin B (60 mg/ml) in dimethylsulphoxide (DMSO) was
prepared and diluted in pipette solution immediately prior to use to yield a final concentration of 198 µg/ml amphotericin B in 0.33% DMSO. Final patch pipette resistance was 1.0-1.3 MΩ to permit maximal electrical access under the present recording configuration. Junction potentials generated by the ions in the pipette and bath solutions were compensated for via the Pipette Offset control of the Axopatch 200B. To test for amphotericin B incorporation into the membrane patch following gigaseal formation, the neurons were held at −60 mV, and 20-ms voltage pulses to −65 mV were applied at 1 Hz. In successful experiments there was an increase in a fast capacitive transient, the appearance of a slow capacitive transient, and a decrease in the series resistance ($R_s$). To minimize voltage error produced by $R_s$, $R_s$ was monitored throughout the experiment, and only cells in which $R_s$ was consistently $\leq$3 MΩ following 50% $R_s$ compensation were used. Also, to minimize space-clamp artifact, only cells with no large visible processes were selected for the experiments.

Depolarization-activated Ca$^{2+}$ channel currents were evoked using voltage jumps from −90 mV to more positive potentials. Capacitive and leak currents were subtracted using the P/4 protocol, which assumes a linear relationship for these currents at voltages less than −60 mV (Xu and Adams, 1992). Membrane currents were amplified using an Axopatch 200B patch-clamp amplifier (Axon Instruments, Union City, CA), filtered at 5 kHz (−3 dB; 4-pole Bessel filter), and digitized at 20 kHz (Digidata 1200B).
Ca\(^{2+}\) currents elicited by long (2 s) depolarizations were fit using single or double exponential functions and the Clampfit 6.0.5 program (Axon Instruments). Activation and steady-state inactivation kinetics were described using Boltzmann distributions, and dose-response curves were fit using the Hill equation. Analysis of these data was conducted using the SigmaPlot 2000 program (SPSS Science, Chicago, IL). Data points represent means ± SE. Statistical difference was determined using paired \(t\)-test for within-group experiments, and unpaired \(t\)-test for between groups experiments, and was considered significant if \(P < 0.05\).

**RT-PCR**

RT-PCR techniques, similar to those previously reported (Cuevas et al. 2000), were used for the detection of sigma-1 receptor expression in autonomic neurons. Total RNA was isolated from ICG ganglia and associated tissue and from SCG (RNeasy, Qiagen, Hilden, Germany). RNA was reverse-transcribed in a 20-µl reaction volume using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, San Diego, CA). As a negative control, a PCR reaction with only water was conducted to eliminate the possibility of false positives due to contaminating cDNA. Primers specific for sigma-1 receptor transcripts were designed to span an intron to discriminate between genomic DNA and cDNA. The sequences of the primers used were: sigma-1\(_{(sense)}\)^−

GTCTTTTGCACGCCTCGCTGTCTGAGTACG, sigma-1\(_{(antisense)}\)^−

ACCCTCTCTGGATGGAGGTGAGTGC, which yielded a product size of 639
base pairs. PCR reactions were conducted using the SuperScript System with Platinum Taq DNA polymerase (Invitrogen). The cycling parameters were one cycle of 94°C for 2 min; 30 cycles of 94°C for 30 s, 61°C for 45 s, and 72°C for 1 min; and 1 cycle of 72°C for 5 min.

For single-cell RT-PCR experiments, SCG and ICG neurons were dissociated, and cytoplasm was extracted from isolated neurons as previously described (Poth et al., 1997). Briefly, the cellular content of individual neurons was harvested using the dialyzing whole cell configuration of the patch-clamp technique. The patch pipettes were filled with 3 µl of 1× SuperScript One-Step RT-PCR Reaction Mix (Invitrogen) containing 1 U/µl RNAsin (Promega, Madison, WI). Following extraction of the cytoplasm, the content of the pipette was expelled into a microfuge tube and quickly frozen on dry ice. Single-cell RT-PCR experiments were conducted immediately following the extraction using SuperScript One-Step RT-PCR with Platinum Taq (Invitrogen). Negative controls for these experiments involved suctioning extracellular solution via a patch pipette located directly above the cells. These controls were carried through all subsequent reactions to rule out the possibility of contamination from cytoplasm from nearby cells or sigma receptor clones isolated in the laboratory. The cycling parameters were 1 cycle of 50°C for 30 min and 95°C for 2 min; 40 cycles of 94°C for 30 s, 61°C for 45 s, and 72°C for 1 min; and 1 cycle of 72°C for 5 min. RT-PCR products were gel purified using a QIAEX II Gel Purification kit
(QIAGEN) and sequenced by the Molecular Biology Core Facility at the H. Lee Moffitt Cancer Center and Research Institute.

**Solutions and Reagents**

The bath solution used in these experiments was a physiological saline solution (PSS) composed of (in mM) 70 NaCl, 70 tetraethylammonium chloride (TEA), 5 BaCl$_2$, 1.2 MgCl$_2$, 7.7 glucose, 0.0005 tetrodotoxin (TTX), and 10 HEPES (pH to 7.2 with NaOH). Barium was used as the charge carrier to maximize Ca$^{2+}$ channel current amplitude, and to minimize any intracellular Ca$^{2+}$-dependent current rundown (Xu and Adams 1992). All drugs, including sigma ligands, were bath applied at room temperature at a rate of ~2 ml/min into a 0.3-ml recording chamber, which permitted rapid exchange of bath solution. The pipette solution used for perforated-patch experiments contained (in mM) 75 Cs$_2$SO$_4$, 55 CsCl, 5 MgSO$_4$, and 10 HEPES (pH to 7.2 with N-methyl-d-glucamine). Block of ionic current through Ca$^{2+}$ channels was achieved via bath application of 100 µM CdCl$_2$ (Xu and Adams 1992). For studies using conventional (dialyzing) whole cell recording configuration, the pipette solution contained (in mM) 140 CsCl, 2 MgCl$_2$, 2 ethylene glycol-bis (β-aminoethyl ether)-$N,N,N',N'$-tetraacetic acid (EGTA), 2 Mg$_2$ATP, 0.1 GTP lithium salt (GTP), and 10 HEPES-CsOH, pH to 7.2. In some experiments GTP was replaced with 100 µM guanosine 5'-O-(2-thiodiphosphate) trilithium salt (GDP-β-S) to inhibit G protein activation.
All chemical reagents used were of analytical grade. Ibogaine hydrochloride, (+)-pentazocine, haloperidol, 1,3-Di-O-tolylguanidine (DTG), metaphit, and tetrodotoxin were purchased from Sigma Chemical (St. Louis, MO).
RESULTS

To determine whether sympathetic and parasympathetic neurons may be the target of sigma receptor ligands, autonomic neurons were first screened for the expression of transcripts encoding the sigma-1 receptor using RT-PCR techniques. Oligonucleotide primers were designed to span introns 2 and 3 of the sigma-1 gene to differentiate between cDNA and genomic DNA. RT-PCR of total RNA extracts from SCG and ICG ganglia and associated tissue (e.g., cardiac myocytes, Schwann cells, and fibroblasts) showed that sigma-1 receptor transcripts are expressed in these cells (Figure 1A). However, since sigma-1 receptors have been found in nonneuronal cells, it seemed prudent to test for the presence of sigma-receptor transcripts at the single-cell level. Using single-cell RT-PCR techniques, transcripts encoding the sigma-1 receptor were shown to be expressed in individual ICG and SCG neurons (Figure 1B). Sigma-1 receptor transcripts were detected in 57% of ICG neurons (4 of 7) and 67% of SCG neurons (4 of 6). Sequencing of the products obtained from individual autonomic neurons indicated exact sequence homology to the known rat brain sigma-1 receptor (Seth et al., 1998). Splice variants of the sigma-1 receptor have been reported in the rat and mouse as submissions to GenBank (accession numbers...
AF087827 and AF226605, respectively). The oligonucleotide primers used here were specifically designed to detect conventional sigma-1 transcripts and both of these sequence variants, but no such isoforms were detected in these cells.

Sigma Receptor-Mediated Attenuation of Ca\(^{2+}\) Channel Currents

Sigma receptors have been shown to modulate calcium homeostasis in various cell types. One of the mechanisms by which sigma receptors appear to modulate cellular calcium is through attenuation of calcium influx through the cell membrane. However, the effects of sigma receptor activation on calcium channel function have not been determined.

Ca\(^{2+}\) channel currents were isolated by inhibiting Na\(^{+}\) currents with extracellular TTX, and K\(^{+}\) channels with intracellular Cs\(^{+}\) and extracellular TEA and Ba\(^{2+}\). Ba\(^{2+}\) was used as the charge carrier through open calcium channels in most experiments for reasons discussed in METHODS. The effect of sigma ligands on the Ba\(^{2+}\) current-voltage (I-V) relationship was examined using brief (250ms) step depolarizations of 10-mV increments (−50 to +90 mV) from a holding potential of −90 mV. Figure 2A shows a family of depolarization-activated Ba\(^{2+}\) currents (\(I_{Ba}\)) recorded from a single SCG neuron in the absence (Control) and presence of the sigma receptor ligand, haloperidol (10 µM). Bath application of haloperidol depressed peak \(I_{Ba}\) amplitude at all potentials positive to −10 mV within 3 min of drug application. Figure 2B shows the average I-V relationship obtained for six neurons before (Control) and after bath application of
10 µM haloperidol. Under control conditions, \( I_{Ba} \) was activated at approximately −30 mV and the \( I-V \) relation was maximal at 0mV, reversing at approximately +50 mV. In the presence of haloperidol, the \( I-V \) relationship exhibited similar voltage dependence, but the peak \( I_{Ba} \) amplitude was reduced at all voltages. At 0 mV, \( I_{Ba} \) decreased from a control value of \(-1,565 ± 76 \text{ pA}\) to \(-969 ± 222 \text{ pA}\) in the presence of 10 µM haloperidol, \((n=6)\). Inhibition of Ca\(^{2+}\) channel currents occurred to a similar degree when Ca\(^{2+}\) was the charge carrier (data not shown).

Bath application of 100 µM Cd\(^{2+}\) completely blocked the depolarization-activated Ba\(^{2+}\) current, both in the absence and the presence of haloperidol (data not shown). The observed Cd\(^{2+}\) block, coupled with the lack of shift in the reversal potential for the depolarization-activated currents (Figure 2B) in the presence of sigma ligands, suggests that these drugs are not activating or inhibiting another membrane conductance.

**Concentration-Dependent Inhibition of \( I_{Ba} \) by Sigma Ligands**

To determine whether the effect of haloperidol on Ca\(^{2+}\) channels is mediated by sigma receptor activation, the ability of various sigma ligands to elicit a similar response was assessed. Figure 3A shows representative currents recorded from three different SCG neurons (1-3) in the absence (Control) and presence of haloperidol, (+)-pentazocine, and DTG. The peak inward \( I_{Ba} \) was measured before and after exposure to various concentrations of the sigma ligands haloperidol, (+)-pentazocine, DTG, and ibogaine. For these experiments,
each cell was exposed to a minimum of three drug concentrations. A plot of the mean peak $I_{\text{Ba}}$ as a function of drug concentration is shown in Figure 3B. Haloperidol had the greatest potency of the ligands tested, and a fit of the data using the Hill equation gave a half-maximal inhibitory concentration ($IC_{50}$) value of 6 µM. Similarly, the $IC_{50}$ values for ibogaine, (+)-pentazocine and DTG were 31, 61, and 133 µM, respectively, and the Hill coefficient was 1.1 for all drugs. Maximum inhibition of $I_{\text{Ba}}$ by all sigma ligands tested was ≥95%.

The effect of sigma ligands on $Ca^{2+}$ channel current amplitude was reversible on wash out. Figure 4A shows a family of $Ba^{2+}$ currents evoked by step depolarizations from a single neuron in the absence (Control), presence (+DTG), and following wash out for the indicated time points of the sigma ligand, DTG. Following inhibition of $I_{\text{Ba}}$, the current recovered to near control levels within 5 min of wash out (Figure 4B). Similar reversal of inhibition was observed for all sigma ligands tested here. No significant rundown of $I_{\text{Ba}}$ was observed in recordings ≤45 min.

**Sigma Receptor Antagonist Depresses the Effect of DTG on $Ca^{2+}$ Channels**

To confirm whether the effect of sigma ligands on $Ca^{2+}$ channels was mediated by activation of a sigma receptor, the irreversible sigma receptor antagonist, metaphit was used. Metaphit is known to rapidly and specifically acetylate sigma receptors, which results in a block of ligand binding (Bluth et al., 1989). Isolated SCG neurons were preincubated in 50 µM metaphit (in PSS) for
10 min at room temperature. Following wash out of drug, Ba$^{2+}$ current amplitude was similar to that recorded in control experiments (no preincubation; Figure 5A), suggesting that preincubation in metaphit alone had no effect on Ca$^{2+}$ channel currents. On application of DTG, Ba$^{2+}$ current amplitude was depressed under both conditions, but in cells preincubated in metaphit the response to DTG was obtunded. Figure 5B shows a bar graph of relative mean $I_{Ba}$ amplitude recorded in the presence of 100 µM DTG in control neurons (DTG; $n = 7$) or neurons preincubated in metaphit (Metaphit+DTG; $n=6$). DTG decreased mean $I_{Ba}$ by 28 ± 4% in cells exposed to metaphit, whereas in control cells the decrease was 49 ± 4%. The difference in DTG attenuation of $I_{Ba}$ under both conditions was statistically significant ($P<0.01$).

**Sigma Receptors Inhibit Ca$^{2+}$ Channels in Parasympathetic Neurons**

To determine whether similar modulation of Ca$^{2+}$ channels also occurs in parasympathetic ICG neurons, the effect of sigma receptor ligands on these channels was studied. For these experiments haloperidol, ibogaine, (+)-pentazocine, and DTG were used at concentrations near the IC$_{50}$ value for $I_{Ba}$ inhibition, as determined in SCG neuron. Figure 6A shows currents evoked from four different neurons in the absence (Control) and presence of the indicated sigma ligands. A plot of the mean inhibition of $I_{Ba}$ evoked by these sigma ligands is shown in Figure 6B and is consistent with the observations made in SCG.
neurons. As in the case of SCG neurons, sigma ligands maximally inhibited peak \( I_{Ba} \) in intrinsic cardiac neurons by \( \geq 95\% \) (data not shown).

