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Store-Operated Calcium Channels in the Function of Intracardiac Neurons

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Store-Operated Calcium Channels in the Function of Intracardiac Neurons

by

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A dissertation submitted in partial fulfillment of the degree of
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Dedication

For my father,

Timothy Howard

November 12, 1939 – January 1, 2012
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ABSTRACT

Proper autonomic regulation of mammalian cardiac function is dependent upon very complex and precise communication among the intracardiac ganglia and individual neurons within the ganglia. An array of neuromodulators is found within the ganglia that direct neuronal activity by modulating the movement of calcium. The current study determines that opioidergic agonists, which have been found to contribute to severe cardiac disease states and intracellular calcium mobilization, are also responsible for changes in the function of the intracardiac neuron via their effects on store-operated calcium channels (SOCs).

Previous studies suggest that phosphorylation plays a role in SOC regulation. Using Fura-2 calcium fluorometry, we determined that protein kinase A (PKA), protein kinase C (PKC), and cyclic adenosine monophosphate (cAMP) had no effect on store-operated calcium entry in the presence of antagonists, phorbol 12, 13 dibutyrate (PDBu), forskolin, and 8-Br cAMP, respectively. We also found pharmacologically that using both electrophysiology and calcium imaging that μ-opioid agonists, met-enkephalin (ME) and endomorphin (EM) depress SOC activity in intracardiac neurons. Arachidonic acid (AA), which
has been found to depress SOC function in rat liver cells and \(\mu\)-opioid receptor activation (MOR), blocked both store-operated calcium entry (SOCE) and the calcium release-activated current \(I_{\text{CRAC}}\) significantly. Contrastingly, AA metabolites, prostaglandin E2 (PGE\(_2\)) and prostaglandin D2 (PGD\(_2\)), do not significantly influence SOCE which suggests that the effects of AA may be direct. The block elicited by EM was partially reversed by pertussis toxin (PTX), indicative of activation of a PTX-sensitive G-protein following MOR activation. Similarly, PLA\(_2\) inhibitors, OBAA and AACOCF\(_3\), decreased the percent block of SOCE due to opioid agonist-induced inhibition.

Using the perforated-patch method of I-clamp electrophysiology, we demonstrated that gadolinium, at low micromolar concentrations, reversibly reduced action potential firing. Importantly, these results suggest that SOCs may influence action potential firing in mammalian intracardiac neurons. Similarly, AA and EM depressed action potential firing. Taken together, these experiments suggest that a pathway involving EM and AA influences repetitive firing through SOC inhibition.

The importance of SOCs in the maintenance of action potential firing and more specifically, the expression and biophysical functionality of the individual pore-forming subunits (Orai1, 2, and 3) in any neuronal cell type has previously not been explored. Quantitative RT-PCR along with I-clamp electrophysiology revealed that Orai3 was exclusive to repetitively firing neurons. As a result, we
hypothesize that robust Ca\(^{2+}\)-dependent fast inactivation, also associated Orai3, is a factor in the maintenance of repetitive action potential firing.

Using Fura-2 calcium fluorometry and patch-clamp electrophysiology, we determined pharmacologically that \(\mu\)-opioid receptor activation precedes an intracellular cascade that is dependent on a PTX-sensitive G-protein and AA but independent of prostaglandin and protein kinase activity. Finally, we used RT-PCR to determine the Orai subunits expressed in the intracardiac neurons and their influence on neuronal firing patterns. This study is the first to determine the role expressed subunits has in the maintenance of the electrical activity of the neuron.
Mammalian Intracardiac Ganglia

The mammalian intracardiac ganglia are composed of clusters of efferent and afferent neurons that serve as the final common pathway for autonomic control of cardiac function. Mediating all parasympathetic input to the heart, these ganglia receive input from the dorsal motor nucleus (DMN) and nucleus ambiguus (NA) via ipsilateral fibers of the right and left vagal nerves (cranial nerve X) (Standish, Enquist et al. 1994). Located superficially on the epicardium within adipose tissue and embedded within the septum between the two atria, the epicardial and intraseptal ganglion, control the sinoatrial (SA) and atrioventricular (AV) nodes, the cardiac myocytes, and the coronary vasculature (Figure 1.1) (Cuevas 1995) (Ardell and Randall 1986; Randall, Milosavljevic et al. 1986).

Each disparate cluster within the cardiac plexus receives input from separate regions of the CNS for specialized coordination of cardiac function. For instance, the SA ganglion, situated at the junction of the right pulmonary vein and left atrial junction, receives input from the NA resulting in a negative chronotropic
effect (Gatti, Johnson et al. 1995; Massari, Johnson et al. 1994; Massari, Johnson et al. 1995). Conversely, the AV ganglion, located at the junction of the left atrium and inferior vena cava, receives input from both the DMN and NA to elicit a negative dromotropic effect (Standish, Enquist et al. 1995; Gatti, Johnson et al. 1995; Massari, Johnson et al. 1994; Massari, Johnson et al. 1995).

The ganglia seem to be interconnected within the plexus by a network of nerve fibers, permitting interganglionic communication (Pardini, Patel et al. 1987). The distribution and quantity of ganglia are different among mammalian species. For example, there are four ganglionic clusters in the atria and three in the ventricles of the dog whereas there are four ganglionic cluster groups totaling 4000 neurons in the atria of the rat heart (Fig 1.2) (Armour and Hopkins 1990; Xu and Adams 1992).

The intrinsic cardiac nerve plexus is by no means a simple relay system like some other autonomic efferent pathways (Gray, Johnson et al. 2004). Emerging studies conclude that this circuit is a part of a ranked series of intrathoracic nested feedback loops including interneurons, afferents, parasympathetic and sympathetic postganglionic efferent neurons, enabling rapid and precise orchestration of cardiac outputs (Ardell, Randall et al. 1988). Furthermore, the complex morphology of the intracardiac ganglia, representative of the organ level of the hierarchy of the cardiac nervous system, strongly suggests that it acts as a major local integration center. This neurocircuitry receives mechanical and chemical information from atrial and ventricular origins,
thus maintaining interganglionic neuronal communication that is also capable of regulation of reflex short-loop and extracardiac sensory feedback (Fig 1.3) (Cuevas 1995).

These various pathways function concomitantly to provide beat-to-beat regulation of the heart. However the intracardiac ganglia are responsible for integrating the information from these feedback loops spatially and temporally for precise and accurate output (Armour 1999). Input from the CNS is not mandatory for the activity of this plexus. Following chronic decentralization of the heart, neural communication returns and management of activities such as contractility, especially, are resumed (Ardell, Butler et al. 1991). The anatomical structure of the rat intracardiac plexus is similar to that of the human. Therefore, the rat is an accurate model for the experiments outlined in this dissertation. The guinea pig cardiac plexus has smaller clusters consisting of about 20 cells whereas the human and rat have clusters of about 200 cells. These neurons are more densely populated in the septum in comparison to the atrial walls. Specifically, more ganglionic neurons are situated near the SA node in the left atrium than in the right (Leger, Croll et al. 1999). The rat has four distinct regions: between the superior vena cava and aorta, within the superior interatrial septum, posterior to the left atrium and the posterior to the inferior interatrial septum and right atrium (Pardini, Patel et al. 1987).

Functionally, there are three types of neurons in the intracardiac ganglia: principal parasympathetic efferent postganglionic neurons (P), interneurons (IN)
that provide intraganglionic and interganglionic communication, and
catacholaminergic small, intensely fluorescent cells (SIF) (Xi, Randall et al.
1991). Principal neurons receive inputs from the CNS, afferent neurons,
sympathetic nerve fibers, INs, and SIFs and synapse on SA and AV nodes, the
myocardium, and the coronary arteries. Receiving inputs from the CNS and
sympathetic efferents, IN synapse on the P and SIF, which release
catecholamines on P (Seabrook, Fieber et al. 1990). In addition, there are
afferent neurons that extend from the ganglia to the central nervous system.

Furthermore, in addition to functionality, these neurons are
morphologically distinct as well. Parasympathetic neurons are usually multipolar
with elongated processes, whereas interneurons are either monopolar or
pseudopolar in appearance (Edwards, Hirst et al. 1995). These cells may also
be categorized by soma diameter as well, with IN being the larger and SIF
having the smallest diameter (Allen and Burnstock 1987; Seabrook, Fieber et al.
1990).

Every cell has a distinct electrical discharge characteristic in addition to
distinct morphological and functional characteristics. In the pig, three distinct
firing patterns have been confirmed: I (phasic), II (accommodating), and III (tonic)
(Smith 1999). In the canine, action potential trains have been described as S, R,
and N, corresponding to phasic firing, tonic firing, and neurons void of firing
activity, respectively (Xi, Thomas et al. 1991; Xi, Randall et al. 1994).
Applicable to the current study, rat cardiac neurons possess three main
ganglionic neuronal types, I$_b$, I$_m$, and II. However, there is some heterogeneity associated with these three types. I$_b$ elicits adapting firing patterns, I$_m$ exhibits tonic firing and Type II is capable of both phasic and tonic firing (Selyanko 1992).

The intracardiac plexus has a complex neurohumoral component in addition to its complex neural network. Indicative of acetylcholine (ACh) dominance as a neurotransmitter, choline acetyl transferase (ChAT) immunoreactivity is present in all postganglionic cells of the guinea pig intrinsic cardiac ganglia (Mawe, Talmage et al. 1996). A wide variety of neurotransmitters and neuropeptides are present in the intrinsic and extrinsic fibers found within the ganglia and ultimately affect neurons and target tissue. Neurotransmitters such as ACh, norepinephrine, and dopamine and neuropeptides such as pituitary adenylate cyclase-activating peptide (PACAP), vasoactive intestinal polypeptide (VIP), substance P, calcitonin gene-related peptide (CGRP), calbindin, and neuropeptide Y found within the ganglia govern the electrical properties of the neurons and in turn, the downstream organs are influenced (Gordon, Polak et al. 1993). There also seems to be subpopulations of cells within the ganglion displaying common functionality based on immunoreactivity implicating similar neurochemistries within a subpopulation. For instance, three distinct neurochemical profiles have been identified within the guinea pig intracardiac ganglion: ChAT/NPY, ChAT/NPY/calbindin and ChAT/NPY/NOS (Richardson, Grkovic et al. 2003).
The study presented here is partially based on the presence of enkephalin-like activity in the rat heart and the hypothesis that it alters parasympathetic control over the effector tissues (Hughes, Kosterlitz et al. 1977; Richardson, Grkovic et al. 2003).

**Autonomic Dysfunction and Heart Disease**

It is well established that in cardiovascular disease states parasympathetic drive is compromised. For example, in early stages of heart failure (HF), changes in vagal discharge can be detected, but the exact mechanism by which this occurs has not been fully described (Kinugawa and Dibner-Dunlap 1995). Under normal conditions, the parasympathetic and sympathetic nervous systems interact by accentuated antagonism in which parasympathetic activation induces sympathetic depression. In healthy human subjects, echocardiogram R-R intervals are augmented in response to subnormal doses of atropine. However, in the case of HF patients, the effect of atropine on R-R intervals are significantly reduced a phenomenon indicative of attenuated parasympathetic control.

Epidemiological studies indicate that decreased parasympathetic tone leads to an increased resting heart rate and a poorer prognosis in heart failure patients (Fox, Borer et al. 2007). A reduction in parasympathetic tone, coupled with an increase in sympathetic tone, leads to an increase risk for ventricular tachycardia, fibrillation, and ultimately sudden cardiac death (Osaka, Saitoh et al. 1996). It is commonly preceded by a decrease in heart rate variability, i.e.
deviations in R-R intervals in sinus rhythm, followed by an electrocardiograph ST segment shift (Pozzati, Pancaldi et al. 1996).

Relevant to the current study, the attenuation of parasympathetic control is due to a defect in communication within the intracardiac ganglia (Bibevski and Dunlap 1999). Studies indicate that during heart failure, the intrinsic cardiac nervous system initially maintains its ability to regulate mechano- and chemosensitive inputs, extracardiac efferent neurons, as well as local circuit intrinsic cardiac neurons. Following disease progression, however, there is restructuring of the circuitry of the ganglia that blunts its modulatory functions (Arora, Cardinal et al. 2003). It has been shown that following myocardial ischemia, the activity of the intrinsic cardiac neurons is also depressed (Huang, Ardell et al. 1993). Furthermore, compromised parasympathetic control is frequently associated with arrhythmias occurring after a myocardial infarction (Kleiger, Stein et al. 2005). Interestingly, there is evidence that the consequences of direct stimulation of the fat pads of the epicardium are a reduction in heart rate and sudden cardiac death in the canine (Wallick and Martin 1990).

In situ, acute myocardial ischemia results in the progression of decreased soma and dendritic function elicited by the accumulation of reactive oxygen species (ROS) and purinergic receptor agonists. Originating from surrounding ischemic myocardial tissue, these factors are then secreted into pericardial fluid (Huang, Sylven et al. 1993; Huang, Sylven et al. 1995; Thompson, Horackova et al. 1998). There is also evidence that ganglionic transmission is enhanced in
response to histamine ligand binding to the H1 receptor of neuronal cells resulting in the activation of surrounding mast cells (Powers, Peterson et al. 2001). Particularly relevant to this study, cardiac vagal afferents are enhanced by prostaglandin and ROS-induced sensitization to bradykinin during the immune response in HF (Schultz 2001). Studies show that VIP regulates vasodilation by binding to the VPAC receptor on intracardiac neurons and enhancing neuroexcitability through intracellular calcium elevations (DeHaven and Cuevas 2004). As a result of this altered parasympathetic regulation and subsequent effects, the myocardium becomes oxygen deprived, contributing to the progression of the disease state (Huang, Shirahase et al. 1993). These examples demonstrate the contribution neurochemistry plays in the pathology of HF.

The significance of this particular study lies in the paucity of information that exists about intrinsic cardiac nervous system, its modes of modulation, and contribution to proper cardiac function. This study contributes to the determination of molecular mechanisms by which intracardiac neuronal activity is modulated by neuromodulators present within the ganglia. Information derived from studies as the current is key in establishing improved understanding of the parasympathetic division of the autonomic nervous system and the methods by which it can be pharmacologically modified (Gheorghiade, van Veldhuisen et al. 2006).
Calcium Regulation and Excitability of Intracardiac Neurons

Calcium regulation plays an important role in many cell types, particularly neuronal cell types because of its capacity to regulate gene expression, Ca\(^{2+}\) dependent ion channels, neurotransmitter release, synaptic plasticity, and excitability. However, mechanisms of intracellular calcium regulation remain to be fully elucidated in intracardiac neurons. Neurotransmitters and neuromodulators such as acetylcholine (Adams 2004), histamine (Hardwick, Kotarski et al. 2006), ATP (Liu, Katnik et al. 2000), PACAP, and VIP (DeHaven and Cuevas 2004) have been shown to modify the activity of these cells by orchestrating transmembrane and intracellular movement of calcium within these cells. In addition to plasma membrane fluxes, rat intrinsic neurons display two intracellular Ca\(^{2+}\) stores, IP\(_3\)-sensitive stores and caffeine/ryanodine-sensitive stores (Smith and Adams 1999; Beker, Weber et al. 2003).

Demonstrated by our group, PACAP was able to induce multiple action potential firing via a calcium-dependent mechanism that required calcium release from ryanodine-sensitive stores (DeHaven and Cuevas 2004). PACAP and VIP evoke both a transient as well as sustained phases of cytosolic calcium entry following VPAC receptor activation. The transient phase is calcium release from ryanodine-sensitive stores and the sustained phase is calcium entry through store-operated calcium channels (SOCs) in neonatal rat intracardiac neurons. VPAC receptor-dependent membrane depolarizations were observed in the
presence of both VIP and PACAP. In contrast, studies in guinea pig intracardiac neurons indicate that neither VIP of PACAP induces the transient phase of intracellular calcium increases. Furthermore, it is the sustained phase not store-release that is responsible for any changes in excitability elicited by PACAP (Tompkins, Hardwick et al. 2006). Taken together, these results are indicative of differential and specific cellular physiological responses following neuropeptide ligand binding, justifying the necessity of extensive exploration of intracellular signaling mechanisms within each species. To date, most of the information that exists regarding calcium homeostasis involves voltage-gated calcium channels as the cardinal supplier of calcium to these cells in the maintenance of excitability. Furthermore, modulation of these channels leads to neurochemical changes in the guinea pig parasympathetic cardiac ganglia. For instance, a decrease in somatostatin gene expression has been attributed to PACAP-dependent Ca\textsuperscript{2+} influx through L-type channels. This finding serves as evidence of the importance of Ca\textsuperscript{2+} as it relates to its influences on the neurochemical composition as well as the electrical activity of the ganglia (Braas, Rossignol et al. 2004).

