PLASTICITY OF ADRENAL CHROMAFFIN CELL FUNCTION DURING INFLAMMATION AND EXPOSURE TO MICROBE-ASSOCIATED MOLECULAR PATTERNS

By

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ABSTRACT

The sympathetic nervous system (SNS) is part of an integrative network that functions to restore homeostasis following injury and infection. The SNS provides negative feedback control over inflammation through the secretion of catecholamines from postganglionic sympathetic neurons and adrenal chromaffin cells (ACCs). Central autonomic structures receive information regarding the inflammatory status of the body and reflexively modulate SNS activity. Evidence suggests that inflammation and infection can also directly regulate ACC function. However, the precise alterations in ACC function that occur in response to regional inflammation, systemic inflammation and exposure to bacterial products have yet to be fully characterized. The present thesis was therefore performed to test the hypothesis that gastrointestinal (GI) and systemic inflammation modulate ACC Ca$^{2+}$ signaling, and that ACCs possess the ability to directly detect microbe-associated molecular patterns (MAMPs).

Ca$^{2+}$ signaling was assessed in single ACCs isolated from control mice and mice with GI or systemic inflammation using Ca$^{2+}$ imaging and perforated patch clamp electrophysiology. Acute and chronic GI inflammation consistently reduced high-K$^+$-stimulated Ca$^{2+}$ transients in ACCs through an inhibition of voltage-gated Ca$^{2+}$ current. In contrast, systemic inflammation significantly enhanced high-K$^+$-stimulated Ca$^{2+}$ transients and catecholamine secretion through an increase in Ca$^{2+}$ release from the endoplasmic reticulum. Incubation of control ACCs in serum obtained from mice with systemic inflammation produced a similar increase in Ca$^{2+}$ signaling, suggesting that circulating mediators play an important role in this response.

To determine whether ACCs can directly detect MAMPs, Ca$^{2+}$ signaling, excitability and neurotransmitter release were assessed in control ACCs and ACCs
incubated in media containing lipopolysaccharide (LPS). Unlike GI and systemic inflammation, LPS did not affect ACC Ca\(^{2+}\) signaling. However, LPS dose- and time-dependently hyperpolarized ACC resting membrane potential and enhanced large conductance Ca\(^{2+}\)-activated K\(^+\) currents. Consistent with membrane hyperpolarization, LPS reduced ACC excitability and inhibited neuropeptide Y release. These effects were mediated by Toll-like receptor 4 and nuclear factor-κB.

In summary, GI and systemic inflammation produce opposite effects on ACC Ca\(^{2+}\) signaling through distinct mechanisms, and ACCs can directly detect MAMPs. These findings extend our knowledge of the complex integration performed by the immune system-nervous system network during health and disease.
Chapter 2: Real-time polymerase chain reaction (PCR) experiments were performed by Shadia Neshat. For all other experiments, data collection, analysis and interpretation were performed by Mark K. Lukewich. The manuscript was written by Mark K. Lukewich with assistance from Dr. Alan E. Lomax and has been published under: Lukewich MK & Lomax AE (2011). Altered adrenal chromaffin cell function during experimental colitis. *Am J Physiol Gastrointest Liver Physiol* **300**, G654-G664.

Chapter 3: Cecal ligation and puncture surgeries were performed by Iva Kosatka. For all other experiments, data collection, analysis and interpretation were performed by Mark K. Lukewich. The manuscript was written by Mark K. Lukewich with assistance from Dr. Alan E. Lomax.

Chapter 4: Reverse transcriptase (RT)-PCR experiments were performed by Shadia Neshat. For all other experiments, data collection, analysis and interpretation were performed by Mark K. Lukewich. The manuscript was written by Mark K. Lukewich with assistance from Dr. Alan E. Lomax and has been published under: Lukewich MK & Lomax AE (2013). Toll-like receptor 4 activation reduces adrenal chromaffin cell excitability through a nuclear factor-κB-dependent pathway. *Endocrinology* **154**, 351-362.

Chapter 5: RT-PCR experiment was performed by Shadia Neshat.
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<th>Description</th>
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<tbody>
<tr>
<td>ACC</td>
<td>adrenal chromaffin cell</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>ANS</td>
<td>autonomic nervous system</td>
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<tr>
<td>AP</td>
<td>action potential</td>
</tr>
<tr>
<td>AR</td>
<td>adrenergic receptor</td>
</tr>
<tr>
<td>BK</td>
<td>large conductance</td>
</tr>
<tr>
<td>[Ca(^{2+})](_i)</td>
<td>intracellular Ca(^{2+}) concentration</td>
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<tr>
<td>CICR</td>
<td>Ca(^{2+})-induced Ca(^{2+}) release</td>
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<tr>
<td>CLP</td>
<td>cecal ligation and puncture</td>
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<tr>
<td>CPA</td>
<td>cyclopiazonic acid</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
</tr>
<tr>
<td>DMPP</td>
<td>1,1-dimethyl-4-phenylpiperazinium iodide</td>
</tr>
<tr>
<td>DNBS</td>
<td>2,4-dinitrobenzene sulfonic acid</td>
</tr>
<tr>
<td>DRG</td>
<td>dorsal root ganglia</td>
</tr>
<tr>
<td>DSS</td>
<td>dextran sulfate sodium</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
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<tr>
<td>Fura-2AM</td>
<td>fura-2 acetoxymethyl ester</td>
</tr>
<tr>
<td>GI</td>
<td>gastrointestinal</td>
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<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
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<tr>
<td>HBSS</td>
<td>Hank’s balanced salt solution</td>
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<td>HMGB1</td>
<td>high mobility group box 1</td>
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<tr>
<td>HPA axis</td>
<td>hypothalamic pituitary adrenal axis</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>HVA</td>
<td>high voltage activated</td>
</tr>
<tr>
<td>IA</td>
<td>rapidly activating and inactivating $K^+$ current</td>
</tr>
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<td>IBD</td>
<td>inflammatory bowel disease</td>
</tr>
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<td>IBK</td>
<td>large conductance $Ca^{2+}$-activated $K^+$ current</td>
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<tr>
<td>ICa</td>
<td>voltage-gated $Ca^{2+}$ current</td>
</tr>
<tr>
<td>IK</td>
<td>delayed-rectifier $K^+$ current</td>
</tr>
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<td>IK2P</td>
<td>two-pore domain $K^+$ current</td>
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<td>ATP-sensitive $K^+$ current</td>
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<td>IκB kinase</td>
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<td>INa</td>
<td>voltage-gated $Na^+$ current</td>
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<td>IP3</td>
<td>inositol 1,4,5-trisphosphate</td>
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<td>small conductance $Ca^{2+}$-activated $K^+$ current</td>
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<td>K$_{ATP}$ channel</td>
<td>ATP-sensitive $K^+$ channel</td>
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<td>LDCV</td>
<td>large dense core vesicle</td>
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<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
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<td>LVA</td>
<td>low voltage activated</td>
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<td>MACHR</td>
<td>muscarinic acetylcholine receptor</td>
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<tr>
<td>MAMP</td>
<td>microbe-associated molecular pattern</td>
</tr>
<tr>
<td>MD-2</td>
<td>myeloid differentiation protein-2</td>
</tr>
<tr>
<td>MPO</td>
<td>myeloperoxidase</td>
</tr>
<tr>
<td>MyD88</td>
<td>myeloid derived factor 88</td>
</tr>
<tr>
<td>NACHR</td>
<td>nicotinic acetylcholine receptor</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor-κB</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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<tr>
<td>NPY</td>
<td>neuropeptide Y</td>
</tr>
<tr>
<td>PAC1</td>
<td>PACAP type 1</td>
</tr>
<tr>
<td>PACAP</td>
<td>pituitary adenylate cyclase activating polypeptide</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>PG</td>
<td>prostaglandin</td>
</tr>
<tr>
<td>PNMT</td>
<td>phenyl-ethanolamine N-methyl transferase</td>
</tr>
<tr>
<td>PNS</td>
<td>parasympathetic nervous system</td>
</tr>
<tr>
<td>RMP</td>
<td>resting membrane potential</td>
</tr>
<tr>
<td>ROI</td>
<td>region of interest</td>
</tr>
<tr>
<td>RT</td>
<td>reverse transcriptase</td>
</tr>
<tr>
<td>RyR</td>
<td>ryanodine receptor</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SERCA</td>
<td>sarcoplasmic/endoplasmic reticulum Ca(^{2+})-ATPase</td>
</tr>
<tr>
<td>SIRS</td>
<td>systemic inflammatory response syndrome</td>
</tr>
<tr>
<td>SK</td>
<td>small conductance</td>
</tr>
<tr>
<td>SNS</td>
<td>sympathetic nervous system</td>
</tr>
<tr>
<td>STIM-1</td>
<td>stromal interaction molecule 1</td>
</tr>
<tr>
<td>TEA</td>
<td>tetraethylammonium</td>
</tr>
<tr>
<td>TH</td>
<td>tyrosine hydroxylase</td>
</tr>
<tr>
<td>TIRF</td>
<td>total internal reflection fluorescence</td>
</tr>
<tr>
<td>TLR</td>
<td>toll-like receptor</td>
</tr>
<tr>
<td>TNBS</td>
<td>2,4,6-trinitrobenzene sulfonic acid</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>TRP</td>
<td>transient receptor potential</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<td>--------------</td>
<td>---------------------------------</td>
</tr>
<tr>
<td>TTX</td>
<td>tetrodotoxin</td>
</tr>
<tr>
<td>VGCC</td>
<td>voltage-gated Ca$^{2+}$ channel</td>
</tr>
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Chapter 1: GENERAL INTRODUCTION
Homeostasis is a term used to describe the maintenance of a stable internal environment (le milieu intérieur) in spite of external and internal challenges (Cannon, 1929). Homeostasis is achieved by the integrated actions of several systems throughout the body that are activated or inhibited by deviations from the homeostatic set point. The autonomic nervous system (ANS) is a fundamental component of the homeostatic network that is comprised of the sympathetic nervous system (SNS), the parasympathetic nervous system (PNS) and the enteric nervous system (ENS). Although homeostatic responses provide short-term beneficial effects, sustained activation of homeostatic pathways can result in pathological alterations in cellular function. Once initiated, the intensity and duration of the homeostatic response is therefore tightly regulated by negative feedback mechanisms.

Inflammation is a homeostatic response to injury and infection. The immune system constantly surveys the body for foreign material, cellular debris, and infected or cancerous cells. Once a potential threat has been detected, the immune system rapidly initiates a localized inflammatory response to eliminate the inciting agent and repair damaged tissue. If left unchecked, localized inflammation can progress to overwhelming systemic inflammatory responses or chronic inflammatory disorders, each of which can generate extensive collateral tissue damage. Fortunately, anti-inflammatory pathways exist that prevent the development of excessive inflammatory responses (see (Kuby, 1997)).

The sympathetic nervous system (SNS) provides important negative feedback regulation of inflammation through the secretion of catecholamines from adrenal chromaffin cells (ACCs) and postganglionic sympathetic neurons (see (Elenkov et al., 2000)). The SNS is activated during inflammation through centrally-mediated reflexes.
and the direct actions of inflammatory mediators on peripheral sympathetic tissues (Rosmaninho-Salgado et al., 2007; Dong et al., 2008; Marom et al., 2011; Vida et al., 2011a). Evidence suggests that ACCs may also possess the ability to directly detect invading micro-organisms and that regional and systemic inflammatory disorders can alter ACC neurophysiology (Qi et al., 1991; Zhou & Jones, 1993; Bornstein et al., 2004; Motagally et al., 2009b). The work described in this thesis was therefore performed to characterize the effects of regional and systemic inflammation on ACC function and to determine whether ACCs can directly detect the microbe-associated molecular pattern (MAMP), lipopolysaccharide (LPS). To provide the appropriate context for the experiments that follow, this literature review covers the cell physiology of the ANS, with particular attention paid to ACCs of the adrenal medulla. Regional and systemic inflammatory disorders are also discussed.

**HISTORY OF THE AUTONOMIC NERVOUS SYSTEM**

Early anatomical descriptions of autonomic structures date back to ancient Greece. However, it was not until the 19th century that our understanding of the function of the ANS began to develop. In 1800, Marie Francois Bichat first hypothesized that the sympathetic ganglia, or “ganglionic nervous system,” could regulate visceral function, which he referred to as “organic life” (Bichat, 1800). In 1846, Ernst and Edward Weber discovered that stimulation of the vagus nerve potently reduced the rate of myocardial contraction (Weber & Weber, 1846). Shortly thereafter, Claude Bernard and Charles-Edouard Brown-Séquard determined that sympathetic neurons promote vasoconstriction (Bernard, 1852; Brown-Sequard, 1852). The first detailed anatomical descriptions of Meisner’s plexus and Auerbach’s plexus of the gastrointestinal (GI) tract and the adrenal
medulla of the adrenal gland also became available around this time (Kolliker, 1852; Meissner, 1857; Auerbach, 1864).

In the late 1800s, Walter Gaskell discovered that neurons within the sympathetic ganglia receive inputs from the thoracolumbar spinal cord that travel through the white rami communicans. After observing a similar organization of myelinated fibers leaving the sacral spinal cord and brainstem, Gaskell hypothesized that the ganglionic nervous system was comprised of a cranial outflow, a thoracolumbar outflow and a sacral outflow. Gaskell also believed that the thoracolumbar outflow was antagonistic to that originating from the cranial and sacral regions (Gaskell, 1886). Shortly thereafter, John Newport Langley began to use pharmacological tools to dissect the functional contributions of each of the outflows identified by Gaskell. He discovered that nicotine inhibited the passage of information within the sympathetic and parasympathetic ganglia, confirming earlier hypotheses that these systems were made up of two sequential neurons, which he named preganglionic and postganglionic (Langley & Dickinson, 1890). Langley then used nicotine to characterize the functions of the sympathetic and parasympathetic nerves in various tissues, many of which he found to be antagonistic (Langley & Dickinson, 1889; Langley & Anderson, 1892). In 1898, Langley coined the term ANS to describe the SNS, the PNS and the ENS (Langley, 1898).

Information regarding the nature of communication between the SNS and PNS and their target tissues soon became available. In 1895, Oliver and Schafer determined that, similar to sympathetic nerves, extracts from the adrenal gland could promote vasoconstriction and increase the rate of myocardial contraction when intravenously injected (Oliver & Schafer, 1895). By 1901, Jokichi Takamine determined the chemical
composition of the major pressor agent found within the adrenal extracts and named it ‘Adrenalin’ (Takamine, 1902). Shortly thereafter, Thomas Renton Elliot was able to show that the application of Adrenalin to sympathetically innervated tissues promoted similar responses to that of stimulated sympathetic nerves (Elliott, 1905). Around this time, Reid Hunt and René deM. Taveau demonstrated that, in contrast to Adrenalin, injection of acetylcholine into experimental animals caused a reduction in heart rate and a decrease in blood pressure (Hunt & Taveau, 1906).

Based on these and other historical observations, we now know that the ANS functions to maintain homeostasis through the integrated actions of the ENS, the PNS and the SNS (Cannon, 1929). The ENS is comprised of a network of neurons located within the submucosal and myenteric plexuses of the GI tract that control GI motility, secretion and blood flow. The PNS is comprised of cholinergic preganglionic parasympathetic neurons that travel through cranial nerves III, VII, IX and X and the pelvic splanchnic nerve to innervate postganglionic parasympathetic neurons. Postganglionic parasympathetic neurons reside within ganglia located on or near their target organs. In general, these neurons function to restore the body’s energy reserves through the release of acetylcholine. In contrast, the SNS performs ‘fight-or-flight’ responses that mobilize the body’s energy stores and increase blood flow to the muscles and heart during stressful situations through the release of catecholamines. The SNS is the primary focus of the present thesis and is therefore discussed in detail below.

THE SYMPATHETIC NERVOUS SYSTEM

The SNS is comprised of preganglionic sympathetic neurons, postganglionic sympathetic neurons and ACCs. Preganglionic sympathetic neurons have cell bodies located within the intermediolateral column, intercalated nucleus, dorsolateral funiculus
and central autonomic area of the thoracolumbar spinal cord (Fenwick et al., 2006). The activity of these neurons is regulated by inputs from central autonomic structures, including the rostral ventrolateral medulla, the ventromedial medulla, the caudal raphe nuclei, A5 cell group and the paraventricular nucleus of the hypothalamus (Strack et al., 1989). Preganglionic sympathetic neurons innervate postganglionic sympathetic neurons that reside within the paravertebral and prevertebral sympathetic ganglia, and ACCs located within the adrenal medullae (Feldberg et al., 1934; Feldberg & Gaddum, 1934). Postganglionic sympathetic neurons innervate a variety of tissues throughout the body where they locally release norepinephrine. In contrast, ACCs are neuroendocrine cells that secrete epinephrine and norepinephrine into the bloodstream.

Catecholamines regulate visceral function through the activation of adrenergic receptors (ARs) expressed by target tissues. ARs are G-protein coupled receptors (GPCRs) comprised of seven transmembrane domains that interact with heterotrimeric G-proteins consisting of α, β and γ subunits. ARs can be structurally and functionally divided into α1- and α2-ARs and β1-, β2- and β3-ARs. At physiological concentrations, both epinephrine and norepinephrine are able to activate each α- and β-AR subtype. However, β2-ARs have a much higher affinity for epinephrine than norepinephrine (see (Dorn, 2010)). Within the cardiovascular system, catecholamines promote vasoconstriction through the activation of α1-ARs and vasodilation through the activation of β2-ARs. Catecholamines also increase the force and rate of myocardial contraction through the activation of β1-ARs (McPherson et al., 1981). Other characteristic sympathetic responses include β2-AR-mediated bronchodilation and glycogenolysis, and α1-AR-mediated dilation of the pupil (Cullum et al., 1969; Arinze & Kawai, 1983; Yu & Koss, 2002). Furthermore, catecholamines can regulate
inflammatory responses by activating α- and β-ARs expressed by immune cells (Elenkov et al., 1995; Ignatowski et al., 1996).

OVERVIEW OF ADRENAL CHROMAFFIN CELL FUNCTION

Catecholamine secretion from ACCs is a Ca^{2+}-dependent process that can be regulated at multiple levels of the stimulus-secretion coupling pathway. In general, acetylcholine released from preganglionic sympathetic neurons innervating ACCs stimulates the generation of action potentials (APs) through the activation of nicotinic acetylcholine receptors (NACHRs) and muscarinic acetylcholine receptors (MACHRs) (Nassar-Gentina et al., 1988; Gonzalez-Rubio et al., 2006). Membrane depolarization stimulates Ca^{2+} influx through voltage-gated Ca^{2+} channels (VGCCs), which elevates intracellular Ca^{2+} concentration ([Ca^{2+}]_i) (Fenwick et al., 1982b). The spatial and temporal characteristics of the initial Ca^{2+} signal are subsequently shaped by intracellular organelles and Ca^{2+} extrusion mechanisms (Cheek et al., 1993; Herrington et al., 1996; Park et al., 1996). Elevated [Ca^{2+}], then stimulates the fusion of large dense core vesicles (LDCVs) with the plasma membrane and the release of catecholamines into the systemic circulation (Douglas & Poisner, 1962; Neher & Marty, 1982).

INNERVATION OF ADRENAL CHROMAFFIN CELLS

Preganglionic sympathetic neurons innervating ACCs possess cell bodies within thoracic spinal cord segments T4-T13 and extend axons through the splanchnic nerve to the adrenal medulla (Kajiwara et al., 1997; Fenwick et al., 2006; Petrovic et al., 2010). Each ACC receives approximately 1-4 synaptic inputs and splanchnic nerve stimulation results in the activation of discrete clusters of 15-20 ACCs (Kajiwara et al., 1997). Under resting conditions, preganglionic sympathetic neurons exhibit irregular firing patterns ranging from approximately 0.2-6 Hz and they predominantly release
acetylcholine (Dembowski et al., 1982; Dembowsky et al., 1986; Morrison & Cao, 2000; Kuri et al., 2009). During an acute stress response, preganglionic sympathetic neuron activity is increased, which promotes the co-release of acetylcholine and pituitary adenylate cyclase-activating polypeptide (PACAP) (Morrison & Cao, 2000; Hamelink et al., 2002; Kuri et al., 2009).

**Muscarinic Acetylcholine Receptors**

MAChRs are GPCRs that can be divided into the M1, M2, M3, M4 and M5 receptor subtypes based on their functional and structural characteristics. Mouse ACCs express all five MAChR subtypes (Wu et al., 2010). MAChR activation results in membrane depolarization and an increase in AP generation (Nassar-Gentina et al., 1988). Although superfusion of MAChR agonists stimulates catecholamine secretion from isolated mouse ACCs, MAChRs do not appear to contribute to the secretory response produced by splanchnic nerve stimulation in adrenal slices (Wu et al., 2010; Petrovic et al., 2010).

**Nicotinic Acetylcholine Receptors**

NACHRs are non-selective ligand-gated cation channels that can pass Na\(^+\), K\(^+\) and Ca\(^{2+}\) ions. Neuronal NACHRs are homopentamers comprised of five α-subunits or heteropentamers comprised of two α- and three β-subunits. Several neuronal NACHR α- and β-subunits have been identified in mammalian tissues, including α\(_1\), α\(_2\), α\(_3\), α\(_4\), α\(_5\), α\(_6\), α\(_7\), α\(_9\), α\(_10\), β\(_2\), β\(_3\) and β\(_4\) (see (Albuquerque et al., 2009)). In bovine and rat ACCs, functional α\(_3\)β\(_4\), α\(_3\)β\(_2\), α\(_4\)β\(_2\) and α\(_3\)α\(_5\)β\(_2\) heteropentamers, and α\(_7\) homopentamers have been shown to contribute to the generation of nicotinic currents (Garcia-Guzman et al., 1995; Campos-Caro et al., 1997; Di Angelantonio et al., 2003). Although mouse ACCs express α\(_1\), α\(_3\), α\(_4\), α\(_5\), α\(_7\), α\(_9\), β\(_1\), β\(_2\) and β\(_4\) mRNA (Wu et al., 2010), it is unclear at this
point which subunit combinations form functional NACHRs within these cells. Nevertheless, NACHR activation potently stimulates catecholamine release from isolated mouse ACCs and adrenal slices (Wu et al., 2010; Petrovic et al., 2010).

**Pituitary Adenylate Cyclase-activating Polypeptide Type 1 Receptor**

Preganglionic sympathetic neurons innervating mouse ACCs co-release PACAP and acetylcholine during periods of sympathetic activation (Hamelink et al., 2002; Kuri et al., 2009). PACAP stimulates catecholamine secretion from ACCs by activating high affinity PACAP type 1 (PAC1) receptors, depolarizing the membrane potential and promoting Ca\(^{2+}\) influx through VGCCs (Kuri et al., 2009; Hill et al., 2011). PACAP-evoked catecholamine secretion has a longer latency period than that of acetylcholine and persists for minutes after PACAP has been removed (Przywara et al., 1996). Interestingly, PACAP-evoked catecholamine secretion can be maintained for several hours in the continued presence of this stimulus, whereas acetylcholine-evoked catecholamine secretion is transient during sustained stimulation (Babinski et al., 1996; Stroth et al., 2013). PACAP is therefore able to maintain catecholamine secretion from mouse ACCs during prolonged high-frequency stimulations in the face of NACHR desensitization (Kuri et al., 2009; Stroth et al., 2013). ACCs also express the non-selective PACAP receptor, VPAC2, which can contribute to the PACAP-mediated secretory responses in ACCs from certain species (Harmar et al., 2004; Valiante et al., 2008).