### Effects of Haloperidol on \( \text{Ca}^{2+} \) Channel Inactivation

Some receptors that modulate \( \text{Ca}^{2+} \) channels, such as \( M_4 \)-muscarinic and \( \alpha_2 \)-adrenergic receptors have been shown to differentially affect the rapid and slow component of \( \text{Ca}^{2+} \) channel current decay (Cuevas and Adams 1997; Xu and Adams 1993). To determine whether sigma receptors have a similar effect on \( \text{Ca}^{2+} \) channel inactivation kinetics, \( \text{Ca}^{2+} \) channel currents were evoked by step depolarization (2 s) to 0 mV from a holding potential of \(-90 \text{ mV}\) in the absence and presence of 10 \( \mu \text{M} \) haloperidol. Figure 7A shows representative responses recorded from a single SCG neuron. Under control conditions, the time-dependent inactivation of \( I_{Ba} \) was biphasic, and best fit by the sum of two exponential functions with time constants of 198 ms (\( \tau_1 \)) and 2.2 s (\( \tau_2 \)). In the presence of haloperidol, the inward current was also best fit by the sum of two exponential functions, but both \( \tau_1 \) and \( \tau_2 \) were decreased to 117 ms and 1.1 s, respectively. Following wash out of haloperidol, \( \tau_1 \) and \( \tau_2 \) returned to near control levels and were 171 ms and 1.5 s, respectively. In five similar experiments, the time course of \( I_{Ba} \) decay was best fit by the sum of two exponential functions with mean time constants of \( 100 \pm 15 \text{ ms} \) (\( \tau_1 \)) and \( 1.2 \pm 0.1 \text{ s} \) (\( \tau_2 \)). In all SCG neurons studied, haloperidol decreased both \( \tau_1 \) and \( \tau_2 \) in a statistically significant manner \((P < 0.01)\), and mean time constants were \( 62 \pm 11 \text{ ms} \) (\( \tau_1 \)) and \( 711 \pm 146 \text{ ms} \) (\( \tau_2 \)).
The peak amplitude of each of the two components of the fit was also depressed, with the amplitude of the first component decreasing by 40 ± 11% and that of the second component by 35 ± 7%. In all cells tested, the effect of haloperidol on the time course of decay of $I_{Ba}$ was reversible on wash out and mimicked by ibogaine (data not shown).

The effect of haloperidol (10 µM) on steady-state inactivation of $Ca^{2+}$ channels in rat SCG neurons was studied using a double pulse protocol. Neurons were initially held at −90 mV, and 10-s prepulses from −120 to +10 mV were applied in 10-mV increments prior to a voltage step to +20 mV (20 ms) to activate (open) the available $Ca^{2+}$ channels. A plot of the relative peak current amplitude [$I_{Ba}/I_{Ba(max)}$] as a function of prepulse voltage is shown in Figure 7B ($n = 6$). The steady-state inactivation of $I_{Ba}$ under control conditions exhibited a sigmoidal dependence on voltage and was best fit with a single Boltzmann function according to the equation

$$I_{Ba} = I_{Ba(max)}/\{1 + \exp[(V-V_h)/k]\}$$

A fit of the mean relative $I-V$ relationship exhibited half-maximal steady-state inactivation ($V_h$) at −27 mV and had a slope parameter ($k$) of −12. In the presence of haloperidol, however, the voltage dependence of steady-state inactivation was best fit by a two-component Boltzmann distribution

$$I_{Ba} = i_1(I_{Ba(max)}/\{1 + \exp[(V_{h1}-V)/k_1]\}) + i_2(I_{Ba(max)}/\{1 + \exp[(V_{h2}-V)/k_2]\})$$

where $i_1$ and $i_2$ represent the fraction contributed by each component to the final function. The values for $i_1$ and $i_2$ were 0.21 and 0.78, respectively. The first
component was half-maximally activated ($V_{h1}$) at $-13$ mV and had a slope parameter ($k_1$) of $-6.3$, whereas the second component was half-maximally activated ($V_{h2}$) at $-53$ mV and had a slope parameter ($k_2$) of $-14.9$.

Effects of Haloperidol on the Voltage Dependence of Ca$^{2+}$ Channel

Activation

The voltage dependence of activation was examined by measuring tail current amplitude. Neurons were held at $-90$ mV, and brief steps (20 ms) to various test potentials ($-50$ to $+100$) were applied prior to repolarization to $-90$ mV. Figure 8A shows Ba$^{2+}$ currents obtained in the absence and presence of haloperidol (10 µM) in response to voltage steps to the indicated potentials and the ensuing tail currents elicited on repolarization to $-90$ mV. The corresponding $I$-$V$ relationship obtained for the peak tail current amplitudes of six neurons is shown in Figure 8B. Haloperidol significantly reduced the peak tail current amplitude at all voltages from $-10$ to $+100$ mV in a reversible manner. However, at 0mV haloperidol decreased peak $I_{Ba}$ tail current amplitude by 57% but by 73% at $+50$ mV. This effect does not appear to be a use-dependent phenomenon since $I_{Ba}$ tail current amplitude did not decrease during a train of brief depolarizations (5 pulses, 20 ms) to $+80$ mV or by reversing the voltage protocol (data not shown). Also, at voltages where Ca$^{2+}$ channels were maximally activated, inhibition by haloperidol was comparable ($+80$ mV, 77%; $+90$ mV, 78%; $+100$ mV, 76%). The reason for greater depression of $I_{Ba}$ tail current amplitude
by haloperidol at higher depolarizations is a drug-induced shift in Ca$^{2+}$ channel activation.

Figure 8C shows a plot of the mean peak $I_{Ba}$ tail current amplitude normalized to maximum $I_{Ba}$ tail current amplitude in the absence and presence of haloperidol. Ca$^{2+}$ channels exhibit sigmoidal activation at potentials positive to $-40$ mV under both conditions. Data points were best fit using a two-component Boltzmann distribution (Eq.2). For control, $i_1$ and $i_2$ were 0.48 and 0.52, respectively, whereas in the presence of haloperidol $i_1$ and $i_2$ were 0.70 and 0.20, respectively. Half-maximal activation of the first component ($V_{h1}$) shifted from $-4$ mV in the absence (control) to $-13$ mV in the presence of haloperidol, while the second component ($V_{h2}$) shifted from $+38$ mV (control) to $+22$ mV (+haloperidol). This sigma receptor-induced shift in the voltage dependence of activation results in tail currents of greater amplitude at more negative potentials and is thus responsible for the difference in percent inhibition of Ba$^{2+}$ tail currents by haloperidol at low and high depolarizations. The effects of haloperidol on the voltage dependence of Ca$^{2+}$ channel steady-state inactivation and activation were reversible on wash out and were mimicked by ibogaine (data not shown).

Effect of Intracellular Dialysis with GTP and GDP-$\beta$-S on Sigma Receptor Inhibition of $I_{Ba}$

In some systems, sigma receptors have been shown to couple to effector targets via a signal transduction cascade involving a G protein. To determine
whether a G protein is involved in the sigma receptor-mediated modulation of Ca\(^{2+}\) channels in autonomic neurons, intrinsic cardiac neurons were dialyzed with pipette solution containing either 100 µM GTP or 100 µM GDP-β-S. In neurons dialyzed with GTP, sigma receptor-induced inhibition of \(I_{Ba}\) was similar to that observed in neurons electrically accessed using the perforated-patch method. Figure 9A shows representative currents in response to step depolarizations from −90 to 0 mV recorded from two neurons dialyzed with either GTP (top traces) or GDP-β-S (bottom traces) in the absence and presence of 10 µM haloperidol. A summary of the peak \(I_{Ba}\) amplitudes elicited on depolarization to 0 mV, normalized to their respective control values, under the different experimental conditions is presented in Figure 9B. Haloperidol decreased \(I_{Ba}\) by 68 ± 8\% (\(n = 5\)) in neurons dialyzed with pipette solution containing GTP and by 62 ± 3\% (\(n = 6\)) in neurons dialyzed with GDP-β-S. The difference between these two experimental groups was not statistically significant. DTG inhibition of \(I_{Ba}\) was reversible on wash out of drug when cells were dialyzed with either GTP or GDP-β-S (data not shown).

To determine whether the dialysis with GDP-β-S was sufficient to block G protein-mediated events, these cells were also exposed to 100 µM ACh to elicit muscarinic receptor-evoked inhibition of Ca\(^{2+}\) channels. Muscarinic receptors have been shown to couple to Ca\(^{2+}\) channels via a pertussis toxin-sensitive G protein in intrinsic cardiac neurons (Cuevas and Adams 1997). Following cell dialysis with GTP containing pipette solution, ACh depressed \(I_{Ba}\) by 40 ± 6\% (\(n = 29\))
3). This ACh-mediated inhibition was reduced to $17 \pm 4$ in cells dialyzed with GDP-β-S ($n = 3$), which was significantly different from the reduction observed when GTP was used.
Figure 2.1 Detection of sigma-1 receptor transcripts in rat ICG and SCG. A: RNA from rat ICG and SCG were reverse transcribed and amplified via PCR using oligonucleotide primers specific for the sigma-1 receptor (sense, GTCTTTTGCACGCCTCGCTGTCTGAGTACG; antisense, ACCCTCTCTGGATGGAGGTGAGTG). B: RT-PCR reaction results for 2 isolated neurons from rat ICG and SCG using the sigma-1 primers. A and B: arrows indicate predicted size for the sigma-1 receptor product (639 bp), and standards are 100 bp ladder.
Figure 2.2 Inhibition of Ca$^{2+}$ channel currents in sympathetic neurons by the sigma receptor agonist, haloperidol. A: family of depolarization-activated Ba$^{2+}$ currents recorded from a single SCG neuron in the absence (Control) and presence of 10 µM haloperidol. B: whole cell current-voltage relation obtained in the absence (○) and presence of 10 µM haloperidol (●). Data points represent means ± SE for 6 cells.
**Figure 2.3.** Dose-dependent inhibition of depolarization-activated Ca\(^{2+}\) channels by sigma receptor ligands in rat sympathetic neurons. *A*: whole cell currents evoked from 3 SCG neurons (1-3) by step depolarizations to 0 mV from a holding potential of −90 mV in the absence (Control) and presence of haloperidol, (+)-pentazocine and 1,3-Di-O-tolyguanidin (DTG) at the indicated concentrations. *B*: peak whole cell \(I_{Ba}\) amplitude, evoked by depolarizing to 0 mV from −90 mV, normalized to control and plotted as a function of sigma ligand concentration. Data points represent means ± SE for 5-7 neurons. The curves represent best fit to the data using the Hill equation. Half-maximal inhibition was 6 µM for haloperidol (■), 31 µM for ibogaine (◊), 61 µM for (+)-pentazocine (●), and 133 µM for DTG (△), and the Hill coefficient was 1.1 for all compounds.
Figure 2.4 Reversibility of DTG-induced block of depolarization-activated Ca\(^{2+}\) channels.  

A: family of depolarization-activated Ca\(^{2+}\) channel currents recorded from a single rat SCG neuron in the absence (Control) and presence of bath-applied 1 mM DTG (+DTG), and following wash out of drug for the indicated time periods.  

B: peak Ba\(^{2+}\) current amplitudes before application (○), during application (●), and after removal (○) of 1 mM DTG for the cell in A.  Values are plotted as a function of time.  Gap represents period during which lower concentrations of DTG were applied, with 1 mM DTG application commencing at t =13 min.
Figure 2.5 Attenuation of DTG-mediated inhibition of Ca\(^{2+}\) channels by the sigma receptor antagonist, metaphit.  

**A**: depolarization activated (−90 to 0 mV) Ba\(^{2+}\) currents recorded from 2 SCG neurons in the absence (Control, Metaphit) and presence of 100 µM DTG (DTG, Metaphit + DTG).  

*Bottom traces* are from a neuron preincubated in metaphit (50 µM) in physiological saline solution (PSS), (10 min).  

**B**: bar graph of the relative mean peak \(I_{Ba}\) (±SE) obtained by step depolarizations (−90 to 0 mV) in control cells (DTG) or cells preincubated in metaphit (50 µM, 10 min; Metaphit + DTG) following bath application of 100 µM DTG.  

Current amplitudes were normalized to their respective controls (absence of DTG).  

Data were collected from 7 neurons for each condition, and asterisk denotes significant difference between the groups \((P < 0.01)\).
Figure 2.6 Inhibition of Ca\(^{2+}\) channel currents in rat ICG neurons by sigma receptor ligands. A: Ba\(^{2+}\) currents evoked by step depolarizations to 0 mV from –90 mV recorded from 4 parasympathetic ICG neurons in the absence (Control) and presence of ibogaine, DTG, (+)-pentazocine, and haloperidol at the indicated concentrations. B: peak whole cell Ba\(^{2+}\) current amplitude normalized to control recorded in the presence of haloperidol (HAL), ibogaine (IBO), (+)-pentazocine (PTZ), and DTG. Bars represent means ± SE for 3-5 ICG neurons.
Figure 2.7 Sigma receptor modulation of time-dependent and steady-state inactivation of Ca\(^{2+}\) channels in SCG neurons.  

A: Ba\(^{2+}\) currents evoked from a single neuron by 2-s depolarizations to 0 mV from a holding potential of \(-90\) mV in the absence (Control), presence of 10 µM haloperidol (Haloperidol), and following wash out of drug (Wash). Solid lines represent a best fit of the time course of current decay with the sum of 2 exponential functions.  

B: relative Ba\(^{2+}\) current amplitude as a function of prepulse amplitude in the absence (○) and presence (●) of 10 µM haloperidol. Data points represent means ± SE for 6 neurons. Solid lines represent best fit to the data with a single (control) or 2-component (haloperidol) Boltzmann distribution.
Figure 2.8 Sigma receptor evoked shift in voltage dependence of Ca$^{2+}$ channel activation. A: Ca$^{2+}$ channel tail currents evoked from a single neuron by repolarization to −90 mV from the indicated potentials in the absence (Control) and presence of 10 µM haloperidol (Haloperidol). The time scale is increased 5-fold at the start of repolarization. Mean peak tail current amplitude (B) and relative peak Ba$^{2+}$ tail current amplitude (C) evoked by repolarization to −90 mV following a brief depolarization to the indicated potentials in the absence (○) and presence (●) of 10 µM haloperidol. Data points represent means ± SE for 6 neurons. Solid lines in C represent best fit to the data using a 2-component Boltzmann distribution.
Figure 2.9 Sigma receptor inhibition of Ca\textsuperscript{2+} channels is not blocked by intracellular GDP-\(\beta\)-S.  

A: depolarization activate (−90 to 0 mV) Ba\textsuperscript{2+} currents recorded from neurons dialyzed with pipette solutions containing either 100 µM GTP (top traces) or GDP-\(\beta\)-S (bottom traces) in the absence (Control) or presence of 10 µM haloperidol (Haloperidol).  

B: peak \(I_{\text{Ba}}\) recorded from neurons dialyzed with either GTP or GDP-\(\beta\)-S in the presence of 10 µM haloperidol or 100 µM ACh.  

Currents are normalized to their respective controls (absence of drug). Asterisk denotes significant difference between groups of cells exposed to ACh.
DISCUSSION

The results presented here provide the first evidence of sigma receptors being expressed in mammalian parasympathetic and sympathetic neurons. Transcripts encoding sigma-1 receptors were detected in individual neurons from ICG and superior cervical ganglia in neonatal rats. Sigma receptors were shown to inhibit all Ca\(^{2+}\) channel subtypes present in neurons of both autonomic ganglia with high efficacy. Furthermore, sigma receptors were demonstrated to differ from other modulators of Ca\(^{2+}\) in these cells on the basis of their effects on the biophysical properties of the channels and the signal transduction cascade coupling them to these Ca\(^{2+}\) channels.