Muscarinic as well as nicotinic synergistic receptor activation leads to calcium release from IP\textsubscript{3} and ryanodine sensitive stores in rat autonomic neurons. Cholinergic receptor activation is capable of converting excitability through membrane depolarizations and Ca\textsuperscript{2+}-dependent K\textsuperscript{+} channels (K\textsubscript{Ca}\textsuperscript{2+}) (Beker, Weber et al. 2003). In addition to Ca\textsuperscript{2+}-induced membrane
depolarization, Ca\textsuperscript{2+}-dependent ion channels contribute to the membrane properties of these cells. Particularly, K\textsubscript{Ca}\textsuperscript{2+} channels have been identified and their function and biophysical properties have been described using electrophysiology (Xu and Adams 1992; Xi-Moy and Dun 1995; Franciolini, Hogg et al. 2001).

Even though at least three types of depolarization-activated calcium channels have been identified in intracardiac neurons, action potential firing occurs quite independently of voltage-gated calcium channel activity (Franciolini, Hogg et al. 2001). Therefore, there must be a depolarization-independent component to calcium influx in intracardiac neurons because bath applied, voltage-gated calcium channel blocker, Cd\textsuperscript{2+} is unsuccessful in significantly reducing action potential or after-hyperpolarization (AHP). It is therefore possible that this component is store-operated (Franciolini, Hogg et al. 2001).

**Store-Operated Calcium Channels**

Acutely dissociated adult rat intracardiac neurons express four different subtypes of HVA (high voltage –activated) Ca\textsuperscript{2+} channels with total contribution to the total voltage current being in the order of N (63%) > Q (19%) > L (11%) > R (7%) (Jeong and Wurster 1997). Franciolini et al. report that selective block of the V-gated calcium channel has no significant effect on the action potential or duration of the AHP within intracardiac neurons (2001). The defining
characteristic of SOCs is their ability to activate in response to the depletion of calcium from the endoplasmic reticulum. Calcium influx through these channels embedded within the plasma membrane then replenishes endoplasmic reticulum (ER) calcium. Primarily, the ER acts as an intracellular sink for $\text{Ca}^{2+}$, and $\text{Ca}^{2+}$ itself is essential for the proper folding of proteins. It also plays a role in various processes, such as vesicular trafficking and cholesterol metabolism, and is a ubiquitous second messenger. When the $\text{Ca}^{2+}$ concentration within the lumen of the ER falls too low, ER stress occurs and may result in apoptosis (Ferri and Kroemer 2001). SOCs are capable of refilling the intracellular stores, preserving protein production, folding, and trafficking (Parekh and Putney 2005).

Capacitative calcium entry (CCE) or store operated calcium entry (SOCE) in response to intracellular store depletion is important for cell survival and function of both excitable and non-excitable cell types (Xue, Zhao et al. 2000; Weigl, Zidar et al. 2003).

To date, the best characterized SOC channel is the channel mediating calcium release activating current ($I_{\text{CRAC}}$). Analogous to SOCE, $I_{\text{CRAC}}$ is evoked following passive calcium store depletion and inwardly-rectifying given its current-voltage relationship. After completing a 1-2s voltage ramp from -100mV to +100mV and returning to a depolarized potential (0 mV), the driving force for $\text{Ca}^{2+}$ ions is greatly reduced (Penner, Matthews et al. 1988; Lewis and Cahalan 1989; Bird, DeHaven et al. 2008). Under physiological conditions the CRAC channel has significantly high conductance for $\text{Ca}^{2+}$ over monovalent ion species.
indicated by its large amplitude at negative potentials and near zero at positive amplitudes (>60 mV). However, \( I_{\text{CRAC}} \) becomes highly selective for \( \text{Na}^+ \) ions in the absence of \( \text{Ca}^{2+} \), potentiating the current (Parekh and Putney 2005; Bird, DeHaven et al. 2008). Furthermore, the CRAC channel has an allosteric binding site for \( \text{Ca}^{2+} \) ions therefore at nanomolar concentrations the \( I_{\text{CRAC}} \) is inhibited.

The pore-forming subunit of the SOC channel, Orai1 or CRACM1, was first identified as non-functional in immune cells of severe combined immune deficiency (SCID) patients (Feske, Gwack et al. 2006). Two other Orai1 homologs, Orai2 and 3, exist with efficacies in the order of Orai1 > Orai2 > Orai3, all capable of regulating and constituting SOCs. The functional properties of these channel subunits differ from one to another. For instance, Orai1 exhibits robust fast \( \text{Ca}^{2+} \)-dependent inactivation whereas Orai2 & 3 do not display fast \( \text{Ca}^{2+} \)-dependent inactivation in stably transfected HEK293 cells (Table 1)(Lis, Peinelt et al. 2007).

Their Orai1-containing channels (Orai1-SOC) are activated in response to a signal generated by the ER calcium sensor STIM1 (Roos, DiGregorio et al. 2005; Spassova, Soboloff et al. 2006). Stim possesses an ER luminal helix-turn-helix \( \text{Ca}^{2+} \)-sensing EF-hand, “E” and “F” representing the two helixes of the \( \text{Ca}^{2+} \)-binding portion of the protein. When ER concentrations fall below 300 \( \mu \text{M} \), Stim1 oligomerizes and translocates near Orai1 embedded the plasma membrane to initiate transmembrane entry of extracellular \( \text{Ca}^{2+} \) (Liou, Kim et al. 2005; Wu, Buchanan et al. 2006). Stim2 however inhibits SOCE only after
coexpression with Stim1 which is indicative the formation of heteromeric puncta formation (Soboloff, Spassova et al. 2006).

Intracardiac neurons, specifically, display a biphasic Ca\(^{2+}\) response to PACAP and VIP application that is indicative of store release and subsequent reentry. More specifically, using the ratiometric calcium imaging along with the add back protocol, we have shown that SOC antagonists such as SKF-96365 and 2- aminoethoxydiphenyl borate (2-APB) and most importantly, Gd\(^{2+}\), are all capable of reducing \([\text{Ca}^{2+}]_i\) during bath application of Ca\(^{2+}\) following passive store depletion caused by thapsigargin. Subsequent whole cell electrophysiological studies indicate that the presence of the \(I_{\text{CRAC}}\) using precisely controlled conditions specific for this relatively elusive current. After evoking the current, application of 5 \(\mu\)M Gd\(^{2+}\) was able to block this current appreciably. Molecular studies using RT-PCR also indicate the expression of Orai1 mRNA transcripts in neonatal rat intracardiac neurons. However Orai2 and 3 expressions have not been confirmed prior to the current study.

The one conundrum that investigators are confronted with in the study of SOCs is that there is not currently an exclusive SOC antagonist. One antagonist, gadolinium, blocks receptor-operated calcium channels (ROCCs) and SOCs. However the differentiation lies within the differences in inhibitory concentrations. Store-operated calcium entry, specifically, is depressed by Gd\(^{3+}\) at concentrations under 10 \(\mu\)M. Other inhibitors such as 2-APB and SKF-96935 inhibit voltage-dependent Ca\(^{2+}\) channels at similar concentrations. As a result
Gd\(^{2+}\), at micromolar concentrations, is accepted as the most selective SOC antagonist to date (Franzius, Hoth et al. 1994).

From one Orai subunit to the next, the pharmacology of each is distinct. For instance, SOC antagonist, 2-APB significantly blocks Orai1 directly and prevents Stim1 puncta formation. In the case of Orai2, 2-APB elicits inhibition to a lesser degree than Orai1 whereas, it potentiates Orai3 in HEK 293 cells transiently transfected with the Orai and Stim (Table 1). Furthermore, we must conclude that 2-APB acts through very intricate mechanisms in the regulation of SOC channels in the exploration of this relatively novel ion channel (DeHaven, Smyth et al. 2008). A Stim1 homolog, Stim2, exhibits dual modes of SOC regulation by store and calmodulin (CaM) dependence. CaM is proposed to have a dual inhibitory role first by its interaction with the Orai-Stim complex and inhibition of the IP\(_3\) receptor (Parvez, Beck et al. 2008).

While these channels have been studied to some extent in non-excitable cells, little is known about the functional properties and cellular role of Orai1-SOC in neurons. In dorsal root ganglion neurons, a Ca\(^{2+}\) current has been evoked following ER store depletion and blocked by 2 µM Ni\(^{2+}\) at hyperpolarized potentials (Usachev and Thayer 1999). For the first time in a neuronal subtype, our group has successfully confirmed molecularly and pharmacologically the presence of functional SOCs. Therefore we propose that SOCs may be as integral as V-gated calcium channels in the regulation of Ca\(^{2+}\)-dependent potassium channels thereby influencing cell-to-cell communication within the
intracardiac ganglia. Additionally, the current study is based on the premise that Orai- SOCs are functionally expressed in neonatal rat intracardiac neurons and \( \mu \)-opioid receptors purported to be expressed in this neuronal cell type regulate SOC channel activity.

\( \mu \)-opioid Receptor Activation

Enkephalins and dynorphins are two classes of opioid agonists that are endogenous to the heart and are potent regulators of cardiac function. Enkephalins have negative effects on heart rate, contractility, and electrical conduction at synapses of vagal preganglionic neurons as well as other targets within the central and peripheral nervous system (Blinder, Johnson et al. 2005; Caffrey, Gaugl et al. 1985). Under normal conditions in the adult mammalian heart, the mRNA encoding for the precursors proteins proenkephalins and prodynorphins are readily detected, but the processed protein end products are sparse (Springhorn and Claycomb 1992; Boluyt, Younes et al. 1993; Caffrey, Boluyt et al. 1994; Barlow, Deo et al. 2006). However, endogenous as well as circulating opioids are elevated in individuals with genetic hypertension, myocardial infarction, and cardiomyopathy and in cardiac transplant patients (Dumont, Ouellette et al. 1991; Ationu, Sorensen et al. 1993; Barlow, Deo et al. 2006). Circulating opioids, specifically, are elevated in patients with congestive heart failure, leading to the exacerbation of the disease state by reducing cardiac
output (Liang, Imai et al. 1987; Fontana, Bernardi et al. 1993; Barlow, Deo et al. 2006).

Opioid antagonist U50,488H promotes bradycardia, arrhythmias, and hypotension associated with myocardial ischemia (Lee, Chen et al. 1992). In a clinical study, met-enkephalin (ME) administered to a group of patients with neurocirculatory dystonia, myocardial ischemia and arterial hypertension nearly doubled sinus node recovery time and shortened the cardiac cycle (Osadchii, Abushkevich et al. 2001). Taken together these studies indicated that as parasympathetic mediators regulating heart rate primarily, intracardiac neurons could possibly be affected by endorphin activity.

Enkephalins injected centrally in the NA and locally in vagal preganglionic nerve fibers have cardioinhibitory effects. (Laubie, Schmitt et al. 1979; Wu and Martin 1983; Blinder, Johnson et al. 2005). Independently, this evidence overwhelmingly suggests that similar effects will be seen in postganglionic intracardiac neurons. On the other hand, opioids are capable of inhibiting catecholamine release and simultaneously being produced in dissociated cardiac myocytes by adrenergic second messengers. Autonomic cross talk is mediated thereby limiting end-organ responses to nerve stimulation through this opioid-induced feedforward effect (Barron and Hexum 1986; Wong and Lee 1987; Wong, Lee et al. 1990). The exact mechanisms by which opioids exert their effects at the tissue and cellular level to produce these cardiovascular effects are poorly understood.
An additional method of opioid-induced modulation of the nervous system is via ion channels, specifically, calcium channels. Previous studies have identified opioid receptor agonists as modulators of ion channels particularly, VGCCs. In parasympathetic neurons of the NA, \( \mu \)-opioid receptor (MOR) activation has been shown to inhibit voltage-gated calcium channels and the rat superior cervical ganglion, respectively (Liu and Rittenhouse 2003). Based upon these findings, opioids may be capable of regulating the activity of the ganglia by modulating calcium channels, specifically.

Pharmacologically, opioidergic agonists function, partly, in the attenuation of pain by reducing pre-synaptic neurotransmitter release, which is dependent upon \( \text{Ca}^{2+} \) currents (Jessell 1977; Wilding, Womack et al. 1995). In adult rat ventricular myocytes, leu-enkephalin dampens L-type calcium channel activity leading to a reduction in the ionotropic response (Xiao, Spurgeon et al. 1993). The greatest contributor to the voltage-gated calcium current (\( I_{\text{CaV}} \)) in adult rat intracardiac neurons, N-type calcium current is significantly reduced by \( \mu \)-opioid specific agonist, ME, in a reduction of the amplitude of the single action potential waveform (Adams and Trequattrini 1998). Also, leu-enkephalin reduces contraction amplitudes in ventricular myocytes through the blockade of L-type calcium channels (Xiao, Spurgeon et al. 1993).

Intracellular pathways are initiated which activate and produce second messengers that the direct consequences on ion channel activity. Through G-protein recruitment, the PLC-IP\(_3\)/DAG and AC-cAMP pathways are initiated and
orchestrate intracellular calcium store release (Johnson, Wang et al. 1994). Relevant to the current study, the Adams group has proposed that opioid ligands induce IP$_3$-mediated calcium mobilization (Smith and Adams 1999) as well as block VGCCs in rat intracardiac ganglion neurons (Adams and Trequattrini 1998). Taken together, it is possible that these agonists may activate an intracellular signaling cascade targeting SOC channels.

Given that G-protein recruitment precedes voltage-gated calcium current inhibition in NG-108 cells transfected with cloned $\mu$-opioid receptors, this study aims to explore the role of G-proteins in an intracellular second messenger system resulting in SOC depression in cardiac neurons (Higashida, Hoshi et al. 1998). Furthermore, MOR activates phospholipase A$_2$ (PLA$_2$), the enzyme responsible for production of the intracellular second messenger AA in pyramidal neurons (Faber and Sah 2004). This finding is relevant to the present study because AA has been shown to be the signaling molecule responsible for SOC inhibition in rat liver cells. This study is novel because it attempts to establish a link among G-protein, PLA$_2$, AA activation and SOC activity reduction in an excitable cell type.

Under normal conditions in mammalian cardiac tissue, unesterified arachidonic acid (AA) concentration is low however following ischemia and reperfusion, AA is produced, having adverse effects on cardiac function (Van der Vusse, Reneman et al. 1997). It is possible that these negative consequences are due the dampening of sarcoplasm Ca$^{2+}$, voltage-gated calcium channels
(VGCCs), K\(^+\), and Na\(^+\) channel function (Huang, Xian et al. 1992; Honore, Barhanin et al. 1994; Kang and Leaf 1996). In non-excitable cells such as rat liver cells, AA is a highly effective inhibitor of SOC function at estimated physiological concentrations (Rychkov, Litjens et al. 2005). However, the effects of AA on SOC channels in neurons remain a mystery.

This study was executed to explore the effect of MOR ligands, AA, and the possibility of the two participating in a common inhibitory pathway for SOC channel activity in the cardiac neuron. It is our hypothesis that opioidergic receptor activation results in the PLA\(_2\)-mediated production of AA only following G-protein recruitment in rat intracardiac neurons. Consequently, the action potential discharge and intraganglionic and interganglionic function is compromised, resulting in altered autonomic regulation of the heart.
Figure 1.1 Schematic diagram of intracardiac ganglia and effector tissues. Note the interganglionic connections. AV, atrioventricular node; SA, sinoatrial node (Cuevas 1995)
Figure 1.2 (A) Representative sketch of the organization of intracardiac neurons within the heart. Each dot is an individual ganglion. Superior view of LA, left atrium and RA, right atrium. Scale bar, 1 µm. PV, pulmonary veins; SVC, superior vena cava.

(B) Intracardiac neurons in vivo under Hoffman modulation contrast optics, Scale bar, 40 µm.