**CATECHOLAMINE SYNTHESIS**

ACCs contain LDCVs filled with the catecholamine neurotransmitters, epinephrine and norepinephrine. Catecholamines are synthesized from the aromatic amino acid, tyrosine, through a series of enzymatic reactions. Tyrosine is taken up into
ACCs and initially converted to dihydroxyphenylalanine by tyrosine hydroxylase (TH), the rate limiting enzyme in catecholamine synthesis (Nagatsu et al., 1964).

Dihydroxyphenylalanine is then converted to dopamine by aromatic L-amino acid decarboxylase (Lovenberg et al., 1962). Dopamine is subsequently taken up into LDCVs via vesicular monoamine transporters, where it is converted to norepinephrine by dopamine β-hydroxylase (Levin et al., 1960; Scherman et al., 1983). In epinephrine secreting ACCs, norepinephrine must leave the LDCV, be converted to epinephrine in the cytosol by phenyl-ethanolamine N-methyl transferase (PNMT), and then re-enter the vesicle prior to being secreted (Connett & Kirshner, 1970).

CATECHOLAMINE SECRETION

Catecholamine secretion from ACCs is achieved through the process of exocytosis. Exocytosis refers to a series of sequential events that bring LDCVs in close proximity to the plasma membrane, assemble the release machinery, and promote the fusion of LDCVs with the plasma membrane in response to elevated [Ca^{2+}]_i (see (Sugita, 2008)). LDCVs in ACCs can be divided into distinct vesicle pools based on their level of maturation and readiness for release. Newly synthesized LDCVs are transported to the reserve vesicle pool located in the cell periphery, which functions to refill more mature vesicle pools as they become depleted (Steyer et al., 1997; Gil et al., 2000). During vesicle refilling, LDCVs are brought into close proximity to the plasma membrane through a process known as docking (Steyer et al., 1997; Giner et al., 2005). Docked vesicles comprise the unprimed vesicle pool and must undergo an additional maturation step, known as priming, before they can be released (Ashery et al., 2000).

Priming is largely mediated by the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) family of proteins (Sollner et al., 1993a; Xu et al.,
The SNARE family of proteins consists of synaptobrevin, syntaxin and synaptosome-associated protein (SNAP)-25 (Sollner et al., 1993b). Snaptobrevin is located on the LDCV membrane, whereas syntaxin and SNAP-25 reside within the plasma membrane (Trimble et al., 1988; Bennett et al., 1992; Gonzalo & Linder, 1998). During priming, synaptobrevin, syntaxin and SNAP-25 tightly connect the LDCV and plasma membrane through the formation of the SNARE complex (see (Sudhof & Rothman, 2009)). Primed LDCVs exist in one of three vesicle pools that are distinguished based on their kinetics of release: the immediately releasable pool, the readily releasable pool and the slowly releasable pool. LDCVs in each of these vesicle pools are released from ACCs in response to elevated [Ca^{2+}]; in the form of an exocytotic burst (Heinemann et al., 1994; Xu et al., 1998; Ashery et al., 2000). During prolonged stimulations, docked LDCVs are continuously primed and released, generating a slow and sustained secretory response (Xu et al., 1999; Ashery et al., 2000).

CATECHOLAMINE REUPTAKE AND DEGRADATION

Catecholamines that have been secreted by ACCs can be taken back up into the cytosol via the norepinephrine transporter (Banerjee et al., 1987; Lingen et al., 1994; Wakade et al., 1996). Once inside the ACC, catecholamines are either recycled into LDCVs or inactivated. Monoamine oxidase is an enzyme located on the outer mitochondrial membrane that inactivates catecholamines by oxidative deamination. Although ACCs can readily take up catecholamines, these cells express type B monoamine oxidase, which is not very effective at catecholamine deamination (Youdim et al., 1984). Catecholamines can also be inactivated through methylation reactions catalyzed by catechol-O-methyltransferase, an enzyme present within the cytosol or
attached to the external surface of the plasma membrane. ACCs predominantly express
the membrane bound form of catechol-O-methyltransferase, which inactivates
extracellular catecholamines (Ellingson et al., 1999). As a result, catecholamines within
the cytosol are likely to be recycled, rather than inactivated in ACCs. In contrast,
secreted catecholamines can be readily inactivated by catechol-O-methyltransferase and
they can also be taken up into adrenal medullary endothelial cells and deaminated by
type A monoamine oxidase, which is expressed by these cells (Youdim et al., 1989).

MEASURING CATECHOLAMINE RELEASE

ACCs are routinely used as a model system to study the mechanisms underlying
neurotransmitter release. ACCs exhibit a spherical shape in culture, and release
catecholamines from their cell bodies, which makes them ideal for electrophysiological,
electrochemical and imaging experiments. Although a variety of techniques have been
developed to measure the secretory responses of ACCs, the two most commonly used
techniques are capacitance measurements and carbon fibre amperometry.

Cell capacitance is directly proportional to the surface area of the plasma
membrane. As LDCVs fuse with the plasma membrane and release their contents, the
capacitance of the cell increases. Changes in capacitance can therefore be used to
measure exocytosis in ACCs (Neher & Marty, 1982). Importantly, capacitance
measurements detect exocytosis that occurs anywhere on the cell membrane and provide
excellent temporal resolution that is ideal for kinetic analyses (Heinemann et al., 1994;
Xu et al., 1998). However, this technique cannot be used to evaluate the amount of
catecholamine released per LDCV and during longer recording periods membrane
endocytosis may obscure the changes in membrane capacitance produced by exocytotic
events (Neher & Marty, 1982).
Carbon fibre amperometry is an electrochemical technique that can be used to detect the release of oxidizable neurotransmitters, including catecholamines. To perform these experiments, a carbon fibre is placed in direct contact with the ACC membrane and is held at a positive potential to promote catecholamine oxidation. Catecholamines that are released from the region of membrane beneath the carbon fibre generate a measurable current (Leszczyszyn et al., 1991). Amperometry has a similar temporal resolution and sensitivity to postsynaptic membranes (Chow et al., 1992; Alvarez deToledo et al., 1993). Importantly, each current spike that is generated represents catecholamine release from a single LDCV. In addition, the overall charge of each amperometric spike is directly proportional to the amount of catecholamine that is released from that LDCV (Wightman et al., 1991). As a result, carbon fibre amperometry can be used to measure the number of secretory events elicited by a given stimulus and the quantal content of each secretory event.

**NEUROPEPTIDE SYNTHESIS AND SECRETION**

In addition to catecholamines, ACCs can also synthesize and secrete a variety of neuropeptides, including neuropeptide Y (NPY), chromogranins and enkephalins (Eiden et al., 1987; Ait-Ali et al., 2004; Whim, 2006). Neuropeptides are synthesised on the rough endoplasmic reticulum and transported to the Golgi apparatus for packaging into LDCVs. Many neuropeptides are synthesized as precursor peptides that are subsequently cleaved within LDCVs into their active form prior to secretion. Neuropeptides are co-packaged with catecholamines in ACC LDCVs (Whim, 2006). Fusion of the LDCV with the plasma membrane results in the generation of a narrow secretory pore, which can readily pass catecholamines but is impermeable to larger polypeptides. Once the secretory pore has been generated, it can rapidly re-seal itself through a process known
as the kiss-and-run mechanism of exocytosis to retain neuropeptides and structural proteins within the LDCV. The secretory pore can also fully expand through the full collapse mechanism of exocytosis to release both catecholamines and neuropeptides into the extracellular space (Alvarez deToledo et al., 1993; Albillos et al., 1997; Fulop & Smith, 2006). In addition to allowing the selective release of catecholamines, the kiss-and-run mechanism also enables LDCVs to be rapidly recycled without having to go through the trans-Golgi network, which is required for LDCVs that have undergone full collapse (Fulop et al., 2005).

The mechanism of exocytosis that ACCs use during a given stimulation is regulated by the intensity of the inciting stimulus and the magnitude of the resultant Ca\textsuperscript{2+} response. During high-frequency stimulations and large elevations in [Ca\textsuperscript{2+}], both catecholamines and neuropeptides are released through the full-collapse mechanism of exocytosis. In contrast, low-frequency stimulations and small elevations in [Ca\textsuperscript{2+}] result in the preferential release of catecholamines alone (Fulop et al., 2005; Elhamdani et al., 2006; Fulop & Smith, 2006). The amount of catecholamine released per LDCV is also dependent upon the rate of ACC stimulation and the magnitude of Ca\textsuperscript{2+} influx. As a result, the quanta of catecholamine released is smaller for lower frequency stimulation patterns and smaller increases in [Ca\textsuperscript{2+}], than it is for high-frequency activation and profound Ca\textsuperscript{2+} influx (Elhamdani et al., 2001; Fulop et al., 2005; Fulop & Smith, 2006). Ca\textsuperscript{2+} dynamics and cellular excitability are therefore important determinants of catecholamine and neuropeptide secretion from ACCs.

**INTRACELLULAR Ca\textsuperscript{2+} DYNAMICS**

Ca\textsuperscript{2+} is a ubiquitous intracellular signaling molecule that can regulate a variety of important functions, including gene expression, excitability, enzyme activity, muscle
contraction and neurotransmitter secretion (Sandow, 1952; Douglas & Rubin, 1961; Morgan & Curran, 1986; Zhang et al., 1993a; Marcantoni et al., 2010). Elevations in 
$[\text{Ca}^{2+}]_i$ can be achieved by $\text{Ca}^{2+}$ influx through ligand-gated ion channels and VGCCs, and $\text{Ca}^{2+}$ release from the endoplasmic reticulum (ER) (Cheek et al., 1993; Mollard et al., 1995; Hernandez-Guijo et al., 1998). The spatiotemporal properties of the resultant $\text{Ca}^{2+}$ signal can be modified by intracellular organelles and the rate of $\text{Ca}^{2+}$ extrusion from the cytosol (Park et al., 1996; Wu et al., 2010). $\text{Ca}^{2+}$ signaling is a plastic process that can become altered during inflammation and infection (Hou & Wang, 2001; Motagally et al., 2009b). In the experiments that follow, ACC $\text{Ca}^{2+}$ dynamics will be assessed during regional inflammation, systemic inflammation and exposure to MAMPs. To provide the appropriate context for these experiments, ACC $\text{Ca}^{2+}$ signaling is discussed in detail in the following sections.

**Nicotinic Acetylcholine Receptors**

Activation of NACHRs generates a large and rapid inward current carried by $\text{Na}^+$ and $\text{Ca}^{2+}$ ions that depolarizes the membrane potential, activates VGCCs and stimulates catecholamine release (Nooney et al., 1992; Gonzalez-Rubio et al., 2006; Wu et al., 2010). Although $\text{Ca}^{2+}$ influx produces only 2.5-5% of the overall current carried by these channels, $\text{Ca}^{2+}$ entering directly through NACHRs can stimulate catecholamine secretion in the absence of membrane depolarization and VGCC activation (Zhou & Neher, 1993; Mollard et al., 1995; Arnaiz-Cot et al., 2008). Furthermore, $\text{Ca}^{2+}$ influx through NACHRs can increase the number of primed LDCVs in ACCs, thereby enhancing the exocytotic burst produced by NACHR-mediated VGCC activation (Arnaiz-Cot et al., 2008; Diego et al., 2008).
**Voltage-gated Ca\(^{2+}\) Channels**

VGCCs are hetero-oligomeric proteins that are made up of an \(\alpha_1\) pore-forming subunit, and auxiliary \(\beta\), \(\alpha_2-\delta\) and \(\gamma\) subunits. The \(\alpha_1\) subunit of VGCCs is comprised of four homologous domains (I-IV), each of which contains six transmembrane segments (S1-S6) and an intracellular ‘P-loop’ connecting S5 and S6. The S5 and S6 segments and the P-loop of each domain create the Ca\(^{2+}\)-permeable pore of the VGCC. The S4 segments contain positively charged amino acids and act as voltage-sensors that drive conformational changes in the \(\alpha_1\) subunit to rapidly open the pore during membrane depolarization (see (Catterall, 2000)). The cytoplasmic \(\beta\) subunit and the membrane-associated \(\alpha_2-\delta\) subunit regulate channel gating, expression and membrane localization to ultimately increase current density (Shistik et al., 1995; Josephson & Varadi, 1996; Felix et al., 1997; Bichet et al., 2000; Sokolov et al., 2000). In contrast, the transmembrane \(\gamma\) subunit has been shown to reduce voltage-gated Ca\(^{2+}\) current (\(I_{Ca}\)) and may function to counteract the modulatory effects of \(\alpha_2-\delta\) (Kang et al., 2001).

VGCCs can be functionally classified as low voltage-activated (LVA) and high voltage-activated (HVA) Ca\(^{2+}\) channels based on their sensitivity to changes in membrane potential. LVA Ca\(^{2+}\) channels open in response to modest depolarizations and are often inactivated at resting membrane potential (RMP) (Carbone & Lux, 1984; Nilius et al., 1985). LVA Ca\(^{2+}\) channels are also referred to as transient, or T-type, Ca\(^{2+}\) channels because they exhibit rapid voltage-dependent inactivation and generate transient \(I_{Ca}\) during sustained depolarizations (Nowycky et al., 1985; Bean, 1985). The \(\alpha_1\) pore-forming subunits of T-type Ca\(^{2+}\) channels are encoded by the \(Ca_v\) 3.1, \(Ca_v\) 3.2 and \(Ca_v\) 3.3 genes (Cribbs et al., 1998; Perez-Reyes et al., 1998; Lee et al., 1999). Under
normal conditions, adult mouse ACCs do not exhibit T-type $I_{Ca}$ (Hernandez-Guijo et al., 1998; Albillos et al., 2000).

As their name implies, HVA Ca$^{2+}$ channels require larger depolarizations for channel activation than LVA Ca$^{2+}$ channels. HVA Ca$^{2+}$ channels can be divided into L-, N-, P/Q- and R-types on the basis of their activation and inactivation kinetics, $\alpha_1$ subunit composition, and susceptibility to pharmacological blockade. L-type Ca$^{2+}$ channels were the first HVA Ca$^{2+}$ channels to be identified and are generally characterized by their large unitary conductance, slow voltage-dependent inactivation, fast deactivation and inhibition by 1,4-dihydropyridine antagonists (Nowycky et al., 1985; Bean, 1985; Nilius et al., 1985; Fox et al., 1987). Four separate genes encode the $\alpha_1$ subunits of L-type VGCCs, including Ca$_{1.1}$, Ca$_{1.2}$, Ca$_{1.3}$ and Ca$_{1.4}$ (Marcantoni et al., 2010). N-type VGCCs were the next HVA Ca$^{2+}$ channels to be discovered and they exhibit several intermediate properties between T-type and L-type Ca$^{2+}$ channels (Nowycky et al., 1985). N-type Ca$^{2+}$ channels are inhibited by $\omega$-conotoxin GVIA and contain $\alpha_1$ subunits encoded by Ca$_{2.2}$ (Williams et al., 1992). P-type Ca$^{2+}$ channels exhibit very slow voltage-dependent inactivation and possess $\alpha_1$ subunits encoded by Ca$_{2.1}$ (Llinas et al., 1989; Pinto et al., 1998). Although Q-type Ca$^{2+}$ channel $\alpha_1$ subunits are also encoded by Ca$_{2.1}$, these channels exhibit a greater degree of voltage-dependent inactivation and require higher concentrations of $\omega$-agatoxin IVA for channel inhibition than P-type Ca$^{2+}$ channels (Randall & Tsien, 1995; Pinto et al., 1998). R-type Ca$^{2+}$ channels are characterized by rapid activation and inactivation kinetics and a resistance to blockade by common HVA Ca$^{2+}$ channel inhibitors (Ellinor et al., 1993; Zhang et al., 1993b; Randall & Tsien, 1995). R-type Ca$^{2+}$ channels have a similar unitary conductance to N-
type Ca\(^{2+}\) channels and possess \(\alpha_1\) subunits encoded by the Ca\(_{v}2.3\) gene (Ellinor et al., 1993; Piedras-Renteria & Tsien, 1998).

Mouse ACCs express all of the HVA Ca\(^{2+}\) channel subtypes. In isolated cells, L-type Ca\(^{2+}\) channels contribute the largest proportion of \(I_{Ca}\) of all of the HVA Ca\(^{2+}\) channels. L-type \(I_{Ca}\) accounts for approximately 40\% of the overall \(I_{Ca}\) elicited during square-pulse depolarizations, whereas N-type and P/Q-type VGCCs contribute 25-35\% and 14-35\%, respectively (Hernandez-Guijo et al., 1998; Aldea et al., 2002). R-type \(I_{Ca}\) has also been observed in isolated ACCs and accounts for approximately 10\% of overall \(I_{Ca}\) (Aldea et al., 2002). Importantly, each HVA Ca\(^{2+}\) channel contributes to exocytosis in proportion to the percentage of overall \(I_{Ca}\) that it generates, suggesting that specific HVA Ca\(^{2+}\) channel subtypes do not preferentially couple to the secretory machinery in isolated mouse ACCs (Aldea et al., 2002). It is important to mention, however, that when the fast component of the exocytotic burst is isolated using 10-50 ms square-pulse depolarizations, P/Q-type Ca\(^{2+}\) channels have been shown to tightly couple to the immediately releasable pool of vesicles (Alvarez et al., 2008).

**Endoplasmic Reticulum Ca\(^{2+}\) Release**

The ER is an important source of intracellular Ca\(^{2+}\). Ca\(^{2+}\) is pumped into the ER lumen by the sarcoplasmic/endoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) to create a large Ca\(^{2+}\) concentration gradient across the ER membrane (Alonso et al., 1999; Villalobos et al., 2002). Ca\(^{2+}\) can be rapidly released from the ER through ryanodine receptors (RyRs) and inositol 1,4,5-trisphosphate receptors (IP\(_3\)Rs), which can deplete ER Ca\(^{2+}\) stores (Cheek et al., 1994; Alonso et al., 1999). Depletion of ER Ca\(^{2+}\) activates store-operated Ca\(^{2+}\) channels and stimulates Ca\(^{2+}\) influx (Hoth & Penner, 1992).
Cytosolic Ca\textsuperscript{2+} is then taken up into the ER through the SERCA to restore ER Ca\textsuperscript{2+} levels back to normal (Alonso \textit{et al.}, 1999; Villalobos \textit{et al.}, 2002).

\textit{Ca}\textsuperscript{2+}-\textit{induced Ca}\textsuperscript{2+} Release

Elevations in [Ca\textsuperscript{2+}]\textsubscript{i} can be amplified by the ER through a process known as Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release (CICR) (Ford & Podolsky, 1970). CICR is mediated by RyRs located on the ER membrane. RyRs exhibit large unitary conductances and allow the passage of both monovalent and divalent cations (Ehrlich & Watras, 1988; Chen \textit{et al.}, 1997; Gao \textit{et al.}, 2000). Three different RyRs have been identified in mammalian tissues and are known as RyR1, RyR2 and RyR3. RyR1 is primarily expressed in skeletal muscle cells where it is physically coupled to the dihydropyridine receptor and participates in excitation-contraction coupling (Penner \textit{et al.}, 1989). RyR2 is the predominant RyR expressed in cardiomyocytes where it stimulates CICR following Ca\textsuperscript{2+} influx through L-type Ca\textsuperscript{2+} channels (Nabauer \textit{et al.}, 1989; Nakai \textit{et al.}, 1990). RyR2 is also the most highly expressed RyR isoform within the brain (McPherson & Campbell, 1993). RyR3 is expressed at low levels in many cell types, including neurons, skeletal muscle cells and smooth muscle cells, where it can also generate CICR responses (Hakamata \textit{et al.}, 1992; Giannini \textit{et al.}, 1995; Chen \textit{et al.}, 1997).

In the early 1970s, Alan Poisner demonstrated that caffeine could stimulate catecholamine secretion from the perfused adrenal gland in the absence of extracellular Ca\textsuperscript{2+}, suggesting that Ca\textsuperscript{2+} release from internal stores can promote exocytosis in ACCs (Poisner, 1973). Shortly thereafter, Ca\textsuperscript{2+} imaging of isolated bovine ACCs revealed that caffeine stimulates transient elevations in [Ca\textsuperscript{2+}]\textsubscript{i} that are inhibited by high concentrations of ryanodine, a RyR antagonist (Teraoka \textit{et al.}, 1991; Cheek \textit{et al.}, 1993). Caffeine- and high-K\textsuperscript{+} have also been directly shown to decrease ER Ca\textsuperscript{2+} levels using
ER-targeted fluorescent Ca\(^{2+}\) probes (Alonso et al., 1998; Alonso et al., 1999; Villalobos et al., 2002). Furthermore, depletion of ER Ca\(^{2+}\) stores decreases depolarization-induced secretion in bovine ACCs, suggesting that CICR triggered by Ca\(^{2+}\) influx can enhance the exocytotic responses of these cells (Lara et al., 1997; Pan & Fox, 2000). Caffeine-sensitive ER Ca\(^{2+}\) stores are functionally discrete, they possess differential sensitivities to caffeine, and they release Ca\(^{2+}\) in an all-or-nothing fashion (Cheek et al., 1993; Cheek et al., 1994; Alonso et al., 1999). In addition, luminal Ca\(^{2+}\) concentration affects the sensitivity of the RyR to caffeine, suggesting that ER Ca\(^{2+}\) stores regulate the magnitude of the CICR response in ACCs (Cheek et al., 1991).

Mouse ACCs express RyR2 and RyR3. RyR2 is primarily located beneath the plasma membrane, whereas RyR3 resides in the perinuclear region (ZhuGe et al., 2006; Wu et al., 2010). Caffeine stimulates [Ca\(^{2+}\)]\(_i\) transients and decreases the ER Ca\(^{2+}\) concentration in mouse ACCs (Wu et al., 2010). Furthermore, these cells exhibit Ca\(^{2+}\) scintillas, which are spontaneous ER Ca\(^{2+}\) release events mediated by RyRs (ZhuGe et al., 2006; Lefkowitz et al., 2009). However, the contribution of CICR to the secretory responses of mouse ACCs is controversial. Initial studies suggested that CICR does not contribute to high-K\(^{+}\)-evoked catecholamine secretion in these cells (Rigual et al., 2002). Caffeine was later shown to stimulate catecholamine secretion, whereas Ca\(^{2+}\) scintillas inhibited spontaneous release events (ZhuGe et al., 2006; Lefkowitz et al., 2009). In more recent experiments, depletion of ER Ca\(^{2+}\) stores was found to drastically decrease catecholamine secretion from mouse ACCs stimulated with high-K\(^{+}\) or NACHR agonists, suggesting that CICR does in fact contribute to the secretory responses of these cells (Wu et al., 2010). Additional studies are required to address these apparent discrepancies.
**Inositol Trisphosphate-stimulated Ca\(^{2+}\) Release**

Inositol 1,4,5-trisphosphate (IP\(_3\)) is an intracellular signaling molecule produced by the enzymatic digestion of plasma membrane phospholipids by phospholipase C (Berridge, 1983). IP\(_3\) production can be enhanced by a variety of stimuli that increase phospholipase C activity, including bradykinin, histamine, angiotensin II and muscarinic agonists (Plevin & Boarder, 1988). IP\(_3\) interacts with IP\(_3\)Rs present on the ER membrane to stimulate Ca\(^{2+}\) release (Streb *et al.*, 1983; Prentki *et al.*, 1984; Streb *et al.*, 1984). IP\(_3\)Rs exhibit large conductances and are relatively non-selective cation channels that can pass monovalent and divalent cations (Mak & Foskett, 1994; Tu *et al.*, 2005). IP\(_3\) binds to the cytosolic surface of the IP\(_3\)R and stimulates channel opening, allowing Ca\(^{2+}\) to flow from the ER lumen to the cytosol (Ehrlich & Watras, 1988). Three IP\(_3\)R isoforms have been identified in mammalian tissues and are known as IP\(_3\)R1, IP\(_3\)R2 and IP\(_3\)R3 (De Smedt *et al.*, 1997).