Two pharmacologically distinct subtypes of sigma receptors have been identified: sigma-1 and sigma-2 receptors, respectively. Photoaffinity labeling experiments with sigma receptor-specific ligands have also revealed the presence of a 25-kDa polypeptide in guinea pig brain corresponding to the sigma-1 receptor and a polypeptide doublet of 18 and 21 kDa that is believed to represent the sigma-2 receptor (Hellewell and Bowen 1990). The mammalian sigma-1 receptor has been cloned in several species including guinea pig, human, and rat (Hanner et al., 1996; Kekuda et al., 1996; Seth et al., 1998).
These receptors are expressed in the brain and in several peripheral tissues and organs (Walker et al., 1990). Because of the presence of sigma-1 receptors in cardiac muscle (Ela et al., 1994), and possibly other tissues associated with ICG ganglia or in support cells in the SCG, we used single-cell RT-PCR techniques to demonstrate that transcripts for this receptor are present specifically in autonomic neurons. The sequences of the sigma-1 receptor transcripts cloned from both ICG and SCG neurons were identical to that reported for the rat brain sigma-1 receptor (Seth et al., 1998). No splice variations of the sigma-1 receptor were detected in these neurons. Sequences for two isoforms of the sigma-1 receptor have been submitted previously to GenBank (Mei and Pasternak 1998; Wang et al., 2000). One of these isoforms, sigma-1β receptor, was cloned from mouse and is reported to have sigma-2-like binding activity (Wang et al., 2000). However, transcripts encoding these truncated forms of the sigma-1 receptor were not observed, indicating that they do not mediate the cellular responses to sigma ligands reported in this study.

In the present study, the use of sigma receptor agonists suggests that sigma receptors inhibit Ca\(^{2+}\) channels in neurons from parasympathetic ICG ganglia and sympathetic superior cervical ganglia. These agonists were shown to inhibit Ca\(^{2+}\) channels in over 90% of the cells tested \((n>100)\). In autonomic neurons, various cell membrane receptors have been shown to be coupled to Ca\(^{2+}\) channels. In mammalian ICG neurons, \(M_4\) muscarinic, α-adrenergic, neuropeptide Y, and µ-opioid receptors have all been shown to depress Ca\(^{2+}\)
channel currents (Adams and Trequattrini 1998; Cuevas and Adams 1997; Jeong and Wurster 1997a; Kennedy et al., 1998; Xu and Adams 1993). However, maximum inhibition of peak Ca\(^{2+}\) by activation of these receptors is ≤75%, and their primary target is N-type calcium channels, which account for ~70% of the whole cell calcium current in these cells (Cuevas and Adams 1997; Jeong and Wurster 1997a; Xu and Adams 1993). In contrast, activation of sigma receptors in these neurons inhibits ≥95% of the peak current, indicating that all Ca\(^{2+}\) channel types are affected. It has been reported that these cells express N-, L-, P/Q-, and R-type calcium channels (Cuevas and Adams 1997; Jeong and Wurster 1997b; Xu and Adams 1993).

The sigma receptor inhibition of heterogeneous populations of Ca\(^{2+}\) may have significant physiological implications. Attenuation of N-type Ca\(^{2+}\) channels by ω-conotoxin GVIA fails to block synaptic transmission in parasympathetic ganglia, but broad-spectrum Ca\(^{2+}\) channel inhibitors, such as cadmium, eliminate excitatory postsynaptic potentials (Seabrook and Adams 1989). Inhibition of multiple classes of Ca\(^{2+}\) channels may contribute to the reported sigma receptor-mediated decrease in guinea pig ileum longitudinal muscle contraction (Campbell et al., 1989; Kinney et al., 1995). It has been proposed that a decrease in ACh release is responsible for this attenuation in muscle contraction (Campbell et al., 1989) and block of presynaptic Ca\(^{2+}\) would depress transmitter release. Therefore activation of sigma receptors may block signaling through autonomic ganglia and inhibit modulation of effector targets by peripheral neurons. In the
cardiovascular system, inhibition of parasympathetic input to the heart may account for the increased heart rate, arrhythmias, and sudden cardiac death observed in some patients during therapy with haloperidol (Mehta et al., 1979; Settle and Ayd 1983; Turbott and Cairns 1984). Furthermore, haloperidol evokes a prolongation of the QT interval in the electrocardiogram (Kriwisky et al., 1990), which is similar to that induced by atropine-mediated vagal block (Annila et al., 1993).

In addition to affecting a broader population of Ca\(^{2+}\) channel types than other endogenous modulators of autonomic Ca\(^{2+}\) channels, activators of sigma receptors have profoundly different effects on Ca\(^{2+}\) channel biophysics. Whereas ACh and norepinephrine (NE), for example, have no effects on the steady-state inactivation of Ca\(^{2+}\) channels (Cuevas and Adams 1997; Xu and Adams 1993), sigma ligands shift the steady-state inactivation curve to more negative potentials. The fact that the voltage dependence of inactivation was best fit by a one-component Boltzmann distribution in the absence of sigma receptor activation but by a two-component Boltzmann distribution in the presence of haloperidol suggests that sigma receptors may not equally modulate steady-state inactivation in all Ca\(^{2+}\) channel subtypes.

Activation of sigma receptors also altered the voltage dependence of activation of Ca\(^{2+}\) channels in a manner distinct from other known Ca\(^{2+}\) channel inhibitors. In the presence of sigma receptor agonists, the Ca\(^{2+}\) channel activation curve was shifted toward more negative potentials. Other inhibitors of
Ca$^{2+}$ channels, such as µ-opioid, muscarinic and α-adrenergic agonists shift the activation curve to more positive potentials (Adams and Trequattrini 1998; Cuevas and Adams 1997; Xu and Adams 1993). Thus stronger depolarizations are required in the presence of these agents to activate the same number of Ca$^{2+}$ channels. The shift in the Ca$^{2+}$ channel activation curve toward more positive potentials has been explained by a "willing-reluctant" model first proposed by Bean (Bean 1989). According to this model, Ca$^{2+}$ channels are converted in the presence of a modulator from a "willing" state to a "reluctant" state that requires stronger depolarization to open the channel. Such a shift in the voltage dependence of activation is not observed here. Further evidence for the lack in willing to reluctant shift in the presence of sigma ligands is provided by experiments in which prolonged depolarizations were applied to activate Ca$^{2+}$ channels. The fast component of the inward Ca$^{2+}$ current ($I_{Ca}$), represents channels in the willing state, and this component was not preferentially inhibited. In contrast, ACh and NE primarily depress the fast inactivating component ($I_{Ca}$) in long depolarizations and have little effect on the amplitude of $I_{Ba}$ (Cuevas and Adams 1997; Xu and Adams 1993). However, ACh also activates "voltage-independent" mechanisms of Ca$^{2+}$ channel inhibition that result in depression of $I_{Ba}$ at all voltages tested (Cuevas and Adams 1997; Mathie et al., 1992), as is observed here. Similarly, NE inhibition of Ca$^{2+}$ channel activation in rat ICG neurons exhibits voltage-dependent and -independent components (Xu and Adams 1993). One of the outcomes of sigma receptor-mediated increase in the
rate of Ca\(^{2+}\) channel inactivation and attenuation of the amplitude of both \(\tau_1\) and \(\tau_2\) is a greater decrease in net Ca\(^{2+}\) entry through the channels compared with other Ca\(^{2+}\) channel inhibitors.

Previous studies have suggested a possible relationship between sigma receptors and calcium channels. Dextromethorphan, a nonselective sigma receptor agonist, decreased K\(^{+}\) depolarization-evoked Ca\(^{2+}\) uptake into brain synaptosomes and PC12 cells (Carpenter et al., 1988). The half-maximal inhibition of Ca\(^{2+}\) uptake by dextromethorphan was consistent with the effect being mediated by a sigma-2 site, and sigma-2 receptors have been reported in brain and PC12 cells (Hellewell and Bowen 1990; Reid et al., 1990). It has also been suggested that some sigma ligands, including dextromethorphan, inhibit Ca\(^{2+}\) currents by directly interacting with Ca\(^{2+}\) channels (Church and Fletcher 1995; Flaim et al., 1985). Conversely, in frog melanotrophs, micromolar concentrations of (+)-pentazocine have been shown to enhance calcium conductances through activation of sigma receptors (Soriani et al., 1999). The present study shows that structurally dissimilar sigma ligands are able to modulate the biophysical properties of Ca\(^{2+}\) channels. Since these ligands have similar effects on the biophysical properties of the channels, it is unlikely that they would be acting on different sites of the channel. Although the exact binding site for these drugs on the cloned sigma-1 receptor has not been identified, any single site that permits binding to such a broad array of drugs is likely to be quite complex (Walker et al., 1990) and conserved. Thus the lack of any significant
homology between Ca\(^{2+}\) channels and the cloned sigma-1 receptor suggests that the effects of sigma ligands are likely mediated by a sigma receptor and not a direct effect on the Ca\(^{2+}\) channel. The argument against a direct effect of sigma ligands on Ca\(^{2+}\) channels is significantly strengthened by the observation that the sigma receptor antagonist, metaphit, blocks the DTG-mediated attenuation of Ca\(^{2+}\) channels.

Consistent with the effects of sigma ligands on Ca\(^{2+}\) channels being mediated by specific binding of the drugs to a sigma receptor is the finding that the rank order potency and IC\(_{50}\) values for the various sigma ligands tested here are in agreement with those reported previously for sigma-2 receptors. Sigma-2 receptors have been shown to modulate Ca\(^{2+}\) release from intracellular stores in human SK-N-SH neuroblastoma cells (Vilner and Bowen 2000). The rank order potency reported in that study, haloperidol > ibogaine > (+)-pentazocine ≈ DTG, and micromolar EC\(_{50}\) values are in agreement with our findings. Sigma-2 receptors have also been reported to mediate the inhibition of guinea pig ileum longitudinal muscle contraction (Kinney et al., 1995). In that study, haloperidol was shown to depress electrically evoked contractions with an IC\(_{50}\) nearly identical to that reported here (~6 µM). In primary cultures of rat frontal cortical neurons, sigma-2 receptor activation blocked N-methyl-D-aspartate (NMDA) mobilization of intracellular free Ca\(^{2+}\) (Hayashi et al., 1995). The IC\(_{50}\) for haloperidol and (+)-pentazocine inhibition of peak free Ca\(^{2+}\) were ~6 and 40 µM, respectively, also in agreement with the values reported here for these drugs.
One of the strongest lines of evidence for sigma-2 receptors mediating the inhibition of Ca\(^{2+}\) channels in autonomic neurons is our observation that ibogaine, a sigma-2-selective agonist (Bowen et al., 1995), exhibits greater potency than (+)-pentazocine. The affinity of sigma-1 receptors for (+)-pentazocine is ~2,000-fold greater than for ibogaine, whereas the affinity of sigma-2 receptors for ibogaine is ~6-fold higher than for (+)-pentazocine (Vilner and Bowen 2000). The IC\(_{50}\) for ibogaine inhibition of \(I_{Ba}\) in these autonomic neurons is two-fold greater than that determined for (+)-pentazocine.

The primary mechanism by which other known modulators of Ca\(^{2+}\) in autonomic neurons depress Ca\(^{2+}\) currents is via the activation of pertussis toxin-sensitive G proteins (Adams and Trequattrini 1998; Cuevas and Adams 1997; Xu and Adams 1993). However, no such G protein appears to be implicated in the signal transduction cascade coupling sigma receptors and Ca\(^{2+}\) channels in these cells, since intracellular dialysis with GDP-β-S failed to inhibit the effects of sigma ligands. Furthermore, the inability of cell dialysis to block the effects of sigma receptors suggests that a diffusible cytosolic second messenger is likely not involved. Similarly, sigma receptors have been shown to modulate K\(^+\) channels in rat pituitary cells through a membrane-delimited signaling pathway that does not incorporate a G protein (Lupardus et al., 2000).

Taken together, molecular biology studies and pharmacological studies conducted here suggest that both sigma-1 and sigma-2 receptors are expressed in ICG and superior cervical ganglion neurons. However, our experiments
indicate that only the sigma-2 receptor, which remains to be cloned, couples to Ca\(^{2+}\) channels in these cells. Given that the sigma-2 receptor has been shown to be a distinct molecular entity (Hellewell and Bowen 1990), it is doubtful that the sigma receptor shown to modulate Ca\(^{2+}\) channels here is a modified form of the sigma-1 receptor gene product. Thus the cellular function of the sigma-1 receptors found in these autonomic neurons remains to be determined.

In conclusion, rat ICG and SCG neurons express sigma-1 and sigma-2 receptors, and activation of these receptors alters the biophysical properties of Ca\(^{2+}\) channels and attenuates whole cell Ca\(^{2+}\) channel currents. Pharmacological experiments suggest that the modulation of Ca\(^{2+}\) channels is mediated by sigma-2 receptors. Because of the importance of Ca\(^{2+}\) channels in the function and regulation of the autonomic nervous system, sigma receptors are likely to have a significant role in the modulation of autonomic nerve activity and thus on regulation of the cardiovascular system and other effector targets.
CHAPTER 3
SIGMA RECEPTOR ACTIVATION BLOCKS POTASSIUM CHANNELS AND
DEPRESSES NEUROEXCITABILITY IN RAT ICG NEURONS