(C) Phase contrast photomicrograph of neurons enzymatically dissociated from rat cardiac ganglia and entered into culture for approximately 48 hours. Scale bar, 100 µm. (Xu and Adams 1992)
Figure 1.3 Schematic diagram of the organization of intrathoracic cardiac plexus. R, Receptor (Cuevas 1995)
Table 1. Functional, Kinetic, and Pharmacological Properties of Orai Channels; adapted from (Lis, Peinelt et al. 2007)

<table>
<thead>
<tr>
<th></th>
<th>Orai1</th>
<th>Orai2</th>
<th>Orai3</th>
</tr>
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<tbody>
<tr>
<td>Store-Operated</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Activation time (t_{1/2})</td>
<td>35 ± 7 s</td>
<td>21 ± 3 s</td>
<td>63 ± 7 s</td>
</tr>
<tr>
<td>Ca^{2+}-dependent inactivation (fast)</td>
<td>Moderate</td>
<td>Moderate</td>
<td>Strong</td>
</tr>
<tr>
<td>Ca^{2+}-dependent inactivation (slow)</td>
<td>Strong</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Ca^{2+}-dependent reactivation</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Selectivity</td>
<td>Ca^{2+}&gt;&gt; Na^+, Ba^{2+}</td>
<td>Ca^{2+}&gt;&gt;Na^+, Ba^{2+}</td>
<td>Ca^{2+}&gt;&gt; Na^+, Ba^{2+}</td>
</tr>
<tr>
<td>Monovalent Permeations in DVF solutions</td>
<td>Moderate</td>
<td>Moderate</td>
<td>Strong</td>
</tr>
<tr>
<td>2-APB at 50 μM</td>
<td>Block</td>
<td>Reduction</td>
<td>Potentiation</td>
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Wong, T. M., A. Y. Lee, et al. (1990). "Effects of drugs interacting with opioid receptors during normal perfusion or ischemia and reperfusion in the isolated rat heart--an attempt to identify cardiac opioid receptor subtype(s) involved in arrhythmogenesis." J Mol Cell Cardiol 22(10): 1167-75.


CHAPTER 2

RECRUITMENT OF INTRACELLULAR KINASES HAS NO APPRECIABLE INFLUENCE ON STORE-OPERATED CALCIUM ENTRY IN INTRINSIC CARDIAC NEURONS

INTRODUCTION

The mammalian intracardiac ganglia mediate parasympathetic input to the heart through very complex neural interactions. However, the cellular mechanisms by which this neural network processes vagal input remain to be fully elucidated. It has been established that these ganglia serve as the final common pathway for the integration of a variety of exogenous inputs ultimately orchestrating end organ function. Communication within the ganglia and target modulation is highly dependent upon the neurochemistry of the ganglia. Similarly, the neurochemical component of this plexus is also very complex and the exact mechanism by which these neuromodulators control the cardiac ganglia is poorly understood.

In order to begin to fully assess the mechanisms by which these neuromodulators exert their effects, we must first explore the effects on the
single neuron. Neuronal excitability is determined by the membrane properties of the cell, biochemical interactions between intracellular and extracellular molecules, and membrane-bound ion channels. Specifically, neuropeptide-induced calcium channel modulation has been implicated in the function of intracardiac neurons. For example, vasoactive intestinal polypeptide (VIP) and pituitary adenylate cyclase-activating polypeptide (PACAP) increase excitability of intracardiac neurons by calcium-induced membrane depolarizations (Smith and Adams 1999; Liu, Cuevas et al. 2000; Liu, Katnik et al. 2000; Beker, Weber et al. 2003; DeHaven and Cuevas 2004). VIP exerts its effects through the mobilization of calcium through vasointestinal pituitary adenylate cyclase receptor (VPAC) activation. Following intracellular store release, there is a sustained phase of the calcium response that is blocked by store-operated calcium channel (SOC) antagonist 2-aminoethoxydiphenyl borate (2-APB). VPAC receptor activation initiates an intracellular signalling cascade that leads to an inositol 1, 3, 4-triphosphate (IP$_3$) -induced store release. Furthermore, previous studies show that VIP-mediated regulation of voltage-gated calcium channels (VGCCs) is cyclic adenosine monophosphate (cAMP)/ protein kinase A (PKA)-dependent. However, the role kinases play in SOC regulation is unclear.

Even though VGCCs have traditionally been thought to be the primary excellular calcium source and modulators of excitability in neuronal cell types, emerging evidence shows that SOCs are expressed and may play role in the function of intracardiac neurons. SOCs are capable of being regulated by the
receptor-operated intracellular second messenger systems. Confirmed to be expressed in intracardiac neurons, muscarinic (M₁ and M₃), P2Y₂, and PAC₁, recruit protein kinase C (PKC), which influences store-operated calcium entry (SOCE) (Strassheim and Williams 2000; Zhou, Yada et al. 2001; Dejda, Jozwiak-Bebenista et al. 2006; Watanabe, Ohba et al. 2006). Pituitary adenylate cyclase (PAC₁) and VPAC₂ receptors couple to adenylate cyclase (AC), which activates kinases. As a consequence, these kinases modulate SOCs directly. (Chatterjee, Sharma et al. 1996; Lutz, Shen et al. 1999).

Protein amino acid sequences may be evaluated and compared to kinase sequences offering a method for determining possible kinase subtypes that are coupled to ion channel subunits. Using Netphosk1.0, we have identified putative PKA and PKC binding sites on the first subtype of the pore-forming subunit of the store-operated calcium channel, Orai1. Preliminary to determining the functional coupling of kinases to the Orai-SOC channel, this finding is also indicative of a possible kinase regulatory component of the sustained phase of Ca²⁺ increase elicited by PACAP.

There is some controversy that exists in the literature regarding the influence that kinases have on SOCE. For instance, PKC has been found to have negative effects on capacitative calcium entry (CCE) in Drosophila photoreceptors and human neutrophils (Hardie, Peretz et al. 1993; Montero, Garcia-Sancho et al. 1994). Studies elucidating the link between SOCE and endothelial cell permeability show that myosin light-chain kinase (MLCK) alone
and not PKA or PKC influences this process (Norwood, Moore et al. 2000). In pulmonary smooth muscle cells, PKA blocks while PKC promotes store-operated calcium channel function (Chen, Dai et al.).

It is important to determine the role of these kinases in regulating SOC in intracardiac neurons because electrophysiological studies done by our group show that SOC function is integral in maintaining the electrical activity of the neuron. Known SOC antagonists, 2-APB and Gd$^{3+}$, at SOC-specific concentrations, attenuate action potential firing in intracardiac neurons, which suggests that repetitive firing is SOC-dependent.

The biophysical properties of these neurons are critical to acetyl choline (ACh) release, which dictates sequential cell-to-cell communication with in the ganglia. These interactions will ultimately influence the efficiency of the integration of information received by the CNS and ultimately affect cholinergic drive to the vasculature, myocytes, and nodal tissue of the heart. This study ascertains, using fura-2 mediated calcium flurometry, that store-depletion calcium entry is unaffected by PKA or PKC.
METHODS

Cell Culture

All animals used in this study were Sprague-Dawley neonatal rats, sacrificed using rapid decapitation at 2-4 days old. All procedures were done in accordance with the regulations of the Institutional Animal Care and Use Committee. Preparation and culture of neurons were followed as previously described (Fieber and Adams 1991). Enzymatically dissociated neurons were fixed on poly-L-lysine coated coverslips and incubated for 2-5 days in 95% air and 5% CO₂ at 37°C. Following cell culture, isolated neurons were used for calcium imaging studies.

Ratiometric Calcium Fluorometry Measurements

Fluctuations in intracellular calcium concentrations were measured using fluorescent imaging techniques. Fura-2-acetoxymethylester (fura-2-AM) was used as the indicator to measure [Ca²⁺]ᵢ in the intracardiac neurons. Cells plated on coverslips were incubated for 1h in physiological saline solution (PSS): 140 mM NaCl, 3 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgCl₂, 7.7 mM glucose and 10 mM Hepes (pH to 7.2 with NaOH) and 3 µM fura-2-AM plus 0.1% dimethyl sulfoxide
(DMSO). All solutions were administered focally via a rapid application system (Cuevas and Berg, 1997).

Methods for calcium imaging experiments were identical to those previously described (DeHaven and Cuevas 2004). Fluorescence emissions were captured using a Sensicam digital CCD camera (Cooke Corp., Auburn Hills, MI) and recorded using Slidebook Version 3 Software (Intelligent Imaging Innovations, Denver, CO). Changes in \([Ca^{2+}]_i\) were calculated using Slidebook 3 software (Intelligent Imaging Innovations, Denver, CO) from the intensity of the emitted fluorescence following excitation with 340- and 380-nM light, respectively using the Grynkiewicz equation:

\[
[Ca^{2+}]_i = K_d Q(R_{min}/(R_{max} - R)) \quad (Eq.1)
\]

Where \(R\) denotes the fluorescence intensity ratio \((F340/F380)\) as determined as a result of experiments, \(Q\) is the ratio of \(F_{min}\) to \(F_{max}\) at 380 nM and \(K_d\) is the \(Ca^{2+}\) dissociation constant for fura-2. Calibration of the system was performed using fura-2 calcium imaging calibration kit (Molecular Probes, Inc., Eugene, OR). These values were determined as follows: \(F_{min}/F_{max} = 224; R_{min} = 0.198977; R_{max} = 7.5354.\)
Reagents and Statistical Analysis

The following agents were used in this study: Forskolin, PDBu, 8-Br-cAMP (Sigma), and fura-2-AM (Molecular Probes, Inc., Eugene, OR). Imaging data files were converted to text format and imported into SigmaPlot 11 and Clampfit 9.2 for analysis. Statistical analysis was performed by SigmaPlot using paired and unpaired t-tests to determine significance within a group. The differences between groups are significant if p<0.05.
RESULTS

PKC activation has no influence on SOCE

To determine whether PKC activation influences SOC activity, fura-2 mediated calcium imaging was used to measure changes in SOCE in the absence and presence of PKC activation by phorbol 12, 13 dibutyrate (PDBu). Cells were preincubated overnight in media containing 1 µM PDBu, or in normal media, and population studies were used to determine the effects of PDBu on SOCE. The add-back protocol was initiated in fura-2 loaded neurons through the activation of passive depletion of intracellular Ca\(^{2+}\) stores by thapsigargin (TG) in a calcium-free PSS solution. Baseline levels of calcium were recorded prior to the introduction of 2.5 mM calcium PSS to the bath. Next, the resultant intracellular calcium increases were measured in neurons that had been exposed to 1 µM PDBu overnight and allowed to wash for 5 min and in naïve neurons in order to evaluate significant changes in SOCE. In Figure 2.1A, representative traces of intracellular calcium concentration as a function of time during Ca\(^{2+}\) add back from two neurons are shown. The solid line is indicative of the add-back response in the absence of PDBu and the dashed line, the presence of PDBu. The intracellular increases in both control and drug treated groups were 146±10
PKA activation does not contribute to the regulation of SOCE

In pulmonary smooth muscle cells, PKA inhibitor H-89 activates SOCE (Chen, Dai et al.). Similarly, NetPhosk1.0 results indicate that Orai1 has putative binding sites for PKA. To determine whether the pore-forming subunit is influenced by the kinase in neonatal intracardiac neurons, calcium imaging with the add-back protocol were used to determine the role PKA has in SOC modulation. The membrane permeable cAMP analog, 8-Br-cAMP, will be used to mimic the effects of cAMP and activate PKA. Fura-2 loaded cells were pre-treated with 4 µM TG to passively deplete the stores. Mean peak calcium levels were recorded and analyzed before (Control) and after (cAMP) drug presentation. Representative traces show qualitatively that application of 8-Br-cAMP has no effects on SOCE, and thus, PKA is unlikely to be affecting SOC (Figure 2.2A). The solid line is indicative of control conditions; while the dashed line is a representative trace from the same cell following 8-Br-cAMP application. The bar graph in Figure 2.2B also shows the mean peak calcium levels for control (162 ±15 nM) and drug-treated groups (151 ±16 nM). These groups were not statistically different. PKA has no considerable influence on SOCE in neonatal rat intracardiac neurons.
**CCE is unaffected by an AC-cAMP pathway**

To confirm the observations made with 8-Br-cAMP, the AC-cAMP pathway was activated to indirectly activate PKA and determine the consequence this pathway has on SOCE. Similar to aforementioned experiments, store-depleted neurons were exposed to a calcium-PSS solution to induce intracellular calcium increases. In this set of experiments, forskolin, an adenylate cyclase activator, was applied to cells to promote the production of cAMP. Figure 2.3 demonstrates the outcome of experiments in which the intracellular calcium concentrations were assessed following add back (solid line) of calcium before and after adenylate cyclase-activator, forskolin (Forsk), application. The mean peak calcium levels were determined to be 128 ± 8 nM for control group and 137 ± 17 nM. Statistical analysis shows that these values are not different. Representative traces of changes in intracellular calcium levels in response to add back are shown (Figure 2.3B). These results confirm that the AC-cAMP-PKA pathway, similar to direct activation of PKC and PKA, have no significant effects CCE.
Figure 2.1. PKC activation has no influence on SOCE. A, Representative traces of $\Delta[\text{Ca}^{2+}]$ for two individual neurons using the add back protocol for control (solid, n=59), a treatment of 1 µM PDBu (dashed, n=42). Line above trace represents 2.5 mM Ca$^{2+}$ application. B, Bar graph of values mean (± SEM) Peak $\Delta[\text{Ca}^{2+}]$ for control, drug-treated, and wash groups for the duration of the add back. There is no significant difference from control group.
Figure 2.2. PKA activation does not contribute to the regulation of SOCE. A, Representative traces of $\Delta[Ca^{2+}]$, for two individual neurons using the add back protocol for control (solid, n=45), a treatment of 100 µM cAMP (dashed, n=27). Line above trace represents 2.5 mM Ca$^{2+}$ application. B, Bar graph of values mean (± SEM) Peak $\Delta[Ca^{2+}]$ for control, drug-treated, and wash groups for the duration of the add back. There is no significant difference from control group.
Figure 2.3. CCE is unaffected by an AC-cAMP pathway. A, Representative traces of $\Delta [\text{Ca}^{2+}]_i$ for two individual neurons using the add back protocol for control (solid, n=72), a treatment of 10 $\mu$M Forskolin (dashed, n=23). Line above trace represents 2.5 mM Ca$^{2+}$ application. B, Bar graph of values mean (± SEM) Peak $\Delta [\text{Ca}^{2+}]_i$ for control, drug-treated, and wash groups for the duration of the add back. There is no significant difference from control group.
DISCUSSION

In this study, we conclude that phosphorylation by PKA and PKC has no appreciable effect on SOCE in intracardiac neurons. This study is novel because published information that exists on phosphorylation of SOCs applies to non-excitable cells. This study attempts to begin to determine possible modes of SOC regulation in excitable cells and the contribution SOCE has on neuronal function.

First, using calcium imaging and PKC activator PDBu, we show that PKC does not potentiate nor does it block intracellular calcium elevations following store depletion in intrinsic cardiac neurons. Our findings concur with studies in other cell types, such as endothelial cells, that cite that PKC has no bearing on the function of SOCs (Norwood, Moore et al. 2000). It is possible that PKC had an indirect effect on store-depletion versus the channel itself in previous studies (Berridge 1995). However, in the present investigation, stores were passsively depleted prior to experiments, consequently this variable was eliminated.

NetPhosk analysis output results indicated that there are putative binding sites for PKC and PKA on the Orai1 subunit. However, NetphosK provides
predicted results based on the amino acid sequences and mathematics. Further, binding studies would be appropriate to confirm protein-protein interaction.

Next we used an identical protocol, but with the membrane permeable cAMP analog, 8-Br-cAMP, to determine if cAMP and/or the downstream kinase, PKA, can affect SOCE activity in these cells. Similarly, no appreciable differences in SOCE could be detected when 8-Br-cAMP was bath applied during the add back. Conversely, Zhou et. al. report that an AC-cAMP-PKA intracellular signalling cascade is initiated following PAC1 receptor binding in neuroepithelia cells (2001). These conflicting findings suggest that the effects elicited by kinases could be dependent upon cell type.

The conclusions drawn from the 8-Br-cAMP experiments were supported by studies using the AC activator, forskolin. Forskolin failed to evoke any changes in SOCE in this system. The published data that exists linking cAMP to SOCE is conflicting. In airway smooth muscle cells, cAMP blocks SOCs (Ay, Iyanoye et al. 2006). However, there is also evidence that this phenomenon could be due to upstream effects on the unloading of the stores. For instance, histamine and ACh hydrolyze IP₃ via cAMP, leading to a decrease in intracellular calcium mobilization, thus causing a decrease in SOC channel modulation (Raeymaekers, Eggermont et al. 1990). In rat aorta, cAMP is a SOC antagonist; while in astrocytes cAMP acts as an agonist for SOCE (Wu, Chen et al. 1999). Taken together, these studies suggest that differences in kinase activity may be due to differences in cell type. More cell type specific studies would need to be
performed to determine these differences in regulation and the factors that contribute to these differences.
REFERENCES


CHAPTER 3

μ-OPIOID RECEPTOR ACTIVATION BLOCKS SOCE AND THE $I_{\text{CRAC}}$ VIA AN ARACHIDONIC ACID MEDIATED PATHWAY IN RAT INTRACARDIAC NEURONS

Introduction

Decreased parasympathetic modulation of the diseased mammalian heart allows sympathetic regulation to predominate, which could lead to ventricular dysrhythmias and ultimately, sudden cardiac death. The mammalian intracardiac ganglia are groups of parasympathetic efferent and afferent neurons that serve as the final common pathway to autonomic control of local cardiac function neurons modulating conduction, contractility, and vascular tone (Ardell 2004). Additionally, intrinsic cardiac neurons function locally to provide rapid sensory feedback and contribute to the maintenance of proper cardiac function independent of the CNS (Ardell 1994). Enkephalins and dynorphins are two classes of opioid agonists that are endogenous to the heart and are potent regulators of cardiac function. Under normal conditions in the adult mammalian
heart, the mRNA encoding for the precursor proteins proenkephalins and prodynorphins are readily detected, but the processed protein end products are sparse (Springhorn and Claycomb 1992; Boluyt, Younes et al. 1993; Caffrey, Boluyt et al. 1994; Barlow, Deo et al. 2006). However, endogenous as well as circulating opioids are elevated in individuals with genetic hypertension and cardiomyopathy and in cardiac transplant patients (Dumont, Ouellette et al. 1991; Ationu, Sorensen et al. 1993; Barlow, Deo et al. 2006). Circulating opioids, specifically, are elevated in patients with congestive heart failure (Liang, Imai et al. 1987; Fontana, Bernardi et al. 1993; Barlow, Deo et al. 2006).