IP\(_3\) stimulates ER Ca\(^{2+}\) release in ACCs through the activation of IP\(_3\)Rs (O'Sullivan *et al.*, 1989; Cheek *et al.*, 1991; Robinson & Burgoyne, 1991; Poulsen *et al.*, 1995; Teraoka *et al.*, 1996; Alonso *et al.*, 1998; Alonso *et al.*, 1999; Huh *et al.*, 2005). IP\(_3\)Rs are present in ER located beneath the plasma membrane and within the perinuclear region. There is considerable co-localization of IP\(_3\)Rs and RyRs in ACCs, suggesting that both of these receptors may be present on the same ER Ca\(^{2+}\) stores (Inoue *et al.*, 2003). Although bovine, rat and mouse ACCs express functional IP\(_3\)Rs, ER Ca\(^{2+}\) release generated by IP\(_3\)R activation does not elicit a sizable secretory response on its own (Schneider *et al.*, 1977; Fisher *et al.*, 1981; Yamagami *et al.*, 1991; Wu *et al.*, 2010).
Store-operated Ca\(^{2+}\) Entry

Stimulation of RyRs and IP\(_3\)Rs can deplete ER Ca\(^{2+}\) stores (Cheek et al., 1994; Alonso et al., 1999). As a result, the ER must be refilled with Ca\(^{2+}\) before it can continue to participate in intracellular Ca\(^{2+}\) signaling during subsequent stimulations. Depletion of ER Ca\(^{2+}\) stores promotes Ca\(^{2+}\) influx through a process known as store-operated Ca\(^{2+}\) entry. Store-operated Ca\(^{2+}\) entry is mediated by the Ca\(^{2+}\)-release activated channel (CRAC), which is a Ca\(^{2+}\)-selective, inwardly rectifying and voltage-independent channel comprised of the Orai family of plasma membrane proteins (Hoth & Penner, 1992; Soboloff et al., 2006; Peinelt et al., 2006; Vig et al., 2006). Following ER Ca\(^{2+}\) depletion, Orai interacts with stromal interaction molecule (STIM)-1, which is present on the ER membrane (Roos et al., 2005; Liou et al., 2005; Penna et al., 2008). STIM1 facilitates the formation of functional Orai channel pores and stimulates Ca\(^{2+}\) entry (Penna et al., 2008; Mignen et al., 2008). Cytosolic Ca\(^{2+}\) is subsequently taken up by the SERCA to replenish the depleted ER Ca\(^{2+}\) stores. Ca\(^{2+}\) entry through the CRAC channel promotes slow and sustained catecholamine secretion in bovine ACCs (Robinson et al., 1992; Sui & Kao, 1994; Fomina & Nowycky, 1999; Zerbes et al., 2001). Mouse ACCs were recently shown to express STIM-1 and Orai and likely also possess functional store-operated Ca\(^{2+}\) entry mechanisms (Wu et al., 2010).

Mitochondrial Ca\(^{2+}\) Uptake

Mitochondria help shape the spatiotemporal properties of intracellular Ca\(^{2+}\) signals by rapidly taking up cytosolic Ca\(^{2+}\) into the mitochondrial matrix (Baker & Schlaepfer, 1978; Rizzuto et al., 1992; Rizzuto et al., 1994; Babcock et al., 1997). Mitochondrial Ca\(^{2+}\) uptake is dependent upon a negative mitochondrial membrane potential generated during cellular respiration and the presence of a Ca\(^{2+}\) uniporter.
The mitochondrial Ca\(^{2+}\) uniporter is a largely inwardly rectifying ion channel located on the inner mitochondrial membrane. The mitochondrial Ca\(^{2+}\) uniporter has a high selectivity for Ca\(^{2+}\) and an open probability of greater than 90\% at resting mitochondrial membrane potentials (Baughman et al., 2011; De Stefani et al., 2011). Ca\(^{2+}\) influx through the mitochondrial Ca\(^{2+}\) uniporter is voltage-dependent and sensitive to cytosolic Ca\(^{2+}\) concentrations (Kirichok et al., 2004). Ca\(^{2+}\) that is taken up into the mitochondria during stimulation is subsequently extruded by the Na\(^{+}\)/Ca\(^{2+}\) exchanger and the H\(^{+}\)/Ca\(^{2+}\) antiporter (Montero et al., 2000; Jiang et al., 2009).

In bovine ACCs, mitochondria rapidly take up Ca\(^{2+}\) entering through VGCCs or released from intracellular stores, thereby shortening the duration of the Ca\(^{2+}\) transient and reducing the amount of catecholamine that is released (Park et al., 1996; Herrington et al., 1996; Babcock et al., 1997; Montero et al., 2000; Ales et al., 2005). However, in mouse ACCs, mitochondrial Ca\(^{2+}\) uptake does not appear to play an important role in shaping stimulus-evoked Ca\(^{2+}\) transients (Wu et al., 2010). In fact, one study has shown that dissipation of the mitochondrial membrane potential actually decreases high-K\(^{+}\)-stimulated Ca\(^{2+}\) transients and inhibits catecholamine secretion in mouse ACCs (Ales et al., 2005). Mitochondrial Ca\(^{2+}\) uptake can reduce Ca\(^{2+}\)-dependent inactivation of VGCCs in ACCs, which may explain this apparent discrepancy (Hernandez-Guijo et al., 2001).

**Ca\(^{2+}\) Extrusion from the Cytosol**

In order for Ca\(^{2+}\) to be an effective intracellular signaling molecule, Ca\(^{2+}\) levels must be tightly regulated. Excitable cells have therefore developed Ca\(^{2+}\) extrusion mechanisms that readily return [Ca\(^{2+}\)]\(_i\) back to resting levels after cell stimulation. In
ACCs, Ca\(^{2+}\) extrusion across the plasma membrane is primarily accomplished by Ca\(^{2+}\)-ATPases and Na\(^{+}/Ca^{2+}\) exchangers (Kao & Cheung, 1990; Chern et al., 1992).

**EXCITABILITY**

Cellular excitability plays an important role in shaping the secretory responses of ACCs to synaptic inputs. Studies have shown that inflammation and infection can modulate the excitable properties of certain neuronal populations (Dong et al., 2008; Ochoa-Cortes et al., 2010). In the experiments that follow, ACC excitability is assessed during GI inflammation, systemic inflammation and exposure to MAMPs. Fundamental aspects of ACC excitability are therefore discussed below to provide the appropriate context for these experiments.

ACCs express voltage-gated Na\(^{+}\) and K\(^{+}\) channels, and can generate trains of APs in response to membrane depolarization. Mouse ACCs exhibit RMPs of approximately -45 to -55 mV that are largely dependent upon basal K\(^{+}\) conductance (Nassar-Gentina et al., 1988; Marcantoni et al., 2010; Mahapatra et al., 2011). The majority of mouse ACCs exhibit a low frequency of spontaneous APs and the rate of AP generation can be readily increased during periods of sympathetic activation (Marcantoni et al., 2009; Marcantoni et al., 2010; Perez-Alvarez et al., 2011). APs elicited by mouse ACCs exhibit a rapid TTX-sensitive upstroke that is produced by Na\(^{+}\) influx through voltage-gated Na\(^{+}\) channels. Ca\(^{2+}\) influx through VGCCs can also generate AP-like events in the absence of Na\(^{+}\) influx (Nassar-Gentina et al., 1988; Perez-Alvarez et al., 2011; Mahapatra et al., 2011). Activation of voltage-gated K\(^{+}\) channels and Ca\(^{2+}\)-activated K\(^{+}\) channels returns the membrane potential to resting levels and often produces a brief afterhyperpolarization (Marcantoni et al., 2010; Vandael et al., 2012).
Voltage-gated Na\textsuperscript{+} Channels

Neuronal voltage-gated Na\textsuperscript{+} channels are hetero-oligomers comprised of a pore-forming \(\alpha\)-subunit and accessory \(\beta\)-subunits that regulate \(\alpha\)-subunit expression, and the voltage-dependence and kinetics of channel gating (Hartshorne et al., 1982; Isom et al., 1992; Isom et al., 1995; Morgan et al., 2000; Yu et al., 2003). Nine \(\alpha\)-subunits have been identified to date and have been designated as Na\textsubscript{\(\alpha\)}1.1-1.9 (Goldin et al., 2000).

ACCs generate voltage-gated Na\textsuperscript{+} currents (\(I_{Na}\)) in response to membrane depolarization. \(I_{Na}\) in ACCs has rapid activation and inactivation kinetics and is TTX-sensitive (Fenwick et al., 1982b; Islas-Suarez et al., 1994; Conceicao et al., 1998; Vandael et al., 2012). Evidence suggests that Na\textsubscript{\(\alpha\)}1.7 is the predominant voltage-gated Na\textsuperscript{+} channel expressed by ACCs (Klugbauer et al., 1995; Yamamoto et al., 1996; Nemoto et al., 2013). Na\textsubscript{\(\alpha\)}1.7 channels exhibit slow rates of recovery from fast inactivation and delayed closed-state inactivation (Cummins et al., 1998; Herzog et al., 2003). Thus, the expression of Na\textsubscript{\(\alpha\)}1.7 channels in ACCs may limit the rate of AP generation and enable these cells to generate APs during gradual membrane depolarizations.

K\textsuperscript{+} Channels

The movement of K\textsuperscript{+} ions across the cell membrane is important for the establishment and maintenance of the RMP and the regulation of cellular excitability. Approximately 80 K\textsuperscript{+} channels have been identified to date and can be structurally and functionally categorized into inwardly rectifying K\textsuperscript{+} channels, two-pore domain K\textsuperscript{+} channels (\(K_{2p}\)), voltage-gated K\textsuperscript{+} channels and Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels (Harmar et al., 2009; Wulff et al., 2009).
Inwardly rectifying K+ channels are voltage-independent and can generate inward K+ currents at membrane potentials negative to the K+ equilibrium potential (Standen & Stanfield, 1978). Guinea pig ACCs express inward rectifier K+ channels that are constitutively active at rest and can be inhibited by GPCR activation (Inoue & Imanaga, 1993). Rat ACCs also express the ATP-sensitive K+ (KATP) channel, which appears to play an important role in O2-sensing in these cells (Mochizuki-Oda et al., 1997; Thompson & Nurse, 1998; Buttigieg et al., 2009; Livermore et al., 2012). However, the function of inwardly rectifying K+ channels in mouse ACCs has not been studied to date.

K2P channels are constitutively open at rest and provide a leak K+ conductance that helps establish RMP (Lesage et al., 1996; Leonoudakis et al., 1998). Guinea pig and rat ACCs express the TWIK-related acid sensitive K+ channel (Inoue et al., 2008; Inoue et al., 2012). However, it is unclear whether mouse ACCs also express K2P channels.

Approximately 40 voltage-gated K+ channels have been identified to date and can be broadly classified as rapidly activating and inactivating K+ channels and delayed-rectifier K+ channels based on their biophysical properties (Harmar et al., 2009). Sheep ACCs exhibit rapidly activating and inactivating K+ current (I\text{A}) and delayed rectifier K+ current (I\text{K}) (Rychkov et al., 1998). Similarly, bovine ACCs express the rapidly activating and inactivating K\text{v}1.4 voltage-gated K+ channel and the delayed rectifier K\text{v}1.5 voltage-gated K+ channel (Garcia-Guzman et al., 1992; Garcia-Guzman et al., 1994a; Garcia-Guzman et al., 1994b). In contrast, I\text{K} predominates in mouse ACCs and functions to repolarize the membrane potential during the downstroke of an AP (Marcantoni et al., 2010; Gavello et al., 2012).
Ca\(^{2+}\)-activated K\(^{+}\) currents account for the majority of outward K\(^{+}\) current elicited at depolarized membrane potentials in mouse ACCs (Marcantoni et al., 2010; Perez-Alvarez et al., 2011). These channels can be divided into small conductance (SK), intermediate conductance and large conductance (BK) K\(^{+}\) channels based on their biophysical and pharmacological properties. BK Ca\(^{2+}\)-activated K\(^{+}\) channels are both voltage- and Ca\(^{2+}\)-dependent, and are selectively inhibited by iberiotoxin (Marty, 1981; Galvez et al., 1990). Inhibition of BK channels increases the frequency of spontaneous APs, decreases the afterhyperpolarization following an AP and delays AP repolarization in mouse ACCs (Marcantoni et al., 2010). SK and intermediate conductance Ca\(^{2+}\)-activated K\(^{+}\) channels are voltage-independent and have a high sensitivity to cytosolic Ca\(^{2+}\) (Leinders & Vijverberg, 1992). Mouse ACCs express SK1, SK2 and SK3 Ca\(^{2+}\)-activated K\(^{+}\) channels, which function to maintain the RMP, increase the afterhyperpolarization following an AP, and reduce the frequency of spontaneous and evoked APs (Vandael et al., 2012).

REGULATION OF ADRENAL CHROMAFFIN CELL FUNCTION

**Immune Mediators**

An intricate and reciprocal relationship exists between the SNS and the immune system, and ACCs are an integral component of this network. Circulating inflammatory mediators readily access ACCs through fenestrated capillaries located throughout the adrenal medulla (Ryan et al., 1975). Adrenal cortical cells and immune cells present within the adrenal medulla can also locally release cytokines that may interact with ACCs (Hume et al., 1984; Gonzalez-Hernandez et al., 1994; Judd, 1998). Several cytokines have been shown to regulate important ACC functions, including Ca\(^{2+}\) signaling, catecholamine secretion, gene expression and neuropeptide release.
(Yanagihara et al., 1994; Ait-Ali et al., 2004; Morita et al., 2004). In mouse ACCs, interleukin (IL)-1β acutely enhances basal catecholamine and NPY release (Rosmaninho-Salgado et al., 2007). Similarly, histamine rapidly stimulates catecholamine secretion and potentiates high-K⁺-evoked catecholamine release (Marom et al., 2011). In contrast, acute application of prostaglandin (PG) E₂ rapidly inhibits $I_{Ca}$ and decreases catecholamine secretion in mouse ACCs (Jewell et al., 2011). These results suggest that inflammatory mediators can produce both excitatory and inhibitory effects in ACCs, which may enable the immune system to co-ordinate catecholamine and neuropeptide release with the specific needs of the inflammatory response.

**Activity-dependent Changes**

Aside from increasing the rate of secretion and promoting the co-release of catecholamines and neuropeptides, high-frequency firing patterns in preganglionic sympathetic neurons can promote long-lasting alterations in ACC function. Repetitive activation of ACCs persistently enhances catecholamine release by elevating the number of secretory events elicited per stimulation and increasing the quanta of catecholamine released per LDCV (Smith, 1999; Park et al., 2006). Chronic sympathetic activation also increases ACC excitability, and enhances catecholamine and neuropeptide synthesis through the upregulation of TH, PNMT and neuropeptide expression (Kanamatsu et al., 1986; Hamelink et al., 2002; Colomer et al., 2008; Stroth et al., 2013). These effects appear to be mediated by a combination of PAC1 receptor and NACHR receptor activation, as well as direct effects of membrane depolarization (Eiden et al., 1984; Craviso et al., 1992; Smith, 1999; Hamelink et al., 2002; Stroth et al., 2013).
POSTGANGLIONIC SYMPATHETIC NEURONS

ACCs and postganglionic sympathetic neurons are derived from the same lineage of progenitor cells of the neural crest during embryological development and share many functional characteristics (Anderson et al., 1991). Acetylcholine released from preganglionic sympathetic neurons activates NACHRs and MACHRs expressed by postganglionic sympathetic neurons to depolarize the membrane potential and promote AP generation (Trinus, 1979). APs that propagate along the axons of postganglionic sympathetic neurons activate VGCCs located within sympathetic varicosities and stimulate norepinephrine release (Macleod et al., 1994; Motagally et al., 2009b). Postganglionic sympathetic neuron function is also regulated by immune mediators and activity-dependent changes (Motagally et al., 2009a; Vargas et al., 2011).

INFLAMMATORY DISORDERS

Inflammation is an adaptive physiological response to injury and infection that functions to neutralize invading micro-organisms and promote tissue repair. Under normal conditions, a delicate balance between proinflammatory and anti-inflammatory signals ensures that the magnitude and duration of the inflammatory response is appropriate for the inciting stimulus. However, if the immune response becomes dysregulated or the infection is too severe, inflammatory mediators and microbial products can damage host tissues.

Inflammatory Bowel Disease

Inflammatory bowel disease (IBD) is a chronic, debilitating condition characterized by recurrent GI inflammation. The two most common forms of IBD include Crohn’s disease and ulcerative colitis (Bernstein et al., 2006). Patients with Crohn’s disease exhibit transmural inflammation that commonly occurs within the distal
ileum and perianal region, but can affect any part of the GI tract. The inflammatory lesions that occur during Crohn’s disease are discontinuous and may result in the development of ulcers, fibrosis, perforations or fistulae. Patients with Crohn’s disease commonly exhibit diarrhea, abdominal pain and weight loss (Stange et al., 2006). In contrast, ulcerative colitis is characterized by mucosal inflammation that predominantly affects the colon and rectum. Inflammatory lesions in patients with ulcerative colitis exhibit a continuous pattern and can lead to the development of crypt abscesses and mucosal ulcerations. Common clinical manifestations of ulcerative colitis include bloody diarrhea, rectal bleeding and rectal urgency (Stange et al., 2008).

Although the etiology of IBD remains elusive, it is generally thought that this condition results from an abnormal immune response to the GI microbiota in a genetically susceptible host. The GI immune system must maintain an intricate balance between tolerance of the intestinal normal flora and the development of rapid and effective immune responses against invading micro-organisms. During IBD, GI immune cells appear to become hyper-responsive and stimulate inflammation in the absence of an overt threat. The inflammatory response that occurs during active IBD is characterized by the infiltration of innate and adaptive immune cells into the intestinal wall. These cells then begin to secrete large amounts of proinflammatory cytokines, which further perpetuate the inflammatory response and promote tissue damage (see (Abraham & Cho, 2009)).

*Animal Models of Inflammatory Bowel Disease*

Animal models of aberrant GI inflammation have been developed to investigate the pathogenesis of IBD. Dextran sulfate sodium (DSS) is a water soluble, sulfated polymer of D-glucose that can be administered to animals through their drinking water
to induce mucosal inflammation of the colon. DSS can be administered according to acute and chronic regimens to induce transient or persistent colitis (Okayasu et al., 1990; Wirtz et al., 2007). 2,4,6-trinitrobenzene sulfonic acid (TNBS) can also be used to induce intestinal inflammation. TNBS is a hapten molecule that can be administered into the GI tract through an intraluminal injection or an enema to induce severe transmural inflammation (Morris et al., 1989; Wirtz et al., 2007).

**Sepsis**

Sepsis is a severe clinical condition characterized by a dysregulated systemic inflammatory response, known as systemic inflammatory response syndrome (SIRS), to an infection. Sepsis is a progressive disorder that is divided into three stages, including sepsis, severe sepsis and septic shock. Patients with sepsis exhibit SIRS and an infection, without any overt changes in mean arterial pressure or tissue perfusion. During severe sepsis, patients develop hypotension, hypoperfusion or organ dysfunction and often require fluid resuscitation (ACCP & SCCM Consensus Conference Committee, 1992). However, as these patients progress to septic shock, fluid resuscitation becomes insufficient to maintain mean arterial pressure and adequate tissue perfusion. These patients must therefore be treated with vasopressors and positive inotropic agents, such as catecholamines, to prevent vascular collapse (ACCP & SCCM Consensus Conference Committee, 1992; Dellinger et al., 2008). The pathological mechanisms responsible for the development and progression of sepsis have been difficult to characterize in human patients. Recent evidence suggests that sepsis is a heterogeneous disease that can result from excessive inflammation, inadequate inflammation, or an appropriate inflammatory response to a severe infection, each of which produces overwhelming tissue damage (see (Adib-Conquy & Cavaillon, 2009)).
Animal Models of Sepsis

A variety of animal models that reflect aspects of sepsis in human patients have been developed to help study the causes, consequences and compensatory mechanisms associated with this disease. The two most common animal models used in sepsis research are endotoxemia and cecal ligation and puncture (CLP). During the endotoxemia model of sepsis, rapid and severe systemic inflammation is induced by intravenous or intraperitoneal injection of LPS, a component of the Gram-negative bacterial cell membrane (Fairchild et al., 2009). During the CLP model of sepsis, the cecum is exteriorized, ligated below the ileocecal junction, punctured with a surgical needle and returned to the peritoneal cavity. Over the ensuing hours, bacteria begin to escape from the damaged cecum and colonize the peritoneal cavity (Wichterman et al., 1980). This produces a form of polymicrobial sepsis resembling sepsis due to perforated diverticulitis or ruptured appendicitis in human patients (Rittirsch et al., 2009). Systemic inflammation produced during the CLP model of sepsis occurs more gradually and is less severe than that of endotoxemia (Fairchild et al., 2009; Leelahavanichkul et al., 2011). Importantly, this model can be used to investigate the progression of sepsis from its early stages to the eventual development of septic shock (Hyde et al., 1990; Ganopolsky & Castellino, 2004).

RATIONALE FOR THESIS

Although several studies have characterized the effects of individual immune mediators on ACC function, the consequences of inflammatory disorders and direct exposure to MAMPs on ACC excitability, Ca^{2+} signaling and exocytosis are not well understood. This is an important area of investigation since catecholamines and neuropeptides secreted from ACCs possess potent immunomodulatory properties that
may alter the course of inflammation and infection. The present thesis was therefore performed to test the hypothesis that GI and systemic inflammation modulate ACC Ca\textsuperscript{2+} signaling, and that ACCs possess the ability to directly detect MAMPs. This hypothesis was addressed through three specific research studies described in chapters 2, 3 and 4.

**Chapter 2**

Animal models of IBD increase postganglionic sympathetic neuron excitability (Dong *et al.*, 2008). However, norepinephrine secretion from postganglionic sympathetic neurons is significantly impaired during colitis through an inhibition of I\textsubscript{Ca} (Motagally *et al.*, 2009b). Importantly, colitis affects postganglionic sympathetic neurons innervating inflamed as well as uninflamed regions of the GI tract (Jacobson *et al.*, 1995; Jacobson *et al.*, 1997; Blandizzi *et al.*, 2003; Motagally *et al.*, 2009b), suggesting that pathological GI inflammation can influence the function of distant sympathetic tissues. Given that ACCs and postganglionic sympathetic neurons have the same embryological origin and share many functional properties (Anderson *et al.*, 1991), chapter 2 of this thesis was performed to test the hypothesis that GI inflammation inhibits I\textsubscript{Ca} in ACCs, similar to postganglionic sympathetic neurons. The specific objectives of chapter 2 were:

a) to determine whether GI inflammation modulates voltage-dependent Ca\textsuperscript{2+} signaling in ACCs and

b) to identify the mechanism underlying altered Ca\textsuperscript{2+} signaling in ACCs during colitis.

**Chapter 3**

Sepsis elevates circulating catecholamine levels through an increase in preganglionic sympathetic neuron activity (Mills, 1990; Tkacs & Strack, 1995). Evidence suggests that sepsis may also directly increase the secretory capacity of ACCs
(Qi et al., 1991; Zhou & Jones, 1993; Jones et al., 1994). However, alterations in ACC function have yet to be thoroughly characterized during this condition. Chapter 3 of this thesis was performed to test the hypothesis that sepsis directly enhances the secretory capacity of ACCs through an increase in intracellular Ca\(^{2+}\) signaling. The specific objectives of chapter 3 were:

a) to determine whether sepsis directly increases the secretory capacity of ACCs,

b) to identify the mechanism underlying enhanced catecholamine secretion from ACCs during sepsis and

c) to characterize the role of circulating factors in the sepsis-induced alterations in ACC function.