INTRODUCTION

Sigma receptors are non-opioid, non-phencyclidine receptors that are distributed throughout the central and peripheral nervous system. These receptors have high affinity for numerous drugs in clinical use including psychotherapeutic agents such as antipsychotics (Su, 1982) and selective serotonin reuptake inhibitors (Narita et al., 1996). At least two subtypes of sigma receptors have been classified based on the findings from biochemical and radioligand binding experiments, and have been designated as sigma-1 and sigma-2 (Hellewell and Bowen, 1990). However, to date, only the sigma-1 receptor has been cloned (Hanner et al., 1996). These receptors have been implicated in physiological processes such as learning and memory and in pathophysiological disorders such as schizophrenia and depression (Snyder and Largent, 1989). More recently, sigma receptors have been linked to the development of drug dependence (Ujike et al., 1996). Sigma receptors also
appear to play a role in the regulation of the cardiovascular system, as suggested by pharmacological studies using sigma receptor ligands. For example, it has been shown that the sigma receptor ligand, DuP 754, raises the threshold of ventricular fibrillation in rats (Lishmanov et al., 1999). Conversely, the sigma receptor agonists, (+) SKF-10,047, 1,3-Di-O-tolyguanidin (DTG), and (+)-3-[3-hydroxyphenyl]-N-(1-propyl)piperidine, have been shown to produce tachycardia by activating central and peripheral sigma receptors (Wu and Martin, 1989; Lishmanov et al., 2000). Some of the effects of sigma receptor ligands on the cardiovascular system are likely due to activation of sigma receptors on cardiac muscle. Sigma receptors have been detected in cardiac myocytes from neonatal and adult rats, and stimulation of these receptors was shown to elicit direct inotropic and chronotropic effects on cardiac muscle (Ela et al., 1994; Novakova et al., 1995). However, sigma receptors have also been found in autonomic neurons that regulate the cardiovascular system and modulation of the electrical activity of these neurons by endogenous or exogenous sigma ligands is likely to have profound effects on cardiovascular function. Mammalian intracardiac ganglia (ICG) mediate all parasympathetic input to the heart and serve as the common final pathway for autonomic regulation of cardiac function. Sigma receptor ligands, including haloperidol, (+) pentazocine, DTG and ibogaine, were shown to inhibit high voltage-activated calcium channels in intrinsic cardiac neurons. The pharmacological profile of the sigma receptor mediating these effects was consistent with a sigma-2 receptor (Zhang and Cuevas, 2002). While
sigma-1 receptor transcripts were detected in ICG neurons, no cellular effects have been attributed to this receptor. One possible role for sigma-1 receptors in these cells is the modulation of voltage-activated K$^+$ channels. Recent experiments have shown that currents mediated by Kv1.4 and Kv1.5 are depressed by the sigma ligand (+)-SKF 10,047 in oocytes injected with Kv subunits and the sigma-1 receptor, but not in oocytes injected with the Kv subunits alone (Aydar et al., 2002). Furthermore, sigma-1 receptors were shown to alter the kinetic properties of Kv1.4 channels in the absence of ligand, and Kv1.4 and Kv1.5 coimmunoprecipitated with the sigma-1 receptor (Aydar et al., 2002), which led those investigators to propose that the sigma-1 receptor functions as a ligand-regulated auxiliary K$^+$ channel subunit. The inhibition of delayed outwardly rectifying K$^+$ channels, such as Kv1.5, by verapamil has been shown to depress AP firing in intrinsic cardiac neurons, and this phenomenon has been implicated in the tachycardia associated with this type IV antiarrhythmic drug (Hogg et al., 1999). However, it remains to be determined if sigma-1 receptors couple to voltage-activated K$^+$ channels in ICG neurons, and if sigma receptor activation can alter the active membrane proprieties of these cells. By regulating the function of K$^+$ channels in parasympathetic ICG neurons, sigma-1 receptors, and therefore drugs that act on these receptors, may have significant influence on the heart. Experiments were undertaken to determine the effects of sigma receptors on currents mediated by voltage-activated K$^+$ channels ($I_{K}$) and the active membrane properties of ICG neurons from neonatal rats. Sigma
Receptors were shown to decrease the peak amplitude of currents mediated by delayed outwardly-rectifying K⁺ channels (I_{K(DR)}), large conductance Ca^{2+}-sensitive K⁺ channels (I_{BK}), and the M-current (I_{M}). The rank-order potency of K⁺ channel inhibition by different sigma ligands suggest that the effect is mediated by sigma-1 receptors. Neither cell dialysis nor intracellular application of GDP-β-S blocked the effects of sigma-1 receptors on I_K, consistent with a direct interaction between sigma-1 receptors and the K⁺ channels. Activation of sigma-1 receptors also decreased AP firing and changed the AP configuration in ICG neurons. Therefore, sigma-1 receptor activation depresses the excitability of ICG neurons and is likely to attenuate parasympathetic input to the heart.
METHODS

Preparation of Cell Culture

The isolation and culture of neurons from neonatal rat ICG ganglia has been described previously (Cuevas and Adams, 1994). Briefly, neonatal rats (2-7 day old) were euthanized by decapitation and the hearts excised and placed in a physiological saline solution containing (mM): 140 NaCl, 3 KCl, 2.5 CaCl₂, 0.6 MgCl₂, 7.7 glucose and 10 histidine; pH to 7.2 with NaOH. Atria were removed and incubated for 1 h at 37 °C in physiological saline solution containing 1 mg/ml collagenase (Type 2, Worthington). Following enzymatic treatment, clusters of ganglia were dissected from the epicardial ganglion plexus and dispersed by trituration in a high glucose culture medium (DMEM), 10% fetal calf serum, 100 units/ml penicillin and 0.1 mg/ml streptomycin. The dissociated neurons were then plated onto glass cover slips coated with poly-L-lysine, and incubated at 37 °C under a 95% air-5% CO₂ atmosphere for 48-72 hours.

Electrical Recordings

Electrophysiological recording methods used were similar to those previously described (Cuevas et al., 1997). Active membrane properties and
voltage-activated K⁺ channel currents in ICG neurons were studied under current-clamp and voltage-clamp mode, respectively, using the whole cell patch-clamp technique. Electrical access was achieved through the use of the amphotericin B perforated-patch method to preserve the intracellular integrity and prevent the loss of cytoplasmic components and subsequent alteration of the functional responses of these neurons (Cuevas and Adams, 1994). For perforated-patch experiments, a stock solution of amphotericin B (60mg/ml) in dimethyl sulfoxide (DMSO) was prepared and diluted in pipette solution immediately prior to use to yield a final concentration of 198 µg/ml amphotericin B in 0.33% DMSO. Final patch pipette resistance was 1.0-1.3 MΩ to permit maximal electrical access under the present recording configuration. Action potentials were elicited by depolarizing current pulses (+100 pA) in absence and presence of sigma ligands. Currents through depolarization-activated K⁺ channels were elicited by step depolarizations from −90 mV to more positive potentials (-50 to +70 mV). For experiments studying the effects of sigma receptor activation on Iₘ, cells were held at -30 mV to activate the channel and repolarized to -60 mV to deactivate the channels. Membrane voltages and currents were amplified using an Axopatch 200B patch-clamp amplifier (Axon Instruments, Union City, CA), filtered at 5 kHz (-3 dB; 4-pole Bessel filter), and digitized at 20 kHz (Digidata 1200 B).
Data Analysis

Analyses of these data were conducted using the SigmaPlot 2000 program (SPSS Science, Chicago, IL). Data points represent means ± SEM. Statistical difference was determined using paired $t$-test for within-group experiments, and unpaired $t$-test for between groups experiments, and was considered significant if $p < 0.05$.

Solutions and Reagents

The bath solution for action potential experiments was a physiological saline solution (PSS) containing (in mM) 140 NaCl, 1.2 MgCl₂, 3 KCl, 2.5 CaCl₂, 7.7 glucose and 10 HEPES, pH to 7.2 with NaOH. Potassium channel currents were isolated by adding tetrodotoxin (TTX, 400nM) and cadmium chloride (CdCl₂, 100 µM) to the PSS to inhibit voltage-activated Na⁺ channel and Ca²⁺ channel currents, respectively. Pipette solution used for perforated-patch experiments contained (in mM): 75 K₂SO₄, 55 KCl, 5 MgSO₄, and 10 HEPES (adjust to pH 7.2 with N-methylglucamine). For studies using conventional (dialyzing) whole cell recording configuration, the pipette solution contained (in mM) 140 KCl, 2 MgCl₂, 2 ethylene glycol-bis (β-aminoethyl ether)-$N,N,N',N'$-tetraacetic acid (EGTA), 2 Mg₂ATP, 0.1 GTP lithium salt (GTP), and 10 HEPES-KOH, pH to 7.2. In some experiments GTP was replaced with 100 µM guanosine 5'-O-(2-thiodiphosphate) trilithium salt (GDP-β-S) to inhibit G protein activation. All chemical reagents used were of analytical grade. Haloperidol; ibogaine hydrochloride; (+)-pentazocine; 1,3-Di-
Otolylguanidine (DTG); 2S-(2a,6a,11R*)-1,2,3,4,5,6-hexahydro-6,11-dimethyl-3-(2-propenyl)-2,6-methano-3-benzazocin-8-ol hydrochloride ( (+)-SKF 10,047); tetrodotoxin (TTX); tetraethylammonium chloride (TEA) and linopirdine were purchased from Sigma Chemical (St. Louis, MO).
RESULTS

Sigma Receptors Activation Inhibit Delayed Outward Rectifying $K^+$ Currents

Mammalian ICG neurons exhibit currents mediated by delayed outwardly rectifying $K^+$ channels (Xi-Moy and Dun, 1995; Hogg et al., 1999) and express various subunits that contribute to these channels, including KCNA5 and KCNA6 (Cuevas, unpublished observation). Given that sigma-1 receptors have been shown to depress heterologously expressed KCNA5 channels (Aydar et al., 2002), and that inhibition of these channels may have significant effects on the function of ICG neurons, it seemed prudent to test the effects of sigma receptor activation on currents mediated by voltage-activated $K^+$ channels in these cells.

Figure 1A shows a family of depolarization-activated $K^+$ currents recorded from a single ICG neuron in the absence and presence of DTG (100µM). Bath application of 100 µM DTG depressed peak voltage-activated $K^+$ current amplitude at potentials positive to +20 mV. The voltage-dependence and kinetics of the currents observed are consistent with the delayed outwardly rectifying $K^+$ current ($I_{K(DR)}$) previously reported in ICG neurons (Xi-Moy and Dun, 1995).

Figure 1B shows a plot of the mean peak $I_{K(DR)}$ amplitude in the absence and presence of DTG as a function of voltage. The DTG-induced block of $I_{K(DR)}$ was
voltage-dependent, with the current being blocked by 26.5 ± 6.2% at +70 mV, but only by 1.5 ± 0.1% at +10 mV (n = 7). This difference was statistically significant ($P < 0.01$).

**Concentration-Dependent Inhibition of $I_{K(DR)}$ by Sigma Ligands**

Further pharmacological experiments were undertaken to confirm that sigma receptors inhibit $I_{K(DR)}$ in ICG neurons and to identify the sigma receptor subtype mediating the observed effects. Figure 2A shows representative currents recorded from three ICG neurons in the absence (Control) and presence of (+) pentazocine, ibogaine, and DTG, respectively, at the indicated concentrations. All of the sigma ligands tested depressed peak $I_{K(DR)}$ in a concentration dependent manner. A plot of the mean peak $I_{K(DR)}$ as a function of drug concentration for several sigma ligands is shown in Figure 2B. Fits of the data using the Hill equation gave half maximal inhibitory concentration (IC50) values for haloperidol, (+)-pentazocine, ibogaine, (+)SKF10,047 and DTG of 9.7 ± 0.5, 76.4 ± 7.7 µM, 218.1 ± 7.0 µM, 295.4 ± 45.4 µM and 341.3 ± 26.0 µM, respectively, and Hill coefficients of ~0.9 for all drugs. Maximum inhibition of $I_{K(DR)}$ by sigma ligands was over 80%. The IC50 values and rank-order potency, in particular the observation that (+) pentazocine is significantly more potent than ibogaine ($p < 0.001$) suggests that the effect on $I_{K(DR)}$ is mediated by activation of sigma-1 receptors.
The Sigma Receptor Antagonist, Metaphit, Depresses the Effect of DTG on K⁺ Channels

To confirm the fact that the effect of sigma ligands on $I_{DR}$ was mediated by activation of sigma receptors and not the result of direct channel block, experiments were done using the irreversible sigma receptor antagonist, metaphit. Metaphit rapidly and specifically acetylates sigma receptors, and inhibits ligand binding to the receptor (Bluth et al., 1989). In ICG neurons, metaphit has been shown to block sigma-2 receptor mediated attenuation of voltage-gated Ca²⁺ channel currents (Zhang & Cuevas, 2002). Isolated ICG neurons were preincubated in 50 µM metaphit (in PSS) for 10 min at room temperature. Following wash out of drug, $I_{DR}$ was activated by depolarizing cells to +50 mV from a holding potential of -90mV in the absence and presence of 100 µM DTG. Figure 3A shows representative currents recorded from two different ICG neurons without (upper) or with (lower) metaphit preincubation. Upon application of DTG, $I_{DR}$ peak amplitude was depressed under both conditions, but in cells preincubated in metaphit the response to DTG was obtunded. Figure 3B shows a bar graph of degree of block of $I_{DR}$ by 100 µM DTG in control neurons (DTG; n=4) or neurons preincubated in metaphit (Metaphit + DTG; n=5). DTG decreased mean $I_{DR}$ by 31 ± 3% control cells, whereas in cells exposed to metaphit the decrease was 18 ± 3%. The difference in DTG attenuation of $I_{DR}$ for the two conditions was statistically significant ($p<0.05$). Several other sigma receptor antagonists
including SM-21, BD-1047 and BD-1067 were tested to determine if these agents could block the effects of DTG on $I_{K(\text{DR})}$. However, all of these drugs themselves depressed peak amplitude of $I_{K(\text{DR})}$, suggesting that they are either acting as partial agonists of the sigma-1 receptors, as reported previously for BD-1047 (Zambon et al., 1997), or having direct effects on the $K^+$ channels.

**Effect of Intracellular Dialysis with GTP and GDP-\(\beta\)-S on Sigma Receptor Inhibition of $I_{K(\text{DR})}$**

Controversy exists in the literature as to whether sigma receptors couple to G proteins and if these second messengers are involved in the regulation of ion channels by sigma receptors. To determine if sigma receptor-mediated inhibition of $K^+$ channels in intrinsic cardiac neurons is dependent on G protein activation, neurons were dialyzed with pipette solution containing either GTP (100 µM) or the G protein inhibitor, GDP-\(\beta\)-S (100 µM). Cell dialysis with 100 µM GDP-\(\beta\)-S has previously been shown to successfully block G protein mediated signal transduction in intrinsic cardiac neurons (Cuevas and Adams, 1994, 1997;)

Figure 4A shows representative currents in response to step depolarizations from -90 to +50 mV recorded from two neurons dialyzed with either GTP (top traces) or GDP-\(\beta\)-S (bottom traces) in the absence and presence of 100 µM DTG. The inhibition of $I_{K(\text{DR})}$ evoked by this concentration of DTG under dialyzing conditions, with GTP in the pipette, was similar to that observed when the intracellular milieu
was preserved (~30%). The DTG induced attenuation of $I_{K(DR)}$ was present when the cells were dialyzed with GDP-β-S (Fig 4A, bottom terraces). A summary of the peak $I_{K(DR)}$ amplitudes elicited upon depolarization to $+50$ mV under the different experimental conditions and normalized to their respective control values is presented in Figure 4B. The difference observed between the two groups was not statistical significant. The I-V relationships for 6 neurons dialyzed with either GTP or GDP-β-S are shown in Figure 4C and Figure 4D, respectively. While dialyzing cells with GDP-β-S decreased the peak $I_{K(DR)}$ amplitude, as compared with the cells dialyzed with GTP, DTG attenuated peak amplitudes under both conditions to a similar extent. These data suggest that neither cell dialysis nor inhibition of G-protein activation blocks sigma-1 receptor-mediated inhibition of $I_{K(DR)}$. Thus, neither a diffusible cytosolic second messenger nor a G protein couples sigma-1 receptors to $I_{K(DR)}$.