Activation of μ-opioid receptors (MOR) in these neurons produce elevations in [Ca^{2+}] but results in depression of excitability (Smith and Adams 1999). In contrast, two neuropeptides found within the mammalian intracardiac ganglion, pituitary adenylate cyclase activating peptide (PACAP) and vasointestinal polypeptide (VIP), have been shown to enhance the excitability of intracardiac neurons via a mechanism dependent on similar elevations of intracellular calcium concentrations ([Ca^{2+}]) (DeHaven and Cuevas 2004). Obviously, these neuromodulators exert their opposing effects on membrane properties of the neuron via different mechanisms. While the MOR agonist depletes intracellular calcium stores, VIP and PACAP elicit a sustained [Ca^{2+}] increase, store release, that precedes a transient intracellular calcium response, influx through channels on the plasma membrane. This phenomenon is capacitative calcium entry (CCE) or store-operate calcium entry (SOCE); that is,
calcium influx regulated by Ca\(^{2+}\) concentrations within the cellular stores. The transient phase is reduced in the presence of SOC antagonist, 50 µM 2-APB, which indicates that VIP and PACAP are able to evoke a SOCE response in rat intracardiac neurons.

The Adams group has proposed that opioid ligands induce IP\(_3\)-mediated calcium mobilization as well as blockade of voltage-gated calcium channels (VGCCs) in rat intracardiac neurons (Adams and Trequattrini 1998; Smith and Adams 1999). Taken together, it is possible that these agonists may play a role in the function of SOC channels. Furthermore, Franciolini et al. report that selective block of the voltage-gated calcium channel has no significant effect on the action potential or duration of the after-hyperpolarization (AHP) within intracardiac neurons (2001). In support of this study, electrophysiology experiments indicate that there is more than one functional type of calcium channel in the intracardiac neuron (Xu and Adams 1992). While VGCCs have been established as the key regulator of calcium mobilization in the intracardiac neurons, these results suggest that SOCs may play a significant role in action potential firing.

SOCs are important because they ensure availability of calcium in response to depletion of intracellular stores. The most concise description of the current as related to SOCE is the calcium release-activating calcium current (I\(_{\text{CRAC}}\)). Evoked following passive calcium store depletion, this current was initially characterized in rat mast cells and human leukemic T cells (Penner,
Matthews et al. 1988; Lewis and Cahalan 1989). Under physiological conditions, the CRAC channels have significantly high preference for Ca\(^{2+}\) over monovalent ion species however \(I_{\text{CRAC}}\) becomes highly selective for Na\(^+\) ions in the absence of Ca\(^{2+}\) (Parekh and Putney 2005; DeHaven, Smyth et al. 2008). Orai-1, one of three pore-forming subunits composing SOC\(\text{s},\) has been shown to be functionally expressed in intracardiac neurons (unpublished data). It was recently characterized in immune cells of severe-combined immune deficiency (SCID) patients that express a mutated Orai-1 protein (Carr, De Costa et al. 1991; Feske, Prakriya et al. 2005). However, its contribution to the function of the cardiovascular system is unknown.

It is important to understand consequences of aberrant SOC channel function on cellular physiology whether by changes in molecular expression or pharmacologically. Therefore this study is based on the premises that opioid peptide production is altered in certain cardiac conditions and these changes could in turn have detrimental consequences on ganglionic function, exacerbating the disease. While the \(\delta\)-opioid receptor is the predominant class of opioid receptor in mostly non-neuronal cardiac tissue (Wittert, Hope et al. 1996; Zimlichman, Gefel et al. 1996; Bell, Sack et al. 2000), the MOR is of particular interest because recent studies in intracardiac neurons suggest that \(\mu\)-opioidergic signaling inhibits N-type calcium channels (Adams and Trequattrini 1998). MOR activation also leads to the recruitment of phospholipase \(A_2\) (PLA\(_2\)), the enzyme responsible for AA production in pyramidal neurons thus altering
action potential discharge (Faber and Sah 2004). In non-excitable cells such as rat liver cells, AA is a highly effective inhibitor of SOC function at estimated physiological concentrations (Rychkov, Litjens et al. 2005). MOR activation and AA production have been shown to inhibit voltage-gated calcium channels in parasympathetic neurons of the nucleus ambiguus (NA) (Irntaten, Aicher et al. 2003) and the rat superior cervical ganglion (Liu and Rittenhouse 2003), respectively. However, the effects of AA on SOC channels in neurons and influences on membrane properties of the neuron remain a mystery. Previous studies done by our group show that two important excitatory neuromodulators, VIP and PACAP, act via a PTX-sensitive G-protein in intracardiac neurons (Liu, Cuevas et al. 2000). Additionally, in pyramidal neurons of the amygdala, it has been shown that a PTX-sensitive G-protein linked to the MOR receptor causes the activation of PLA$_2$ when the G-protein subunits dissociate (Faber and Sah 2004). Relevant to the current study, we hypothesize that a G-protein is involved in the signaling cascade coupling MOR to SOC. For instance, Jelson et.al report changes in the action potential waveform following cardiac ganglionic mast cell degranulation as the result of the inhibition of small-conductance calcium channels (SK$_{Ca}^{2+}$) by prostaglandins. However it remains unclear whether prostaglandins inhibit the channel directly or via Ca$_{2+}$ channels.

We show that MOR ligands and arachidonic acid are involved in a common inhibitory pathway for SOC channel regulation in cardiac neurons and could possibly be influential in the neuromodulation of cardiac function. First,
using ratiometric calcium imaging, we show that SOCE is attenuated by MOR agonists and AA and linoleic acid (LA) alike. However these effects of MOR activation are reduced in the presence of the PLA$_2$ antagonist, 4-(4-Octadecylalkybenzoylacrylic acid (OBAA), suggesting that AA production follows receptor activation in a common signaling cascade. AA metabolites, PGE$_2$ and PGD$_2$, present in the cardiac ganglia have no apparent effects on SOC function, indicating that they do not function downstream of AA in the depression of SOC activity. Finally, using whole-cell patch clamp electrophysiology, the I$_{CRAC}$ is reduced by AA and endomorphin-1 (EM), concluding that Orai-SOCs are the specific targets in the MOR-mediated intracellular pathway.
METHODS

Cell Culture

All animals used in this study were Sprague-Dawley neonatal rats, sacrificed using rapid decapitation at 2-4 days old. All procedures were done in accordance with the regulations of the Institutional Animal Care and Use Committee. Preparation and culture of neurons were followed as previously described (Fieber and Adams 1991). Enzymatically dissociated neurons were fixed on poly-L-lysine coated coverslips and incubated for 1-5 days in 95% air and 5% CO$_2$ at 37°C. Following cell culture, isolated neurons were used for both calcium imaging and electrophysiological studies.

Ratiometric Calcium Fluorometry Measurements

Fluctuations in intracellular calcium concentrations were measured using florescent imaging techniques. Fura-2-acetoxyethylster (fura-2-AM), was used as the indicator to measure [Ca$^{2+}$]$_i$ in the intracardiac neurons. Cells plated on coverslips were incubated for 1hr. in physiological saline solution (PSS): 140 mM NaCl, 3 mM KCl, 2.5 mM CaCl$_2$, 1.2 mM MgCl$_2$, 7.7 glucose and 10 mM Hepes (pH to 7.2 with NaOH) and 3µM fura-2-AM plus 0.1% dimethyl sulfoxide
(DMSO). All solutions were administered focally via a rapid application system (Cuevas and Berg, 1997).

Specifications for calcium imaging experiments are as previously described (DeHaven and Cuevas 2004). Fluorescence emissions were captured using a Sensicam digital CCD camera (Cooke Corp., Auburn Hills, MI) and recorded using Slidebook Version 3 Software (Intelligent Imaging Innovations, Denver, CO). Changes in $[\text{Ca}^{2+}]_i$ were calculated using Slidebook 3 software from the intensity of the emitted fluorescence following excitation with 340- and 380-nM light, respectively using the Grynkiewicz equation:

$$[\text{Ca}^{2+}]_i = K_d Q(R - R_{\text{min}})/(R_{\text{max}} - R) \quad (\text{Eq. 1})$$

where $R$ denotes the fluorescence intensity ratio ($F_{340}/F_{380}$) as determined as a result of experiments, $Q$ is the ratio of $F_{\text{min}}$ to $F_{\text{max}}$ at 380 nM and $K_d$ is the $\text{Ca}^{2+}$ dissociation constant for fura-2. Calibration of the system was performed using fura-2 calcium imaging calibration kit (Molecular Probes, Inc., Eugene, OR). These values were determined as follows: $F_{\text{min}}/F_{\text{max}} = 224$; $R_{\text{min}} = 0.198977$; $R_{\text{max}} = 7.5354$. 
Whole-Cell Patch Clamp Electrophysiology

Coverslips containing neurons cultured as described above were transferred to a recording chamber mounted on a phase contrast microscope and visualized at 400X. Intracardiac neurons were electrically accessed using whole cell patch clamp recording techniques as described previously (Cuevas and Adams 1994). Cells were exposed to normal PSS, PSS nominally calcium free (NCF), which contained no added CaCl$_2$ or divalent-free PSS (DVF), which contained no added CaCl$_2$ and 1 mM EDTA. The pipette solution consisted of (in mM): 75 CsSO$_4$, 55 CsCl, 10 EGTA, 5 MgSO$_4$ and 10 Hepes. All recordings were made at room temperature. Currents were amplified and filtered using an Axoclamp-200B Amplifier, digitized with a 1322A DigiData digitizer (20 kHz), and collected using a computer running the Clampex 9.2 software (Axon Instruments, Inc., Foster City, CA, USA). Data were subsequently analyzed using Clampfit 9.2 (Axon Instruments, Inc.).

Reagents and Statistical Analysis

The following drugs were used in this study: arachidonic acid (AA), thapsigargin (TG) (Alamone Labs, Jerusalem, Israel) endomorphin-1 (EM), met-enkephalin (ME), PGD$_2$ and PGE$_2$ (Cayman Chemical, Ann Arbor, MI), OBAA (Santa Cruz, Santa Cruz, CA) and fura-2-AM (Molecular Probes, Inc., Eugene, OR). Imaging data files were converted to text format and imported into SigmaPlot 11 and Clampfit 9.2 for analysis. Statistical analysis was performed by SigmaPlot 11.
using paired and unpaired $t$-tests and Two-Way ANOVAs to determine significance within a group or between groups. The differences between groups are significant if $p<0.05$. 
RESULTS

Opioid Receptor Agonists Inhibit SOC Activity

Experiments were carried out to determine the effects of μ-opioid receptor activation in rat intracardiac neurons. Intracardiac neurons were incubated in 4 µM thapsigarin (TG), an inhibitor of sarco-endoplasmic reticulum calcium-ATPase (SERCA), to passively deplete the endoplasmic reticulum of calcium and elicit CCE. Cells were initially bathed in divalent-free PSS, followed by a 2 min application of normal PSS. The subsequent increase in intracellular calcium concentration ([Ca$^{2+}$]) is defined as SOCE or CCE. Figure 3.1 shows quantitatively the effects of EM (D) and ME (B) on CCE and representative traces of [Ca$^{2+}$]$_i$ at each sampling interval (A&C). Cardiac neurons were pre-incubated in met-enkephalin (ME), a μ-opioid specific agonist, for a minute and applied during the reintroduction of a 2.5 mM calcium solution (Figure 3.1 A-B). ME depressed SOC activity considerably, decreasing peak calcium influx from 272 ±17 nM to 107 ±19 nM. Endomorphin-1 (EM-1) was more potent than met-enkephalin, reducing mean Peak Δ[Ca$^{2+}$]$_i$ from 141±6 nM to 6±1 nM even at 1 µM (Figure 3.1 C-D). Pronounced effects of EM were reversible following a 5min PSS wash (220 ±11 nM). When the two different opioid receptor ligands
were applied to the neurons their SOC activity was attenuated comparably. Therefore it is possible that the MOR is coupled to a G-protein, activating an inhibitory signaling cascade.

**A PTX-sensitive G-protein is Recruited Following μ-opioid Receptor Activation**

Stimulation of MOR in intracardiac neurons has been shown to result in the activation of a PTX-sensitive G-protein. To determine if a PTX-sensitive G protein couples μ-opioid receptors to SOC, we obtained values for $\Delta[Ca^{2+}]_i$ using the add back protocol (as previously described in Figure 3.1) before and after PTX-treated (200ng/µL) cells were exposed to EM. By blocking G-protein activation via PTX, EM-induced depression of SOC activity was reduced, indicative a reduction of percent block from control levels (Figure 3.2). Percent block under control conditions was established by evaluating $\Delta[Ca^{2+}]_i$ following Ca$^{2+}$ add back in naïve intracardiac neurons before and after EM application in the absence of PTX. As shown in Figure 3.1 C-D and Figure 3.2 A, C, and D, EM elicits about a 52 ± 8% block of normal CCE however in populations pre-treated with PTX this block was reduced to about 27 ± 9%. This is substantial evidence that a PTX-sensitive G-protein is an integral component within a MOR-initiated inhibitory pathway that could include PLA$_2$ initiation downstream.
The MOR and AA are Part of a Common Signaling Mechanism that Results in the Loss of SOC Function.

Figure 3.4 includes calcium-imaging experiments that determine if this pathway could be a regulatory mechanism for the CCE. OBAA is unique in comparison to other PLA₂ inhibitors because it lacks structural resemblance to AA itself. Traditional PLA₂ inhibitors often cause pharmacological effects similar to AA due to their structural similarity to AA. Therefore cultured intracardiac neurons were store-depleted as previously described and pre-incubated with 50 µM OBAA for 1 hr and the EM-induced block of CCE was compared to control groups. As in Figure 3.2, control percent block was determined by measuring $\Delta [\text{Ca}^{2+}]_i$ before and during EM application in the absence of PTX. The opioid agonist elicited a 73.96 ± 4.44 % block in the absence of the inhibitor however in the presence of OBAA this block was reduced appreciably to 21 ± 3%.

Arachidonic Acid Pathway Inhibits CCE

To further dissect the second messenger system linking MOR to SOC in intracardiac neurons, we examined the role of PLA₂ in this cascade. It is important to note that the PTX-sensitive $G_{i/o}$ has been linked to PLA₂ activation (Faber and Sah 2004). Representative traces of $\Delta [\text{Ca}^{2+}]_i$ and quantitative data in Figure 3.4 A-B show that application 6 µM AA markedly reduces SOC activity. AA evokes an 80% block of CCE and this effect was readily reversible upon PSS wash, tripling peak $\Delta [\text{Ca}^{2+}]_i$ levels during the add back following the low AA level
exposure (102 ± 6 nM to 31.5 nM). Also, AA precursor linoleic acid (LA) decreased CCE (245 ± 15 to 132 ± 21). Unlike AA, the effects of LA were not reversible upon wash-out using PSS (data not shown). The effect was only reversible upon application of BSA (Figure 3.4C&D).

Prostaglandins Show No Significant Effect on SOCE

As shown in Figure 3.4, the AA pathway seems to have an appreciable effect calcium entry following store depletion. Therefore, it is imperative to explore whether AA and/or prostaglandins have a direct effect on the SOC channel. To test whether SOCs are influenced by prostaglandins, rat intracardiac neurons were depleted using TG as described before and mean peak calcium concentration was measured intracellularly following calcium add back in the absence and presence of PGD₂ (Figure 3.5 A-B) and PGE₂ (Figure 3.5 C-D). The results suggest that neither prostanoid has the ability to effect SOC channel activity.