**Chapter 4**

Growing evidence suggests that neurons and endocrine cells can directly detect MAMPs through the activation of pattern recognition receptors, such as TLR-4 (Hou & Wang, 2001; Ochoa-Cortes et al., 2010; Liu et al., 2011). Although ACCs were recently shown to express TLR-4 (Bornstein et al., 2004), the effects of TLR-4 activation on ACC function have yet to be investigated. IBD and sepsis are associated with increased bacterial translocation from the GI tract and elevated circulating levels of MAMPs (Casey et al., 1993; Martinez-Moya et al., 2012). Direct exposure of ACCs to MAMPs may therefore contribute to the alterations in ACC function that occur during GI and systemic inflammation. Chapter 4 of this thesis was performed to test the hypothesis that ACCs possess the ability to directly detect LPS and that TLR-4 activation by LPS contributes to the changes in ACC Ca\(^{2+}\) signaling produced by regional and systemic inflammation. The specific objectives of this chapter were:

a) to examine the expression of TLR-4 in ACCs,
b) to characterize the effects of TLR-4 activation on ACC excitability, Ca\textsuperscript{2+} signaling and neurohormone secretion and

c) to determine the role of NF-\kappaB in the LPS-induced alterations in ACC function.
Chapter 2: ALTERED ADRENAL CHROMAFFIN CELL FUNCTION DURING EXPERIMENTAL COLITIS
ABSTRACT

The sympathetic nervous system regulates visceral function through the release of catecholamines and co-transmitters from postganglionic sympathetic neurons and adrenal chromaffin cells (ACCs). Previous studies have shown that norepinephrine secretion is decreased during experimental colitis due to the inhibition of voltage-gated Ca\(^{2+}\) current (\(I_{Ca}\)) in postganglionic sympathetic neurons. The present study examined whether colonic inflammation causes a similar impairment in depolarization-induced Ca\(^{2+}\) influx in ACCs using the dextran sulfate sodium (DSS) model of acute colitis in mice. Alterations in ACC function during colitis were assessed using Ca\(^{2+}\) imaging and perforated patch clamp electrophysiology. ACCs isolated from mice with DSS-induced acute colitis exhibited significantly smaller high-K\(^+\)-stimulated Ca\(^{2+}\) transients compared to ACCs isolated from water-treated, time matched controls. Acute colitis caused a 10 mV hyperpolarization of the resting membrane potential, without significantly affecting ACC excitability. Delayed rectifier voltage-gated K\(^+\) current and voltage-gated Na\(^+\) current were significantly enhanced in ACCs from mice with DSS-induced acute colitis, with peak current densities of 154% and 144% that of controls, respectively. Importantly, acute colitis significantly inhibited \(I_{Ca}\) in ACCs between -25 and +20 mV. Peak \(I_{Ca}\) density in ACCs from mice with DSS-induced acute colitis was 61% that of controls. High-K\(^+\)-stimulated Ca\(^{2+}\) transients were also reduced in ACCs from mice with 2,4,6-trinitrobenzene sulfonic acid-induced acute colitis and DSS-induced chronic colitis to 68% and 78% of the control responses, respectively. Our results suggest that during colitis, voltage-dependent Ca\(^{2+}\) influx is impaired in ACCs through an inhibition of \(I_{Ca}\). Given the importance of Ca\(^{2+}\) signaling in exocytosis, these alterations likely decrease
the secretion of catecholamines from ACCs during colitis. This is the first demonstration of aberrant ACC function during experimental colitis.

**INTRODUCTION**

Inflammatory bowel disease (IBD) is a term used to describe a group of chronic disorders that are characterized by intermittent bouts of gastrointestinal (GI) inflammation. Crohn’s disease and ulcerative colitis are the two most prevalent forms of IBD that collectively place a substantial socioeconomic burden on developed countries around the world (Bernstein et al., 2006; Bernstein & Shanahan, 2008; Kappelman et al., 2008). Although the precise etiology of IBD remains elusive, evidence suggests that aberrant sympathetic nervous system (SNS) function may contribute to symptom generation during the course of this disease (Furlan et al., 2006; Straub et al., 2008).

Sympathetic hyperexcitability has been observed in patients with active ulcerative colitis using direct recordings from postganglionic sympathetic axons and power spectrum analysis of heart rate variability (Furlan et al., 2006; Maule et al., 2007; Ganguli et al., 2007). However, plasma epinephrine and norepinephrine levels are not significantly altered during ulcerative colitis, suggesting that the release of sympathoadrenal transmitters may be impaired during this condition (Furlan et al., 2006). In animal models of IBD, GI inflammation has been shown to affect various aspects of postganglionic sympathetic neuron function. Hyperexcitability has been observed in tonically firing neurons of the guinea pig celiac ganglion during 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced ileitis (Dong et al., 2008). However, reduced norepinephrine secretion from sympathetic varicosities has been described in the acute dextran sulfate sodium (DSS), 2,4-dinitrobenzene sulfonic acid (DNBS), TNBS and *Trichinella Spiralis* models of GI inflammation (Swain et al., 1991; Jacobson et al.,
DSS-induced acute colitis in mice inhibits N-type voltage-gated Ca$^{2+}$ current (I$_{Ca}$) in postganglionic sympathetic neurons of the superior mesenteric ganglion and may underlie the reduced norepinephrine secretion that is observed (Motagally et al., 2009b). An upregulation of $\alpha_2$-adrenergic receptors (ARs) has also been described during DNBS colitis and may increase presynaptic inhibition of norepinephrine secretion from sympathetic varicosities (Blandizzi et al., 2003). Importantly, norepinephrine secretion from sympathetic neurons was found to be decreased in both inflamed and uninflamed regions of the GI tract (Jacobson et al., 1995; Jacobson et al., 1997; Blandizzi et al., 2003; Motagally et al., 2009b), suggesting that localized inflammation can modulate the function of distant sympathetic tissues. Although it is clear that sympathetic neurophysiology is altered during experimental colitis, it is currently unknown whether adrenal chromaffin cells (ACCs), which secrete catecholamines into the systemic circulation, are also modulated by GI inflammation.

ACCs are excitable neuroendocrine cells that express voltage-gated Na$^+$, K$^+$ and Ca$^{2+}$ channels, and can generate trains of action potentials (APs) in response to stimulation (Biales et al., 1976; Fenwick et al., 1982b; Marty & Neher, 1985). ACCs are located within the adrenal medullae, highly vascularized structures that contain fenestrated capillaries (Ryan et al., 1975), which are permeable to large molecules, such as cytokines. ACCs express receptors for cytokines that are systemically elevated during IBD, including tumour necrosis factor (TNF)-$\alpha$ and interleukin (IL)-6 (Mahida et al., 1991; Gadient et al., 1995; Komatsu et al., 2001; Ait-Ali et al., 2004; Ait-Ali et al., 2008). Furthermore, inflammatory mediators have been shown to alter I$_{Ca}$ and
catecholamine secretion in ACCs in vitro (Tachikawa et al., 1997; Morita et al., 2004). Given these findings, we hypothesized that $I_{ca}$ is inhibited in ACCs during experimental colitis, similar to postganglionic sympathetic neurons (Motagally et al., 2009b). To test this hypothesis, colonic inflammation was induced in mice through the administration of DSS or TNBS, and changes in ACC function were assessed using Ca$^{2+}$ imaging and perforated patch clamp electrophysiology.

**MATERIALS AND METHODS**

**Mice and Reagents**

Male CD1 (25-35 g) and C57BL/6 (20-25 g) mice (Charles River, Saint-Constant, QC, Canada) were housed in a controlled environment where they were kept on a 12:12 hour light-dark cycle and allowed access to food and water *ad libitum*. Procedures performed on animals were in accordance with the principles and guidelines of the Canadian Council on Animal Care and Queen’s University Animal Care Committee. All reagents used during this study were purchased from Sigma Aldrich (St. Louis, MO) and a minimum of 3 mice were used in each experimental group, unless otherwise stated. Electrophysiological and Ca$^{2+}$ imaging experiments were performed at room temperature (22-24ºC).

**Induction of Colitis**

Acute colitis was induced in CD1 mice through the administration of 5% DSS (wt/vol; MP Biomedicals, Solon, OH) in drinking water for 5 days, followed by 2-3 days of normal water (Motagally et al., 2009b). Age-matched littermates were given normal drinking water for 7-8 days and used as controls. Acute colitis was also achieved following intracolonic injection of TNBS. CD1 mice were anaesthetized by intraperitoneal injection of ketamine (1.66 mg/10 g body weight) and xylazine (0.113
mg/10 g body weight), and a midline incision was performed. The descending colon was exposed and 4.5 mg of TNBS dissolved in 0.1 mL of 40% ethanol was injected directly into the lumen of the colon. Surgical incisions were closed in layers and the animals were euthanized 6-7 days later. In control mice, a midline incision was performed, the colon was exposed and an equal amount of 0.9% sterile saline was injected directly into the lumen of the colon. In a separate set of experiments, chronic colitis was induced in C57BL/6 mice through 3 consecutive cycles of DSS treatment, each consisting of 5 days of 2% DSS in drinking water followed by 5 days of normal drinking water. Age-matched littermates were given normal drinking water for 30 days and used as controls. The DSS- and TNBS-treated mice were monitored daily for signs of sickness behaviour.

**Measurement of Myeloperoxidase Activity**

Mice were deeply anaesthetized by isoflurane inhalation and euthanized by cervical dislocation. A laparotomy was performed and the colon was exposed. Segments of proximal colon were obtained from mice with DSS-induced acute colitis and water-treated controls, and segments of distal colon were excised from mice that received intracolonic injections of TNBS or saline. Fecal contents were removed, and each colon sample was blotted dry, weighed, flash-frozen in liquid nitrogen and stored at -80°C. Myeloperoxidase (MPO) activity was assayed within 2 weeks of colon extraction and used as an estimate of neutrophil infiltration and intestinal inflammation, as previously described (Bradley et al., 1982). Values are expressed as units of MPO activity per gram of tissue. One unit of MPO is defined as the amount that degrades 1 μmol of hydrogen peroxide per minute.
**Isolation of Adrenal Chromaffin Cells**

The methods used for isolating mouse ACCs have been adapted from Kolski-Andreaco et al. (2007). Briefly, mice were deeply anaesthetized by isoflurane inhalation and euthanized by cervical dislocation. A laparotomy was performed and the adrenal glands were excised. The outer cortex of each adrenal gland was removed under a dissecting microscope and the remaining adrenal medullae were placed in ice cold Hank’s Balanced Salt Solution (HBSS). After being washed three times, adrenal medullae were enzymatically dissociated by incubation in HBSS containing papain (30 U/mL; Worthington Biomedical Corporation, Lakewood, NJ) and L-cysteine (0.67 mg/mL) for 35 minutes at 37˚C. The papain solution was then removed and residual enzyme was neutralized by the addition of Dulbecco’s Modified Eagle’s Medium (DMEM; Invitrogen, Carlsbad, CA) containing 15% fetal bovine serum (FBS). Adrenal medullae were physically dissociated by gentle trituration through a series of plastic and fire-polished glass pipettes of decreasing internal diameter. Isolated ACCs were plated on 16 mm glass coverslips coated with poly-D lysine (2 mg/mL) and laminin (20 µg/mL). Cells were cultured overnight at 37˚C and 5% CO₂ in DMEM supplemented with 15% FBS and 2500 IU penicillin-streptomycin. Electrophysiological and Ca²⁺ imaging experiments were performed on the following day.

**Intracellular Ca²⁺ Imaging**

Intracellular Ca²⁺ concentration ([Ca²⁺]ᵢ) was monitored in isolated ACCs using ratiometric analysis of the fluorescent Ca²⁺ indicator dye, Fura-2 acetoxymethyl ester (AM). ACCs were incubated in DMEM containing 2.5 µM Fura-2AM (dissolved in DMSO; Invitrogen Carlsbad, CA) for 30 minutes at 37˚C to allow for uptake of the dye.
Coverslips containing Fura-2AM-loaded ACCs were placed in a recording chamber on an inverted microscope (Olympus IX71, Markham, ON, Canada) and ACCs were superfused at a rate of 2 mL/min with a normal HEPES-buffered saline solution containing (mM) 150 NaCl, 10 glucose, 10 HEPES, 2 MgCl₂, 2.5 CaCl₂, 2.8 KCl, with pH adjusted to 7.4 with NaOH (Fulop et al., 2005). ACCs were superfused with normal saline for 20 minutes to remove excess dye and allow for intracellular de-esterification. The Fura-2 loaded cells were then illuminated with a DeltaRamV high-speed random access monochromator at 340 and 380 nm. Fluorescence emitted at 510 nm was detected by a Photometrics Cascade 512B CCD camera (Photon Technology International, London, ON, Canada) and recorded on a PC using ImageMaster 5.0 software. A region of interest (ROI) was defined within the cytoplasm of each ACC and the ratio of fluorescence generated by excitation at 340 and 380 nm was measured for each ROI every second and used as an estimate of [Ca²⁺]ᵢ. Baseline [Ca²⁺]ᵢ was monitored for 3 minutes and ACCs were subsequently stimulated for 1 minute with a high K⁺ extracellular solution of the following composition (mM): 20 KCl, 132.8 NaCl, 10 glucose, 10 HEPES, 2 MgCl₂, 2.5 CaCl₂, pH adjusted to 7.4 with NaOH. In a separate set of experiments, ACCs were superfused for 1 minute with the nicotinic acetylcholine receptor (NACHr) agonist, 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP; 20 µM) to assess NACHr function. A 5 minute washout period followed each stimulus to ensure that [Ca²⁺]ᵢ returned to baseline levels. Approximately 20 ACCs were recorded from during each stimulation period. Baseline fluorescence was averaged over 30 340/380 excitation cycles acquired prior to cell stimulation. To compare changes in [Ca²⁺]ᵢ in response to application of high K⁺ or DMPP between cell populations, the peak change
in 340/380 fluorescence ratio was expressed as a percentage change of the baseline fluorescence prior to stimulation (Motagally et al., 2009b).

**Electrophysiological Recordings**

Patch pipettes were fabricated from borosilicate glass capillaries (Warner Instruments, Hamden, CT) and polished to a final resistance of 1.5-3 MΩ when filled with an internal solution containing (mM) 110 K-gluconate, 20 KCl, 10 EGTA, 10 HEPES, 4 Na₂ATP, 1 CaCl₂, 1 MgCl₂, 0.2 NaGTP, with pH adjusted to 7.2 with KOH. Isolated ACCs were viewed through an inverted microscope and superfused at a rate of 2 mL/min with a normal HEPES-buffered saline solution. The perforated patch clamp configuration was achieved by backfilling pipettes with an internal solution containing amphotericin B. A stock solution of amphotericin (60 mg/ml) was prepared fresh each morning and diluted with internal solution to a final concentration of 240 µg/mL before use. After a GΩ seal was acquired, the cell membrane was gradually perforated over 10-15 minutes until a stable access resistance between 7 and 20 MΩ was achieved. Leak subtraction and liquid junction potential compensation were not performed. For voltage clamp experiments, membrane capacitance and series resistance were electronically compensated by 70-80%, and current amplitude was normalized to cell capacitance.

Current-clamp experiments were performed on isolated ACCs to assess parameters of excitability. Resting membrane potential (RMP) was monitored and ACCs were stimulated with 500 ms current injections ranging from -14 to +24 pA in 2 pA increments, once every 5 seconds. Current-clamp recordings were also used to measure the amplitude of depolarization produced by a 1 minute superfusion of high K⁺ or DMPP.
To assess voltage-gated Na\(^+\) and K\(^+\) currents, ACCs were held at -90 mV and stimulated with 500 ms command potentials ranging from -100 to +50 mV in 10 mV increments, once every 5 seconds. The peak amplitude of voltage-gated Na\(^+\) current (I\(_{Na}\)) was measured within the first 3 ms of each voltage step, while delayed-rectifier voltage-gated K\(^+\) current (I\(_{K}\)) was measured within the last 100 ms. A 500 ms test pulse to 0 mV was applied following each command potential to examine the voltage-dependence of current inactivation. To quantify DMPP-induced inward current amplitude, ACCs were held at -50 mV to represent the average RMP, and DMPP was superfused for 1 minute.

For experiments measuring I\(_{Ca}\), patch pipettes were filled with an internal solution containing (mM) 108 Cs-methanesulfonate, 20 CsCl, 10 HEPES, 1 MgCl\(_2\), 4 MgATP, 0.3 NaGTP, 8 NaCl, 10 EGTA, with pH adjusted to 7.2 with CsOH. ACCs were superfused with an extracellular solution containing (mM) 100 NaCl, 45 tetraethylammonium (TEA)-Cl, 10 glucose, 10 HEPES, 2 MgCl\(_2\), 5 BaCl\(_2\), 0.0003 tetrodotoxin (TTX), with pH adjusted to 7.4 with NaOH. Ba\(^{2+}\) was used as the primary charge carrier through voltage-gated Ca\(^{2+}\) channels (VGCCs) to minimize Ca\(^{2+}\)-dependent inactivation (Hernandez-Guijo et al., 1998). I\(_{Ca}\) carried by Ba\(^{2+}\) was measured in response to 100 ms command potentials ranging from -60 to +55 mV in 5 mV increments from a holding potential of -90 mV, once every 10 seconds.

Electrophysiological recordings were performed using a MultiClamp 700B amplifier. Data were acquired at 10 kHz using a Digidata 1440A data acquisition system and recorded on a PC using pClamp software (all from MDS Analytical Technologies, Mississauga, ON, Canada). Data were analyzed offline using Clampfit 10.0 (MDS Analytical Technologies). For activation and inactivation profiles, data were normalized to peak current density (I/I\(_{max}\)) and fit to a Boltzmann function of the form: I/I\(_{max}\) = 1/(1
I = \frac{I_\text{max}}{1 + \exp\left[\frac{V_{50} - V_m}{k}\right]}, \text{ where } I \text{ is current, } V_m \text{ is membrane potential, } V_{50} \text{ is the voltage of half-maximal current, } k \text{ is the slope factor and } I_\text{max} \text{ is the maximum current.}

**Real-time Polymerase Chain Reaction Analysis of Ca^{2+} Channel \( \alpha_1 \)-Subunit Expression**

RNA was extracted from adrenal medullae of control and DSS-treated mice using the Trizol method (Invitrogen). cDNA was then reverse transcribed from 2 μg of total RNA using Superscript III Reverse Transcriptase (Roche, Mississauga, Canada) and intron-spanning oligo(dT) primers (Invitrogen; Table 2-1). Subsequently, real-time polymerase chain reaction (PCR) was performed using a Roche Lightcycler and a Quantitech Sybrgreen PCR Kit (Qiagen, Mississauga, Canada) with GAPDH as the reference gene. The target-to-reference ratio was then calculated using Relquant software (Roche).

**Statistical Analysis**

Statistical analysis was performed using GraphPad Prism 4. Unpaired t-tests were used to compare parametric data and Mann-Whitney tests were used to compare non-parametric data. Proportional data were compared using the Fisher’s exact test. Two-way analyses of variance (ANOVAs) with Bonferroni post-tests were used to compare current-voltage relation data. N values refer to the number of animals and n values refer to the number of cells used in a given experiment. Statistical significance was inferred when p < 0.05. Population data are expressed as mean ± standard error of the mean (S.E.M).
<table>
<thead>
<tr>
<th>Gene Product</th>
<th>Primers</th>
<th>Product Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH (NM_008084)</td>
<td>F: TAG ACA AAA TGG TGA AGG TCG G R: AGT TGA GGT CAA TGA AGG GGT</td>
<td>130</td>
</tr>
<tr>
<td>CaV1.2 (NM_009781)</td>
<td>F: CCA TTG CCT CCG AAC ATT AC R: CTG AAG ACC CAG GCT GTA CAT</td>
<td>125</td>
</tr>
<tr>
<td>CaV1.3 (NM_028981)</td>
<td>F: CAA GCG AAG CAC CTT TGA CAA C R: GAT ACT GAC AAT CAT CCC GGA AG</td>
<td>140</td>
</tr>
<tr>
<td>CaV2.1 (NM_007578)</td>
<td>F: CAT GGA AGG CTG GACTGA T R: AAA GGA GCC GAT GAT GAT AGG</td>
<td>105</td>
</tr>
<tr>
<td>CaV2.2 (NM_007579)</td>
<td>F: AGC CCT CAG ATC CCA GCA R: GCC TCC TTC TTG CCC TCT</td>
<td>124</td>
</tr>
<tr>
<td>CaV2.3 (NM_009782)</td>
<td>F: GTG GCC ATT GTT CATCAC AAC R: TGA GTG AAAATA GAG GCG TGG C</td>
<td>138</td>
</tr>
</tbody>
</table>

**Table 2-1.** Intron-spanning primers used for real-time PCR. F, forward primer; R, reverse primer; bp, base pairs.
RESULTS

Assessment of inflammation

Mice with acute DSS-, chronic DSS- and TNBS-induced colitis frequently displayed well characterized manifestations of colonic inflammation, including diarrhea, rectal bleeding, adhesions and dilation of the colon, all of which were absent in control animals. Acute administration of DSS resulted in significant weight loss. By day 7, mice with DSS-induced acute colitis had lost 6.6 ± 2.6% of their initial body weight, while control mice exhibited a 10.1 ± 1.3% increase in body weight (control, N = 9; DSS, N = 13; p < 0.01). Significant weight loss was also observed in mice with TNBS colitis. By day 1, TNBS-treated mice exhibited an 8.4 ± 1.4% reduction in body weight compared to a 1.4 ± 0.9% decrease in control mice. On days 3 and 6, mice with TNBS colitis exhibited 15.7 ± 0.9% and 10.7 ± 1.6% reductions in body weight, respectively, whereas the weights of control mice had marginally increased (control, N = 3; TNBS, N = 3; p < 0.001 at each time point).

Inflammation was assessed in mice with acute DSS- and TNBS-induced colitis using an assay for MPO activity. Colon samples from mice with DSS-induced acute colitis exhibited significantly elevated MPO activity compared to controls (control, 0.64 ± 0.22 units/g, N = 10; DSS, 4.41 ± 0.49 units/g, N = 12; p < 0.001). Mice with TNBS colitis also exhibited significantly higher levels of colonic MPO activity compared to sham operated animals (control, 0.40 ± 0.18 units/g, N = 3; TNBS, 11.3 ± 3.5 units/g, N = 3; p < 0.05).

Acute and chronic colitis inhibited depolarization-induced Ca^{2+} influx in ACCs

Changes in [Ca^{2+}]_{i} can modulate important cellular processes, including gene expression, excitability, enzyme activity, muscle contraction and neurotransmitter
secretion (Sandow, 1952; Douglas & Rubin, 1961; Morgan & Curran, 1986; Zhang et al., 1993a; Marcantoni et al., 2010). [Ca^{2+}]_{i} can be rapidly elevated in ACCs through the activation of VGCCs and the influx of extracellular Ca^{2+}. Our laboratory has previously shown that DSS-induced acute colitis inhibits depolarization-evoked Ca^{2+} influx in postganglionic sympathetic neurons (Motagally et al., 2009b). In the present study, we wanted to assess whether voltage-dependent Ca^{2+} signaling was similarly impaired in ACCs during colitis. [Ca^{2+}]_{i} was monitored using fluorescence imaging of the Ca^{2+} indicator dye, Fura-2AM. ACCs were stimulated with a high-K^{+} extracellular solution to depolarize the membrane potential, stimulate AP generation and activate VGCCs. During superfusion with high-K^{+} extracellular saline, [Ca^{2+}]_{i} was transiently but substantially increased in ACCs from control mice and mice with DSS-induced acute colitis (Figure 2-1A). However, the amplitudes of high-K^{+}-stimulated Ca^{2+} transients were significantly reduced in ACCs from mice with DSS-induced acute colitis to 74.3% of the control response (Figure 2-1B). No significant difference was observed in baseline 340/380 fluorescence ratios between groups, indicating that DSS-induced acute colitis did not affect resting [Ca^{2+}]_{i} in ACCs (control, 0.203 ± 0.003, n = 267; DSS, 0.198 ± 0.002, n = 585; p > 0.05).