**Effects of Sigma Receptor Ligands on AP Firing**

Studies have shown that inhibition of $I_{K(DR)}$ by verapamil in ICG neurons can depress AP firing (Hogg et al., 1999). Experiments were thus conducted to determine if sigma receptor modulation $I_{K(DR)}$ has a similar effect on neuroexcitability in these cells. The effects of sigma ligands on the active membrane properties of isolated ICG neurons were studied using the amphotericin B perforated-patch method under current clamp mode. Figure 5A shows a family of APs elicited from a single ICG neuron in response to
depolarizing current pulses (100 pA; 300 ms) in the absence (control) and presence of 100 µM DTG (DTG), and following washout of drug (wash). DTG depolarized the neuron and decreased the number of APs evoked by the current injection in a rapidly reversible manner. In similar experiments, 100 µM DTG depolarized neurons from a control value of 50.8 ± 2.1 mV to -49.2 ± 2.4 mV (n = 9). DTG also decreased the number of APs evoked by depolarizing membrane pulses by 85% (Figure 5B). Both of these changes were statistically significant (p<0.01 and p<0.001, respectively). DTG also altered the AP configuration by decreasing both the AP overshoot and afterhyperpolarization, and by slowing both the rate of depolarization and rate of repolarization (inset of figure 5A and table1). The effects of DTG were mimicked by haloperidol (10 µM), ibogaine (200 µM), and (+) pentazocine (50 µM), which depressed AP firing by 65%, 85% and 65%, respectively and changed the configurations of the waveform in a reversible manner. The effects of the sigma ligands on the AP firing and configuration are summarized in Table1.

Role of M-Currents in the Effects of Sigma Receptor Activation.

One possible mechanism by which sigma-1 receptor activation depolarizes ICG neurons is via a block of the M-current. Previous studies have shown that inhibition of I_M by muscarinic receptor activation depolarizes ICG neurons (Cuevas et al., 1997). The M channels mediating I_M are non-inactivating and close slowly in response to membrane repolarization from depolarizing
holding potentials. The closing of these channels in ICG neurons upon repolarization results in a characteristic inward current relaxation (Cuevas et al., 1997). Figure 6A shows whole-cell currents recorded from a single cell in response to repolarizing steps to -60 mV from a holding potential of -30 mV in the absence (Control) and presence of 1 mM DTG. The inward relaxation observed under control conditions is abolished by application of DTG. Similarly, linopirdine (20 µM), a specific blocker of I\textsubscript{M} (Wallace et al., 2002), eliminates the inward relaxation (Figure 6B). Figures 6C and 6D show bar graphs of mean peak I\textsubscript{M} recorded under control conditions (absence of drug) and when either DTG (n = 4) or linopirdine (n = 6) were added, respectively. I\textsubscript{M} was completely blocked by application of either drug, and this effect was reversible after wash out in both cases (data not shown).

**Effects of Sigma Receptor Activation on BK Currents**

In central and peripheral neurons, AP afterhyperpolarizations (AHP) are mediated by the opening of Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels (I\textsubscript{K(Ca)}). Thus far, three types of Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels have been identified on the basis of their pharmacology and single channel conductances. These channels have been named according to their unitary conductance: large (big) conductance K\textsubscript{Ca} channels (BK), small conductance K\textsubscript{Ca} channels (SK) and intermediate conductance (IK). While both BK and SK channels have been found in mammalian ICG neurons, neonatal rat ICG neurons only express BK channels.
(Franciolini et al., 2001; Jelson et al., 2003). The fact that sigma receptor activation decreases the AHP suggests that sigma receptors may modulate these channels. TEA, at micromolar concentrations (200-500 µM), has been shown to preferentially block BK channels in ICG neurons and to block the AHP more effectively than either charybdotoxin or iberiotoxin (Franciolini et al., 2001). Thus, 500 µM TEA was used to distinguish between outward K⁺ currents mediated by BK and other K⁺ channel types (i.e. I_{K(DR)}, I_{M}). Outward currents were evoked by stepping to +50 mV from a -90 mV holding potential in the absence and presence of 500 µM TEA. The net TEA-sensitive current, and thus the current mediated by BK channels, was determined by subtracting the current remaining after TEA application from that observed under control conditions (absence of TEA). Two distinct TEA-sensitive currents, which differed in their time-dependent inactivation kinetics, were seen in our experiments. These currents were expressed in different populations of neurons, with the fast inactivating current (BK_i) being expressed in 6 of 10 cells and the slowly-inactivating or noninactivating currents (BK_s) being expressed in the remaining 4 cells. Currents representative of each BK channel subtype are shown in Figures 7A and 7B. Both the rapidly decaying and the slowly decaying TEA-sensitive currents were blocked by 1 mM DTG. Figure 7C and 7D show bar graphs of the mean peak and sustained current amplitude obtained from the BK_i (n = 6) and BK_s (n = 4) species, respectively. Both phases of the current were reduced in the two current types when 1 mM of DTG was applied.
Figure 3.1 Sigma receptor ligands inhibit $I_{k(DR)}$ in rat ICG neurons. A: Depolarization activated (-50 to +70 mV; 300 ms) K$^+$ currents recorded from a single neuron held at -90 mV in the absence (Control) and presence of 100 µM DTG (DTG) or after washout of drug (Wash). B: Peak $I_{k(DR)}$ current density as a function of voltage recorded in a similar manner from 7 neurons in the absence (o) and presence (●) of 100 µM DTG.
Figure 3.2. Dose-dependent inhibition of $I_{K(DR)}$ by sigma receptor ligands in rat ICG neurons. A: Whole-cell $K^+$ currents evoked from 3 ICG neuron by step depolarizations to $+50$ mV from a holding potential of $-90$ mV in the absence (Control) and presence of (+) pentazocine, ibogaine, and DTG at the indicated concentrations. B: Peak whole-cell $I_{K(DR)}$ current amplitude, evoked by depolarizing to $+50$ mV from $-90$ mV, normalized to control and plotted as a function of sigma ligands concentration. Data points represent mean ± SEM for ≥4 observations. The curves represent best fit to the data using the Hill equation. Half-maximal inhibition was $9.7 \pm 0.5$ µM for haloperidol, $76.4 \pm 7.7$ µM for (+)-pentazocine, $218.1 \pm 7.0$ µM for ibogaine, $295.4 \pm 45.4$ µM for SKF10,047 and $341.3 \pm 26.0$ µM for DTG. The corresponding Hill coefficients were $1.01 \pm 0.05$, $0.69 \pm 0.05$, $1.14 \pm 0.04$, $0.84 \pm 0.11$, $0.97 \pm 0.07$, respectively.
Figure 3.3 Attenuation of DTG-mediated inhibition of $I_{K(DR)}$ by the sigma receptor antagonist, metaphit.  A: depolarization-activated (-90 to 50mV) $I_{K(DR)}$ recorded from 2 ICG neurons in the absence (Control) and presence of 100µM DTG (DTG).  Bottom traces are from a neuron preincubated in metaphit (50 µM in PSS, 10 min).  B: bar graph of the percent inhibition of mean peak $I_{K(DR)}$ (± SE) produced by 100 µM DTG in control cells that were not exposed to metaphit (DTG) or cells preincubated in metaphit (50 µM, 10 min; Metaphit + DTG).  $I_{K(DR)}$ was evoked by step depolarizations (-90 to +50 mV).  Data were collected from 7 neurons for each condition, and asterisk denotes significant difference between the groups ($p<0.05$)
Figure 3.4 Sigma receptor inhibition of $I_{K(\text{DR})}$ is not blocked by intracellular GDP-β-S. A: Depolarization activate (-90 to +50 mV) $K^+$ currents recorded from neurons dialyzed with pipette solutions containing either 100 µM GTP or GDP-β-S in the absence (Control) or presence of 100 µM of DTG (DTG). B: Mean (± SEM) peak $I_{K(\text{DR})}$ recorded from neurons dialyzed with either GTP (n=6) or GDP-β-S (n=6) in the presence of 100 µM DTG. Currents are normalized to their respective controls (absence of drug). Mean (± SE) peak $I_{K(\text{DR})}$ current density as a function of depolarization voltage (-90 mV, holding potential) recorded from neurons dialyzed with pipette solution containing either 100 µM GTP (C, n = 6) or 100 µM GDP-β-S (D, n = 6) in the absence (○) and presence (●) of 100 µM DTG.
Figure 3.5 Inhibition of AP firing by sigma receptor ligand, DTG, in rat ICG neurons. A: APs fired from an isolated ICG neuron in response to 100 pA depolarizing current pulses in the absence (Control) and presence of 50 µM DTG (DTG), and following washout of drug (Wash). Inset is superimposition of first AP from Control and DTG traces. B: Bar graph of the number of APs fired in the absence (Control) or presence of 100 µM DTG (DTG) and following washout of the drug (Wash). Asterisk denotes significant difference from Control and DTG (p<0.01) (n=7).
Figure 3.6 The sigma receptor ligand, DTG, inhibits the M-current in rat ICG neurons. A: Slow inward current relaxation (M-current) induced by repolarizing step (-30 mV to -60 mV, 250 ms) recorded from a single ICG neuron before (Control) and after application of 1mM of DTG (DTG). B: M-current recorded in the absence (Control) and in the presence of 20 µM linopirdine (Linopirdine). Bar graph of mean current amplitudes in the absence (Control) or presence of 1mM DTG (C, n = 4) and 20 µM linopirdine (D, n= 6). The amplitude of the M current was defined as the difference between the peak current observed at the start of the relaxation (panel A, left trace, black arrow) and the holding current at the end of the 250 ms repolarizing step (panel A, left trace, gray arrow). Asterisks denote significant difference from Control (p<0.001).
Figure 3.7 Sigma receptor activation blocks large conductance Ca\(^{2+}\)-activated K\(^{-}\) channel (BK) in rat ICG neurons. Traces of BK mediated currents recorded from two neurons, expressing fast inactivating (A) and slowly-inactivating BK channels (B), in the absence (Control) and presence of 1 mM DTG (DTG). BK mediated currents were evoked by step depolarizations to +50 mV from a holding potential of –90 mV, and defined as the net TEA sensitive current. The net TEA-sensitive current was determined by subtracting the outward current recorded in the presence of 500 µM TEA (with or without DTG) from the outward current obtained in the absence of TEA (with or without DTG). Bar graph of the peak and sustained BK current amplitudes recorded in the absence (Control) and presence of DTG (DTG, 1mM) in cells expressing fast-inactivating (C, n = 6) or slowly-inactivating (D, n = 4) BK channels. Asterisks denote significant difference from control (p<0.05).
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<th>Decay Slope (mV/sec)</th>
<th>After Hyperpolarization(mV)</th>
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**Table 3.1** Effects of sigma ligands on the resting membrane potential and AP waveform parameters. APs were evoked by 100 pA current injections. All drugs were bath applied and the data are shown as: mean ± SE (n=5-6). Asterisks denote significant difference. (*: p<0.05 and **: p<0.01).
DISCUSSION

The results presented here show that in neonatal rat ICG neurons activation of sigma-1 receptors reversibly inhibited $I_{K(DR)}$, $I_{K(Ca)}$ and $I_M$ through a pathway that involved neither a diffusible cytosolic second messenger nor a G-protein. Furthermore, activation of sigma receptors resulted in the depolarization of the neurons and depression of neuroexcitability. It has been shown that sigma-1 receptors are expressed in parasympathetic ICG neurons, but the cellular function of these receptors remained unknown. Whereas sigma-2 receptors in these cells couple to $Ca^{2+}$ channels, sigma-1 receptors modulate the function of various $K^+$ channels in these neurons. While the modulation of $Ca^{2+}$ channels in ICG neurons by neurotransmitters is well documented, less is known about the regulation of $K^+$ channels in these cells (Adams and Cuevas, 2004). Activation of M1 muscarinic receptors in neonatal rat ICG neurons has been shown to block $I_M$ in these cells (Cuevas et al., 1997), and activation of a PGE2 receptor inhibits a small-conductance $I_{K(Ca)}$ in adult guinea pig ICG neurons (Jelson et al., 2003). Sigma-1 receptor activation, however, inhibits multiple $K^+$ channels subtypes in ICG neurons. The modulation of $K^+$ channels by sigma receptors has been reported in mouse sympathetic neurons, frog melanotrophs, and mouse neurohypophysial nerve terminals (Kennedy and Henderson, 1990;
Soriani et al., 1998; Soriani et al., 1999a&amp;b; Wilke et al., 1999). The K⁺ channel types that have been shown to be modulated by sigma receptor activation include A-Type K⁺ channels (Iₐ), I₉(K(Ca)), I₉(K(DR)), and I₉(M), and multiple K⁺ channel types are frequently affected in individual cells (Soriani et al., 1999a, Wilke et al., 1999a, Soriani et al., 1999b). All of those K⁺ channel subtypes, except A-Type K⁺ channels which are not expressed in ICG neurons, were shown to be affected by sigma receptor activation in our study. While sigma receptors have been shown to block K⁺ channels in native cells, the sigma receptor subtype mediating these effects has not been definitively identified. The rank order potency for I₉ inhibition by sigma ligands reported here, haloperidol > (+)-pentazocine > ibogaine > DTG, suggests that the effect is mediated by sigma-1 receptor activation. In contrast, the rank order potency for sigma ligand inhibition of Ca²⁺ channels in ICG neurons is: haloperidol > ibogaine > (+)-pentazocine > DTG, which is consistent with a sigma-2 receptor mediated effect. The IC₅₀ values for the various sigma ligands tested here are in agreement with those reported in the literature for modulations of voltage-gated K⁺ channels. For example, in frog pituitary melanotrophs, (+)-pentazocine inhibited delayed outwardly rectifying K⁺ channels with an IC₅₀ of 37 µM, compared to the IC₅₀ of 42 µM reported here. Similarly, 100 µM (+)-SKF10047 blocked approximately 50% of the voltage-activated K⁺ currents recorded in neurohypophysial nerve terminals (Wilke et al., 1999a). Thus, sigma-1 receptors are likely to be responsible for inhibition of K⁺ channels in ICG neurons and in other native cells studied. The conclusion that
sigma receptors mediate the inhibition of $I_k$ by sigma ligands in ICG neurons is strengthened by the observation that metaphit, an irreversible, antagonist of sigma receptors, blocks the effects of DTG on $I_k$ in these cells. Considerable controversy exists as to the mechanisms by which sigma receptors modulate $K^+$ channels. In frog pituitary melanotrophs, cell dialysis with GTP-β-S and preincubation in cholera toxin were shown to inhibit sigma receptor effects on $I_A$ and $I_{K(DR)}$ (Soriani et al., 1999 a&b), suggesting that sigma receptors couple to the $K^+$ channels via a G protein. G proteins have also been implicated in both sigma receptor-mediated activation of phospholipase C and regulation of $Ca^{2+}$ release from intracellular stores (Morin-Surun et al., 1999; Hayashi et al., 2000). However, neither cell dialysis nor application of intracellular GTP-β-S blocked the inhibition of $I_k$ by sigma-1 receptors in ICG neurons. Thus, sigma receptors couple to $K^+$ channels in these cells via a membrane-delimited signal transduction cascade that does not involve a G protein. Our observation is in agreement with the direct protein-protein interaction between Sigma-1 receptors and $K^+$ channels shown by Jackson and colleagues (Lupardus et al., 2000; Aydar et al., 2002). The net effect of sigma receptor modulation of ion channels in ICG neurons at rest is depolarization of the membrane potential. We have previously shown that the $I_M$ contributes to the resting membrane potential of ICG neurons and that inhibition of this channel depolarizes the cells (Cuevas et al., 1997). Given that application of DTG blocks $I_M$, sigma receptor modulation of these channels likely accounts for the depolarizations reported here. Further support
for this conclusion comes from the fact that neither inhibition $I_{K(DR)}$ nor $I_{K(Ca)}$ has been linked to changes in the resting membrane potential of these neurons (Hogg et al., 1999; Xu and Adams, 1992; Jelson et al., 2003). Inhibition of voltage gated Ca$^{2+}$ channels by sigma-2 receptor activation is also unlikely to account for this depolarization since removal of extracellular Ca$^{2+}$ does not alter the resting membrane potential of these cells (DeHaven and Cuevas, 2004). The block of $I_K$ by sigma receptors in ICG neuron is associated with decreased AP firing, whereas previous studies have shown that sigma receptor block of $I_K$ enhances neuroexcitability (Soriani et al., 1998). Our laboratory has shown that inhibition of $I_M$ is associated with increased excitability of ICG neurons (Cuevas et al., 1997), and thus sigma receptor modulation of non-$I_M$ K$^+$ channels must be responsible for the decreased excitability observed. The depressed neuroexcitability reported here likely results from modulation of $I_{K(DR)}$ by sigma receptors. Verapamil has been shown to convert tonic and adapting ICG neurons into phasic neurons via a direct block of $I_{K(DR)}$ (Hogg et al., 1999). However, high concentrations of verapamil failed to abolish AP firing in ICG neurons, whereas high concentrations of sigma ligands completely blocked the genesis of APs in these cells. The concentrations of (+) pentazocine (50 µM) and ibogaine (200 µM) required to appreciably decrease AP firing and alter the AP configuration in our study suggest that the effects of sigma ligands on the active membrane properties of these cells are primarily mediated by sigma-1 receptors. However, inhibition of voltage-activated K$^+$ channels alone cannot
explain the complete block of AP firing at high concentrations of sigma ligands, and thus sigma receptors are likely to affect other channel types that regulate AP firing (e.g. voltage-gated sodium channels). The inhibition of \( I_{K(Ca)} \) by sigma receptors is also likely to contribute to changes in the active membrane properties of ICG neurons. Two types of sigma receptor-regulated BK currents were found in rat ICG neurons, one which exhibited rapid time-dependent inactivation, BKi, and a second which showed little or no inactivation, BKs. The presence of distinct subpopulations of BK channels with dissimilar inactivation kinetics has been reported in rat adrenal chromaffin cells and mouse neocortical pyramidal neurons and (Solaro et al., 1995; Sun et al., 2003). The inhibition of BK currents by sigma receptors would account, at least in part, for the increase in AP duration and decrease in AHP reported here, since inhibition of BK by 200 µM TEA produces similar effects on the APs of these cells (Franciolini et al., 2001). While inhibition of voltage-gated \( Ca^{2+} \) channels may contribute to the decrease in \( I_{K(Ca)} \) amplitude observed in our studies, it is unlikely to exclusively account for the block of BK channels observed here. Evidence for this conclusion comes from the fact that concentrations of \( Cd^{2+} \) (100 µM) that inhibit all \( Ca^{2+} \) channels in these neurons (Cuevas and Adams, 1997) fail to eliminate the AHP in these cells (Franciolini et al., 2001). Furthermore, in our study, 100 µM \( Cd^{2+} \) blocked < 5% of \( I_K \), whereas 500 µM TEA or \( Ca^{2+}\)-free extracellular solution blocked ~25% of \( I_K \) (data not shown). Taken together, these data suggest that sigma-1 receptors are likely having a direct effect on BK channels in
ICG neurons. However, sigma receptor modulation of other mechanisms of Ca\(^{2+}\) entry or homeostasis must also be examined to confirm a direct effect on \(I_{K(Ca)}\).