Opioid Agonists Block the I_{CRAC}

Whole-cell patch clamp electrophysiological experiments were used to gain further insight into the specific ion channel modulated by MOR. It is important to note that certain conditions were followed to ensure that the I_{CRAC} only, as opposed to another calcium current, was evoked. First, neurons were
hyperpolarized using voltage steps of -60mV to -100mV, below range for activation of VGCCs. Second, stores were depleted by bath application of 10 mM caffeine and calcium chelator EGTA (1 mM) in the pipette solution. A nominally free calcium solution was able to block the current, indicative of the $I_{\text{CRAC}}$.

Figure 3.6 shows a time course of the changes in mean peak current in response to differences in the contents of the applied bath solutions. First, electrical access is gained in the 2.5 mM Ca$^{2+}$ solution, then store depletion and stimulation of the CRAC current is achieved using caffeine (10 mM) in a NCF for about 5 min. Then, the currents were evoked using a Na$^{+}$-rich divalent free solution. To induce MOR activation, 1 µM EM and 10 µM ME were added to the bath solution. The mean peak current is greatly diminished from -151 ± 15 pA to -95 ± 15 pA (n=6) upon application of EM and -199 ± 43 pA to -82 ± 16 pA with ME (n=5) (Figure 3.7). Reintroduction of a DVF solution reverses this response. These results are similar to the changes in [Ca$^{2+}$]$_{i}$ seen Figure 3.1.

**AA Inhibits the $I_{\text{CRAC}}$ in Intracardiac Neurons similar to Opioidergic Agonists**

To determine if AA can mimic the effects of MOR activation, a similar time course was used as in Figure 3.6 and 3.7. We applied 6 µM AA to the bath to test proposed SOC inhibition due to activation of an intracellular opioid signaling cascade. Similar to results demonstrated in Figures 3.6 and 3.7, mean peak
currents were diminished from maximum of $-116 \pm 16$ pA during the time course to $-40 \pm 8$ pA. In addition, to eliminate the possibility that arachidonic acid-regulated calcium currents (ARCs) are being activated during the AA application, AA was added to the inhibitory NCF solution following AA depression of the current. AA was unable to potentiate any calcium current in the NCF solution (Figure 3.9). As previously seen in prior experiments, the NCF solution, even in the presence of AA, was attenuated ($105 \pm 37$ pA to $78 \pm 12$ pA) the current (Figure 3.8).
Figure 3.1. MOR agonists depress CCE.  

A, Representative traces of Δ[Ca$^{2+}$] from a single neuron for before (control, solid) and after a 5 min met-enkephalin (10 µM) treatment (ME, dotted) for calcium add-back. Line above trace represents a 2 min 2.5 mM Ca$^{2+}$ application.  

B, Bar graph of mean (± SEM) peak Δ[Ca$^{2+}$] for control and met-enkephalin (ME)-treated groups in response to calcium add-back. Asterisk above ME bar indicates significant difference from control condition (n=27, P < 0.001).  

C, Representative traces of Δ[Ca$^{2+}$] evoked using the add-back protocol for control (solid), 1 µM endomorphin-1 (EM, dotted), and following a 5 min PSS wash of the µ-opioid receptor agonist (Wash, dashed). Line above trace represents a 2 min 2.5 mM Ca$^{2+}$ application.  

D, Bar graph of mean (± SEM) values of peak Δ[Ca$^{2+}$] obtained using the add-back protocol and the same conditions as (C) (n=50). Asterisk above EM bar indicates significant difference from control group (P < 0.001).
Figure 3.2. A PTX-sensitive G-protein is Recruited Following MOR Activation. A, Representative traces of $\Delta[Ca^{2+}]_i$ for a single neuron recorded in the absence (solid, PSS) and presence (dashed, EM) of 1 µM EM. Line above trace represents a 2 min 2.5 mM Ca$^{2+}$ application. B, Representative traces of $\Delta[Ca^{2+}]_i$ for a single neuron exposed to 200 ng/mL overnight, PTX alone (solid, PTX), and PTX plus 1 µM endomorphin-1 (dashed, PTX + EM). C. Bar graph of mean (± SEM) values of peak $\Delta[Ca^{2+}]_i$ obtained from cells (n=21) in PSS (black, PSS) in the absence (Control) and presence of endomorphin-1 or following preincubation (n=23) in PTX (PTX, grey) in the absence (Control) and presence (EM) of EM. Asterisk above EM in PSS shows significance from the PSS Control (P<0.05). Pound above PTX in PSS shows significance from PSS (P<0.05). The two-Way ANOVA method of statistical analysis was used to determine significance. D, Bar graph of mean percent inhibition of peak $\Delta[Ca^{2+}]_i$ observed when endomorphin-1 was applied alone (PSS, n = 21) or following preincubation in PTX (PTX+EM, n=23). Asterisk above PTX+EM bar indicates significant difference from PTX group (P < 0.001).
Figure 3.3. OBAA Reverses MOR-Induced Inhibition of SOCE.  A, Representative traces of $\Delta [Ca^{2+}]_i$ for a single neuron recorded in the absence (solid, PSS) and presence (dashed, EM) of 10 $\mu$M EM. Line above trace represents a 2 min 2.5 mM Ca$^{2+}$ application.  B, Representative traces of $\Delta [Ca^{2+}]_i$ for a single neuron exposed to the PLA$_2$ inhibitor, 50 $\mu$M OBAA (solid, OBAA), and the PLA$_2$ inhibitor plus 10 $\mu$M EM (dashed, OBAA + EM). Cell was exposed to OBAA for 1 hr prior to the experiments being carried out.  C, Bar graph of mean (± SEM) values of peak $\Delta [Ca^{2+}]_i$ obtained from cells (n=67) in PSS (black, PSS) in the absence (Control) and presence of EM or following preincubation (n=41) in OBAA (OBAA, grey) in the absence of EM (Control) and presence of EM (EM). Asterisks above EM in PSS shows significance from the PSS Control (P<0.05). Asterisk above OBAA bar indicates significant difference from Control group (P<0.001).
Figure 3.4. AA and LA Inhibit CCE. A, Representative traces of $\Delta$[Ca$^{2+}$]$_{i}$ for a single neuron using the add back protocol for control (solid, Control), a treatment of 6 µM AA (dashed, AA), and a PSS wash (dotted, Wash) for add back duration. Line above trace represents 2.5 mM Ca$^{2+}$ application. B, Bar graph of values mean (± SEM) Peak $\Delta$[Ca$^{2+}$]$_{i}$ for control, drug-treated, and wash groups (n=15). Asterisk above AA and Wash bars indicate significant difference from control group (p <0.001). C, Representative traces of $\Delta$[Ca$^{2+}$]$_{i}$ from a single neuron following Ca$^{2+}$ add back before (solid, Control) and after LA 6 µM (LA) exposure (dotted, LA) and following 0.05 mg/mL BSA wash out (dashed, Wash). D, Bar graph of values mean (± SEM) peak $\Delta$[Ca$^{2+}$]$_{i}$ for control, drug-treated, and wash groups for the duration of the add back (n=30). Asterisk above LA bar indicates significant difference from control group.
Figure 3.5. Prostaglandins Show No Significant Effect on SOCE. A, Representative traces of $\Delta [\text{Ca}^{2+}]_i$ from a single neuron for before (solid, control), after a 5 min PGD$_2$ (1 µM) treatment (dotted, PGD$_2$), and wash (dashed, Wash). Line above trace represents a 2 min 2.5 mM Ca$^{2+}$ application. B, Bar graph of mean (± SEM) peak $\Delta [\text{Ca}^{2+}]_i$ for control and PGD$_2$ treated groups in response to calcium add-back (n=139). C, Representative traces of $\Delta [\text{Ca}^{2+}]_i$ evoked using the add-back protocol for control, 1 µM PGE$_2$ (dotted, PGE$_2$), and following a 5 min PSS wash of the prostanoid (dashed, Wash). Line above trace represents a 2 min 2.5 mM Ca$^{2+}$ application. D, Bar graph of mean (± SEM) values of peak $\Delta [\text{Ca}^{2+}]_i$ obtained using the add-back protocol and the same conditions as (C) (n=48).
Figure 3.6. EM blocks the CRAC. Mean Peak currents were measured from intracardiac neurons (n=6). Electrical access was obtained in a 2.5 mM calcium-containing bath solution with a holding current of -60 pA. Cells were dialyzed with a 75 mM CsCl/55 mM CsSO4/10 mM EDTA patch pipette solution and stepped from -60 mV to -100 mV. Then a nominally calcium-free (NCF) solution containing 10 mM caffeine was applied to the bath to assist with the depletion of intracellular stores for 5 min. Then, the divalent-free solution (DVF) was bath applied to replace any Ca$^{2+}$ ions with sodium ions so that Soc activity could then be measured as function of current carried by Na$^+$ ions. The Na$^+$-$I_{\text{CRAC}}$ was attenuated by bath application of EM-1 (1 µM) in a DVF solution for 2 min following by a 2 min wash-out. Then a nominally calcium free solution was applied to ensure the presence of the $I_{\text{CRAC}}$ only and no other current was evoked during the course of the recording.
Figure 3.7. ME blocks the CRAC current. Mean Peak currents were measured from intracardiac neurons (n=5). Electrical access was obtained in normal 2.5 mM calcium containing bath solution with a holding current of -60 pA. Cells were dialyzed with a 75 mM CsCl/55 mM CsSO4/10 mM EDTA patch pipette solution and stepped from -60 mV to -100 mV. Then a nominally-free calcium solution containing 10 mM caffeine to was used assist with the depletion of intracellular stores for 5 min. Then, the divalent-free solution was bath applied to replace any Ca\(^{2+}\) ions with sodium ions. The Na\(^{+}\)-I\(_{CRAC}\) was attenuated by bath application of ME (10 \(\mu\)M) in a DVF solution for 2 min following by a 2 min wash-out.
Figure 3.8. AA Inhibits the $I_{\text{CRAC}}$ similar to Opioidergic Agonists. Mean Peak currents were measured from intracardiac neurons ($n=5$). Electrical access was obtained in normal 2.5 mM calcium containing bath solution with a holding current of -60 pA. Cells were dialyzed with a 75 mM CsCl/ 55 mM CsSO₄/ 10 mM EDTA patch pipette solution and stepped from -60 mV to -100 mV. Then a nominally-free calcium solution containing 10 mM caffeine was used to assist with the depletion of intracellular stores for 5 min. Then, the divalent-free solution was bath applied to replace any Ca$^{2+}$ ions with sodium ions so that Soc activity could then be measured as function of current carried by Na$^+$ ions. The Na$^+$-$I_{\text{CRAC}}$ was attenuated by bath application of AA (3 µM) in a DVF solution for 2 min following by a 2 min wash-out.
Figure 3.9. AA Failed to Activate any Calcium Current. Mean Peak currents were measured from intracardiac neurons (n=5). Electrical access was obtained in normal 2.5 mM calcium containing bath solution with a holding current of -60 pA. Cells were dialyzed with a 75 mM CsCl/ 55 mM CsSO₄/ 10 mM EDTA patch pipette solution and stepped from -60 mV to -100 mV. Then a nominally-free calcium solution containing 10 mM caffeine was used to assist with the depletion of intracellular stores for 5 min. Then, the divalent-free solution was bath applied to replace any Ca²⁺ ions with sodium ions. The Na⁺-ICRAC was attenuated by bath application of AA (3 µM) in a DVF solution for 2 min following by a NCF solution containing AA to ensure that there were no AA-modulated currents evoked during the electrophysiological recordings involving AA.
Discussion

The outcome of this study supports the hypothesis that MOR activation and AA acid inhibit SOC channel activity. Additionally, it is likely that opioid receptor activation exerts its inhibitory effects through the production of AA via a PTX-sensitive G-protein. Calcium imaging and electrophysiology confirms the hypothesis that MOR agonists inhibit SOC function and the \( I_{\text{CRAC}} \) (Figures 3.1, 3.6 and 3.7). This finding along with studies that suggest SOC activity regulates action potential firing indicates that MOR activation may also suppress intracardiac neuronal function (DeHaven and Cuevas 2004). While it has been firmly established that opioids exert their cardioinhibitory effects primarily via the CNS, studies involving intracoronary administration of opioid receptor antagonist naloxone suggest that opioids may operate more locally to modulate cardiac function (Caffrey, Gaugl et al. 1985). Results in the current study and the fact that peripheral opioid concentrations spike in cases of congestive heart failure and circulatory shock align with the results of the aforementioned study (Barlow, Deo et al. 2006; Vargish, Reynolds et al. 1980; Schadt and York 1981; Curtis and Lefer 1982).
Studies in rat amygdala pyramidal cells and in rat supraoptic nucleus neurons suggest that MOR activation changes excitability in these neurons through a PTX-sensitive G-protein therefore we hypothesize that MOR in intracardiac neurons may also be G-protein linked (Brown, Johnstone et al. 2000). In Figure 3.2 SOC activity was restored once the G-protein was inactivated suggesting that a PTX-sensitive peptide must be activated upstream of \( \text{PLA}_2 \) induction.

Application of AA inhibitor, OBAA, was used in conjunction with receptor agonist treatment to ascertain whether \( \text{PLA}_2 \) activation is a result of MOR activation. A comparison of the percent block exhibited for the PSS control group and percent block for the groups pre-treated with the inhibitor before and after opioid application was established to account for any differences in basal SOC activity endogenous to the two groups. In accordance with the current findings, AA production following opioid receptor activation has been discovered in rat amygdala pyramidal neurons. Their studies also show, following receptor activation, \( G_{i/o} \) is activated and as a result \( \text{PLA}_2 \) is induced (Faber and Sah 2004).

Furthermore, similar imaging experiments also show that application of 30\( \mu \)M AA abolishes the SOC current induced following store depletion with TG; its effects were not readily reversible. A normal PSS wash was unsuccessful in reversing \( I_{soc} \) depression; bovine serum albumin (BSA), which binds free fatty acids, was mandatory to restore normal channel activity without any direct effects
on the channel itself (*data not shown*). Previous studies have shown similar inhibition of SOC activity with lower concentration therefore after a 10-fold decrease in AA concentration, effects were indeed reversible with a PSS wash (Rychkov, Litjens et al. 2005). Similarly, the $I_{\text{CRAC}}$ was blocked following 6 $\mu$M AA application in rat intracardiac neurons. Successive I-clamp electrophysiology studies would need to be performed to determine if there is a consequence on membrane properties of the neuron in the presence of AA. However this finding is novel because, to date, it has not been displayed in excitable cells.

Membrane phospholipid degradation is altered during myocardial ischemia shown by the alteration of fatty acid concentrations in vivo. Interestingly, AA and its precursor LA are both upregulated in cardiac tissue following ischemia and reperfusion. Therefore in Figure 3.4 A-B the essential dietary fatty acid was used to determine its effects on the intracardiac neurons that are closely associated with the myocardium. LA blocked SOCE similar to AA which could suggest a compounded loss of function of the SOC channel than seen in the presence of AA alone during ischemic damage.

Studies in guinea pig intracardiac ganglion show that prostaglandins, by-products of AA, alter the electrical activity of the neurons by attenuating the after-hyperpolarization due in part to a decrease in small –conductance $K_{\text{Ca}}^{2+}$ currents (Jelson, DeMasi et al. 2003). It is also possible that AA and/or prostaglandins may be acting as a modulator of electrical activity by depression of SOC activity via direct channel binding. However in the current study, using the rat as the
model, we show that the identical prostaglandins used in the former investigation, in fact, do not modulate the SOC channels.

There is evidence of a non-capacitive arachidonic acid-mediated current \( (I_{ARC}) \) existing in cell types such as fibroblasts and smooth muscle cells (Munaron, 1997; Broad, 1999). To ensure that no part of the mean peak current is due to a contribution of an \( I_{ARC} \), we omitted the wash-out and inserted NCF with 3 \( \mu \text{M} \) AA into the conclusion of the time course (Figure 3.9). Inhibition in the DVF solution by AA was similar (Figure 3.8), most importantly, when AA was introduced into the bath in a NCF solution there was no increase in the mean peak current. Therefore we conclude that the total current evoked in the DVF solution does not contain any AA-modulated current.