In order to determine whether voltage-dependent Ca^{2+} signaling was affected during other models of colitis, Ca^{2+} imaging experiments were performed on ACCs from mice with TNBS-induced acute colitis and DSS-induced chronic colitis. We found that TNBS colitis inhibited the Ca^{2+} transients produced during high-K^{+} stimulation of ACCs to a similar extent to DSS-induced acute colitis. The high-K^{+}-stimulated increase in [Ca^{2+}]_{i} in ACCs from TNBS-treated mice was only 68.3% that of sham operated controls (Figure 2-1C). DSS-induced chronic colitis also resulted in significantly blunted
Ca\(^{2+}\) responses to high-K\(^+\) superfusion in ACCs. The amplitude of increase in [Ca\(^{2+}\)]\(_i\) in ACCs from mice with DSS-induced chronic colitis was reduced to 78.4% of the control response to high-K\(^+\) stimulation (Figure 2-1D). Since DSS-induced acute and chronic colitis, and TNBS-induced colitis each had similar inhibitory effects on voltage-dependent Ca\(^{2+}\) influx, subsequent mechanistic experiments focussed on the DSS-induced acute colitis model, henceforth referred to in the results section as DSS-colitis.

**DSS-colitis hyperpolarized ACC RMP**

It is possible that differences in the amplitude of the depolarization produced by high-K\(^+\) stimulation could have contributed to the reduced Ca\(^{2+}\) signaling observed in ACCs from mice with colitis. Current-clamp recordings were therefore used to measure high-K\(^+\)-induced depolarizations in ACCs from each group. The RMPs of ACCs from mice with DSS-colitis were found to be significantly hyperpolarized compared to ACCs from untreated control mice (Figure 2-2A). Superfusion of high-K\(^+\) extracellular saline resulted in transient depolarizations in ACCs from control mice (Figure 2-2B) and mice with DSS-colitis. No significant difference in the amplitude of depolarization was observed between groups (Figure 2-2C). However, the most depolarized membrane potential reached during high-K\(^+\) stimulation was significantly hyperpolarized in ACCs from mice with DSS-colitis compared to controls (Figure 2-2D).

**ACC excitability was not affected by DSS-colitis**

APs fired by ACCs transiently depolarize the membrane potential and activate VGCCs to increase [Ca\(^{2+}\)]\(_i\) (Chan *et al.*, 2005). Changes in cellular excitability may therefore have important effects on Ca\(^{2+}\) signaling in ACCs. Current-clamp electrophysiological recordings were performed on ACCs isolated from control mice.
Figure 2-1. Acute and chronic colitis inhibited depolarization-induced Ca$^{2+}$ influx in ACCs. A) Ca$^{2+}$ imaging recordings of high-K$^+$-induced increases in [Ca$^{2+}$]$_i$ in ACCs from control mice and mice with DSS-induced acute colitis. B) Mean ± S.E.M. graph depicting the peak percent change in 340:380 ratio from baseline during high-K$^+$ stimulation. ACCs from mice with DSS-induced acute colitis exhibited significantly reduced Ca$^{2+}$ influx compared to controls. Control, n = 267; DSS, n = 585; **p < 0.001. C) Mean ± S.E.M. graph of the peak increase in [Ca$^{2+}$]$_i$ relative to baseline during high-K$^+$ stimulation of ACCs from control and TNBS-treated animals. Ca$^{2+}$ influx was significantly inhibited in ACCs from mice with TNBS-induced colitis. Control, n = 327; TNBS, n = 321; **p < 0.001. D) Graph of the mean ± S.E.M. high-K$^+$-induced peak percent change in [Ca$^{2+}$]$_i$ relative to baseline in ACCs from control mice and mice with DSS-induced chronic colitis (cDSS). DSS-induced chronic colitis led to a significant inhibition of voltage-dependent Ca$^{2+}$ influx in ACCs. Control, n = 116; chronic DSS colitis, n = 322; **p < 0.001.
Figure 2-2. DSS-colitis hyperpolarized ACC RMP. A) ACCs from mice with DSS-induced acute colitis exhibited significantly hyperpolarized RMPs compared to controls. Control, n = 29; DSS, n = 32; **p < 0.001. B) Current clamp recording of a high-K⁺-induced depolarization in a control ACC. C) Mean ± S.E.M. graph illustrating the amplitude of depolarization in ACCs from control mice and mice with DSS-induced acute colitis following the application of high-K⁺. No significant difference was observed between groups. Control, n = 10/N = 3; DSS, n = 10/N = 2; p > 0.05. D) Mean ± S.E.M. peak membrane potential achieved during high-K⁺-stimulation. The most depolarized membrane potential reached during high-K⁺ administration was significantly hyperpolarized in ACCs from mice with DSS-induced acute colitis compared to controls. Control, n = 10/N = 3; DSS, n = 10/N = 2; *p < 0.01.
and mice with DSS-colitis to assess several parameters of excitability. ACCs from both
groups had large input resistances and fired APs in response to small depolarizing
current injections. Spontaneous APs were frequently observed at rest. These general
observations are in accordance with previous reports on isolated bovine, rat and mouse
ACCs (Fenwick et al., 1982a; Lovell et al., 2004; Vandael et al., 2012). Although DSS-
colitis produced a significant hyperpolarization of ACC RMP, no change in cellular
excitability was observed between groups. Input resistance, rheobase, the number of APs
elicited at rheobase, the number of APs elicited at 2x rheobase, AP amplitude, AP half-
width, and the proportion of cells exhibiting spontaneous APs were not significantly
different between ACCs from control mice and mice with DSS-colitis (Table 2-2).

ACC RMP can be modulated by changes in K⁺ conductance (Lee et al., 2000;
Bournaud et al., 2007). Voltage-clamp experiments were therefore performed to
determine whether alterations in voltage-gated K⁺ currents may have contributed to the
hyperpolarized RMP that was observed during DSS-colitis. Similar to previous reports
(Thompson et al., 1997), I_K was elicited by step depolarizations in all ACCs tested,
whereas rapidly activating and inactivating K⁺ current (I_A) was not detectable (Figure 2-
3A and B). In ACCs from mice with DSS-colitis, the amplitude of I_K was significantly
enhanced between +20 and +50 mV (Figure 2-3C), and peak I_K density was 154.3% that
of controls (control, 1116.05 ± 71.4 pA/pF, n = 30; DSS, 1722.1 ± 100.7 pA/pF, n = 13).

Step depolarizations also elicited I_Na in ACCs from control mice and mice with
DSS-colitis. The amplitude of I_Na was found to be significantly larger in ACCs from
mice with DSS-colitis compared to controls at membrane potentials between -20 and 0
mV (Figure 2-4A). Peak I_Na density in ACCs from mice with DSS-colitis occurred at 0
<table>
<thead>
<tr>
<th></th>
<th>Control (n = 29)</th>
<th>DSS (n = 32)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Input Resistance (GΩ)</td>
<td>4.86 ± 0.38</td>
<td>5.54 ± 0.41</td>
</tr>
<tr>
<td>Rheobase (pA)</td>
<td>1.93 ± 0.46</td>
<td>2.50 ± 0.48</td>
</tr>
<tr>
<td>Number of APs at Rheobase</td>
<td>1.59 ± 0.16</td>
<td>1.72 ± 0.19</td>
</tr>
<tr>
<td>Number of APs at 2x Rheobase</td>
<td>3.24 ± 0.22</td>
<td>3.06 ± 0.27</td>
</tr>
<tr>
<td>AP Amplitude (mV)</td>
<td>70.92 ± 3.19</td>
<td>73.86 ± 3.59</td>
</tr>
<tr>
<td>AP Half-width (ms)</td>
<td>5.53 ± 0.56</td>
<td>8.04 ± 1.43</td>
</tr>
<tr>
<td>Percentage of Cells Exhibiting</td>
<td>55.2</td>
<td>46.9</td>
</tr>
<tr>
<td>Spontaneous APs (%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Table 2-2. Excitability parameters of ACCs from control mice and mice with DSS-colitis. DSS-colitis did not alter ACC excitability. Values are expressed as mean ± S.E.M. AP, action potential. p > 0.05 for each parameter of ACC excitability.*
Figure 2-3. $I_K$ was enhanced in ACCs during DSS-colitis. Voltage clamp recordings of $I_K$ in ACCs from control mice (A) and mice with DSS-colitis (B). Stimulus waveform is illustrated below. C) Mean ± S.E.M. graph depicting the I-V relation of $I_K$ in ACCs from control mice and mice with DSS-colitis. DSS-colitis caused a significant increase in $I_K$ at membrane potentials between +20 and +50 mV. Control, n = 30; DSS, n = 13; *p < 0.01, **p < 0.001.
mV and was 144.3% that of the control peak current density occurring at +10 mV (control, 162.9 ± 18 pA/pF, n = 30; DSS, 235 ± 22.2 pA/pF, n = 13). To determine whether changes in channel gating may contribute to the enhanced Na\(^+\) conductance observed in ACCs from mice with DSS-colitis, the voltage-dependences of activation and inactivation of I\(_{\text{Na}}\) were examined. ACCs from mice with DSS-colitis exhibited a leftward shift in the activation profile of I\(_{\text{Na}}\) (Figure 2-4B) and a hyperpolarized voltage of half-maximal activation (Figure 2-4C). The slope factor for activation was not significantly different between ACCs from control mice and mice with DSS-colitis (control, 4.4 ± 0.2, n = 30; DSS, 3.9 ± 0.2, n = 13; p > 0.05). No significant difference in the inactivation profile, the voltage of half-inactivation (Figure 4D), or the slope factor for inactivation (control, 6.53 ± 0.33, n = 30; DSS, 7.16 ± 0.55, n = 13; p > 0.05) was observed between groups.

**I\(_{\text{Ca}}\) was inhibited in ACCs during DSS-colitis**

DSS-colitis has previously been shown to inhibit I\(_{\text{Ca}}\) in postganglionic sympathetic neurons (Motagally *et al.*, 2009b). We therefore performed voltage-clamp analysis of I\(_{\text{Ca}}\) to determine whether similar alterations occur in ACCs. We found that there was a significant inhibition of I\(_{\text{Ca}}\) in ACCs from mice with DSS-colitis at membrane potentials between -25 and +20 mV (Figure 2-5C). Peak I\(_{\text{Ca}}\) density in ACCs from mice with DSS-colitis at 0 mV was only 61.6% that of peak I\(_{\text{Ca}}\) density in control ACCs produced at -5 mV (control, -62.5 ± 4.4 pA/pF, n = 24; DSS, -38.5 ± 2.8 pA/pF, n = 29), a similar percentage inhibition to that seen in the Ca\(^{2+}\) imaging experiments. The voltage-dependence of activation of I\(_{\text{Ca}}\) was measured in ACCs from control mice and mice with DSS-colitis. The activation profile of I\(_{\text{Ca}}\) did not differ between groups (Figure 2-5D). No significant difference in the voltage of half-maximal activation
Figure 2-4. DSS-colitis increased $I_{Na}$ in ACCs and caused a hyperpolarizing shift in its voltage-dependence of activation. A) Mean ± S.E.M. I-V relation of $I_{Na}$ in ACCs from control mice and mice with DSS-colitis. $I_{Na}$ was significantly enhanced in ACCs from mice with DSS-colitis between -20 and 0 mV. B) Voltage-dependence of activation of $I_{Na}$. DSS-colitis caused a hyperpolarizing shift in the activation profile of $I_{Na}$. C) Mean ± S.E.M. voltage of half-maximal activation for $I_{Na}$ in ACCs from control mice and mice with DSS-colitis. ACCs from mice with DSS-colitis had a significantly hyperpolarized voltage of half-maximal activation compared to controls. D) Mean ± S.E.M. voltage of half-maximal inactivation for $I_{Na}$. The voltage of half-maximal inactivation for $I_{Na}$ was not significantly different between groups. $p > 0.05$. For A-D, control, $n = 30$; DSS, $n = 13$; **$p < 0.001$. 
Figure 2-5. *DSS-colitis inhibited I_{Ca} in ACCs.* Representative voltage clamp recordings of I_{Ca} in ACCs from control mice (A) and mice with DSS-colitis (B). Stimulus waveform is given in inset. C) Mean ± S.E.M. I-V relation of I_{Ca}. DSS-colitis produced a significant inhibition of I_{Ca} in ACCs at membrane potentials between -25 and +20 mV. *p < 0.01, **p < 0.001. D) Mean ± S.E.M. graph illustrating the voltage-dependence of activation of I_{Ca} in ACCs from control mice and mice with DSS-colitis. For C and D, control, n = 24; DSS, n = 29.
(control, $-21.1 \pm 1.8 \text{ mV, } n = 24$; DSS, $-20.1 \pm 1.9 \text{ mV, } n = 29$; $p > 0.05$) or the slope factor for activation (control, $5.7 \pm 0.2$, $n = 24$; DSS, $5.6 \pm 0.1$, $n = 29$; $p > 0.05$) was detected between ACCs from control mice and mice with DSS-colitis.

**Ca$^{2+}$ channel subunit expression during DSS-colitis**

We have previously described a decrease in $I_{\text{Ca}}$ in postganglionic sympathetic neurons during colitis that was due to the selective inhibition of N-type Ca$^{2+}$ channels accompanied by a reduction in mRNA encoding the channel $\alpha_1$-subunit, Cav2.2 (Motagally et al., 2009b). Therefore, we examined whether the inhibition of $I_{\text{Ca}}$ in ACC was associated with a selective reduction in mRNA encoding VGCC $\alpha_1$ subunits in these cells: Cav1.2, Cav1.3, Cav2.1, Cav2.2, and Cav2.3 (Hernandez-Guijo et al., 1998; Aldea et al., 2002; Chan et al., 2005; Wu et al., 2010). Colitis caused a slight but statistically insignificant decrease in mRNA encoding Cav2.2, which underlies N-type Ca$^{2+}$ channels, as well as Cav2.1, which underlies P/Q-type Ca$^{2+}$ channels (Figure 2-6C and D). Interestingly, Cav1.2 mRNA was significantly increased in medullae from mice with colitis (Figure 2-6A). The implication of this finding is unclear, however, given that $I_{\text{Ca}}$ and voltage-dependent Ca$^{2+}$ influx were reduced in ACCs in this model of colitis.

mRNA for Cav1.3 (another L-type channel subunit in ACCs) was not affected by colitis (Figure 2-6B). Although Cav2.3 (R-type channels) mRNA was detectable in mouse adrenal medullae by RT-PCR, the amount of mRNA present for this channel was too low to accurately perform real-time PCR analysis.

**DSS-colitis reduced DMPP-stimulated Ca$^{2+}$ influx in ACCs**

Acetylcholine released by preganglionic sympathetic neurons provides the primary stimulus for ACC activation *in vivo* (Petrovic et al., 2010). We therefore assessed whether Ca$^{2+}$ signaling in response to stimulation with DMPP, a NACHR
Figure 2-6. Effects of DSS-colitis on mRNA expression of VGCC $\alpha_1$-subunits, as measured using real-time PCR. Adrenal medullae were harvested from 7 control mice and 7 mice with DSS-colitis to quantify the expression of mRNA encoding Cav1.2 (A), Cav1.3 (B), Cav2.1 (C), and Cav2.2 (D). * $p < 0.05$. 

agonist, was also affected during colitis. NACHRs are ligand-gated cation channels that generate a net inward cation current and a depolarization of the membrane potential when activated from the RMP (Douglas et al., 1967a; Douglas et al., 1967b). Similar to high-K⁺ extracellular saline, DMPP produced transient elevations in [Ca²⁺], that were significantly reduced in ACCs from mice with DSS-colitis compared to controls (Figure 2-7A). However, there was no significant difference in the amplitude of depolarization (Figure 2-7B) or the peak current produced by DMPP superfusion between groups (Figure 2-7C).

DISCUSSION

Intricate and reciprocal interactions occur between the SNS and the immune system during health and disease. Studies have shown that inflammatory mediators can alter sympathetic neurophysiology and, in turn, sympathetic neurotransmitters can modulate immune cell activation and cytokine secretion (Severn et al., 1992; Morita et al., 2004; Motagally et al., 2009a). However, the effects of colitis on SNS function are currently not well defined. In the present study, we examined ACC function in mouse models of colitis. We found that depolarization-induced Ca²⁺ influx was reduced in ACCs during acute DSS-, chronic DSS- and TNBS-induced colitis through an inhibition of Iₖₐ.

Stimulus-secretion coupling is a term used to describe the ability of Ca²⁺ to couple cellular excitation with exocytosis (Douglas & Rubin, 1963). Acetylcholine released from preganglionic sympathetic neurons activates NACHRs expressed by ACCs to depolarize the membrane potential and initiate APs. These APs provide large, transient depolarizations that rapidly activate VGCCs to increase [Ca²⁺]ᵢ (Chan et al., 2005). Ca²⁺-sensing molecules associated with the exocytotic machinery detect
Figure 2-7. DSS-colitis inhibited DMPP-induced Ca$^{2+}$ influx in ACCs. A) Mean ± S.E.M. peak percent change in [Ca$^{2+}$] from baseline in ACCs from control mice and mice with DSS-colitis. DSS-colitis inhibited DMPP-stimulated Ca$^{2+}$ influx in ACCs. Control, n = 235; DSS, n = 404; *p < 0.01. B) Mean ± S.E.M. amplitude of depolarization produced by DMPP superfusion. No significant difference was observed between groups. Control, n = 11/N = 3; DSS, n = 10/N = 2; p > 0.05. C) Mean ± S.E.M. graph illustrating the peak inward current produced in ACCs from control mice and mice with DSS-induced acute colitis during DMPP stimulation. No significant difference was observed between groups. Control, n = 12/N = 1; DSS, n = 8/N = 2; p > 0.05.
elevations in [Ca\textsuperscript{2+}]i and promote the fusion of catecholamine-filled LDCVs with the plasma membrane, leading to the release of catecholamines into the extracellular space (Schonn et al., 2008). The number of exocytotic events that occur in response to stimulation is proportional to the final [Ca\textsuperscript{2+}]i that is achieved (Engisch & Nowycky, 1996). Therefore, alterations in ACC Ca\textsuperscript{2+} signaling can have large effects on systemic catecholamine secretion.

Using Ca\textsuperscript{2+} imaging techniques, we found that depolarization-induced increases in [Ca\textsuperscript{2+}]i were significantly smaller in ACCs from mice with acute DSS-, chronic DSS- and TNBS-induced colitis compared to controls. Given that Ca\textsuperscript{2+} imaging of Fura-2AM assesses global changes in [Ca\textsuperscript{2+}]i, this effect may have resulted from a decrease in the amplitude of high-K\textsuperscript{+}-stimulated depolarization, a reduction in ACC excitability, inhibition of I\textsubscript{Ca}, or a decrease in Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release (CICR) in ACCs from mice with colitis.

DSS-induced acute colitis resulted in a 10 mV hyperpolarization of ACC RMP. Although high-K\textsuperscript{+} administration produced a similar amplitude of depolarization in ACCs from both groups, the peak depolarized membrane potential reached by ACCs from mice with DSS-induced acute colitis during high-K\textsuperscript{+} stimulation remained significantly hyperpolarized compared to controls. It is therefore possible that high-K\textsuperscript{+}-induced depolarization provided a weaker stimulus for VGCC activation in ACCs from mice with DSS-induced acute colitis.

Experimental models of IBD have been shown to alter the excitability of several neuronal populations that innervate the GI tract. TNBS ileitis was found to decrease rheobase and increase the number of APs fired by postganglionic sympathetic neurons of the guinea pig celiac ganglion in response to depolarization (Dong et al., 2008).
Following TNBS colitis, guinea pig myenteric and submucosal AH neurons were found to fire more APs in response to stimulation and have reduced afterhyperpolarizations (Linden et al., 2003; Lomax et al., 2005). In sensory neurons of the dorsal root ganglia (DRG) of mice and guinea pigs, TNBS-induced GI inflammation decreased AP threshold and increased AP generation (Moore et al., 2002; Stewart et al., 2003; Beyak et al., 2004). The increased excitability observed in enteric, DRG and postganglionic sympathetic neurons during TNBS-induced inflammation occurred without significant alterations in RMP (Moore et al., 2002; Linden et al., 2003; Lomax et al., 2005; Dong et al., 2008). In the present study, we found that the opposite was true for ACCs; DSS-induced acute colitis hyperpolarized ACC RMP without significantly affecting cellular excitability.

Alterations in K+ conductance can affect the RMP of ACCs (Lee et al., 2000; Bournaud et al., 2007). In the present study, I_K was enhanced in ACCs during DSS-induced acute colitis at membrane potentials between +20 and +50 mV. Although the voltage range over which I_K was found to be increased was depolarized in relation to ACC RMP, it is possible that enhanced K+ conductance contributed to the hyperpolarization that was observed during colitis. I_Na was also found to be increased in ACCs during DSS-induced acute colitis. Enhanced I_Na may have compensated for the increased K+ conductance and membrane hyperpolarization that occurred during colitis to maintain ACC excitability. The reason for simultaneous enhancement of two opposing conductances is unclear, but may suggest that voltage-gated Na+ and K+ channels are susceptible to modulation by similar intracellular signaling pathways during inflammation. This is in contrast to DRG neurons, where I_Na is enhanced and
voltage-gated $K^+$ conductances are decreased during GI inflammation (Stewart et al., 2003; Beyak et al., 2004).

VGCCs allow $Ca^{2+}$ to rapidly flow into the cell during membrane depolarization. In the present study, DSS-induced acute colitis was found to inhibit $I_{Ca}$ in ACCs. Adult mouse ACCs express all high-voltage activated (HVA) $Ca^{2+}$ channel subtypes and each subtype contributes to exocytosis in proportion to the $I_{Ca}$ that it carries (Aldea et al., 2002). As a result, there does not appear to be any preferential coupling of specific HVA $Ca^{2+}$ channel subtypes to the exocytotic machinery. Although the present study did not determine which HVA $Ca^{2+}$ channel subtype was responsible for the reduced $I_{Ca}$, given the relationship between changes in $[Ca^{2+}]_i$ and the degree of exocytosis (Engisch & Nowycky, 1996), it is likely that the observed inhibition of $I_{Ca}$ in ACCs will result in decreased systemic catecholamine secretion.

Alterations in $I_{Ca}$ have also been observed in postganglionic sympathetic neurons and smooth muscle cells during experimental colitis. In postganglionic sympathetic neurons, $I_{Ca}$ is primarily carried by N-type and L-type HVA $Ca^{2+}$ channels, with N-type channels providing the major stimulus for exocytosis (Brock & Cunnane, 1999; Motagally et al., 2009b). During DSS-induced acute colitis in mice, norepinephrine secretion from postganglionic sympathetic varicosities innervating the GI tract is reduced through an inhibition of N-type $I_{Ca}$ (Motagally et al., 2009b). In circular smooth muscle cells, depolarization of the membrane potential results in $Ca^{2+}$ influx through L-type $Ca^{2+}$ channels. During TNBS- or acetic acid-induced colitis, L-type $I_{Ca}$ was found to be inhibited in colonic circular smooth muscle cells, and may have contributed to the aberrant smooth muscle contraction that was observed (Liu et al., 2001; Kinoshita et al., 2003). It is important to note, however, that the effects of GI inflammation on $Ca^{2+}$
signaling are not necessarily always inhibitory. For example, during TNBS-colitis in guinea pigs, facilitation of synaptic transmission was observed within the submucosal and myenteric plexuses (Lomax et al., 2005; Krauter et al., 2007), which is unlikely to have resulted from decreased $I_{Ca}$.