There has been considerable speculation about the role of sigma receptors in the cardiovascular system. While the endogenous ligand responsible for activating sigma receptors under physiological and pathophysiological conditions remains to be determined, there is data suggesting that some putative sigma receptor ligands may affect the heart and coronary vasculature. For example, pregnenolone, a putative sigma ligand (Maurice et al., 2001), increases heart rate and cardiac output in anesthetized dogs (Hogskilde et al., 1991). Such effects may in part be due to activation of sigma receptors on ICG neurons. Similarly, various drugs that act on sigma receptors, such as haloperidol, have significant effects on the heart (Monassier and Bousquet, 2002). Our observations raise the possibility that the cardiovascular effects of these compounds may be mediated by their modulation of the electrical activity of intrinsic cardiac neurons. In conclusion, stimulation of sigma-1 receptors inhibits multiple voltage-gated K\(^+\) channel subtypes and depresses excitability in ICG neurons. Thus, activation of sigma-1 receptors in these cells is likely to attenuate parasympathetic input to the heart and, consequently, affect cardiovascular function.
CHAPTER 4
SIGMA RECEPTORS ACTIVATION INHIBIT VOLTAGE-GATED SODIUM CHANNELS IN RAT ICG NEURONS

INTRODUCTION

Sigma receptors have been shown to modulate a variety of cell membrane ion channels, including voltage-gated K⁺ (Kennedy and Henderson, 1990; Soriani et al., 1998; Wilke et al., 1999; Zhang and Cuevas, 2005) and Ca²⁺ channels (Soriani et al., 1999b; Zhang and Cuevas, 2002). The effects of sigma receptors on voltage-gated Na⁺ channels, which play very important role in cellular functions of central and peripheral neurons, as well as muscle cells, have never been studied. We have shown that sigma receptors activation not only inhibits the action potential (AP) firing but also decreases the amplitude of AP overshoot (Zhang and Cuevas, 2005). Inhibition of K⁺ currents alone cannot explain these effects, which suggests other channels are involved and Na⁺ channels could be one of them.

Voltage-gated Na⁺ channels are responsible for the initiation and propagation of APs in both nerve and muscle cells (Hille 2001). Modulation of
Na\(^+\) channels will influence the threshold of AP generation, as well as the frequency of AP firing. So voltage-gated Na\(^+\) channels are primary targets for the antiepileptic drugs and are used for identification and development of new anticonvulsants (Ragsdale and Avoli, 1998; Chao and Alzheimer, 1995; Taylor and Narasimhan, 1997). In addition to the antiepileptic action of voltage-gated Na\(^+\) channels blockers, some other Na\(^+\) channel blockers are also used in the treatments of neuropathic pain (Deffois et al., 1996), arrhythmia (Catterall 1981), head trauma and stroke (Baumgold and Spector 1987; Meldrum 1994; Taylor and Narasimhan 1997).

Voltage-gated Na\(^+\) channels also play important roles in cardiac functions and the modulators of voltage-gated Na\(^+\) channels have been shown enormous potential for therapeutic advances in the treatment of heart failure (Doggrell et al., 1994; Doggrell and Liang, 1998). Mammalian intracardiac ganglia (ICG) are thought to exert local regulation over cardiac function by integrating information from efferent and afferent pathways of both parasympathetic and sympathetic origin (Moravec and Moravec, 1987) and the ICG neurons express similar types of Na\(^+\) channels as CNS. So investigating the effects of sigma receptors on voltage-gated Na\(^+\) channels in ICG neurons will help understanding the cellular effects of these receptors, as well as providing more information on the mechanisms of the effects of these receptors, especially in the cardiovascular system.
Experiments were undertaken to determine the effects of sigma receptors on voltage-gated Na\(^+\) channels and the AP firing latency of ICG neurons from neonatal rats. The sigma ligands used here include: DTG, ibogaine, (+) pentazocine and BD1047. These compounds have been selected because they are structurally dissimilar, which will help to rule out other nonspecific effects, for example, direct binding to Na\(^+\) channels or through other receptors. Also these compounds have different selectivities for two subtypes of sigma receptors, which will help to differentiate the receptor subtype that mediates the modulations of Na\(^+\) channels. Sigma receptor ligands were shown to have inhibitory effects on the current mediated by voltage-gated Na\(^+\) channels (I\(_{Na}\)) and shifted the steady-state inactivation of Na\(^+\) channels to more negative potentials. Activation of sigma receptors also increased the latency of AP generation produced by depolarizing current ramps.
METHODS

Preparation of Cell Culture

The isolation and culture of neurons from neonatal rat ICG ganglia has been described previously (Cuevas and Adams, 1994). Dissociated neurons were plated onto poly-L-lysine coated glass coverslips and incubated at 37°C for 36-72 hr under a 95% air, 5% CO₂ environment prior to the experiments.

Electrical Recordings

Electrophysiological recording methods used were similar to those previously described (Cuevas et al., 1997). Voltage-activated Na⁺ channel currents in ICG neurons were studied by using the whole cell patch-clamp technique under voltage-clamp mode. The conventional (dialyzing) whole cell recording technique was used in the experiments studying current-voltage relationship, while the amphotericin B perforated-patch method was used in other experiments. For perforated-patch experiments, a stock solution of amphotericin B (60mg/ml) in dimethyl sulfoxide (DMSO) was prepared and diluted in pipette solution immediately prior to use to yield a final concentration of 198 µg/ml amphotericin B in 0.33% DMSO. Final patch pipette resistance was 1.0-1.3 MΩ
to permit maximal electrical access under the present recording configuration. Currents through depolarization-activated Na\(^+\) channels were elicited by step depolarizations from \(-90\) mV to more positive potentials (-50 to +100 mV). For experiments studying the effects of sigma receptor on steady state inactivation of Na\(^+\) channel, cells were prepulsed to different voltage (-120 mV to 0 mV) from holding potential of -90 mV and then depolarized to 0 mV to activate the channel. AP was evoked by current ramp (0 to 200 nA, 400 ms) and the latency of AP generation was determined as the time interval from onset of the current ramp to the point at which the rising phase of the AP crossed 0 mV. Membrane voltages and currents were amplified using an Axopatch 200B patch-clamp amplifier (Axon Instruments, Union City, CA), filtered at 5 kHz (-3 dB; 4-pole Bessel filter), and digitized at 20 kHz (Digidata 1200 B).

**Solutions and Reagents**

The bath solution used in Na\(^+\) channel experiments was a physiological saline solution (PSS) composed of (in mM): 70 NaCl, 70 tetraethylammonium chloride (TEA), 5 BaCl\(_2\), 1.2 MgCl\(_2\), 7.7 glucose, 0.1 cadmium chloride (CdCl\(_2\)), and 10 HEPES (pH to 7.2 with NaOH). Barium and TEA were used to block voltage-gated K\(^+\) channels and cadmium was used to block voltage-gated Ca\(^{2+}\) channels. Pipette solution used for Na\(^+\) channel experiments contained (in mM): 130 CsCl, 10 NaCl, and 10 HEPES (adjust to pH 7.2 with CsOH). The bath solution for AP experiments was a physiological saline solution (PSS) containing (in mM) 140
NaCl, 1.2 MgCl₂, 3 KCl, 2.5 CaCl₂, 7.7 glucose and 10 HEPES, pH to 7.2 with NaOH. Pipette solution used for AP experiments contained (in mM): 75 K₂SO₄, 55 KCl, 5 MgSO₄, and 10 HEPES (adjust to pH 7.2 with N-methyl-d-glucamine). All chemical reagents used were of analytical grade. Ibogaine hydrochloride; (+)-pentazocine; 1,3-Di-O-tolylguanidine (DTG); BD1047, and tetraethylammonium chloride (TEA) were purchased from Sigma Chemical (St. Louis, MO).

**Data Analysis**

Analyses of these data were conducted using the SigmaPlot 2000 program (SPSS Science, Chicago, IL). Data points represent means ± SEM. Statistical difference was determined using paired t-test, and was considered significant if \( p < 0.05 \).
RESULTS

Sigma Receptor Activation Inhibits Voltage-Gated Na\(^+\) Channel Currents.

Sigma receptors have been shown to inhibit AP firing and decrease AP overshoot. One of the mechanisms by which sigma receptors may modulate AP firing is through inhibition of voltage-gated Na\(^+\) channels. Voltage-gated Na\(^+\) channel currents were isolated by inhibiting Ca\(^{2+}\) currents with extracellular Cd\(^{2+}\), and K\(^+\) currents with extracellular TEA and intracellular Cs\(^+\). Figure 1A shows a family of depolarization-activated Na\(^+\) currents recorded from a single ICG neuron in the absence and presence of 100 µM ibogaine. Under control conditions, I\(_{Na}\) was activated at approximately -30mV and the I-V relation was maximal at -10 mV, reversing at approximately +70 mV. The voltage dependence and kinetics of the currents observed are consistent with voltage-gated Na\(^+\) channel currents characterized in ICG neurons (Xu and Adams, 1992). Bath application of 100 µM of ibogaine depressed the peak I\(_{Na}\) at potentials positive to -20 mV and shifted the reversing potential from +70 mV to +50 mV. Sigma-1 receptor agonist, pentazocine, at 100 µM concentration, had similar effects (data not shown).
Concentration-dependent Inhibition of $I_{Na}$ by Sigma Ligands

Further pharmacological experiments were undertaken to confirm that sigma receptor activation mediated the inhibitory effects of $I_{Na}$ of sigma ligands and to identify the sigma receptor subtype mediating the observed effects. Figure 2A shows representative currents recorded from single ICG neuron in the absence (control) and presence of (+)-pentazocine (PTZ), at the indicated concentration. A plot of the mean peak $I_{Na}$ as a function of drug concentration for several sigma ligands is shown in Figure 2B. All of the sigma ligands tested depressed peak $I_{Na}$ in a concentration-dependent manner. Fits of the data using the Hill equation gave half-maximal inhibitory concentration ($IC_{50}$) values for DTG, ibogaine and pentazocine of 30.0 µM, 30.9 µM and 50.8 µM, respectively, and Hill coefficients of ~1.0 for all drugs. Maximal inhibition of $I_{Na}$ was more than 90%.

BD 1047 was claimed as sigma-1 receptor antagonist (Matsumoto et al., 1995), however, it was also reported as partial agonist of sigma receptors (Zambon et al., 1997). In our system, we have shown that BD1047 had similar effects as sigma receptors agonist as to block $K^+$ channel currents (data not shown). Effects of BD1047 on voltage-gated $Na^+$ channels are shown in Figure 3. Figure 3A shows representative currents recorded from single neuron in the absence and presence of different concentrations of BD1047, as indicated. Similar as sigma receptor agonists, BD1047 dose-dependently depressed $I_{Na}$ and
the IC$_{50}$ value for BD1047 was 54.8 µM, which suggests it is either a partial agonist of the sigma receptors or has direct effects on the Na$^+$ channels.

The effect of sigma ligands on I$_{Na}$ amplitude was reversible on wash out. Figure 4A shows a family of Na$^+$ currents evoked by step depolarization from a single neuron in the absence (control), presence (+DTG), and following wash out of the sigma ligand, DTG (wash). Following inhibition of I$_{Na}$, the current recovered gradually and similar reversal of inhibition was observed for all sigma ligands tested here (data not shown).