It has become evident that opiates exacerbate conditions such as shock (Holaday 1983; Liang, Imai et al. 1987), heart failure (Liang, Imai et al. 1987), and myocardial ischemia (Fagbemi, Lepran et al. 1982). However the exact mechanism by which this phenomenon occurs is unclear. Electrophysiological studies would be necessary to confirm that modulation of SOC activity via MOR activation indeed compromises excitability, even though previous studies indicate that it is probable (Smith and Adams 1999). This study implicates two potent inhibitors of parasympathetic input to the heart that may be a part of a common pathway for inhibition of this critical local circuit.
REFERENCES


CHAPTER 4

NEUROCHEMICAL AND GENETIC DETERMINANTS OF EXCITABILITY OF INTRACARDIAC NEURONS

INTRODUCTION

There are a wide variety neuropeptides within the ganglia that control communication within the ganglia by modulating neurotransmitter release. Among these neuropeptides, cardiac met-enkephalin (ME) production is increased in spontaneous hypertensive rats (SHR) in comparison to normotensive rats (Dumont, Ouellette et al. 1991). Similarly, the circulating levels of the peptide, endomorphin (EM), are increased in the ventricles of heart transplant patients (Ationu, Sorensen et al. 1993). Given that both of these peptides activate $\mu$-opioid receptors, the possibility exists that these peptides may affect cardiac function in diseased hearts. It is therefore of interest to determine if $\mu$-opioid receptor (MOR) activation can alter the function of intracardiac neurons and affect parasympathetic input to the heart. Pituitary adenylate cyclase activating peptide (PACAP) which is found within the
mammalian intracardiac ganglion, promotes neuroexcitability through intracellular calcium mobilization in neurons from this ganglion (DeHaven and Cuevas 2004). In contrast, ME elicits elevations in intracellular calcium in intracardiac neurons by promoting Ca\(^{2+}\) release from intracellular stores (Smith and Adams 1999), but has been suggested to depress the excitability of these cells (Adams and Trequattrini 1998). The study by Adams and Trequattrini, however, focused primarily on \(\mu\)-opioid receptor regulation of N-type calcium channels (1998). The effects on excitability were largely inferred by studies on single action potential firing (Adams and Trequattrini 1998).

We have determined that there is a store-dependent, inwardly rectifying current that is carried by Na\(^+\) ions in the absence of extracellular calcium in individual neurons. This current, \(I_{\text{CRAC}}\), is inhibited by the SOC antagonist, Gd\(^{3+}\) at exclusive concentrations as well as arachidonic acid, as shown in Chapter 3. The PLA\(_2\)–dependent free fatty acid has been also responsible for depression of the \(I_{\text{SOC}}\) in rat liver cells and HEK293 cells (Rychkov, Litjens et al. 2005; Luo, Broad et al. 2001).

The central hypothesis of this study is based upon the studies that report that both \(\mu\)-opioid receptor agonists and AA are present in the heart at supranormal levels following a myocardial infarction and could be responsible for abnormal cardiac function through the alterations in intracellular calcium concentration (Barlow, Deo et al. 2006; Van der Vusse, Reneman et al. 1997). In addition to AA and opioidergic agonists having analogous consequences on
cardiovascular function, there is also the possibility that they are a part of a common pathway in the intracardiac neuron, as observed in other excitable cell types such as periaqueductal grey and pyramidal neurons (Vaughan, Ingram et al. 1997; Faber and Sah 2004).

While this and other studies suggest that both AA and opioid receptor agonists have deleterious effects on Ca$_{2+}$ channel conductance and the activity of excitable cells, the physiological consequences of SOC inhibition, specifically, by way of AA and $\mu$-opioid activity in excitable cell types are unknown. This is not surprising considering the lack of conclusive information relating to SOC expression and function in neuronal cell types.

Studies similar to the current examining modes of SOC modulation in excitable and non-excitable cell types are beginning to emerge. The pore-forming subunit of SOCs, the Orai protein was previously thought to absent in neuronal cell types. Therefore, to date, there have not been any comparative assessments of Orai channel subunit expression among neuronal subpopulations partly because SOC-dependent modulation of excitability is a fairly novel concept. Even though there are a plethora of factors that direct action potential discharge such as neurochemical influences and synaptic inputs, understanding ion channel protein expression is mandatory in assembling a comprehensive analysis of neuronal function.

There are individual morphologies associated with guinea pig intracardiac neurons: unipolar, multipolar, and bipolar termed S, SAH, and P, respectively.
Each cell type also has disparate electrophysiological profiles. For instance, P cells display mostly tonic firing whereas S and SAH cell are more associated with either single or adaptive firing. Relevant to the current study, there are two main electrophysiological subtypes of rat intracardiac ganglionic neurons, short (I) and prolonged after-hyperpolarizations (II). Type I neurons are further subdivided into I_b and I_m, based on the action potential bursts and multiple firing, respectively, in response to prolonged membrane depolarizations. Constituting only 8% of the ganglionic population, Type II neurons fire tonically, similar to I_m. The exact mechanisms by which these neurons manifest these intrinsic firing patterns independent of factors such as neurotransmitter release have not been fully described.

This study explores the possibility of Orai-SOC channel expression influencing endogenous firing patterns in neonatal rat intracardiac neurons. Orai1 or CRACM1, the pore-forming subunit of the CRAC channel, was discovered as a result of its central role in the pathology of severe combined immune deficiency (SCID). T-cells expressing mutated Orai protein sequences are incapable of Ca^{2+}-dependent activation, rendering the cells incapable of cell proliferation (Thompson, Mignen et al. 2009). While the importance of Orai channel expression and functionality has been explored in immune cells, little is known about CRAC channel contribution to neuronal function. The lack of information is due, in part, to the fact that VGCC have been viewed as the primary channels involved in Ca^{2+} entry into excitable cells.
Orai subunit expression differs among tissue types. Orai1 and 3 are found predominately in lymphoid organs, skeletal muscle, hepatocytes, and skin (Gwack, Srikanth et al. 2008; Mignen, Thompson et al. 2008). Contrastingly, Orai2 is found mostly in the brain, kidney, lung and spleen (Gross, Wissenbach et al. 2007; Gwack, Srikanth et al. 2007). However Orai expression among neuronal subpopulations remains a mystery. Orai1 is capable of homodimerization and can assemble with Orai2 and Orai3 to form heteromultimers (Gwack, Srikanth et al. 2007). On the other hand, only Orai1 has been identified as functional in native cells.

Three Orai subunits each have different pharmacologies and biophysical properties. Overexpression of Orai1 in HEK 293 cells yields 3-fold higher current amplitude and displays a more robust Ca\(^{2+}\)-dependent inactivation than Orai2 and 3 expressed in the same cells. Conversely, fast Ca\(^{2+}\)-dependent inactivation is pronounced in cells overexpressing Orai3 while Orai1 and 2 display more moderate fast Ca\(^{2+}\)-dependent inactivation. Furthermore, the SOC antagonist 2-aminoethoxyphenyl borate (2-APB) elicits its effects on Orai-SOCs through multifaceted mechanisms including modulation of the intracellular Ca\(^{2+}\) sensor Stim as well as direct influences on Orai subunits. Orai1 and 3 are potentiated through direct interactions in transfected HEK293 cells whereas Orai1 and Orai2- I\(_{\text{CRAC}}\) currents are decreased in cells transfected Stim1. This finding implies that the kinetics could vary considerably depending on subunit expression. Considering the potential alterations channel expression could have
on cellular function, we must explore the possibility of modified cellular function with respect to subunit expression (Lis, Peinelt et al. 2007).

First, this study determines the possible contribution of SOCs to excitation of the neuron; second we used analogous experimental conditions to elucidate whether AA and opioid agonists alter action potential firing in dissociated intracardiac neurons. Finally, this study serves as an electrophysiological and molecular study of the contribution Orai expression has on the innate electrical activity of intracardiac ganglionic neuronal subtypes.
METHODS

Cell Culture

All animals used in this study were Sprague-Dawley neonatal rats, sacrificed using rapid decapitation at 2-5 days old. All procedures were done in accordance with the regulations of the Institutional Animal Care and Use Committee. Preparation and culture of neurons were followed as previously described (Fieber and Adams 1991). Enzymatically dissociated neurons were fixed on poly-L-lysine coated coverslips and incubated for 2-5 days in 95% air and 5% CO₂ at 37°C. Following cell culture, isolated neurons were used for electrophysiological studies.

Whole-Cell Patch Clamp Electrophysiology

Neurons from neonatal rats were enzymatically dissociated and mounted on poly-L-lysine coated coverslip as previously described. The coverslips were transferred to the recording chamber of a phase contrast microscope (400X). Voltages were measured at room temperature in PSS: 140 mM NaCl, 3 mM KCl, 2.5 MgCl₂, glucose, 10 mM HEPES, and 2.5mM CaCl₂. Whole-cell perforated patch clamp technique was utilized as previously described to prevent loss of
cytoplasmic contents and preserve native physiological responses (Rae, Cooper et al. 1991; Xu and Adams 1992; Cuevas and Adams 1996). The pipette filling solution contained (mM): 75 K$_2$SO$_4$, 55 KCl, 5 MgSO$_4$, 10 Hepes, 198 µg/mL amphotericin B, and 0.4% DMSO. The dialyzing method was used for PCR experiments; therefore amphotericin was omitted from the filling solution and action potentials were recorded no longer than 5 min following gaining electrical access (Cuevas, Harper et al. 1997). Currents were amplified and filtered using an Axoclamp-200B Amplifier, digitized with a 1322A DigiData digitizer (20 kHZ), and collected. Clampex 9 (Axon Instruments, Inc., Foster City, CA, USA). Clampfit, pClamp 9 program was used for analysis.

**Reagents and Statistical Analysis**

The following drugs were used in this study: arachidonic acid (AA), (Alamone Labs, Jerusalem, Israel) endomorphin-1 (EM) and Gd$^{3+}$ (Sigma), Files were converted to text format and imported into SigmaPlot 11 and Clampfit 9.2 for analysis. Statistical analysis was performed by SigmaPlot using repeat measures One-Way ANOVA on Ranks non-parametric analysis, used for multiple group comparisons.

**Cytoplasm Harvest of Individual Neurons**

Cytoplasmic contents of individual neurons were isolated using negative pressure applied to the pipette following electrical recordings. Pipettes were
filled with ~ 5 μL of filtered pipette solution. Aspirated cellular contents were swiftly expelled by gently scraping the pipette tip along the side of the microcentrifuge tube and carefully applying positive pressure. Individual neurons that were determined by electrophysiological recordings to be of the same subpopulation based on firing patterns (single, adapting, and tonic) were included in the same tube to pool and concentrate genetic material. 5-7 cells were included in every isolation. Due to the small amount of genetic material expected, 1U RNase inhibitor (Promega, Madison, WI) was added to optimize yield.

**Quantitative Real-Time Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR)**

Gene transcripts were detected using qRT-PCR with Orai1, 2, and 3-specific primers (Table 2) (Invitrogen). Using the RNeasy mini kit (Qiagen, Mississauga, ON), RNA was isolated, purified, and used in a two-step reverse transcription and polymerase chain reactions. The Sensiscript Reverse Transcriptase Kit (Qiagen, Mississauga, ON) for reverse transcription reactions of less than 50 ng of RNA was utilized to convert ~45ng of RNA to cDNA. The oligo-dT priming method, more specific than the random hexamer, was used to hybridize and prime poly A tails of mRNA for transcription. Per 20 μL reaction, 1 μL Sensiscript reverse transcriptase, 1 μM oligo (dT) primer, 5 μM dNTP, Sensiscript reaction buffer and 1U RNase inhibitor (Promega, Madison, WI)
were added to water and allowed to incubate at 37°C for 60 min. Gene amplification was achieved using the SYBR® Green JumpStart™ Taq ReadyMix™ (Sigma, St. Louis, MO) and 500 nM primers for each Orai gene. The reaction was performed in an Applied Biosystems StepOnePlus™ Real-Time PCR System (Foster City, CA) at 95°C for 15 min, followed by 60 cycles at 95°C for 1 min and 53°C for 30s. No template controls were included for each gene and melt curves yielded a single peak, indicating specific amplification. Endogenous control, GAPDH was included to account for sample-to-sample variations in reaction performance and errors in sample quantification.

The comparative cycle threshold (Ct) method was employed to determine relative gene expression. Each reaction was performed in triplicate and mean cycle threshold values were determined. Mean delta cycle threshold (ΔCt) values are determined as the difference between the Ct value obtained from the GAPDH control sample for each neuronal subtype and the Ct value generated from the genes of interest (Orai1, Orai2, Orai3) for each neuronal subtype:

\[
(\Delta Ct = Ct_{(Orai)} - Ct_{(GAPDH)})
\]

(Eq. 2)

Significance was determined using the repeat measures One-Way ANOVA on ranks non-parametric statistical analysis.
Table 2. Specific primer sets for Orai 1, 2, and 3 (Ohana, Newell et al. 2009)

<table>
<thead>
<tr>
<th>Orai</th>
<th>F:</th>
<th>R:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Orai1</td>
<td>GACTGGATCGGCCAGAGTT</td>
<td>GAGAGCGAGGAGGAGGTTTC</td>
</tr>
<tr>
<td>Orai2</td>
<td>CCGTGAGCAACATCCACA</td>
<td>CAGCCAGGAAAAGCAGGA</td>
</tr>
<tr>
<td>Orai3</td>
<td>CCACCAGTCACCACACCA</td>
<td>CCAGCCCACCCAAAAACAAAC</td>
</tr>
</tbody>
</table>
RESULTS

**Gadolinium Reduces Action Potential Firing at SOC-specific Concentrations**

To determine whether action potential firing is SOC-dependent, we applied 5 \( \mu \text{M} \) Gd\(^{3+} \) to repetitively firing neurons. Figure 4.1 shows representative traces of action potentials evoked in response to a depolarizing current pulse (500 ms, +100 pA) before (Control), during (Gd\(^{3+} \)) and after wash-out (Wash) of 5 \( \mu \text{M} \) Gd\(^{3+} \). Quantitative data shows a reduction from 7.0 ± 0.4 (Control) to 2.6 ± 0.9 action potentials (Gd\(^{3+} \)), then back to 4.2 ± 1.8 upon wash-out of gadolinium. The difference from control to drug-treated groups is statistically different. Action potential discharge was appreciably reduced in the presence of 5 \( \mu \text{M} \) Gd\(^{3+} \). Upon analysis of the single waveform, there seems to be no effect on the after-hyperpolarization upon application of Gd\(^{3+} \), however the action potential amplitude was increased (Figure 4.1C).

**Tonic Action Potential Firing is Attenuated upon \( \mu \)-Opioid Receptor Activation**

We used whole-cell perforated patch clamp electrophysiology in the current clamp mode to investigate whether opioid agonist, EM-1, has any effect on action potential firing. Figure 4.2A shows a family of voltage responses elicited by 500 msec, +100 pA depolarizing current pulses from a single neuron.
Voltage changes were recorded in the absence (Control), presence (EM-1) and following wash-out (Wash) of 1 µM EM. EM blocked action potential firing appreciably and the inhibitory effects were easily reversible upon wash-out. The opioid agonist reduced the mean number of action potentials from 3.6 ± 0.7 under control conditions to 1.4 ± 0.6 in the presence of the drug. Following wash-out action potential firing was restored to 2.9 ± 0.7 demonstrating the reversibility of the drug (Figure 4.2). The single action potential waveform remained unchanged following EM treatment (Figure 4.2C).

**Arachidonic Acid Inhibits Repetitive Action Potential Firing in Rat Intracardiac Neurons**

The central hypothesis of this study predicates that µ-opioid receptor activation leads to AA production and subsequently, SOCE inhibition. To test whether AA has any influence on tonic firing, we used the whole-cell patch clamp in the perforated patch clamp configuration in current clamp mode to evoke action potentials by using depolarizing current pulses (500 ms, +100 pA). In Figure 4.3, action potential traces recorded before (Control), during (AA) and following wash-out (Wash) of 3 µM AA are shown.

Prior to AA application, the neurons fired 3.6 ± 0.2 mean action potentials. However following a 5 min application of AA, this mean number decreased significantly to 1.2 ± 0.4 and following a 5 min wash-out of the free fatty acid, the neurons fired a mean number of 3.0 ± 0.8 action potentials over the same
amount of time. There is an increase in the after-hyperpolarization and decrease in the amplitude when the single action potential waveforms are transposed upon one another for analysis.