Acetylcholine provides the primary physiological stimulus for catecholamine release from ACCs (Petrovic et al., 2010). In the present study, we found that NACHr activation resulted in significantly smaller elevations in $[Ca^{2+}]_i$ in ACCs from mice with DSS-induced acute colitis. DMPP-stimulated increases in $[Ca^{2+}]_i$ result from $Ca^{2+}$ influx through NACHRs, depolarization-induced $Ca^{2+}$ influx through VGCCs, as well as $Ca^{2+}$ release from intracellular stores (Arnaiz-Cot et al., 2008; Wu et al., 2010). Given that the amplitude of depolarization and the peak inward current produced by DMPP stimulation was similar in ACCs from control mice and mice with DSS-induced acute colitis, it does not appear that NACHr function was altered during colitis. Rather, inhibition of $I_{Ca}$ likely caused the reduction in DMPP-induced $Ca^{2+}$ signaling in ACCs.

In many excitable cell types, increases in $[Ca^{2+}]_i$ can promote CICR through the activation of ryanodine receptors (RyRs) on the endoplasmic reticulum (ER) membrane (Nabauer et al., 1989; Smith & Cunnane, 1996). Initial reports suggested that CICR does not play a significant role in the amplification of $Ca^{2+}$ signals in mouse ACCs (Rigual et al., 2002). However, more recently, mouse ACCs were found to express RyR2 and RyR3, and CICR was shown to contribute to high-$K^+$- and DMPP-induced increases in $[Ca^{2+}]_i$ (Wu et al., 2010). Although the present study did not assess the contribution of CICR to $Ca^{2+}$ signaling in ACCs during colitis, our results suggest that inhibition of $I_{Ca}$ is likely to be the primary cause of decreased $[Ca^{2+}]_i$ elevations.
Several animal models can be used to reproduce aspects of IBD. Important information can be gained by comparing the effects of models that differ with respect to the mechanism through which inflammation is achieved or the duration of the inflammatory insult. In the present study, we observed a similar inhibition of high-K\(^+\)-induced increases in \([\text{Ca}^{2+}]_i\) in ACCs from animals with acute DSS- and TNBS-induced colitis. Our results therefore suggest that impaired voltage-dependent \(\text{Ca}^{2+}\) signaling is a consistent feature of colitis in rodents. Furthermore, the continuous presence of colonic inflammation can sustain the inhibition of depolarization-induced increases in \([\text{Ca}^{2+}]_i\) during DSS-induced chronic colitis.

During IBD and experimental models of colitis, inflammatory mediators are elevated at sites of active inflammation and may spill over into the systemic circulation (Mahida et al., 1991; Reimund et al., 1996; Komatsu et al., 2001; Vasina et al., 2008; Alex et al., 2009). Enteric, DRG and postganglionic sympathetic neurons provide extensive innervation of the GI tract and may therefore come into contact with both sources of inflammatory mediators. Local and systemic cytokines may contribute to the aberrant postganglionic sympathetic neuron function that occurs during colitis, as altered norepinephrine secretion has been observed in both inflamed and uninflamed regions of the GI tract (Jacobson et al., 1995; Jacobson et al., 1997; Blandizzi et al., 2003; Motagally et al., 2009b). In addition, overnight incubation of postganglionic sympathetic neurons in TNF-\(\alpha\), an important proinflammatory cytokine in IBD pathogenesis, causes a similar inhibition of N-type \(I_{\text{Ca}}\) as DSS-induced acute colitis (Motagally et al., 2009a). Local and systemic factors may also promote altered DRG neuron function during GI inflammation. For example, colitis has been shown to promote hyperexcitability in DRG neurons innervating inflamed regions of the GI tract,
as well as increase $I_{\text{Na}}$ in urinary bladder sensory afferents (Beyak et al., 2004; Malykhina et al., 2004).

ACCs do not directly innervate the GI tract and therefore cannot interact with local cytokines during IBD. The alterations in ACC function that we observed during experimental colitis provide compelling evidence that GI inflammation can affect distant tissues. It is possible that circulating inflammatory mediators may contribute to this response, as several cytokines have been shown to modulate $Ca^{2+}$ signaling and exocytosis in ACCs in vitro. For example, acute application of IL-1α or IL-1β was found to reduce stimulus-induced catecholamine secretion in bovine ACCs through inhibition of P/Q-type $Ca^{2+}$ channels (Morita et al., 2004). A similar inhibition of acetylcholine-induced $Ca^{2+}$ signaling and catecholamine release has been observed following chronic treatment of bovine ACCs with interferon-α (Tachikawa et al., 1997). ACCs are also susceptible to activity-dependent changes (Stachowiak et al., 1990; Desnos et al., 1992) that may have contributed to the changes in ACC function that we observed.

**Potential Significance**

In the present study, colonic inflammation was found to inhibit depolarization-induced $Ca^{2+}$ influx in ACCs. Given the importance of changes in $[Ca^{2+}]$, in the process of exocytosis (Engisch & Nowycky, 1996), it is likely that systemic catecholamine levels will be decreased during colitis. Catecholamines have been shown to possess important immunomodulatory properties. For example, β-AR activation inhibits TNF-α and nitric oxide secretion, and enhances the release IL-10 from lipopolysaccharide (LPS)-stimulated peritoneal macrophages (Severn et al., 1992; Zinyama et al., 2001). In addition, leukocytes obtained from individuals with high systemic epinephrine levels exhibit reduced proinflammatory and enhanced anti-inflammatory cytokine secretion in
response to stimulation with LPS (Elenkov et al., 2008). In the DNBS model of colitis, administration of a β3-AR agonist was found to inhibit proinflammatory cytokine secretion and limit colonic damage, highlighting the anti-inflammatory capabilities of sympathomimetics in vivo (Vasina et al., 2008). In contrast, α2-AR activation has been shown to enhance proinflammatory cytokine secretion from isolated peritoneal macrophages and worsen the severity of colitis in mice (Spengler et al., 1990; Ignatowski et al., 1996; Bai et al., 2009). Reduced catecholamine secretion from ACCs may therefore have important effects on the inflammatory responses associated with experimental colitis.

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Chapter 3: ENDOTOXEMIA ENHANCES CATECHOLAMINE SECRETION FROM ADRENAL CHROMAFFIN CELLS THROUGH AN INCREASE IN Ca^{2+} RELEASE FROM THE ENDOPLASMIC RETICULUM
ABSTRACT

Enhanced epinephrine secretion from adrenal chromaffin cells (ACCs) is an important homeostatic response to severe systemic inflammation during sepsis. However, the mechanisms underlying elevated epinephrine secretion during this condition are not fully understood. We hypothesized that sepsis directly enhances epinephrine secretion from ACCs by increasing intracellular Ca\(^{2+}\) signaling. Plasma epinephrine concentration was increased 5-fold in the lipopolysaccharide-induced endotoxemia model of sepsis, compared to saline-treated controls. ACCs from endotoxemic mice exhibited significantly enhanced high-K\(^{+}\)-stimulated epinephrine secretion. Carbon fiber amperometry revealed an increase in the number of secretory events elicited by high-K\(^{+}\) during endotoxemia, without significant changes in spike amplitude, half-width or quantal content. ACCs isolated up to 12 hours after the induction of endotoxemia exhibited significantly larger high-K\(^{+}\)-stimulated Ca\(^{2+}\) transients compared to controls. The cecal ligation and puncture (CLP) model of sepsis also significantly increased Ca\(^{2+}\) signaling in ACCs. High-K\(^{+}\)-stimulated Ca\(^{2+}\) transients were significantly enhanced in naïve ACCs incubated overnight in sera from endotoxemic and CLP mice compared to ACCs incubated in control serum. Although sepsis did not significantly affect ACC excitability or voltage-gated Ca\(^{2+}\) current, a 2-fold increase in caffeine (10 mM)-stimulated Ca\(^{2+}\) transients was observed during endotoxemia. Depletion of endoplasmic reticulum (ER) Ca\(^{2+}\) stores using cyclopiazonic acid (10 µM) abolished the effects of endotoxemia on catecholamine secretion from ACCs. Our results suggest that sepsis enhances catecholamine secretion from ACCs through an increase in Ca\(^{2+}\) release from the ER. These effects appear to be at least partly mediated by circulating factors.
INTRODUCTION

Sepsis is characterized by an aberrant systemic inflammatory response to infection. As sepsis progresses to severe sepsis and septic shock, systemic inflammation leads to profound hypotension, hypoperfusion and organ dysfunction that threaten patient survival (Martin et al., 2003; Vincent et al., 2006; Moreno et al., 2008). Sepsis promotes a rapid and sustained activation of the sympathetic nervous system (SNS) that results in increased circulating levels of endogenous catecholamines (Groves et al., 1973; Benedict & Grahame-Smith, 1978; Benedict & Rose, 1992; Annane et al., 1999; Lin et al., 2005). A similar activation of the SNS also occurs during the endotoxemia and cecal ligation and puncture (CLP) models of sepsis (Kovarik et al., 1987; Qi et al., 1991; Zhou et al., 1991; Hahn et al., 1995; Wang et al., 2002).

Activation of the SNS is thought to be a protective response to the systemic inflammation produced during sepsis. Elevated circulating levels of endogenous catecholamines counteract sepsis-induced hypotension and decrease mortality rates in animal models (Falk et al., 1983; McKechnie et al., 1985; Zhou & Jones, 1993; Jones et al., 1994). Consistent with this, septic shock patients are routinely administered catecholamines to mimic the sympathetic response, increase vascular resistance, improve cardiac output and enhance global O₂ delivery (Dellinger et al., 2008). Catecholamines also possess immunomodulatory properties that may alter the course of inflammation during this condition (Elenkov et al., 1995; Rosas-Ballina et al., 2008; Vida et al., 2011b). Despite the important compensatory effects produced by catecholamines, the cellular mechanisms underlying enhanced catecholamine secretion during sepsis are not well understood.
Adrenal chromaffin cells (ACCs) are the cellular source of the elevated circulating levels of epinephrine that are observed during animal models of sepsis (Spink et al., 1966; Prager et al., 1975; McKechnie et al., 1985). Although increased activation of ACCs by preganglionic sympathetic neurons during sepsis certainly contributes to this response (Mills, 1990; Zhou & Jones, 1993; Tkacs & Strack, 1995), direct sepsis-induced alterations in ACC function are also thought to enhance catecholamine release. When preganglionic sympathetic neurons are stimulated at a constant frequency throughout endotoxemia in pithed rats, increased circulating epinephrine levels are still observed (Zhou & Jones, 1993; Jones et al., 1994). Similarly, transient stimulation of preganglionic sympathetic neurons innervating the adrenal medulla results in greater epinephrine secretion in endotoxemic animals compared to controls (Qi et al., 1991). However, direct evidence of altered ACC function during sepsis is currently lacking.

Catecholamine secretion from ACCs is stimulated by elevations in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) (Douglas & Rubin, 1961; Heinemann et al., 1994; Engisch & Nowycky, 1996). Importantly, Ca\(^{2+}\) signaling and catecholamine secretion are plastic processes that can be regulated by inflammation and cytokines (Morita et al., 2004; Feng et al., 2010; Lukewich & Lomax, 2011). The present study was therefore performed to test the hypothesis that sepsis directly increases catecholamine secretion from ACCs through an enhancement of intracellular Ca\(^{2+}\) signaling.

**MATERIALS AND METHODS**

**Mice and Reagents**

Male C57BL/6 mice (20-25 g) were obtained from Charles River (Saint-Constant, Quebec, Canada). Mice were placed on a 12 hour light, 12 hour dark cycle and provided free access to food and water. All procedures performed on animals were
approved by the Queen’s University Animal Care Committee and conformed to the principles and guidelines of the Canadian Council on Animal Care. Unless otherwise stated, reagents used during this study were obtained from Sigma Aldrich (St. Louis, MO). A minimum of 3 mice were used for each experimental group and in vitro recordings were performed at room temperature (22-24°C).

**Models of Sepsis**

Endotoxemia was induced through the intraperitoneal injection of ultrapure lipopolysaccharide (LPS; from *Escherichia coli* serotype 055:B5) dissolved in 0.1 mL of sterile saline (5 mg/kg body weight). Control mice received intraperitoneal injections of 0.1 mL of sterile saline alone. Unless otherwise stated, the 6 hour time point was used for endotoxemia experiments to achieve peak circulating levels of inflammatory mediators and LPS prior to euthanization (Gautier et al., 2008; Fairchild et al., 2009).

For the CLP model of sepsis, mice were anaesthetized using ketamine (166 mg/kg body weight) and xylazine (11.3 mg/kg body weight) and a midline incision was performed. The cecum was exteriorized, ligated below the ileocecal junction and punctured twice with a 21-gauge needle, as previously described (Rittirsch et al., 2009). Sham surgeries were performed on control mice where the cecum was exteriorized and manipulated, but no ligation or puncture was performed. Following the CLP and sham surgeries, all mice received a subcutaneous injection of 1 mL of lactated Ringer’s solution. Tissue was obtained 6 or 12 hours after CLP to represent early sepsis and the onset of septic shock, respectively (Hyde et al., 1990; Ganopolsky & Castellino, 2004).

**Isolation of Adrenal Chromaffin Cells**

The procedure used to isolate mouse ACCs was adapted from Kolski-Andreaco et al., (2007). Mice were deeply anaesthetized using isoflurane inhalation and...
euthanized by cervical dislocation. Adrenal medullae were obtained and digested in Hank’s Balanced Salt Solution (HBSS) containing papain (30 U/mL; Worthington Biomedical Corp., Lakewood, NJ) and L-cysteine (0.67 mg/mL) at 37°C for 35 minutes. The enzyme solution was replaced with Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with fetal bovine serum (FBS; 15% by volume) and penicillin/streptomycin (2500 IU/mL). Adrenal medullae were gently triturated through a series of glass pipettes of decreasing internal diameter. Isolated ACCs were plated on glass coverslips coated with poly-D lysine (2 mg/mL) and laminin (20 µg/mL), and incubated at 37°C and 5% CO2 overnight.

**Plasma Epinephrine and Interleukin-6 Measurements**

Blood was obtained through the orbital sinuses of anaesthetized mice. Blood samples were immediately placed on ice, and sodium metabisulfite (4 mM) and EDTA (1 mM) were added to inhibit epinephrine degradation (Boomsma et al., 1993). Plasma was obtained following centrifugation at 3000 g for 10 minutes and stored at -80°C. Plasma epinephrine concentrations were measured using an enzyme-linked immunosorbent assay (ELISA) according to the manufacturer’s instructions (Rocky Mountain Diagnostics, Inc., Colorado Springs, CO) (Siraskar et al., 2011). The epinephrine ELISA had a minimum detection threshold of 120 pg/mL and a cross-reactivity for norepinephrine of 0.14%. Plasma interleukin (IL)-6 concentrations were measured as an assay of inflammation using an IL-6 ELISA according to the manufacturer’s instructions (R&D Systems, Minneapolis, MN) (Deng et al., 2012). The IL-6 ELISA had a minimum detection threshold of 1.6 pg/mL.
**In Vitro Epinephrine Release Assay**

Coverslips containing $4 \times 10^3$ isolated ACCs were placed in physiological saline containing (in mM) 150 NaCl, 10 glucose, 10 HEPES, 2.8 KCl, 2.5 CaCl$_2$ and 2 MgCl$_2$ with pH adjusted to 7.4 using NaOH. Following several washes, ACCs were stimulated for 5 minutes with a 20 mM K$^+$ solution, prepared by equivalent substitution of KCl for NaCl, and the supernatant was collected. In previous studies (Lukewich & Lomax, 2011), 20 mM high-K$^+$ elicited an approximately 20 mV depolarization in ACC membrane potential, which was sufficient to stimulate Ca$^{2+}$ influx through voltage-gated Ca$^{2+}$ channels (VGCCs). As a result, high-K$^+$ was the primary stimulus used to characterize changes in ACC function during sepsis in the present study. To measure intracellular epinephrine content, unstimulated ACCs were scraped from their coverslips and sonicated. Sodium metabisulfite (4 mM) and EDTA (1 mM) were added to each sample to inhibit epinephrine degradation (Boomsma et al., 1993) and the samples were stored at -80ºC. Epinephrine concentrations were determined using an epinephrine ELISA according to the manufacturer’s instructions (Siraskar et al., 2011). To compare between populations, epinephrine secretion and intracellular epinephrine content were normalized to the number ACCs present on each coverslip.

**Carbon Fiber Amperometry**

Glass-encased carbon fiber electrodes were fabricated as previously described (Machado et al., 2008). Briefly, a 5 µm diameter carbon fiber (Delta Scientific, Mississauga, ON, Canada) was aspirated into a glass capillary tube (World Precision Instruments, Sarasota, FL) and heated using a pipette puller (Narishige, East Meadow, NY). The glass seal was broken by pulling on the exposed carbon fiber and the tip of the electrode was dipped in epoxy comprised of Epon 828 resin (Miller-Stephenson...
Chemical Company, Inc., Toronto, ON, Canada) and m-phenylenediamine (15% by weight). The epoxy was cured for 2 hours at 100ºC followed by 2 hours at 150ºC. Prior to recording, the carbon fiber electrode was bevelled at 45º using an EG-400 microgrinder (Narishige) and dipped in isopropanol for at least 30 minutes. The electrode was backfilled with 3 M KCl and held at +700 mV.

Coverslips containing isolated ACCs were placed in a recording chamber on an inverted microscope and superfused with physiological saline. Catecholamine secretion was stimulated by 30 second application of 70 mM K+, prepared by equivalent substitution of KCl for NaCl (Michelena et al., 1997; Desnos et al., 2003). High-K+-induced catecholamine secretion was analyzed over a 2 minute period beginning at the onset of stimulation. For experiments assessing the contribution of endoplasmic reticulum (ER) Ca2+ stores to high-K+-stimulated catecholamine secretion, ACCs were incubated in DMEM containing cyclopiazonic acid (CPA; 10 µM), an inhibitor of the sarcoplasmic/endoplasmic reticulum Ca2+-ATPase (SERCA) (Seidler et al., 1989), for 40 minutes in the dark to deplete ER Ca2+ stores (Kachoei et al., 2006). ACCs were superfused with nominally Ca2+-free saline containing CPA before and after stimulation to minimize store-operated Ca2+ entry. Catecholamine secretion was stimulated by 30 second application of 70 mM K+ in the presence of CPA and 2.5 mM Ca2+, and high-K+-induced catecholamine secretion was analyzed over a 2 minute period beginning at the onset of stimulation.

Recordings were performed using an Axopatch 200B amplifier and a Digidata 1440A data acquisition system (MDS Analytical Technologies, Mississauga, Ontario, Canada). Data were acquired using pClamp software at 10 kHz, low-pass filtered at 1 kHz and analyzed offline using IGOR Pro 6 software (WaveMetrics, Portland, OR).
Spike analysis was performed using an open-source program written in IGOR Pro by Dr. Eugene Mosharov (http://www.sulzerlab.org/download.html). The threshold for spike detection was set to 3 pA and only cells that exhibited at least 20 spikes during the recording period were included in the spike analysis (Mosharov & Sulzer, 2005).

Isolation of Postganglionic Sympathetic Neurons

Mice were deeply anaesthetized by isofluorane inhalation and euthanized by cervical dislocation. A laparotomy was performed and the superior mesenteric ganglion was carefully dissected and placed in ice cold HBSS. Following several washes, the ganglion was incubated in HBSS containing papain (40 U/mL; Worthington Biomedical Corp., Lakewood, NJ) and L-cysteine (0.67 mg/mL) for 20 minutes at 37ºC. The ganglion was then incubated in HBSS containing collagenase type II (4 mg/mL; Worthington Biomedical Corp.) and dispase type II (4.5 mg/mL; Roche, Mississauga, Ontario, Canada) for 15 minutes at 37ºC (Malin et al., 2007). The HBSS solution was replaced by DMEM containing FBS (10% by volume), penicillin/streptomycin (1250 IU/mL) and nerve growth factor (50 ng/mL). The ganglion was gently triturated using glass pipettes of decreasing internal diameter. Isolated postganglionic sympathetic neurons were plated on glass coverslips coated with poly-D lysine (2 mg/mL) and laminin (20 µg/mL) and cultured at 37ºC and 5% CO₂ overnight.

Ca²⁺ Imaging

Ratiometric Ca²⁺ imaging using the membrane permeable Ca²⁺ indicator, Fura-2 acetoxyethyl ester (AM), was performed on isolated ACCs and postganglionic sympathetic neurons, as previously described (Motagally et al., 2009a; Lukewich & Lomax, 2011). ACCs were stimulated with either 20 mM K⁺, carbachol (100 µM) or caffeine (10 mM) for 1 minute, and postganglionic sympathetic neurons were stimulated
with 70 mM K⁺ for 1 minute. To make comparisons between populations, the peak percentage change in 340:380 from baseline was calculated for each cell.

**Incubation of Adrenal Chromaffin Cells in Serum and Cytokines**

Trunk blood was obtained through decapitation of anaesthetized mice and kept at room temperature for 15-20 minutes to promote coagulation. Serum was obtained following centrifugation at 3000 g for 10 minutes and stored at -80°C. ACCs were incubated overnight in culture medium containing 5% mouse serum and 10% FBS. Sera from at least 3 animals were used for each experimental group. To determine the effects of specific cytokines on ACC function, cells were incubated overnight in media containing recombinant mouse tumor necrosis factor (TNF)-α (PeproTech, Rocky Hill, NJ; 1 nM), prostaglandin (PG) E₂ ethanolamide (Cedarlane Labs, Burlington, Ontario, Canada; 10 µM), IL-6 (Cedarlane; 100 ng/mL), IL-1β (2 ng/mL), IL-10 (10 ng/mL) or a combination of IL-6 and IL-1β.

**Electrophysiology**

Patch pipettes were pulled from borosilicate glass capillary tubes (Warner Instruments, Hamden, CT) and polished to a final internal resistance of 1.5-3 MΩ. For experiments assessing ACC excitability, ACCs were superfused with physiological saline and patch pipettes were filled with an internal solution of the following composition (in mM) 110 K-gluconate, 20 KCl, 10 EGTA, 10 HEPES, 4 Na₂ATP, 1 CaCl₂, 1 MgCl₂ and 0.2 Na₂GTP, with pH adjusted to 7.2 using KOH. Membrane perforation to a stable access resistance of 7 to 15 MΩ was achieved using amphotericin B (240 µg/mL). Resting membrane potential (RMP) was monitored and ACCs were stimulated with current injections from -10 to +30 pA in 1 pA increments, once every 5 seconds. Rheobase was defined as the minimum amount of current required to elicit an
action potential (AP). AP amplitude and half-width were measured in the first AP
generated at rheobase. Voltage-gated $K^+$ and $Na^+$ currents were evoked in ACCs held at
-90 mV using 500 ms voltage steps ranging from -100 to +50 mV in 10 mV intervals,
onece every 5 seconds. Voltage-gated $Na^+$ current ($I_{Na}$) was measured within the first 3 ms and delayed-rectifier voltage-gated $K^+$ current ($I_K$) was measured during the last 100 ms of each voltage step.

Voltage-gated $Ca^{2+}$ current ($I_{Ca}$) was assessed using voltage-clamp
electrophysiology. ACCs were superfused with saline containing (in mM) 100 NaCl, 45
TEA-Cl, 10 glucose, 10 HEPES, 5 CaCl$_2$, 2 MgCl$_2$ and 0.0003 TTX, with pH adjusted to
7.4 using NaOH. Patch pipettes were filled with an internal solution of the following
composition (in mM): 20 CsCl, 108 Cs-methane-sulfonate, 10 HEPES, 1 MgCl$_2$, 4
MgATP, 0.3 Na$_2$GTP, 8 NaCl, and 10 EGTA, with pH adjusted to 7.2 using CsOH. $I_{Ca}$
was elicited in ACCs held at -90 mV by 100 ms voltage steps from -60 to +55 mV in 5
mV increments, once every 5 seconds.