**Effects of Sigma Receptors Activation on Voltage-gated Na$^+$ Channel**

**Inactivation**

The effects of DTG (30 µM) on steady-state inactivation of Na$^+$ channels in rat ICG neurons was studied using a double pulse protocol. Neurons were initially held at -90 mV, and 50 ms prepulses from -120 to 0 mV were applied in 10 mV increments prior to a voltage step to 0mV (50 ms) to activate the available Na$^+$ channels. A plot of the relative peak current amplitude (I/I$_{max}$) as a function of prepulse voltage is shown in Figure 5. The steady-state inactivation of I$_{Na}$ exhibited a sigmoidal dependence on voltage and was best fit with a single Boltzmann function according to the equation

$$I_{Na} = I_{Na(max)}/\{1 + \exp[(V-V_h)/k]\}$$

In the absence of DTG, a fit of the mean relative I-V relationship exhibited half-maximal steady-state inactivation ($V_h$) at -50 mV and had a slope parameter (k)
of 7.5. While in the presence of DTG, a fit of mean relative I-V relationship exhibited $V_h$ at -64 mV and $k$ of 15.

Effects of Sigma Receptors on the Latency of AP Generation

One of the important functions of voltage-gated Na$^+$ channel is the initiation and propagation of AP. Effects of sigma ligands on the latency of AP generation were tested during a 400 ms depolarizing current ramp by using the perforated patch whole cell recording configuration under current clamp mode. Figure 6A shows a family of AP in response to depolarizing current ramp in the absence and presence of 30 µM of DTG. The latency of AP determined as the time interval from onset of the current ramp to the point at which the rising phase of the AP crossed 0 mV (Parson et al., 2002) was significantly increased by DTG (~35%). DTG also decreased the AP firing, as well as altered AP configuration as reported before (Zhang and Cuevas, 2005). The effects of DTG were mimicked by 50 µM of ibogaine, (+)-pentazocine and BD1047, which increased the latency of AP firing by 15%, 20% and 15% respectively and the effects of all sigma ligands are reversible after wash out of the drug (data not shown).
Figure 4.1 Inhibition of Na\(^+\) channel currents in rat ICG neurons by the sigma receptor agonist, DTG. A: Whole-cell, Na\(^+\) currents evoked by depolarizing test pulses (-50 to +100 mV) from a holding potential of -90 mV in the absence (Control) or presence of 100 µM ibogaine (Ibogaine). B: Whole cell current-voltage relation obtained in the absence and presence of 100 µM DTG. C: Whole cell current-voltage relation obtained in the absence and presence of 100 µM ibogaine. Data points represent means ± SE for 3-4 cells..
Figure 4.2 Dose-dependant inhibitions of depolarization-activated Na\(^+\) channel currents by sigma receptors agonists in rat ICG neurons. A: Whole cell Na\(^+\) currents evoked from a ICG neuron by step depolarizations to -10 mV from a holding potential of -90 mV in the absence (Control) and presence of (+)-pentazocine (PTZ) at the indicated concentration. B: Peak whole cell \(I_{Na}\) amplitude, evoked by depolarizing to -10 mV from -90 mV, normalized to control and plotted as a function of sigma receptors agonists, (+)-pentazocine (PTZ), ibogaine (IBO) and DTG, concentrations. Data points represent mean ± SE for 4-5 neurons. The curves represent best fit to the data using the Hill equation. Half-maximal inhibition was 50.8 µM for (+)-pentazocine, 30.9 µM for ibogaine and 30.0 µM for DTG and the hill coefficient was 1.0 for all compounds.
A

PTZ

300 µM

100 µM

30 µM

10 µM

3 µM

Control

0.5 nA

2 ms

B

\[ \frac{I}{I_{\text{max}}} \]

Concentration (µM)

PTZ

IBO

DTG

102
Figure 4.3 Dose-dependant inhibitions of depolarization-activated Na\(^+\) channel currents by sigma receptors antagonist or partial agonist, BD1047 in rat ICG neurons. A: Whole cell Na\(^+\) currents evoked from a ICG neuron by step depolarizations to -10 mV from a holding potential of -90 mV in the absence (Control) and presence of BD1047 at the indicated concentration. B: Peak whole cell I\(_{Na}\) amplitude, evoked by depolarizing to -10 mV from -90 mV, normalized to control and plotted as a function of concentration of BD1047. Data points represent mean ± SE for 4 neurons. The curves represent best fit to the data using the Hill equation. Half-maximal inhibition was 54.8 µM and the hill coefficient was 1.0.
Figure 4.4 Reversibility of DTG-induced block of voltage-gated Na⁺ channels. A: Family of depolarization-activated Na⁺ channel currents recorded from a single ICG neurons in the absence (Control) and presence of bath-applied 300 µM DTG (+DTG), and following wash out of drug (Wash). B: Peak Na⁺ channel current amplitude before and during application of 300 µM DTG and after removal of DTG for the cell in A. Values are plotted as a function of time. Gap represents period during which lower concentrations of DTG were applied, with 300 µM DTG application commencing at t=20 min.
Figure 4.5 Sigma receptor modulation of steady-state inactivation of Na⁺ channels in rat ICG neurons. Relative Na⁺ current amplitude as a function of prepulse amplitude in the absence (control) and presence of 30 μM DTG (DTG). Data points represent means ± SE for 7 neurons. Solid lines represent best fit to the data with a single component Boltzmann distribution.
Figure 4.6 Sigma receptors ligands increased the latency of AP generation in rat ICG neurons.  A: Example recording of AP generated by a 400 ms depolarizing current ramp from one neuron prior to (solid line) and during DTG (30 µM) (dash line) treatment.  B: Bar graphs summarized the AP latency for multiple neurons before (Control) and after application of sigma receptors ligands as indicated. The concentrations for sigma ligands are: 30 µM of DTG, and 50 µM for pentazocine (PTZ), ibogaine (IBO) and BD 1047.  Asterisks indicated a significant difference between conditions using a paired $t$-test. (*: $p<0.05$, **: $p<0.01$)
DISCUSSION

The results presented in this chapter provide the first evidence of modulation of voltage-gated Na\(^+\) channels by sigma receptor ligands. Sigma ligands dose-dependently inhibited $I_{\text{Na}}$ in a reversible manner and shifted the steady-state inactivation to more negative potentials. Furthermore, activation of sigma receptors increased the latency of AP generation.

It has been shown in ICG neurons that sigma receptor ligands inhibit voltage-gated K\(^+\) and Ca\(^{2+}\) channels through sigma-1 and sigma-2 receptor activation, respectively. Previous experiments also showed that sigma receptor ligands, at high concentration, abolish AP firing in these neurons. Although the inhibition of K\(^+\) and Ca\(^{2+}\) channel currents contribute to the effects on AP firing, it can not explain the significant decrease in the amplitude of the first AP overshoot and complete block of AP firing at higher concentrations. Another possible target of modulation by sigma receptors is voltage-gated Na\(^+\) channels, which are responsible for the generation and conduction of AP in neurons. Like rat sympathetic neurons, voltage-gated Na\(^+\) channels in rat intracardiac neurons are TTX sensitive, with complete blockade at 300 nM TTX (Xu and Adams, 1992). The data presented here show that sigma ligands inhibit the voltage-gated Na\(^+\) channels.

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channels in a voltage-dependent manner. A shift in the reversal potential of Na\(^+\) channel to less positive potentials was also observed. One reason for this shift is incomplete blockade of outward K\(^+\) currents by TEA (extracellular) and Cs\(^{2+}\) (intracellular). Compared with Na\(^+\) channel inhibition, both DTG and ibogaine have less potency for blocking K\(^+\) channels and at 100 µm of concentration, they only block about 20% of total outward K\(^+\) currents (Zhang and Cuevas, 2005). So the outward current seen at +70 mV in the presence of DTG and ibogaine is an artifact due to K\(^+\) current contamination. In contrast, pentazocine, which has similar potency of blocking Na\(^+\) and K\(^+\) channels, does not shift the reversal potential of Na\(^+\) channels (data not shown). The similar IC\(_{50}\) values of blocking Na\(^+\) and Ca\(^{2+}\) channels of selective sigma-1 and sigma-2 receptor ligands, (+) pentazocine (51 µM compared with 61 µM) and ibogaine (31 µM compared with 31 µM) suggest that like blocking of voltage-gated Ca\(^{2+}\) channels, sigma ligands inhibit the voltage-gated Na\(^+\) channels also through sigma-2 receptor activation. However this cannot explain the high potency of DTG which has equal affinity for sigma-1 and sigma-2 receptors of blocking Na\(^+\) channels (IC\(_{50}\) = 30 µM), compared with Ca\(^{2+}\) (133 µM) and K\(^+\) channels (341 µM) inhibition. One possibility is that DTG has direct effect on voltage-gated Na\(^+\) channels. This is consistent with our observations that the inhibitory effect of DTG on Na\(^+\) channels is not inhibited by the sigma receptor antagonist, metaphit (data not shown) which blocked the inhibitory effects of DTG on voltage-gated Ca\(^{2+}\) and K\(^+\) channels (Zhang and Cuevas, 2002; Zhang and Cuevas, 2005).
Voltage-gated Na\(^+\) channels are known to undergo two distinct inactivation processes, a fast and a slow inactivation process, which occur within a few milliseconds and a few seconds, respectively. In ICG neurons, the fast inactivation process, which is mainly involved in the current shape and the \(I_{\text{Na}}\text{max}\) (Ruff et al., 1996), was determined by double-pulse protocol in our experiments. Our results show that DTG shift the steady-state inactivation to more negative potentials. This effect is also seen as other sigma ligands (data not shown). Thus the shifting of the steady-state inactivation of Na\(^+\) channels to more negative potentials attribute at least in part to the inhibition of \(I_{\text{Na}}\) by sigma receptors activation.

AP generation is determined by complex interaction of the voltage- and time-dependent properties of primarily Na\(^+\) and K\(^+\) selective ion channels (Hille, 2001). In different types of neurons, the contribution of the generation of AP firing by different ion channels is different. For example, in guinea-pig sympathetic neurons (Locknar et al., 2004) and mudpuppy parasympathetic neurons (Parsons et al., 2002; Barstow et al., 2004), the outward current which is activated by Ca\(^{2+}\)-induced calcium release (CICR) at membrane voltages approaching the threshold for AP initiation regulates AP generation, and the disruption of CICR by thapsigargin, ryanodine and Cd\(^{2+}\) decreased the latency of AP generation in these neurons. Parson et al proposed the mechanism of this effect that when membrane depolarization approach the threshold for AP generation, Ca\(^{2+}\) influx through voltage-dependent Ca\(^{2+}\) channels initiates CICR.
The Ca$^{2+}$ released from internal stores raises the intracellular Ca$^{2+}$ concentrations in domains between the ER and the plasma membrane to levels high enough (≥40 µM) to activate membrane outward currents that decrease the effectiveness of the depolarizing current (Parsons et al., 2002). Also in these neurons, big Ca$^{2+}$-activated K$^+$ channels (BK), which are at least in part activated by CICR, regulate the generation of AP as well, and blocking the BK by iberiotoxin or 500 µM of TEA decrease the latency of AP generation (Parsons et al., 2002; Locknar et al., 2004). The common property of these two types of neurons is that none of them express M-currents or express M-currents in only a small subpopulation of cells. While in mammalian intracardiac neurons that express M-current, CICR inhibition did not affect the latency of AP generation (K.L. Barstow & R.L. Parson, unpublished observations; Dehaven and Cuevas, 2004). Therefore in rat ICG neurons, the initiation of AP firing is determined by voltage-dependent Na$^+$ channels and M-channels. Since blocking M-current, which opposes depolarizing stimuli, would decrease the latency of AP firing, the increased latency of AP firing by sigma receptors ligands must be through the inhibition of voltage-gated Na$^+$ channels. At normal resting membrane potential, in the presence of sigma ligands, a smaller portion of Na$^+$ channels is available for activation by excitatory depolarization.

Sigma receptor ligands have been shown to exert neuroprotective effects both in vivo and in vitro (Lobner and Lipton 1990; Poignet et al., 1992; Takahashi et al., 1996; Lockhart et al., 1995; Kimura et al., 1998 Goyagi et al., 2001;). but
the mechanisms of this effect are not well known. Disturbance in intracellular ion homeostasis induced by ischemia is implicated in the initiation and progression of neuronal injury. It has been proposed that the mechanism of neuronal toxicity during oxygen/glucose deprivation is the increase of glutamate efflux into the intracellular space by overactivating glutamate receptors, calcium channels and sodium channels (Kimura et al., 1998). The neuroprotective effects of the Na$^+$ channel blocker TTX have been demonstrated both in vivo and in vitro (Prenen et al., 1998; Boening et al., 1989; Yamasaki et al., 1991; Weber and Taylor, 1994; Xie et al., 1994). Even though there is no clear evidence shown that sigma receptors modulate voltage-gated Na$^+$ channels in central nervous system, in rat hippocampus, sigma ligands have been shown to suppress CA1 neuronal activity by increasing the threshold of action potential firing (Ishihara and Sasa, 2002). Inhibition of voltage-gated Na$^+$ channels may mediate this effect and these effects on the hippocampal neurons may contribute to the neuroprotective effects of sigma ligands.

The present data indicate that sigma ligands have inhibitory effects on voltage-gated Na$^+$ channels and this effect causes the threshold of AP generation to increase, as well as the amplitude of AP overshoot to decrease. In parasympathetic neurons, the inhibition of the AP firing in response to depolarizing stimuli may be expected to reduce the bradycardia associated with vagal nerve stimulation in vivo, leading to tachycardia. One of the common side effects of local anesthetics, such as ketamine, is increasing the heart rate. It has
been shown that ketamine inhibits Na\textsuperscript{+} but not K\textsuperscript{+} channels in brainstem parasympathetic cardiac neurons and this action may mediate the decrease in parasympathetic cardiac activity and increase the heart rate as seen in surgical patients (Irnaten et al., 2002). Thus, our data suggest that the tachycardia effect caused by sigma ligands (+)-SKF-10,047, DTG and (+)-3-[3-hydroxyphenyl]-N-(1-propyl)piperidine (Wu and Martin, 1989; Lishmanov et al., 2000b) may be mediated in part by the inhibition of voltage-gated Na\textsuperscript{+} channels in rat ICG neurons via the activation of sigma receptors. Further, these results on peripheral neurons have broad implications in CNS and stroke.
CHAPTER 5
SUMMARY

The effects of sigma receptors activation on voltage-gated ion channels, including \( \text{Ca}^{2+} \), \( \text{K}^{+} \) and \( \text{Na}^{+} \) channel, were investigated in neonatal rat autonomic neurons. The results presented here provide the first evidence that sigma-1 receptor transcripts are expressed in superior cervical ganglia (SCG) and intracardiac ganglia (ICG) neurons, which mediate sympathetic and parasympathetic input to the heart, respectively. Furthermore, activation of sigma-1 or sigma-2 receptors modulates voltage-gated ion channels and decreases the excitability of these cells.

The sigma-1 receptor has been cloned in several species and these receptors are expressed in brain and various peripheral tissues and organs (Walker et al., 1990). By using single cell RT-PCR techniques, we found that sigma-1 receptor transcripts are present specifically in both SCG and ICG neurons, with only the conventional isoform expressed in these cells.