**Action Potential Firing is Dependent upon Orai Subtype Expression**

Previous electrophysiological studies suggest that there is a Ca\(^{2+}\) store-dependent component to action potential firing in neonatal rat intracardiac neurons. We hypothesize that differences in intrinsic firing patterns may be attributed to differences in Orai subunit expression. We used RT-PCR to determine whether variations in Orai subunit expression contribute to differences in firing patterns. Following electrophysiological data collection, RNA was extracted and purified from neurons. RNA from neurons displaying similar firing patterns was reverse transcribed into cDNA. Then, the cDNA template from each of the experimental groups, single, adapting, and repetitively firing neurons, was submitted to separate qRT-PCR reactions. Specific primers (Table 2) were used to determine the relative levels of mRNA expression of Orai1, 2, and 3. Contrastingly, Orai3 mRNA expression was relatively low for single and adapting neurons for each isolation (n=3). For tonically firing neurons, Orai3 mRNA transcripts were high when compared to the other two neuronal subtypes. However, subunit expression combinations were inconsistent among the isolations (n=3). We can conclude that tonically firing neurons are unique in that they express elevated Orai3 mRNA (Figure 4.4).
Figure 4.1 Low-dose Gadolinium Blocks Action Potential Firing. Representative traces of action potential bursts recorded from a single cell in response to 500 ms, +100 pA current pulses before (Control, black trace), during (Gadolinium, red trace) and following washout (Wash, blue trace) of 5 µM gadolinium (A). Bar graph of the mean number of action potentials ± SEM recorded from 5 neurons under identical conditions as in A (B). Asterisk denotes significant difference from control group (p<0.05). Single action potential wave forms before (Control, black trace), during (Gadolinium, red trace) and following washout (Wash, blue trace) of gadolinium (C). Electrical access was achieved using the perforated patch configuration of the whole cell technique.
Figure 4.2 Tonic Action Potential Firing is attenuated upon MOR Activation

Representative traces of action potential bursts recorded from a single cell in response to 500 ms, +100 pA current pulses before (Control, black trace), during (EM, red trace) and following washout (Wash, blue trace) of 1 μM endomorphin-1 (A). Bar graph of the mean number of action potentials ± SEM recorded from 5 neurons under identical conditions as in A (B). Asterisk denotes significant difference from control group (p<0.05).

Single action potential wave forms before (Control, black trace), during (EM, red trace) and following washout (Wash, blue trace) of EM(C). Electrical access was achieved using the perforated patch configuration of the whole cell technique.
Figure 4.3 AA Inhibits Repetitive Action Potential Firing. Representative traces of action potential bursts recorded from a single cell in response to 500 ms, +100 pA current pulses before (Control, black trace), during (AA, red trace) and following washout (Wash, blue trace) of 3 µM arachidonic acid (A). Bar graph of the mean number of action potentials ± SEM recorded from 5 neurons under identical conditions as in A (B). Asterisks denotes significant difference from control group (p<0.05). Single action potential wave forms before (Control, black trace), during (AA, red trace) and following washout (Wash, blue trace) of the fatty acid (C). Electrical access was achieved using the perforated patch configuration of the whole cell technique.
Figure 4.4 Comparative qRT-PCR Results Orai 1, 2, and 3 mRNA expression for Single, Adapting, and Multiple Firing Neurons. Mean $\Delta$Ct values were determined from $\Delta$Ct values from 3 separate RNA extractions for each neuronal subtype. Asterisk denotes significant difference of Orai3 subtype mRNA expression from the multiple firing group in comparison to the other neuronal subtypes ($p<0.05$).
Discussion

The main discovery of this study is, first, that store-operated calcium channels contribute to repetitive action potential firing. Second, AA and EM both decrease the number of action potentials fired per unit time in cardiac neurons. These findings suggest that the reduction of SOC activity via the activation of a μ-opioid receptor-mediated pathway has the ability to affect the electrical properties of the neuron. This study is novel because most of the previous studies of SOCs and their consequences on cellular physiology are in non-excitable cell types. Now we can begin to fully understand the functional significance and mechanism of action of SOCs in the modulation of action potential firing not only in intracardiac neurons but all excitable cell types.

The most accepted antagonist for SOCE, Gd$^{3+}$ at low micromolar concentrations, was able to reduce action potential firing which suggests that SOC channels have at least some contribution to action potential firing. We may exclude the possibility of the VGCC blockade because of their decreased sensitivity to Gd$^{3+}$. Our lab has shown that Gd$^{3+}$ at low micromolar concentrations (1 -10 μM) is able to reduce capacitative calcium entry in a dose dependent manner (unpublished data). Also, it has been shown to block action
potential firing in baroreceptor neurons at millimolar concentrations through the inhibition of stretch-activated ion channels (Hajduczok, Chapleau et al. 1994). Future studies of the effects on possible SOC-dependent action potential firing may include other direct SOC antagonists such as 2-APB and SKF-96365 because these antagonists have been proven to be responsible for the inhibition of CCE. Store-dependent calcium entry is due to the interaction of an endoplasmic reticulum associated protein, Stim, to the Orai subunit. Therefore Stim1 antagonist ML-9 may be used in current-clamp experiments as an indirect method of testing SOC effects on action potential firing.

Interestingly Cd\(^{2+}\)-induced blockade of voltage dependent Ca\(^{2+}\) channels reduced the after-hyperpolarization peak but not the duration of the AHP of the action potential; which suggests that there may be an unidentified calcium source in neonatal rat intracardiac neurons (Franciolini, Hogg et al. 2001). It is our hypothesis that SOCs are likely to be the calcium source that is responsible for the maintenance of repetitive firing in mammalian intrinsic cardiac neurons. There is evidence that intracellular calcium oscillations, that is, movement across that endoplasmic reticulum and plasma membranes by SOCs, particularly, lead to long-term repetitive action potential firing (Kusters, Dernison et al. 2005).

These results are analogous to those seen as a result of EM-induced \(\mu\)-opioid receptor activation in the supraoptic nucleus (SON), nucleus tractus solitarius (NTS) and dorsal motor nucleus of the vagus (DMV) (Doi, Brown et al. 2001; Glatzer, Derbenev et al. 2007). It is important to note, relevant to the
current study, that the intracadiac ganglia receives input from the CNS from the DMV therefore it would be likely that EM would play an analogous inhibitory role in the cardiac ganglia.

In addition to Gd$^{3+}$, the consequences of EM and AA on repetitive firing are examined in this study as a part of the central hypothesis which claims that EM-induced attenuation of intrinsic cardiac neuronal activity is a result of the direct action of AA. To test the validity of this theory, EM and AA were applied at concentrations that were shown to block SOCE in imaging experiments, 1µM and 6µM, respectively. Both blocked repetitive firing appreciably, which supports the original hypothesis.

Using qRT-PCR we found that Orai2 was expressed most frequently across multiple RNA isolations, consistently in single and adapting neuronal subtypes, which concurs with previous studies that report Orai2 as being the most abundant in murine brain cells (Gwack, Srikanth et al. 2007). However, a separate study purports mRNA Orai3 only as commonly expressed in human brain cells (Gwack, Srikanth et al. 2007). Orai3 was expressed at high levels in repetitive firing primary neonatal rat intracardiac neurons in comparison to the other subtypes. Proper control of calcium cycling is needed to avoid apoptosis and maintain firing frequency, in the case of excitable cell types. Initiated by Ca$^{2+}$ in the vicinity (3-4nM) of the pore the calcium channel, Ca$^{2+}$-dependent inactivation is a negative feedback mechanism which limits global calcium overload (Zweifach and Lewis 1995; Frischauf, Muik et al. 2009). Fast Ca$^{2+}$-
dependent inactivation is most pronounced for the Orai3-ICRAC in comparison to the remaining two subtypes. Orai1-ICRAC is unique in that it displays slow reactivation whereas Orai2-ICRAC and Orai3-ICRAC exhibit subsequent slow inactivation (Lis, Peinelt et al. 2007). Therefore SOC-dependent action potential firing may be dependent on its inactivation. Using a quantitative model, Kusters et al. demonstrates a SOC-dependent model in the maintenance of intracellular calcium cycling and action potential firing (2005). Furthermore, it has been proposed that fast inactivation of SOCs serves to diminish the role of fast Ca\(^{2+}\)-activated K\(^+\) currents in the Ca\(^{2+}\) oscillation mechanism by reducing the voltage gated calcium component of calcium entry during the after-hyperpolarization (Zweifach and Lewis 1995).

V-clamp experiments would need to be performed to definitively assess the contribution of various SOC inactivations on excitability in these primary cultured neurons. As most of the previous studies involve expression systems, the results may vary greatly. Intracellular calcium sensor, Stim interacts with Orai subunits to activate SOCs and exists in two different species, Stim1 and 2. The current study does not take into account differences in Stim expression. Therefore when interpreting the results presented here, one must keep in mind that Stim1 alone or in combination with Stim2 could also be playing a role in SOC-dependent action potential firing. The limitations associated with this study may help explain inconsistent molecular data within the experimental groups. However it maintains its significance in that it exists as the first of its kind to
determine the potential effect of protein expression, namely a non-voltage activated calcium channel, on action potential discharge within parasympathetic subtypes (Won, Whang et al. 2006).

The neurohumoral component plays a central role in the regulation of the activity of these ganglia and these neuropeptides function in altering the membrane properties of individual neurons through their differential influences on Ca\(^{2+}\) channels (Adams and Trequattrini 1998; DeHaven and Cuevas 2004). Each Orai subunit has distinct biophysical properties; therefore we hypothesize that differences in expression may contribute uniquely to the action potential waveform, namely the AHP via large conductance calcium-activated potassium channel (BK\(_{Ca}^{2+}\)), and ultimately firing frequency.

Serving as the final common circuit for the processing of parasympathetic input, the mammalian intracardiac ganglia exists as a very intricate network of postganglionic neurons within the cardiac nerve plexus. There are intraganglionic as well as interganglionic connections that orchestrate this integration as well as providing a local means for regulation of the end organ. In disease states such as congestive heart failure, proper integration of this information becomes disrupted, having deleterious effects on cardiac function. The likelihood of this study having overwhelming implications in the pathology of heart failure is small considering the complex architecture and neurochemistry associated with these ganglia that may also be influencing firing frequencies. However these findings could provide information towards the subsequent
exploration of Orai channels of the cardiac plexus and their regulatory mechanisms as potential therapeutic targets for heart failure.
REFERENCES


CHAPTER 5

SUMMARY

The data presented here serve as a comprehensive study of the contribution store-operated calcium channels (SOCs) have to neuronal activity along with the consequences of opioidergic regulation of SOC channels. While an excitatory pathway involving SOC activity has been described in the modulation of intracardiac neurons (DeHaven and Cuevas 2004), this study offers an inhibitory mode of regulation for SOC channels mediated by μ-opioid receptor (MOR) activation. Furthermore, a comparative study was conducted to determine the link between Orai expression and action potential discharge. We concluded that repetitive firing may be dependent upon Orai3 expression within intracardiac neuronal subpopulations.

The current study is motivated by previous work elucidating the effects of various ganglionic neuropeptides on cellular function. Cuevas and Adams found that VIP potentiates ACh-evoked currents in rat intracardiac neurons via activation of a PTX-sensitive G-protein (1996). Contrary to previous studies that claim cAMP is produced following Gs recruitment in neural and epithelial cells,
the study was also able to determine that diffusible second messenger cAMP was not activated following vasointestinal polypeptide (VIP) receptor activation in rat neonatal parasympathetic neurons. In order to study single channel conductance, the study utilized excised membrane patch experiments. Outside-out membrane patches involve the detachment of small sections of the membrane with the outside of the membrane facing away from the electrode to allow the evaluation of single channel properties. This electrophysiological method revealed that VIP augments the ACh receptor channel open probability. This phenomenon is thought to be responsible for the effects of VIP on cardiac function (Cuevas and Adams 1996). Modulating nicotinic cholinergic receptors (nAChRs), VIP and pituitary adenylate cyclase-activating peptide (PACAP) initiate pathways that have a common G-protein (G_o) in common. Using PACAP6-38, a PAC_1 and VPAC_2 antagonist, this group also found that VIP elicits its synergistic effects via its binding to the VPAC_1 receptor (Liu, Cuevas et al. 2000). Contrastingly, exogenous VIP enhances muscarinic synaptic transmission and excitatory responses within superior cervical ganglia (Kawatani, Rutigliano et al. 1985).

One study in particular done by our lab, explores the effects of cardiac peptides VIP and PACAP on neuronal function and the mechanisms by which they exert their effects. The study concluded that PACAP and VIP promoted action potential firing via a mechanism dependent on the mobilization of calcium from intracellular stores. Using fura-2 calcium imaging, a biphasic response
could be observed, intracellular store release followed by an influx into the cellular cytosol. The latter phenomenon was suggestive of store-operated calcium entry.

In a recent study, muscarinic and nicotinic cholinergic receptors have differential effects on calcium mobilization. The Adams group shows pharmacologically that the nACh elicits an ionotropic response involving calcium influx and subsequent calcium-induced calcium release (CICR) from ryanodine-sensitive stores. On the other hand, mACh induces a metabotropic response that initiates phospholipase C (PLC), leading to the activation of phosphatidylinositol 4,5-bisphosphate (PIP$_2$), and production of IP$_3$ and DAG. The intracellular second messenger responsible Ca$^{2+}$ mobilization from the ER, IP$_3$ binds to the IP$_3$ receptor channel. At this point, Ca$^{2+}$, now cytoplasmic as opposed to membrane bound, rapidly activates Ca$^{2+}$-dependent K$^+$ channels before being buffered by the mitochondria or actively resubmitted into the ER via Ca$^{2+}$-ATPase pump (Beker, Weber et al. 2003). Similary, µ-opioid receptor agonists have been implicated in the production of inositol trisphosphate (IP$_3$) to have similar effects on calcium mobilization in rat intracardiac neurons (Smith and Adams, 1999).

Previous studies confirm that of the five muscarinic receptor subtypes, mRNA transcripts for M1-M4 are present in rat and guinea pig (Hassall, Stanford et al. 1993; Hoover, Baisden et al. 1994). Interestingly, chiefly parasympathetic, the intracardiac neuron is capable of coexpressing more subtypes of the
muscarinic receptor than any other native cell type. Furthermore, electrophysiological studies confirm that M4 receptor activation, specifically, modulates voltage-gated calcium channel activity via a PTX-sensitive G-protein (Cuevas and Adams 1997). Analogous to outcomes reported here, studies previous to the latter suggest that muscarinic receptor modulation occurs through mechanisms that exclude prokein kinase C (PKC), diacylglycerol (DAG), and adenylate cyclase (AC) (Xu and Adams 1993). Also, the M1 receptor has been implicated in the inhibition of $I_m$ in a variety of autonomic neuronal types (Bernheim, Mathie et al. 1992; Cuevas and Adams 1997; Xi-Moy and Dun 1995). Whereas $K^+$ channel activity is suppressed following M2 receptor activation in rat intracardiac neurons through G-protein coupled mechanisms which concurs with signaling cascades that result in the inhibition of the SOC (Allen and Burnstock 1990; Xi-Moy, Randall et al. 1993).

Vasointestinal pituitary adenylate cyclase (VPAC$_1$) and pituitary adenylate cyclase (PAC$_2$) receptor activation has been shown to modulate intracellular kinases such as protein kinase A (PKA) and protein kinase C (PKC), respectively, in certain cell types. In addition, NetPhosK 1.0 predicted PKA and PKC binding sites on the Orai1 subunit. Therefore we hypothesized that these kinases, along with cAMP, have an influence on CCE. Using calcium imaging, we measured changes in neuronal $[Ca^{2+}]_i$ before and after incubation in 1 $\mu$M phorbol 12, 13 dibutyrate (PDBu) using a conventional calcium add back protocol following passive store depletion by thapsigargin (TG). The PKC
activator did not induce any appreciable deviations in $\Delta [\text{Ca}^{2+}]$, from the control group. Similarly, membrane permeable cAMP analog, 8-Br-cAMP and, adenylate cyclase (AC) activator, forskolin did not influence SOCE upon Ca$^{2+}$ add back. Both cAMP and AC are a part of a common pathway to PKA activation therefore we have concluded that PKA does not have an effect on Orai-SOC channels.

Much variability exists in regard to the reported influences of kinases on capacitative calcium entry (CCE). The differences are largely dependent upon cell type and the kinase subtype. Mechanistically, the conflicts could be due to indirect effects on the plasma membrane-bound channel by store-depletion. In the present study, Ca$^{2+}$ stores are depleted prior to drug treatment to ensure that all effects are direct. However, this study is the first to examine kinase-regulated SOC activity in excitable cell types and subsequent experiments are required to fully describe the absolute consequences of kinase activity. Opioid agonist, met-enkphalin depletes IP$_3$ sensitive stores as well as blocks N-type voltage-gated calcium channels (VGCCs) in intracardiac neurons, specifically (Adams and Trequattrini 1998; Smith and Adams 1999). Therefore, we hypothesized that MOR agonists may have an effect on CCE. To test this hypothesis, we employed the add back protocol as previously described, before and after 10 $\mu$M ME and EM applications (Figure 3.1). As a result, we were able to conclude that opioidergic activation potently inhibits CCE in rat intracardiac neurons.
This finding is paramount considering that enkephalins have been found to be a component of the neurochemical profile of the intracardiac ganglionic neurons. However, the methods by which these peptides modulate cellular function have not been fully described. While the data presented here provides only a singular component of the composite effects of opioidergic activity within intracardiac ganglia, this study is based on the novel premise that SOCs are expressed in neuronal subtypes as opposed to non-excitable cell types and are functional and capable of modulation by cardiac peptides. There are a plethora of reports citing opioidergic inhibition of VGCCs (Jessel 1977; Wilding, Womack et al. 1995; Adams and Trequattrini 1998). However prior to the present study, no information concerning SOCs and opioidergic signaling has existed.