Electrophysiological recordings were performed using a MultiClamp 700B
amplifier and a Digidata 1440A data acquisition system. Data were acquired at 10 kHz
and recorded onto a PC using pClamp software. Analysis was performed offline using
Clampfit 10.0 (all from MDS Analytical Technologies). Leak subtraction and liquid
junction potential correction were not performed.

**Statistical Analysis**

Data are presented as mean ± standard error of the mean (S.E.M.). N represents
the number of animals and n represents the number of ACCs used in each experiment.
Parametric data were compared using unpaired t-tests and non-parametric data were
compared using Mann-Whitney tests. Multiple samples of parametric data were
analyzed using one-way analyses of variance (ANOVAs) with Bonferroni post-tests, except in the case of current-voltage relation data, which were compared using two-way ANOVAs with Bonferroni post-tests. Proportion data were compared using the Fisher’s exact test. All statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA) and statistical significance was inferred when p < 0.05.

RESULTS

Assessment of inflammation

Serum concentrations of IL-6 are often used as a marker of sepsis severity (Harbarth et al., 2001). Similar to previous reports (Fairchild et al., 2009), LPS-induced endotoxemia led to an approximately 600-fold increase in plasma IL-6 concentration compared to saline-injected, time-matched control mice (control, 0.025 ± 0.02 ng/mL, N = 5; 6 hour endotoxemic, 15.00 ± 2.4 ng/mL, N = 8; p < 0.001). Endotoxemic mice also displayed behavioural manifestations of systemic inflammation, including decreased activity, piloerection and hunched posture (Wichterman et al., 1980), all of which were absent in control mice.

Endotoxemia increased catecholamine secretion from ACCs

Activation of the SNS is a prominent feature of sepsis in human patients, as well as animal models of sepsis (Zhou et al., 1991; Hahn et al., 1995; Annane et al., 1999; Lin et al., 2005). In the present study, an approximately 5-fold increase in plasma epinephrine concentration was observed following 6 hours of endotoxemia (Figure 3-1A). To assess whether alterations in ACC function contributed to this response, epinephrine secretion was measured in ACCs isolated from 6 hour endotoxemic mice and control mice \textit{in vitro}. ACCs isolated from endotoxemic mice exhibited significantly
Figure 3-1. Epinephrine secretion was increased during endotoxemia. A) Plasma epinephrine concentrations were significantly elevated following 6 hours of endotoxemia. Control, N = 3; endotoxemic, N = 5; * p < 0.05. B) ACCs isolated from 6 hour endotoxemic mice exhibited enhanced epinephrine release during a 5 minute stimulation with high-K⁺. C) Intracellular epinephrine content was significantly greater in ACCs isolated from 6 hour endotoxemic mice compared to saline-treated controls. For panels B and C, control, N = 3; endotoxemic, N = 3; * p < 0.05.
enhanced high-K\(^+\)–stimulated epinephrine secretion compared to controls (Figure 3-1B). Endotoxemia also significantly increased the intracellular epinephrine concentration (Figure 3-1C).

Catecholamine secretion from ACCs was further assessed using amperometry, an electrochemical technique that detects the secretion of individual catecholamine-containing vesicles from single cells (Wightman et al., 1991). Upon stimulation with high-K\(^+\), numerous secretory events were detected in both control ACCs (Figure 3-2A) and ACCs from 6 hour endotoxemic mice (Figure 3-2B). In line with our previous experiments performed using populations of isolated ACCs (Figure 3-1B), endotoxemia significantly increased cumulative catecholamine secretion from individual ACCs stimulated with high-K\(^+\) for 30 seconds (Figure 3-2C and D). The number of secretory events elicited by high-K\(^+\) application was significantly greater in ACCs from endotoxemic mice compared to controls (Figure 3-2E). However, no significant difference in spike amplitude (Figure 3-2F), half-width (Figure 3-2G) or quantal content (Figure 3-2H and I) was observed between groups.

**Intracellular Ca\(^{2+}\) signaling was enhanced in ACCs during sepsis**

Given the importance of [Ca\(^{2+}\)], in the regulation of catecholamine secretion (Douglas & Rubin, 1961; Heinemann et al., 1994; Engisch & Nowycky, 1996), intracellular Ca\(^{2+}\) signaling was measured in isolated ACCs using Fura-2 ratiometric Ca\(^{2+}\) imaging. Similar to previous reports (Lukewich & Lomax, 2011), high-K\(^+\)-stimulation resulted in a large, transient elevation in 340:380 in control ACCs. A slight decrease in basal 340:380 was observed in ACCs from endotoxemic mice compared to controls (control, 0.273 ± 0.003, n = 491; endotoxemic, 0.256 ± 0.01, n = 379; p < 0.01). Importantly, ACCs from endotoxemic mice exhibited significantly larger high-K\(^+\)-
Figure 3-2. *Endotoxemia enhanced the number of high-K⁺-stimulated exocytotic events in ACCs.* Representative traces of high-K⁺-stimulated catecholamine secretion from a control ACC (A) and an ACC isolated from a 6 hour endotoxemic mouse (B). C) High-K⁺-stimulated cumulative catecholamine secretion was significantly enhanced in ACCs from 6 hour endotoxemic mice compared to controls. ACCs were stimulated for 30 seconds with 70 mM K⁺ and catecholamine secretion was measured for 2 minutes beginning at the onset of the high-K⁺ stimulation. D) Graph depicting the time course of high-K⁺-stimulated catecholamine release from all control ACCs and ACCs from endotoxemic mice. Cumulative catecholamine release was normalized to the number of cells in each group. E) Endotoxemia significantly increased the number of high-K⁺-stimulated exocytotic events in ACCs. No significant difference in spike amplitude (F), half-width (G) or quantal content (H) was observed between groups. For panels C-H, control, n = 26; endotoxemic, n = 24; * p < 0.05 and ** p < 0.01. I) Distribution of the cubic root of spike charge for secretory events stimulated in control ACCs and ACCs from 6 hour endotoxemic mice. Control, n = 907 spikes from 26 cells; endotoxemic, n = 1321 spikes from 24 cells.
stimulated \( \text{Ca}^{2+} \) transients compared to ACCs from control mice (Figure 3-3A). This was evident despite the finding that the amplitude of depolarization produced during high-\( \text{K}^+ \) application was not significantly different between groups (Figure 3-3B). The amplitudes of \( \text{Ca}^{2+} \) transients elicited by the application of carbachol, a non-selective acetylcholine receptor agonist, were also significantly increased in ACCs from endotoxemic mice (Figure 3-3C). The time course of the endotoxemia-induced enhancement of \( \text{Ca}^{2+} \) signaling was also determined. Following 1-12 hours of endotoxemia, ACCs exhibited significantly larger high-\( \text{K}^+ \)-stimulated \( \text{Ca}^{2+} \) transients compared to controls (Figure 3-3D). To examine whether these changes were restricted to the endotoxemia model of sepsis, we measured \( \text{Ca}^{2+} \) signaling in the immunologically distinct CLP model of sepsis. Enhanced high-\( \text{K}^+ \) stimulated \( \text{Ca}^{2+} \) transients were also observed in ACCs from mice with CLP compared to sham-operated controls (Figure 3-3E).

**Circulating mediators promoted the sepsis-induced increase in ACC \( \text{Ca}^{2+} \) signaling**

Sepsis is characterized by an aberrant systemic inflammatory response that results in elevated levels of circulating inflammatory mediators and increased preganglionic stimulation of ACCs (Mills, 1990; Tkacs & Strack, 1995; Fairchild et al., 2009). To determine whether circulating mediators could recapitulate the effect of sepsis on ACCs without any increase in preganglionic activity, \( \text{Ca}^{2+} \) signaling was measured in naïve ACCs incubated overnight in sera obtained from either control mice, mice with endotoxemia, or mice that had undergone CLP. High-\( \text{K}^+ \)-stimulated \( \text{Ca}^{2+} \) transients were significantly larger in naïve ACCs incubated in sera from 3 hour and 6 hour endotoxemic mice compared to ACCs incubated in control serum (Figure 3-4A). Sera from 6 hour and 12 hour CLP mice were also found to significantly enhance high-\( \text{K}^+ \)-
**Figure 3-3.** Stimulus-evoked $Ca^{2+}$ transients were elevated in ACCs during sepsis.

A) High-$K^+$ stimulated $Ca^{2+}$ transients were significantly enhanced in ACCs from 6 hour endotoxemic mice compared to controls. Control, $n = 491$; endotoxemic, $n = 379$; *** $p < 0.001$.

B) No significant difference in the amplitude of high-$K^+$-induced depolarization was observed between groups. Control, $n = 17$; endotoxemic, $n = 18$.

C) ACCs from 6 hour endotoxemic mice exhibited significantly increased carbachol (100 µM)-induced $Ca^{2+}$ transients compared to controls. Control, $n = 173$; endotoxemic, $n = 173$; *** $p < 0.001$.

D) Graph depicting the time course of enhanced $Ca^{2+}$ signaling during endotoxemia. Following 1-12 hours of endotoxemia, ACCs exhibited significantly increased high-$K^+$-stimulated $Ca^{2+}$ transients compared to controls. Control, $n = 219$; 1 hour, $n = 148$; 3 hours, $n = 290$; 12 hours, $n = 347$; 24 hours, $n = 328$; ** $p < 0.01$ and *** $p < 0.001$.

E) High-$K^+$-induced $Ca^{2+}$ transients were significantly elevated in ACCs from 12 hour CLP mice compared to sham operated controls. 6 hour control surgery, $n = 236$; 6 hour CLP, $n = 360$; 12 hour control surgery, $n = 249$; 12 hour CLP, $n = 277$; *** $p < 0.001$. 

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induced Ca\(^{2+}\) transients in ACCs (Figure 3-4B). Overnight incubation of ACCs in cytokines associated with sepsis, including TNF-\(\alpha\), PGE\(_2\) ethanolamide, IL-1\(\beta\), IL-6, IL-10, or a combination of IL-1\(\beta\) and IL-6, at concentrations resembling their systemic levels during sepsis (Fairchild et al., 2009), did not significantly affect high-K\(^{+}\)-stimulated Ca\(^{2+}\) transients (Figure 3-4C).

**Endotoxemia enhanced Ca\(^{2+}\) release from the endoplasmic reticulum**

Depolarization-induced elevations in [Ca\(^{2+}\)], and subsequent catecholamine secretion can be altered through changes in excitability, I\(_{Ca}\) and ER Ca\(^{2+}\) release. We examined the contribution of each of these parameters to the sepsis-induced changes in ACC function. Similar to previous reports (Lukewich & Lomax, 2011), control ACCs exhibited large input resistances and fired APs in response to small depolarizing current injections. Endotoxemia had no effect on RMP, input resistance, rheobase, the number of APs fired at 2x rheobase, AP amplitude, AP half-width, the proportion of ACCs exhibiting spontaneous APs or the proportion of ACCs exhibiting anodal break APs (Table 3-1). These results suggest that ACC excitability and viability were not altered during sepsis. Endotoxemia produced a slight inhibition of I\(_K\) in ACCs between +20 and +50 mV (Figure 3-5A). No significant difference in I\(_{Na}\) (Figure 3-5B) or I\(_{Ca}\) (Figure 3-5C) amplitude was observed between groups.

Elevations in [Ca\(^{2+}\)], can stimulate Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR) from the ER (Ford & Podolsky, 1970). CICR is mediated by ryanodine receptors (RyRs) on the ER membrane that open in response to increases in [Ca\(^{2+}\)], and allow Ca\(^{2+}\) stored within the ER to enter the cytosol (Chen et al., 1997). Caffeine reduces the threshold for RyR activation and is commonly used to assess RyR-mediated ER Ca\(^{2+}\) release.
Figure 3-4. Circulating mediators released during experimental models of sepsis enhanced Ca$^{2+}$ signaling in ACCs. A) High-K$^+$-stimulated [Ca$^{2+}$]$_i$ transients were significantly increased in naïve ACCs incubated in sera from 3 and 6 hour endotoxemic mice compared to ACCs incubated in control serum. Control, n = 229; 3 hours, n = 121; 6 hours, n = 84; 12 hours, n = 144; ** p < 0.01 and *** p < 0.001. B) Sera from 6 and 12 hour CLP mice also enhanced Ca$^{2+}$ signaling in ACCs. Control, n = 87; 6 hour CLP, n = 110; 12 hour CLP, n = 87; ** p < 0.01 and *** p < 0.001. C) Overnight incubation of ACCs in TNF-α, PGE$_2$, IL-6, IL-1β, IL-10 or a combination of IL-6 + IL-1β did not significantly affect high-K$^+$-stimulated Ca$^{2+}$ transients. Data were normalized to the high-K$^+$-stimulated Ca$^{2+}$ transients exhibited by the control ACCs for each cytokine application group. TNF-α, n = 142 cells from 2 animals; PGE$_2$, n = 190; IL-6, n = 71; IL-1β, n = 70; IL-10, n = 61; IL-6 + IL-1β, n = 143.
<table>
<thead>
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<th>Parameter</th>
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<th>Endotoxemic (n = 62)</th>
</tr>
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<td>Resting membrane potential (mV)</td>
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<td>-53.2 ± 1.6</td>
</tr>
<tr>
<td>Input resistance (GΩ)</td>
<td>7.0 ± 0.4</td>
<td>6.4 ± 0.3</td>
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<tr>
<td>Rheobase (pA)</td>
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<td>1.7 ± 0.3</td>
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<td>Number of APs generated at 2x rheobase</td>
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<td>2.5 ± 0.1</td>
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<td>AP amplitude (mV)</td>
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<td>73.8 ± 3.0</td>
</tr>
<tr>
<td>AP half-width (ms)</td>
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<td>8.7 ± 3.0</td>
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<tr>
<td>Percentage of cells exhibiting spontaneous APs (%)</td>
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<td>60</td>
</tr>
<tr>
<td>Percentage of cells exhibiting anodal break APs (%)</td>
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<td>38.7</td>
</tr>
</tbody>
</table>

**Table 3-1. Parameters of ACC excitability.** ACC excitability was not altered following 6 hours of endotoxemia. Values are expressed as mean ± S.E.M. AP, action potential. p > 0.05 for each parameter of ACC excitability.
Figure 3-5. Endotoxemia did not affect $I_{Na}$ or $I_{Ca}$ in ACCs. A) I-V curve of $I_K$ in ACCs from control and 6 hour endotoxemic mice. Endotoxemia slightly inhibited $K^+$ conductance in ACCs. Control, n = 38; endotoxemic, n = 48; * p < 0.05 and ** p < 0.01. B) Endotoxemia did not significantly affect $I_{Na}$ in ACCs. Control, n = 38; endotoxemic, n = 48. C) I-V curve of $I_{Ca}$ elicited in control ACCs and ACCs from 6 hour endotoxemic mice. No significant difference in $Ca^{2+}$ current was observed between groups. Control, n = 29; endotoxemic, n = 28.
Upon superfusion of 10 mM caffeine, transient elevations in $[\text{Ca}^{2+}]$, were observed in control ACCs and ACCs from endotoxemic mice (Figure 3-6A). Endotoxemia significantly enhanced the amplitude of caffeine-induced $\text{Ca}^{2+}$ transients in ACCs (Figure 3-6B). To determine the functional significance of the sepsis-induced increase in ER $\text{Ca}^{2+}$ release, catecholamine secretion was assessed in control ACCs and ACCs from endotoxemic mice following depletion of ER $\text{Ca}^{2+}$ stores using CPA, a selective SERCA inhibitor (Seidler et al., 1989). In the presence of CPA, no significant difference in high-$K^+$-stimulated catecholamine secretion was observed between control ACCs and ACCs isolated from endotoxemic mice (Figure 3-6E and F).

**Ca$^{2+}$ signaling was enhanced in postganglionic sympathetic neurons during sepsis**

Evidence suggests that alterations in postganglionic sympathetic neuron function contribute to the enhanced circulating norepinephrine levels that are observed during sepsis (Jones et al., 1994). To determine whether animal models of sepsis promote a similar increase in postganglionic sympathetic neuron $\text{Ca}^{2+}$ signaling to ACCs, $\text{Ca}^{2+}$ imaging experiments were performed on postganglionic sympathetic neurons isolated from control and endotoxemic mice. Similar to previous reports (Motagally et al., 2009a), high-$K^+$ stimulated transient elevations in $[\text{Ca}^{2+}]$, in control postganglionic sympathetic neurons (Figure 3-7A). Postganglionic sympathetic neurons isolated from 1-24 hour endotoxemic mice exhibited significantly enhanced high-$K^+$-stimulated $\text{Ca}^{2+}$ transients compared to controls (Figure 3-7B). The CLP model of sepsis also increased high-$K^+$-evoked $\text{Ca}^{2+}$ transients in postganglionic sympathetic neurons at the 12 hour time point (Figure 3-7C).
Figure 3-6. Endotoxemia enhanced catecholamine secretion from ACCs through an increase in Ca\(^{2+}\) release from the ER. **A)** Representative Ca\(^{2+}\) imaging recordings of caffeine-stimulated Ca\(^{2+}\) transients in ACCs from control and 6 hour endotoxemic mice. B) Endotoxemia significantly enhanced caffeine-stimulated Ca\(^{2+}\) transients in ACCs. Control, n = 290; endotoxemic, n = 333; *** p < 0.001. Representative recordings of high-K\(^{+}\)-stimulated catecholamine secretion in ACCs from control (**C**) and 6 hour endotoxemic mice (**D**) pre-treated with CPA (10 µM) for 40 minutes. Depletion of ER Ca\(^{2+}\) stores using CPA abolished the effects of endotoxemia on peak cumulative catecholamine secretion (**E**) and the number of exocytotic events (**F**) stimulated by high-K\(^{+}\). For panels **E** and **F**, control, n = 16; endotoxemic, n = 25.
Figure 3-7. High-K⁺-stimulated Ca²⁺ transients were enhanced in postganglionic sympathetic neurons during animal models of sepsis. A) Ca²⁺ imaging tracing from a control postganglionic sympathetic neuron stimulated with high-K⁺. B) Postganglionic sympathetic neurons isolated from 1-24 hour endotoxemic mice exhibited enhanced high-K⁺-stimulated Ca²⁺ transients compared to controls. Control, n = 457; 1 hour, n = 108; 3 hours, n = 173; 6 hours, n = 135; 12 hours, n = 206; 24 hours, n = 186; *** p < 0.001. C) High-K⁺-stimulated Ca²⁺ transients were significantly enhanced in postganglionic sympathetic neurons from 12 hour CLP mice compared to sham operated controls. 6 hour control surgery, n = 290; 6 hour CLP, n = 167; 12 hour control surgery, n = 273; 12 hour CLP, n = 219; *** p < 0.001.
DISCUSSION

Previous studies have suggested that altered ACC function contributes to the enhanced catecholamine secretion observed during sepsis (Qi et al., 1991; Zhou & Jones, 1993; Jones et al., 1994). However, prior to the present work, this has not been directly demonstrated and the cellular mechanisms mediating this response have not been characterized. The results from the present study suggest that aberrant systemic inflammation enhances catecholamine secretion from ACCs primarily through an increase in Ca\textsuperscript{2+} release from the ER (Figure 3-8). These effects appear to be prolonged, as enhanced Ca\textsuperscript{2+} signaling was observed following 1-12 hours of endotoxemia and persisted during overnight culture. Importantly, two immunologically distinct models of sepsis could elicit a similar increase in depolarization-induced Ca\textsuperscript{2+} transients in ACCs. Circulating factors are likely to contribute to this response, as incubation of ACCs in serum from septic mice also enhanced Ca\textsuperscript{2+} signaling in the absence of the increased preganglionic stimulation that would occur in vivo during sepsis. A similar enhancement in Ca\textsuperscript{2+} signaling was also observed in postganglionic sympathetic neurons during the endotoxemia and CLP models of sepsis.

Time course of enhanced Ca\textsuperscript{2+} signaling

A variety of animal models have been developed to study the pathophysiology of sepsis in human patients. The endotoxemia model of sepsis uses LPS, a potent activator of the innate immune system, to generate rapid and severe systemic inflammation (Fairchild et al., 2009). In contrast, CLP induces a form of polymicrobial sepsis that more gradually produces systemic inflammation (Leelahavanichkul et al., 2011). Enhanced depolarization-induced Ca\textsuperscript{2+} signaling was observed in ACCs isolated from
Figure 3-8. Schematic model depicting the proposed mechanism of the endotoxemia-induced enhancement of catecholamine secretion from ACCs. Our data suggest that circulating mediators released during the endotoxemia and CLP models of sepsis enhance ACC Ca$^{2+}$ signaling through an increase in Ca$^{2+}$ release from the ER. The larger Ca$^{2+}$ transients produced during sepsis enhance catecholamine secretion by increasing the number of catecholamine-containing vesicles released in response to a given stimulus.
both endotoxemic and CLP mice, suggesting that these effects are independent of the method used to induce systemic inflammation. However, the time course associated with the development of increased Ca\(^{2+}\) signaling was quite different for each of these models. In accordance with the more rapid onset of systemic inflammation characteristic of endotoxemia, altered ACC function was apparent within 1 hour of LPS administration. In contrast, the CLP-induced increase in Ca\(^{2+}\) signaling was not evident until the 12 hour time point, likely reflecting the more gradual development of systemic inflammation characteristic of this model of sepsis (Fairchild et al., 2009; Leelahavanichkul et al., 2011). Importantly, enhanced ACC Ca\(^{2+}\) signaling persisted during overnight culture, suggesting that the sepsis-induced alterations in ACC function are stable for many hours. This is in line with previous studies showing that elevated circulating catecholamine levels persist upwards of 16 and 40 hours after the induction of endotoxemia and CLP, respectively (Kovarik et al., 1987; Hahn et al., 1995).

**Role of circulating mediators**

The alterations in ACC function observed during endotoxemia and CLP followed similar time courses to the development of severe systemic inflammation characteristic of these models of sepsis (Remick et al., 2000), suggesting that inflammatory mediators may contribute to this response. Consistent with this concept, exposure of naïve ACCs to serum obtained from endotoxemic mice enhanced Ca\(^{2+}\) signaling in a similar manner to endotoxemia *in vivo*. The circulating factor responsible for mediating this effect was present following 3 hours and 6 hours of endotoxemia. However, by the 12 hour time point, endotoxemic serum was no longer able to modulate ACC Ca\(^{2+}\) signaling. Although enhanced intracellular Ca\(^{2+}\) signaling was not apparent until the 12 hour time point in the CLP model of sepsis, sera from both 6 hour and 12 hour CLP mice increased
the amplitudes of high-K⁺-stimulated Ca²⁺ transients in ACCs. The circulating factor mediating this response must therefore be present at the 6 hour time point. However, ACCs may require a longer exposure to this factor in vivo before the effects on Ca²⁺ signaling become apparent.

Overnight incubation of ACCs in a variety of cytokines at concentrations resembling their systemic levels during endotoxemia and CLP (Osuchowski et al., 2006; Fairchild et al., 2009) did not affect ACC Ca²⁺ signaling. LPS is also unlikely to contribute to the effect of septic serum on Ca²⁺ signaling, as we have recently reported that LPS decreases ACC excitability without altering high-K⁺-stimulated Ca²⁺ transients (Lukewich & Lomax, 2012). The factor mediating enhanced Ca²⁺ signaling during sepsis therefore remains to be identified. It is possible that a combination of several cytokines is required to facilitate ACC Ca²⁺ signaling during sepsis or that factors other than cytokines, such as reactive oxygen species, that are known to be elevated during sepsis contribute to this response. Nonetheless, our data strongly suggest that enhanced preganglionic sympathetic input during sepsis cannot, on its own, account for the sepsis-induced increase in catecholamine secretion.