The effects of sigma ligands, (+)-pentazocine (selective sigma-1 receptor agonist), ibogaine (selective sigma-2 receptor agonist), DTG (sigma receptor agonist with equal affinity of sigma-1 and sigma-2) and haloperidol (sigma-2
receptor agonist), on voltage-gated Ca\textsuperscript{2+} channel currents were studied by using whole cell patch-clamp technique under voltage-clamp mode in SCG and ICG neurons. Our data showed that sigma receptor agonists inhibit Ca\textsuperscript{2+} channel currents in a dose-dependent manner and reversibly, with maximal inhibition of over 95% of the peak current amplitude, indicating that all Ca\textsuperscript{2+} channel types which include N-, L-, P/Q, and R-type Ca\textsuperscript{2+} channels are affected. In addition to affecting a broad population of Ca\textsuperscript{2+} channel types, sigma ligands have significant effects on the kinetic properties of Ca\textsuperscript{2+} channels. In the presence of sigma ligands, the steady-state inactivation of Ca\textsuperscript{2+} channels shifted to more negative potentials. In control cells, the inactivation curve was best fit by a one-component Boltzmann distribution, but by a two-component Boltzmann distribution in the presence of sigma ligands. This observation suggests that sigma receptors may not equally modulate steady-state inactivation in all Ca\textsuperscript{2+} channel subtypes found in these cells. Sigma receptors activation also altered the voltage-dependence of activation of Ca\textsuperscript{2+} in a manner distinct from other known Ca\textsuperscript{2+} channel inhibitors. In the presence of sigma receptor ligands, the Ca\textsuperscript{2+} activation curve was shifted toward more negative potentials. In contrast, other inhibitors of Ca\textsuperscript{2+} channels, such as muscarinic and \(\alpha\)-adrenergic agonists, shift the activation curve to more positive potentials (Cuevas and Admas 1997; Xu and Admas 1993). Thus, stronger depolarizations are required in the presence of these agents to activate the same number of Ca\textsuperscript{2+} channels. The structure dissimilarity of sigma ligands and the lack of any significant homology
between Ca\(^{2+}\) channels and the cloned sigma-1 receptor suggest that the effects of sigma ligands are likely mediated by a sigma receptor and not a direct effect on the Ca\(^{2+}\) channel. This is also approved by the observation that the sigma receptor antagonist, metaphit that specifically acetylate sigma receptors and block ligand binding (Bluth et al., 1989), blocked the DTG-mediated attenuation of Ca\(^{2+}\) channels. The half-maximal inhibitory concentration (IC\(_{50}\)) for haloperidol, ibogaine, (+)-pentazocine and DTG were 6, 31, 61 and 133 µM, respectively. The rank order potency of haloperidol > ibogaine > (+)-pentazocine > DTG is consistent with the effects on Ca\(^{2+}\) channels being mediated by a sigma-2 receptor. The primary mechanism by which other known modulators of Ca\(^{2+}\) in autonomic neurons depress Ca\(^{2+}\) current is via the activation of pertussis toxin-sensitive G protein (Cuevas and Adams, 1997). However, no such G-protein appears to be implicated in the signal transduction cascade coupling sigma receptors and Ca\(^{2+}\) channels in these cells, since intracellular dialysis with GDP-β-S failed to inhibit the effects of sigma ligands. Furthermore, the inability of cell dialysis to block the effects of sigma receptors suggests that a diffusible cytosolic second messenger is likely not involved.

Sigma receptors have been shown to modulate K\(^{+}\) channels in various cell types. Mammalian ICG neurons exhibit currents mediated by delayed outwardly rectifying K\(^{+}\) channels (I\(_{K(DR)}\)) (Xi-Moy and Dun, 1995; Hogg et al., 1999) and express various subunits that contribute to these channels, including KCNA5 and KCNA6 (Cuevas, unpublished observation). In neonatal rat ICG neurons, effects
of sigma receptor activation on voltage-dependent K$^+$ channels were studied by whole cell patch-clamp techniques under voltage-clamp mode. Sigma receptor activation inhibits multiple K$^+$ channel subtypes in ICG neurons, which include $K_{(DR)}$, large conductance Ca$^{2+}$-activated K (BK), and M channels, with maximal inhibition > 80%. The inhibition on $I_{K(DR)}$ is dose-dependent and IC$_{50}$ values for (+)-pentazocine, ibogaine and DTG are 76.4, 218 and 341 µM, respectively. The rank order of potency of (+)-pentazocine > ibogaine > DTG suggests that the effect is mediated by sigma-1 receptor activation. The IC$_{50}$ values for the various sigma ligands tested here are in agreement with those reported in the literature for modulation of voltage-activated K$^+$ channels. The inhibitory effect of $I_K$ of DTG is blocked by pretreatment with metaphit, an irreversible sigma receptor antagonist, which strengthens the observation that sigma receptors mediate the effects of DTG on $I_K$ in these cells. To determine whether sigma receptor-mediated inhibition of K$^+$ channels in ICG neurons is dependent on G-protein activation, neurons are dialyzed with pipette solution containing either 100 µM GTP or 100 µM GDP-β-S. Intracellular application of GDP-β-S fails to block sigma-1 receptor-mediated inhibition of $I_{K(DR)}$. Thus neither a diffusible cytosolic second messenger nor a G-protein couples sigma-1 receptors to $I_{K(DR)}$, which is in agreement with the direct protein-protein interaction between sigma-1 receptors and K$^+$ channels shown by Jackson and colleagues (Lupardus et al., 2000; Aydar et al., 2002). Application of sigma ligands depolarizes cell membrane of ICG neurons at rest and this effect is thought through the inhibition
of M-current which contributes to the resting membrane potential of ICG neurons and that inhibition of this channel depolarizes the cells (Cuevas et al., 1997). In presence of sigma ligands, the number of action potentials (AP) fired by ICG neurons in response to depolarizing current pulses is decreased and depressed neuroexcitability likely results from modulation of $I_{K(DR)}$ by sigma receptors. In addition to decreased AP firing, sigma receptor activation also alters the AP configuration by decreasing both the AP overshoot and afterhyperpolarization (AHP) and by slowing both the rate of depolarization. These latter effects result in the increase in AP duration. The inhibition of BK currents by sigma ligands would account, at least in part, for the increase in AP duration and decrease in AHPs.

In ICG neurons, the inhibition of voltage-gated Ca$^{2+}$ and K$^+$ channels cannot explain the significant decrease in the amplitude of the first AP overshoot and complete block of the firing at higher concentration of sigma ligands. Effects of sigma receptor activation on voltage-gated Na$^+$ channels were investigated in these neurons. The voltage-activated Na$^+$ channel in ICG neurons is TTX-sensitive, with complete blockade at 300 nM TTX. Bath application of sigma ligands inhibits $I_{Na}$ in a reversible dose-dependent manner. Based on the fact that the potency (IC$_{50}$ values) of blocking Na$^+$ channels is similar as Ca$^{2+}$ channels inhibition by selective sigma-1 and sigma-2 receptor ligands, (+)pentazocine (51 µM compared with 61 µM) and ibogaine (31 µM compared with 31 µM) (Table 5.1), the inhibition of Na$^+$ channels by sigma ligands is also
through sigma-2 receptor activation. The fast inactivation of Na\(^+\), which was
determined by double-pulse protocol, shifts to more negative potentials in the
presence of sigma ligand, DTG. Since the fast inactivation of Na\(^+\) channel is
mainly involved in the current and the peak amplitude of I\(_{\text{Na}}\), the shifting of the
steady-state inactivation of Na\(^+\) to more negative potential attribute at least in
part to the inhibition of I\(_{\text{Na}}\) by sigma receptors activation. One of the effects that
results from the inhibition of voltage-gated Na\(^+\) channels by sigma receptors
activation is an increase in the threshold of AP generation in response to a 400 ms
current ramp. Based on our and other group findings, we believe that in rat ICG
neurons, the initiation of AP firing in response to current ramp is determined by
voltage-gated Na\(^+\) channels and M-channels. Since blocking M-channels, which
opposes depolarizing stimuli, would decrease the threshold of AP firing, the
increase of threshold (latency) of AP firing is likely through the inhibition of Na\(^+\)
channels.

The present data show that sigma-2 receptors modulate high-voltage-
gated Ca\(^{2+}\) channels in both neonatal rat SCG and ICG neurons. In rat ICG
neurons, sigma ligands have inhibitory effects on various types of K\(^+\) channels,
as well as voltage-gated Na\(^+\) channels, mediated by sigma-1 receptor and sigma-
2 receptor activation, respectively. The net effect of sigma receptor modulation
of ion channels in ICG neurons is decreased neuroexcitability in these neurons
suggesting that the activation of sigma receptors in ICG neurons will likely to
attenuate parasympathetic input to the heart, and consequently, will affect cardiovascular function.

Table 5.1 IC$_{50}$ values of selective sigma-1 receptor agonist, (+)-pentazocine and sigma-2 receptor agonist, ibogaine on voltage-gated Ca$^{2+}$, K$^+$ and Na$^+$ channels.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Ca$^{2+}$ channel</th>
<th>K$^+$ channel</th>
<th>Na$^+$ channel</th>
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</thead>
<tbody>
<tr>
<td>(+)-Pentazocine</td>
<td>61 µM</td>
<td>76 µM</td>
<td>51 µM</td>
</tr>
<tr>
<td>Ibogaine</td>
<td>31 µM</td>
<td>218 µM</td>
<td>31 µM</td>
</tr>
</tbody>
</table>
CONCLUSION

Since sigma receptors were first identified in the central nervous system, they have been surrounded with a considerable amount of controversy and confusion in their physiological functions. The distribution of these receptors appears ubiquitous, and besides the CNS, they have been shown to be present in numerous peripheral tissues. Furthermore, sigma receptors are found to be a distinct pharmacological entity distinguished by their unusually promiscuous ability to bind a wide variety of drugs and their unique distribution in subcellular fractions. While sigma receptors have been implicated in a variety of physiological and pathological conditions, the cellular function of these receptors remains poorly understood.

Several studies have shown that sigma receptors may be involved in the regulation of cardiovascular function. Cardiac performance is continuously modulated by both sympathetic and parasympathetic branches of the autonomic nervous system. With respect to neural control of the heart, rat superior cervical ganglia (SCG) contain sympathetic efferent postganglionic neurons, which innervate various regions of the heart, and intracardiac ganglia (ICG) form the principal final common pathway for autonomic modulation of regional cardiac
function (Armour, 1994). Investigating the effects of sigma receptor activation on these neurons will help us understand the function of these receptors in the cardiovascular system. Results reported here using structurally dissimilar sigma ligands show that sigma receptor activation inhibits multiple voltage-gated ion channels in autonomic neurons. The overall effect of blocking these channels is an inhibition of neuroexcitability in these cells. These data indicate that activation of sigma receptors may block signal transduction through autonomic ganglia and onto the heart. Thus the deleterious cardiac side effects induced by recreational drugs (e.g. PCP and cocaine) or clinical drugs (e.g. antipsychotic and antidepressants), which have high affinity with sigma receptors, may be due to their interaction with sigma receptors in autonomic neurons. Also the effects of sigma receptors on these autonomic neurons suggest that sigma receptors may mediate the cardiovascular effects of progesterone, a putative for the endogenous sigma ligand.

The data reported here will not only serve to increase our understanding of sigma receptors in the heart, but also on the cellular function of these receptors in other tissues and organs. For example, both subtypes of sigma receptors have been found highly expressed in tumor cell lines from various human cancer tissues, and some studies demonstrate that sigma ligands inhibited the proliferation of tumor cells in vitro (Brent and Pang, 1995; Moody et al., 2000; Crawford and Bowen, 2002). The mechanism(s) underlying the inhibitory effect of sigma drugs on tumor cell proliferation has not fully been
discerned. Ion channels are expressed in several cancer cell lines and play an important role in metastasis (Fraser et al., 2003; Wonderlin et al., 1996; Wilke et al., 1999). The observations that sigma receptors are overexpressed in tumor cells and modulate various voltage-gated ion channels as seen in this report provide more evidence to support the potential of sigma ligands as anticancer drugs. In CNS, sigma ligands have been shown to exert neuroprotective effects both in vivo and in vitro (Lobner and Lipton 1990; Poignet et al., 1992; Takahashi et al., 1996; Lockhart et al., 1995; Kimura et al., 1998 Goyagi et al., 2001;). but the mechanisms of this effect are not well known. The modulations of ion channels by sigma receptors may mediate, at least in part, the neuroprotective effects of sigma ligands during ischemia.

The most interesting unresolved questions from the sigma receptors research are the mechanism(s) of signal transduction. Based on the results seen in the study of the effect of sigma-1 receptor on K+ channels, Jackson and colleagues have elucidated a novel mechanism of cellular signal transduction. In this model, they proposed that in response to excellular signals, sigma receptors are capable of modulating ion channels function by interacting either with channel proteins or some very closely associated membrane constituent (Aydar et al., 2002). This behavior of sigma receptors can be explained by ‘three-state allosteric’ mechanism (Leff et al., 1997). The model of protein-protein interaction could be used to explain the results we show here. First, our data show that neither a diffusible cytosolic second messenger nor a G-protein is involved in the
modulations of ion channels by sigma receptor ligands. Second, some of the sigma receptor antagonist, such as, BD1063 and BD1047, act as sigma receptor agonist in the modulation of ion channels in autonomic neurons. This phenomenon suggests that the cellular effects of sigma ligands depend on the effector target. The conformational changes of sigma receptors in response to the ligand binding can cause agonist-like effect or antagonist-like effect, which is dependent on the effector targets that are close to sigma receptors in the microdomains of the cell membrane.

In conclusion, our studies of the effects of sigma receptors activation on voltage-gated ion channels in rat SCG and ICG neurons not only increase the knowledge of sigma receptors in the heart, but also have implications for a broad range of physiological and pathophysiological processes, such as cancer and stroke.
WORK CITED


Goyagi T, Goto S, Bhardwaj A, Dawson VL, Hurn PD, and Kirsch JR (2001) Neuroprotective effect of sigma(1)-receptor ligand 4-phenyl-1-(4-phenylbutyl)
piperidine (PPBP) is linked to reduced neuronal nitric oxide production. *Stroke* 32(7):1613-20.


Hellewell SB and Bowen WD (1990) A sigma-like binding site in rat pheochromocytoma (PC12) cells: decreased affinity for (+)-benzomorphans and lower molecular weight suggest a different sigma receptor form from that of guinea pig brain. *Brain Res* 527:244–253.


Hille B (2001) *Ion channels of excitable membranes* Sunderland, Massachusetts


channels in rat neurohypophysial terminals requires neither G-protein nor ATP. J Physiol (Lond) 526:527–539.


Soriani O, Vaudry H, Mei YA, Roman F, and Cazin L (1998) Sigma ligands stimulate the electrical activity of frog pituitary melanotrope cells through a G-


Vilner BJ, John CS, and Bowen WD (1995) Sigma-1 and sigma-2 receptors are expressed in a wide variety of human and rodent tumor cell line. *Cancer Res.* 55, 408-413.


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