Following MOR activation, an intracellular signaling pathway is initiated, having direct effects on the channel protein subunit. In NG-108 cells overexpressing MOR receptors, PTX was able to attenuate EM-induced depression of VGCCs, indicating G-protein coupling. To elucidate whether a PTX-sensitive G-protein is activated as a result of ligand binding, cells were pre-treated with PTX before applying EM. PTX was able to prevent EM-induced SOCE, proving that a $G_{i/o}$ protein becomes active in response to opioid peptide interacting with the MOR. Similarly, in amygdala pyramidal neurons, it has been shown that a PTX-sensitive G-protein linked to the MOR receptor causes the activation of PLA$_2$ (Faber and Sah 2004).
We concluded that there may be a possibility that PLA$_2$ may be a second messenger in the signaling cascade currently under investigation. First we used the ketone, AACOCF$_3$ to bind and antagonize PLA$_2$ prior to MOR recruitment (data not shown). However, we found that the AA analogue had direct effects on CCE. Therefore we decided to use OBAA a potent, structurally disparate PLA$_2$ inhibitor that does not exhibit direct effects (Kohler, Friedrich et al. 1991). Both agents were able to attenuate percent inhibition of SOCE due to MOR activity (Figure 3.1). These findings suggest that the PLA$_2$ plays a central role in eliciting MOR-induced reduction in calcium influx following ER depletion.

PLA$_2$ is the key enzyme responsible for phospholipid membrane cleavage in the production of AA; it is then probable that AA is a subsequent second messenger in this particular intracellular pathway. An AA-induced decrease in mean peak SOC currents has been documented in rat liver cells. Therefore using calcium imaging, I tested the probability that AA could have similar consequences on CCE in neonatal rat intracardiac neurons. AA was applied to cells following passive store-depletion with TG in zero Ca$^{2+}$. AA attenuated [Ca$^{2+}$], increases upon addition of 2.5 mM Ca$^{2+}$ to the bath. During this investigation, we observed that the two lipids AA and its analogue AACOCF$_3$ had negative consequences on the SOC channel. Therefore LA was presented to the neurons to elucidate further whether the lipid structure was responsible for this inhibitory phenomenon. In the same manner, LA was able to block the
activity of the channel indicative of a lipid-protein interaction, detrimental to channel function.

Along with neural control, the intracardiac ganglia are capable of control by immune responses. Secreted by interganglionic mast cells, prostaglandins, which are AA metabolites, are a class of immune mediators that elicit rapid modulation of the electrical properties of neighboring neurons (Jelson, DeMasi et al. 2003). Two prostaglandin isoforms in particular, PGE$_2$ and PGD$_2$, reduced the after-hyperpolarization duration and amplitude via the attenuation of the small-conductance K$^+$ channels (SK$_{Ca^{2+}}$). However whether they have direct effects or indirect effects via Ca$^{2+}$ has not been determined although prostaglandins have been previously reported to attenuate Ca$^{2+}$ channels in other neuronal preparations (Mo, Ammari et al. 1985; Ikeda 1992; Ito, Murai et al. 2000).

We explored the possibility that this phenomenon may be due to inhibition of SOCs using the add back protocol as previously described. In the presence of both PGE$_2$ and PGD$_2$, there was no difference in $\Delta$[Ca$^{2+}$], from control values. Therefore we concluded that these particular prostaglandin subtypes do not have an influence on SOCE in rat intracardiac neurons. It is important to note that differences between the rat and the guinea pig should be considered when making a comparison between any physiological systems because of the evolutionary hypoxic Andean environment of the guinea pig (Turek, Ringnalda et
al. 1980). Therefore one must be cautious when establishing function
correlations between the two animal models.

The initial portion of this study used calcium imaging as a tool to assess
the effects of MOR agonists and second messengers in our proposed inhibitory
pathway on CCE. We have proven that MOR-specific agonists EM and ME
both depress SOC activity following G-protein recruitment. The G-protein is
responsible for activating PLA$_2$, which subsequently cleaves AA from the plasma
membrane to directly interact with the SOC channel.

Using calcium imaging, we assessed the effects of the MOR-mediated
pathway on SOCE through intracellular calcium elevations following store
depletion. However, in order to make a complete assessment we must consider
the Ca$^{2+}$ current evoked across the plasma membrane in response to store
depletion, $I_{\text{CRAC}}$. A relatively small current, the $I_{\text{CRAC}}$ is blocked at low nM
calcium concentrations; however it is highly selectively for Ca$^{2+}$ ions. Some
other biophysical properties of the $I_{\text{CRAC}}$ are that it is potentiated when Ca$^{2+}$ is
completely removed from the bath solution and evoked in response to store
depletion by caffeine and EGTA. Using the appropriate pipette and bath
solutions to detect the $I_{\text{CRAC}}$, I studied the consequences of our proposed
inhibitory signaling cascade on $I_{\text{CRAC}}$ using the V-clamp mode of whole cell patch
clamp electrophysiology.

Enkephalins have been shown to block voltage dependent calcium
currents and ME blocks VGCC in rat neonatal intracardiac neurons, specifically
(Jessell 1977; Wilding, Womack et al. 1995; Xiao, Spurgeon et al. 1993; Adams and Trequattrini 1998). First, we determined whether EM had a similar effect on $I_{CRAC}$ as the voltage-dependent calcium current and CCE. After store-depletion by 10 mM caffeine, a -160 pA current was evoked in a divalent free (DVF) solution. However when 1 µM EM was introduced to the bath, this current was reduced to about -95 pA (Figure 3.6). Similarly, 10 µM ME was able to depress the $I_{CRAC}$ from -225 pA to -75 pA. In both instances, this effect was readily reversible upon a DVF physiological saline solution PSS wash (Figure 3.7). These findings are evidence that MOR activity is responsible for a common inhibitory thread extending from VGCCs to SOC channels and provide an explanation for the neurosuppression that occurs following release of these peptides within the mammalian heart.

Next, we used our proposed model created in accordance with the imaging results to predict that AA would behave similar to the EM and ME in the control of SOCs. Additionally, AA has been shown to inhibit $I_{SOC}$ in rat liver cells (Rychkov, Litjens et al. 2005). To test this hypothesis, we applied our protocol as previously described in the case of EM and ME in the V-clamp electrophysiological studies. AA was able to attenuate the $I_{CRAC}$ by two-thirds upon bath application (Figure 3.8), an outcome which is analogous to the results obtained from the imaging experiments presented previously.

While AA causes a reduction in this capacitative $\text{Ca}^{2+}$ current, a non-capacitative $\text{Ca}^{2+}$ current has been identified that is modulated by AA, $I_{ARC}$,
arachidonic acid regulated current. Interestingly, $I_{ARC}$ seems to be evolutionarily related yet functionally distinct from other types of calcium channels (Shuttleworth 2009). In order to differentiate between the $I_{ARC}$ and $I_{CRAC}$ in the current study, AA in a NCF PSS was added to the bath following AA-induced inhibition of the $I_{CRAC}$. There was no subsequent current evoked in NCF solution therefore we have concluded that the current evoked is purely due to calcium release (Figure 3.9). Taken together, these electrophysiological experiments concur with the results of fura-2 calcium experiments that indicate a MOR-initiated signaling cascade consisting of the production of intracellular second messengers, leading to SOC inhibition. Electrophysiology provides a more precise method which CCE can be studied because we are able to control precisely the ionic environment according to the biophysical properties of the SOC channel. By studying the changes in mean peak current densities in response to drug bath application, this method offers a more exact and conclusive method of studying Orai-SOCs, in particular.

Next we asked: Does this current play a functional role in the regulation of electrical activity of the neuron? The question under investigation is very unique considering that no other Ca$^{2+}$ channel aside from VGCC currents has been found to be integral in the modulation of the membrane properties of the neuron. However, results of previous studies have suggested VGCCs are not responsible for modifications in the after-hyperpolarization (AHP) in rat intracardiac neurons (Franciolini, Hogg et al. 2001). To explore the possibility of
the involvement of SOCs, we used the most SOC-specific antagonist, 5 μM Gd$^{3+}$, to elucidate its influences on repetitive firing in dissociated neonatal rat intrinsic cardiac neurons. Using the I-clamp mode of the perforated patch configuration of patch clamp electrophysiology, we have shown that multiple action potential firing is converted to adaptive firing in the presence of 5 μM Gd$^{3+}$, which is indicative of SOC-dependent control of action potential discharge in these neurons. Experiments similar to these using other SOC antagonists such as SKF-96365 should be performed to confirm Orai-SOC suppression as Gd$^{3+}$ is a promiscuous antagonist of cationic channels. However the current results definitely justify further investigation of the potential role of SOCs as regulators of membrane properties of the neuron.

We repeated the same I-clamp experiment with EM to determine whether EM would block action potential (AP) firing similarly. EM was effective in reducing action potential firing appreciably from 3.6 ± 0.6 to 1.4 ± 0.6. The results of our calcium imaging and V-clamp electrophysiology experiments with AA indicate first, that AA suppresses SOCE as well as the $I_{\text{CRAC}}$ in intracardiac neurons. We therefore predicted that AP firing will be suppressed upon application of AA. Next, we applied the same protocol to elucidate the effects of AA on AP discharge and found that AP firing was reduced from tonic to adaptive firing.

So far, we have established that a MOR-mediated pathway is capable of eliciting a potent reduction of repetitive firing through the $I_{\text{CRAC}}$. However the
molecular composition of these channels has not been identified in this or any other neuronal subtype. Furthermore, no prior information exists on the molecular contribution of the individual channel subunits to cell excitability because $\text{Ca}^{2+}$ regulation of membrane properties has been attributed to the activity of VGCCs. This study is novel in that its hypothesis is based on recent studies that indicate there is an alternative source of $\text{Ca}^{2+}$ in these ganglionic neurons and this source is calcium release-activated not voltage-dependent.

Using I-clamp electrophysiology and RT-PCR, we found that Orai3 was present in comparably high levels in multiple firing neurons (Figure 4.4). The fact the Orai3 is associated with robust $\text{Ca}^{2+}$-dependent fast inactivation, exclusively, suggests that repetitively firing neurons display more robust fast-inactivation of SOCE than single and adaptive firing neurons (Lis, Peinelt et al. 2007).
CONCLUSIONS

In several cardiac disease states, such as congestive heart failure (CHF) and post-myocardial infarction arrhythmias, decreased parasympathetic and increased sympathetic tones are observed resulting in sudden cardiac death. The final common pathway for autonomic regulation of the heart, the mammalian intracardiac ganglia serve as a major integration center for all parasympathetic inputs into the heart and are capable of local control over the heart independently of the CNS. However studies in the canine model indicate that this local control is compromised in the case of CHF. Contemporary studies indicate that the intracardiac ganglia function within the cardiac plexus as a key player in a series of nested feedback loops rather than linearly. The loss of parasympathetic control in the case of heart failure, particularly, is at the level of the intracardiac ganglia.

Due to the complex architecture of the cardiac nervous system, intracardiac neurons are able to play both a causative as well as an effector role in progression and development of cardiac dysfunction. For instance, cardiac neurons promote coronary vasodilation and dysregulation of coronary blood flow in disease states. Furthermore, changes in intracardiac neuronal function are
observed following myocardial ischemia. Considering the integral role the parasympathetic autonomic nervous system plays in the maintenance of proper cardiac function, the amount of information that exists within the current literature pertaining to its exact mechanisms of regulation are deficient. It is through these studies that we are able to devise pharmacological agents to safely and effectively treat certain cardiac diseases.

The most commonly prescribed pharmacological therapy for CHF in particular, digoxin, elicits its negative chronotropic effects by facilitating parasympathetic tone to the heart (Khand, Rankin et al. 2003; Gheorghiade, Adams et al. 2004). Extreme caution must be practiced when prescribing digitalis because of the narrow therapeutic window associated with digitalis. Consequently, the development of alternative therapeutic agents is necessary to facilitate safe and effective management of critical cardiac disease states.

The complexity of the neurocircuitry as well as the neurochemistry of these ganglia demands comprehensive studies of its modes of regulation in the complete assessment of its contribution to cardiac function. Along with changes in neural transmission, there are also changes in the neurohumoral component of the intrinsic cardiac ganglia associated with an array of cardiac conditions. Relevant to the current study, cardiac opioid and enkephalin production is transformed in an array of diseases such as cardiomyopathy, heart failure, and genetic hypertension resulting in disease progression. Additionally, in the dog, the severity and incidence of cardiac arrhythmias were attenuated following
opioid receptor antagonism. The mechanisms by which these peptides exert their effects are poorly understood. The conclusions attained in the current endeavor align with those that claim MOR stimulation is responsible for the inhibition of VGCCs in parasympathetic neurons of the nucleus ambiguus and the superior cervical ganglionic neurons. Therefore, this study may provide additional insight into a possible method by which these peptides cause detrimental alterations to cardiac physiology.

Experiments executed here are paramount in that they contribute to the understanding of a voltage-independent source of calcium to excitable cells, because SOC channels were previously thought to be restricted to non-excitatory cell types. We prove that Orai-SOC channels, particularly, are not only expressed but they are indeed functional in a neuronal cell and contribute appreciably to cellular excitability. The comparative evaluation of molecular and electrophysiological properties serves as the first to establish a link between Orai expression and membrane properties within neuronal subtypes. Furthermore, our observations in intracardiac neurons could pioneer similar research on store-operated methods of calcium regulation in other neuronal types. Future studies would reveal additional modes of modulation for these channels and could lead to unique, new pharmacological interventions as that target the regulation of intracellular calcium concentrations.

Our proposed pathway indicates AA as the singular and direct second messenger leading to SOC antagonism. Considering the fact that AA is
upregulated following ischemia and reperfusion in vitro, the phospholipid could lead to disruption of normal autonomic function independent of MOR activity therefore the implications for potential therapies are compounded in this respect.
REFERENCES


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2-APB - aminoethoxydiphenyl borate
OBAA - 4-4- Octadecylalkybenzoylacrylic acid
ACh - acetylcholine
AC - adenylate cyclase
AHP - after-hyperpolarization
AA - arachidonic acid
AV - atrioventricular
BSA - bovine serum albumin
$K_{Ca}^{2+}$ - calcium-dependent potassium channels
$I_{CRAC}$ - calcium release-activated current
CICR - calcium-induced calcium release
CaM - calmodulin
CCE - capacitative calcium entry
ChAT - choline acetyl transferase
CHF - congestive heart failure
cAMP - cyclic adenosine monophosphate
DAG - diacylglycerol
DMSO - dimethyl sulfoxide
DVF - divalent-free
DMN - dorsal motor nucleus
P - efferent postganglionic neurons
EM - endomorphin
HF - heart failure
HVA - high voltage –activated
IP₃ - inositol trisphosphate
IN - interneurons
[Ca²⁺]ᵢ - intracellular calcium concentrations
BKᵦCa²⁺ - large conductance calcium-activated potassium channel
LA - linoleic acid
ME - met-enkephalin
MLCK - myosin light-chain kinase
mAChRs – muscarinic cholinergic receptors
nAChRs - nicotinic cholinergic receptors
nAChRs - nominally calcium free (NCF)
NA - nucleus ambiguus
NCF-nominally calcium-free
NTS - nucleus tractus solitarius
PDBu - phorbol 12, 13 dibutyrate
PIP₂ - phosphatidylinositol 4,5-bisphosphate
PLA₂ - phospholipase A₂
PLC - phospholipase C
PSS - physiological saline solution
PAC - pituitary adenylate cyclase
PACAP - pituitary adenylate cyclase-activating peptide
PGD₂ - prostaglandin D2
PGE$_2$ - prostaglandin E2
PKA - protein kinase A
PKC - protein kinase C
ROS - reactive oxygen species
ROCCs - receptor-operated calcium channels
SCID - severe combined immune deficiency
SA - sinoatrial
SIF - small, intensely fluorescent cells
SK$_{Ca}^{2+}$ - small-conductance calcium channels
SOCs - store-operated calcium channels
SOCE - store-operated calcium entry
SON - supraoptic nucleus
TG - thapsigargin
VIP - vasoactive intestinal polypeptide
VPAC - vasointestinal pituitary adenylate cyclase receptor
VGCCs - voltage-gated calcium channels
About the Author

Timetria Bonds was born in Montgomery, AL to Debra Ann Bonds and Timothy Howard on July 13, 1983. She graduated from Sidney Lanier High School where she ranked among the top five in the class of 2001. After receiving her high school diploma, Timetria completed the requirements for the Bachelor’s of Science degree in Biology from Alabama State University in the Fall of 2005.

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