Plasticity of Ca²⁺ release from the endoplasmic reticulum

Intracellular organelles, such as the ER and mitochondria, play important roles in shaping spatiotemporal Ca²⁺ signals and regulating neurotransmitter release (see (Garcia et al., 2006)). The initial Ca²⁺ signal produced by Ca²⁺ influx through VGCCs or nicotinic acetylcholine receptors can be amplified by the ER through a process known as CICR, whereby elevations in [Ca²⁺]ᵢ activate RyRs and promote ER Ca²⁺ release (Fill & Copello, 2002; Wu et al., 2010). CICR has been shown to modulate the secretory responses of several neuronal and neuroendocrine cell types. Within the central nervous
system, CICR participates in short term and long term synaptic plasticity by enhancing Ca\textsuperscript{2+} transients produced in presynaptic nerve terminals during successive APs and increasing the probability of neurotransmitter release (Emptage et al., 2001; Shimizu et al., 2008; Qin et al., 2012). A similar CICR-mediated enhancement in synaptic strength is produced in motor axons and postganglionic sympathetic varicosities during presynaptic AP trains (Onodera, 1973; Smith & Cunnane, 1996; Narita et al., 2000).

ACCs also exhibit CICR responses that enhance catecholamine secretion (Ohta et al., 2001; Inoue et al., 2003; Milla et al., 2011). However, these responses vary in ACCs from different animal species and the role of CICR in mouse ACCs remains controversial (Rigual et al., 2002; Wu et al., 2010). In the present study, caffeine-stimulated Ca\textsuperscript{2+} transients were observed in control mouse ACCs, suggesting that RyRs are functional in these cells. Importantly, sepsis was found to enhance Ca\textsuperscript{2+} signaling and catecholamine secretion in ACCs through increased Ca\textsuperscript{2+} release from the ER. Stimulation of RyR-mediated ER Ca\textsuperscript{2+} release using caffeine resulted in larger Ca\textsuperscript{2+} transients in ACCs from endotoxemic mice compared to controls. Furthermore, depletion of ER Ca\textsuperscript{2+} stores abolished the increase in catecholamine secretion observed during sepsis. The effects of endotoxemia on ER Ca\textsuperscript{2+} release appear to be cell-type specific, as animal models of sepsis inhibit ER Ca\textsuperscript{2+} release in cardiac and skeletal muscle cells (Liu & Wu, 1991; Zhu et al., 2005; Zink et al., 2008).

**Potential significance**

Activation of the SNS has been shown to play a protective role during the initial stages of sepsis. ACCs make an important contribution to this compensatory response, as adrenal demedullation, adrenalectomy, adrenal denervation and ligation of the adrenal vein are associated with more severe hypotension and higher mortality rates during
endotoxemia (Spink et al., 1966; Falk et al., 1983; McKechnie et al., 1985; Zhou & Jones, 1993; Jones et al., 1994). Similarly, the mortality rates of CLP are significantly higher in β-adrenergic receptor knock-out mice and mice that have been administered β-adrenergic receptor antagonists (Schmitz et al., 2007; Walker-Brown & Roberts, 2009).

Although the SNS plays an important role during pathological systemic inflammation, this is the first study to characterize the cellular mechanisms underlying altered ACC function during sepsis. The alterations in ACC function that occur during sepsis would be expected to amplify the effects of increased preganglionic sympathetic neuron activity on catecholamine secretion (Mills, 1990; Zhou & Jones, 1993; Tkacs & Strack, 1995).

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Chapter 4: TOLL-LIKE RECEPTOR 4 ACTIVATION REDUCES ADRENAL CHROMAFFIN CELL EXCITABILITY THROUGH A NUCLEAR FACTOR-KAPPA B-DEPENDENT PATHWAY
ABSTRACT

The adrenal medulla contains fenestrated capillaries that allow catecholamines and neuropeptides secreted by adrenal chromaffin cells (ACCs) to readily access the circulation. These capillaries may also allow microbe-associated molecular patterns to enter the adrenal medulla and interact with ACCs during infection. One potential mediator of this interaction is toll-like receptor 4 (TLR-4), a pattern recognition receptor that detects lipopolysaccharide (LPS) from Gram-negative bacteria. Evidence suggests that excitable cells can express TLR-4, and that LPS can modulate important neuronal and endocrine functions. The present study was therefore performed to test the hypothesis that TLR-4 activation by LPS affects ACC excitability and secretory output. RT-PCR revealed that TLR-4, CD-14, myeloid differentiation protein (MD)-2 and myeloid derived factor 88 (MyD88) are expressed within mouse adrenal medullae. TLR-4 immunoreactivity was observed within all tyrosine hydroxylase-immunoreactive ACCs. Incubation of isolated ACCs in LPS dose-dependently hyperpolarized the resting membrane potential and enhanced large-conductance Ca$^{2+}$-activated K$^+$ current (I_{BK}). LPS (10 µg/mL) also increased rheobase, decreased the number of action potentials (APs) fired at rheobase, and reduced the percentage of ACCs exhibiting spontaneous and anodal break APs. Although catecholamine release was unaltered, LPS significantly reduced high-K$^+$-stimulated neuropeptide Y (NPY) release from isolated ACCs. LPS did not alter the excitability of ACCs from TLR-4$^{-/-}$ mice. Inhibition of nuclear factor (NF)-κB signaling with SC-514 (20µM) abolished the effects of LPS on ACC excitability. Our findings suggest that LPS acts at TLR-4 to reduce ACC excitability and NPY release through an NF-κB-dependent pathway.
INTRODUCTION

Systemic secretion of catecholamines and neuropeptides from adrenal chromaffin cells (ACCs) is facilitated by the presence of fenestrated capillaries throughout the adrenal medulla (Ryan et al., 1975). However, during infection and inflammation, these capillaries may allow the direct exposure of ACCs to blood-borne macromolecules, such as cytokines and microbe-associated molecular patterns (MAMPs). Although it is well established that inflammatory mediators can modulate the electrophysiological properties and neurochemistry of ACCs (Tachikawa et al., 1997; Morita et al., 2004; Ait-Ali et al., 2004), it is less clear what effect, if any, MAMPs have on ACC function. This is an important issue because several diseases associated with bacterial translocation into the circulation, including sepsis and inflammatory bowel disease (IBD), alter the function of ACCs (Qi et al., 1991; Zhou & Jones, 1993; Lukewich & Lomax, 2011). It is possible that direct effects of MAMPs on ACCs account for some of these changes.

Toll-like receptors (TLRs) are a family of evolutionarily-conserved pattern recognition receptors that detect MAMPs and contribute to the development of immune responses (see (Iwasaki & Medzhitov, 2004)). TLR-4 is a well-characterized member of the TLR family that primarily detects lipopolysaccharide (LPS), a component of the outer cell membrane of Gram-negative bacteria (Poltorak et al., 1998; Qureshi et al., 1999). Effective detection of LPS by TLR-4 requires the expression of several accessory molecules, including CD-14 and myeloid differentiation protein (MD)-2 (Wright et al., 1990; Shimazu et al., 1999). Activation of TLR-4 initiates an intracellular signaling cascade involving adaptor proteins, such as myeloid derived factor 88 (MyD88), and the transcription factor nuclear factor (NF)-κB (Sen & Baltimore, 1986; Kawai et al., 1999).
Under basal conditions, NF-κB is largely confined to the cytoplasm of the cell by inhibitory IκB proteins. However, upon TLR-4 activation, IκB is phosphorylated by IκB kinase (IKK), poly-ubiquitinated and degraded, which allows NF-κB to translocate to the nucleus and alter gene expression (see (Verstrepen et al., 2008)).

Although the effects of TLR-4 activation have primarily been characterized in immune cells, growing evidence suggests that TLR-4 can also regulate important properties of non-immune cells. A variety of neuronal populations, including cortical neurons, enteric neurons, sensory neurons, and ventral motor neurons express functional TLR-4 signaling complexes (Wadachi & Hargreaves, 2006; Tang et al., 2007; Barajon et al., 2009; Ochoa-Cortes et al., 2010; Goethals et al., 2010), with activation leading to changes in neuronal excitability, Ca^{2+} signaling and neuropeptide secretion (Hou & Wang, 2001; Ochoa-Cortes et al., 2010). TLR-4 expression has been observed within the adrenal medulla (Bornstein et al., 2004), suggesting that ACCs may possess the ability to directly detect MAMPs, such as LPS. The goal of the present study was therefore to test the hypothesis that ACCs express functional TLR-4 and to characterize the effects of TLR-4 activation on ACC excitability and secretory output.

MATERIALS AND METHODS

Mice and Reagents

Male CD1 (25 - 30 g) and C57BL/6 (20-25 g) mice were obtained from Charles River (Saint-Constant, QC, Canada). TLR-4^-/- mice (B6.B10ScN-Tlr4^{lps-del}/JthJ) with a C57BL/6 background were obtained from Jackson Laboratories (Bar Harbor, ME). All mice were kept on a 12 hour light/12 hour dark cycle and provided with food and water ad libitum. All procedures performed on animals conformed to the principles and guidelines of the Canadian Council on Animal Care and were approved by the Queen’s
University Animal Care Committee. Unless stated otherwise, cells or tissues from a minimum of three animals were used for each experiment and all recordings were performed at room temperature (22-24°C). All reagents used in this study were obtained from Sigma Aldrich (St. Louis, MO), unless otherwise specified.

**Reverse-transcriptase Polymerase Chain Reaction**

Male CD1 mice were deeply anaesthetized by isofluorane inhalation and euthanized by cervical dislocation. Following a laparotomy, the adrenal glands were excised and the adrenal medullae were isolated, placed in ice-cold Trizol solution (Invitrogen, Carlsbad, CA), homogenized and subsequently frozen at -80°C. RNA was extracted using the Trizol method according to the manufacturer’s instructions (Invitrogen). cDNA was reverse transcribed from 1 μg of total RNA using Expand Reverse Transcriptase (Roche, Mississauga, ON, Canada) and oligo(dT) primers (Invitrogen). cDNA was amplified by polymerase chain reaction (PCR) at an annealing temperature of 60°C for 35 cycles followed by a final elongation at 72°C for 10 min. The primers for TLR-4, MD-2, CD-14 and MyD88 were designed using Primer 3 software and are listed in Table 4-1. The amplicons from each primer set were sequenced (ACGT Corp., Toronto, ON, Canada) to confirm product identity.

**Adrenal Chromaffin Cell Dissociation**

The methods used for dissociating mouse ACCs have been adapted from Kolski-Andreaco et al, (2007). Adrenal medullae were obtained and enzymatically digested in Hank’s Balanced Salt Solution (HBSS) containing papain (30 U/mL; Worthington Biomedical Corporation, Lakewood, NJ) and L-cysteine (0.67 mg/mL) for 35 minutes at 37°C. The enzyme solution was then replaced with Dulbecco’s Modified Eagle’s Medium (DMEM; Invitrogen) supplemented with 15% fetal bovine serum (FBS) and
<table>
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<th>Gene Product</th>
<th>Primers</th>
<th>Product Size (bp)</th>
</tr>
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<tbody>
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<td>TLR-4: NM_021297</td>
<td>F: ACC TGG CTG GTT TAC ACG TC R: CTG CCA GAG ACA TTG CAG AA</td>
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<tr>
<td>CD-14: NM_009841.3</td>
<td>F: GTC AGG AAC TCT GGC TTT GC R: GGA GTG AGT TTT CCC CTT CC</td>
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<tr>
<td>MyD88: NM_010851</td>
<td>F: TGG CCT GAG CAA CTA GGA CT R: CGT GCC ACT ACC TGT AGC AA</td>
<td>217</td>
</tr>
</tbody>
</table>

**Table 4-1.** *Intron-spanning primers used for RT-PCR.* F, forward primer; R, reverse primer; bp, base pairs.
2500 IU penicillin/streptomycin. Adrenal medullae were trituated using a series of plastic and fire-polished glass pipettes of decreasing internal diameter. Isolated ACCs were plated on glass coverslips coated with Poly-D lysine (2 mg/mL) and laminin (20 µg/mL), and cultured overnight at 37°C and 5% CO₂ in supplemented DMEM. To investigate the effects of LPS on ACC function, coverslips were incubated overnight in media containing 0.01, 0.1, 1, 10 or 100 µg/mL ultrapure LPS from E. Coli serotype 055:B5. Control ACCs were incubated in media alone. The time course of the LPS-induced alterations in ACC function was determined by incubating cells in 10 µg/mL LPS for 3, 6, 12 or 24 hours. For experiments assessing the role of NF-κB activation, ACCs were treated with SC-514 (20 µM), a selective IKK-2 inhibitor (Kishore et al., 2003), two hours prior to and throughout the incubation in LPS.

**Immunohistochemistry**

Male CD1 mice were intraperitoneally injected with ketamine (1.66 mg/10 g body weight) and xylazine (0.113 mg/10 g body weight). The heart was exposed and 0.1 mL of heparin was injected into the left ventricle. The right atrium was cut and the mouse was perfused with 30 mL of ice-cold 4% paraformaldehyde through the left ventricle. Following perfusion, the adrenal glands were removed and placed in 4% paraformaldehyde at 4°C overnight. Fixative was then replaced by a 30% sucrose solution in phosphate buffered saline (PBS) over a 24 hour period. Dehydrated adrenal glands were immersed in Shandon cryomatrix (Thermo Scientific, Kalamazoo, MI), flash-frozen using 2-methylbutane (Mallinckrodt Baker, Inc., Phillipsburg, NJ) and stored at -80°C. 10 µm sections of adrenal glands were cut using a Shandon Cryotome SME Cryostat (Thermo Scientific) and placed onto Superfrost Plus microscope slides (Fisher Scientific, Ottawa, ON, Canada). Slides were washed several times with PBS.
containing 0.01% Tween to remove the cryomatrix. After blocking for 1 hour in 5% donkey serum (Jackson ImmunoResearch, West Grove, PA), slides were washed and subsequently co-incubated in rabbit anti-mouse TLR-4 (1:100, ab13867; abcam, Cambridge, MA) (Good et al., 2012) and sheep anti-mouse tyrosine hydroxylase (TH; 1:500; Millipore, Temecula, CA) (Ghasemlou et al., 2004) primary antisera for 24 hours. Slides were then washed with PBS-Tween and incubated for 2 hours in DyLight 549-conjugated affinipure donkey anti-goat IgG (1:800; Jackson ImmunoResearch Laboratories). After three washes with PBS-Tween, the slides were incubated for 2 hours in DyLight 488-conjugated affinipure donkey anti-rabbit IgG (1:800; Jackson ImmunoResearch Laboratories). Coverslips were mounted onto the slides using Dako Fluorescence Mounting Medium (Dako North America, Inc., Carpinteria, CA). TLR-4 and TH immunoreactivities were observed using an epifluorescence microscope (Olympus BX51). Images were acquired at 60X magnification using a Photometrics CoolSNAP fx CCD camera and Image-Pro Plus 6.0 software (Media Cybernetics, Bethesda, MD). In a subset of experiments, coverslips containing isolated ACCs were fixed using 4% paraformaldehyde at room temperature for 10 minutes and immunolabelled as described above. Confocal micrographs of isolated ACCs of 0.5 µm optical section thickness were acquired using a Leica TCS SP2 confocal microscope (Leica Microsystems Inc., Concord, ON, Canada) and converted to z-stacks with Leica Confocal Software.

**Electrophysiology**

Coverslips containing isolated ACCs were placed in a recording chamber on an inverted microscope and superfused with saline solution containing (in mM) 150 NaCl, 10 glucose, 10 HEPES, 2 MgCl₂, 2.5 CaCl₂ and 2.8 KCl, with pH adjusted to 7.4 using
NaOH. Patch pipettes were pulled from borosilicate capillary tubes (Warner Instruments, Hamden, CT) and polished to a final resistance of 2-3 MΩ when filled with an internal pipette solution of the following composition (mM): 110 K-gluconate, 20 KCl, 10 EGTA, 10 HEPES, 4 Na₂ATP, 1 CaCl₂, 1 MgCl₂ and 0.2 Na₂GTP, with pH adjusted to 7.2 using KOH. Electrophysiological recordings were performed using the perforated patch clamp configuration. Amphotericin B (240 µg/mL) was added to the pipette solution to achieve a stable access resistance between 7 and 15 MΩ. Membrane capacitance and series resistance were compensated by 70-80%.

To assess parameters of cellular excitability, ACC membrane potential was monitored while cells were stimulated with 500 ms current injections ranging from -14 to +20 pA in 2 pA increments, once every 5 seconds. Rheobase was defined as the smallest depolarizing current injection that elicited an action potential (AP). In many ACCs, more than one AP was fired at rheobase despite the very small current increments used to estimate rheobase, presumably due to the very high input resistances of these cells. AP amplitude and half-width were measured in the first AP generated at rheobase.

K⁺ currents were evoked in ACCs held at -90 mV using 500 ms voltage steps ranging from -100 mV to +50 mV in 10 mV intervals, once every 5 seconds. Delayed-rectifier voltage-gated K⁺ current (I_K) was measured during the last 100 ms of each 500 ms stimulus waveform. For experiments assessing Ca²⁺-activated K⁺ currents, apamin (500 nM) or iberiotoxin (50 nM; Tocris Bioscience, Minneapolis, MN) was bath applied for 5 minutes to inhibit small conductance (SK) or large-conductance (BK) Ca²⁺-activated K⁺ channels, respectively (Blatz & Magleby, 1986; Galvez et al., 1990). Apamin- and iberiotoxin-sensitive currents were isolated by subtracting I_K generated in the presence of the inhibitor from I_K generated before the inhibitor was applied. Two-
pore domain K⁺ current (I_{K2P}) was assessed using the two-pore domain K⁺ (K_{2P}) channel inhibitor, bupivacaine (100 µM) (Kindler et al., 1999). The contribution of ATP-sensitive K⁺ current (I_{KATP}) to I_K was estimated using the ATP-sensitive K⁺ (K_{ATP}) channel inhibitor glibenclamide (50 µM) (Buttigieg et al., 2009).

For experiments assessing voltage-gated Ca²⁺ current (I_{Ca}), patch pipettes were filled with an internal solution containing (in mM) 20 CsCl, 108 Cs-methane-sulfonate, 10 HEPES, 1 MgCl₂, 4 MgATP, 0.3 Na₂GTP, 8 NaCl and 10 EGTA, with pH adjusted to 7.2 using CsOH, and ACCs were superfused with an external solution of the following composition (mM): 100 NaCl, 45 TEA-Cl, 10 glucose, 10 HEPES, 2 MgCl₂, 5 BaCl₂ and 0.0003 tetrodotoxin TTX, with pH adjusted to 7.4 using NaOH. Ba²⁺ was used as the primary charge carrier through voltage-gated Ca²⁺ channels (VGCCs) to reduce Ca²⁺-dependent inactivation of I_{Ca} (Hernandez-Guijo et al., 1998). Peak I_{Ca} was measured in ACCs held at -90 mV in response to 100 ms voltage steps ranging from -60 mV to +55 mV in 5 mV increments, once every 5 seconds. In a separate set of experiments I_{Ca} was elicited by stimulus waveforms modeling APs from control and LPS-treated ACCs.

Electrophysiological recordings were performed using a MultiClamp 700B amplifier and data were acquired at 10 kHz using a Digidata 1440A data acquisition system. Data were recorded onto a PC using pClamp software and analyzed offline using Clampfit 10.0 (all from MDS Analytical Technologies, Mississauga, ON, Canada). Leak subtraction and liquid junction potential correction were not performed.

**Trypan Blue Exclusion Experiments**

To examine cell viability, a trypan blue exclusion assay was performed, as previously described (Motagally et al., 2009a). Viable ACCs were defined as cells that
excluded trypan blue from their cytoplasm. The number of viable cells present on each coverslip was calculated as a percentage of the total cell population.

**Ca²⁺ Imaging**

Ratiometric Ca²⁺ imaging using the membrane permeable Ca²⁺ indicator, Fura-2 acetoxymethyl ester (AM), was performed on isolated ACCs, as previously described (Lukewich & Lomax, 2011). Baseline 340:380 was monitored for 2 minutes and ACCs were stimulated for 1 minute with a 20 mM high-K⁺ saline solution containing (mM): 20 KCl, 132.8 NaCl, 10 glucose, 10 HEPES, 2 MgCl₂ and 2.5 CaCl₂, with pH adjusted to 7.4 using NaOH. To make comparisons between populations, the peak percentage change in 340:380 from baseline was calculated for each cell.

**Catecholamine and Neuropeptide Y Release Assays**

Coverslips containing isolated ACCs were stimulated for 5 minutes with 20 mM high-K⁺ and supernatants were collected. To prevent catecholamine degradation, sodium metabisulfite (4 mM) and EDTA (1 mM) were immediately added to each supernatant (Boomsma *et al.*, 1993). Aprotinin (500 KIU/mL) and DPP IV inhibitor (50 µM; Millipore) were added to each solution used in the neuropeptide Y (NPY) assay experiments to prevent NPY degradation. Samples were stored at -80°C until catecholamine (Rocky Mountain Diagnostics, Inc., Colorado Springs, CO) (Siraskar *et al.*, 2011) and NPY (Millipore) (Makinde *et al.*, 2012) content were measured using enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturers’ instructions. Data were normalized to the number of cells on each coverslip.

**Carbon Fiber Amperometry**

Glass-encased carbon fiber electrodes were fabricated as previously described (Machado *et al.*, 2008). Briefly, a 5 µm diameter carbon fiber (Delta Scientific,
Mississauga, ON, Canada) was aspirated into a glass capillary tube (World Precision Instruments, Sarasota, FL) and heated using a pipette puller (Narishige, East Meadow, NY). The glass seal was broken by pulling on the exposed carbon fiber and the tip of the electrode was dipped in epoxy comprised of Epon 828 resin (Miller-Stephenson Chemical Company, Inc., Toronto, ON, Canada) and m-phenylenediamine (15% by weight). The epoxy was cured for 2 hours at 100ºC followed by 2 hours at 150ºC. Prior to recording, the carbon fiber electrode was bevelled at 45º using an EG-400 microgrinder (Narishige) and dipped in isopropanol for at least 30 minutes. The electrode was backfilled with 3 M KCl and held at +700 mV.

Coverslips containing isolated ACCs were placed in a recording chamber on an inverted microscope and superfused with physiological saline. Catecholamine secretion was stimulated by 15 second application of 70 mM K+, prepared by equivalent substitution of KCl for NaCl. High-K⁺-induced catecholamine secretion was analyzed over a 30 second period beginning at the onset of stimulation.

Recordings were performed using an Axopatch 200B amplifier and a Digidata 1440A data acquisition system (MDS Analytical Technologies, Mississauga, Ontario, Canada). Data were acquired using pClamp software at 10 kHz, low-pass filtered at 1 kHz and analyzed offline using IGOR Pro 6 software (WaveMetrics, Portland, OR). Spike analysis was performed using an open-source program written in IGOR Pro by Dr. Eugene Mosharov (http://www.sulzerlab.org/download.html). The threshold for spike detection was set to 3 pA and only cells that exhibited at least 10 spikes during the recording period were included in the spike analysis (Mosharov & Sulzer, 2005).
**Statistical Analysis**

Data are presented as mean ± standard error of the mean (S.E.M.). N represents the number of animals and n represents the number of ACCs used in each experiment. Parametric data were compared using unpaired t-tests and non-parametric data were compared using Mann-Whitney tests. Multiple samples of parametric data were analyzed using one-way analyses of variance (ANOVAs) with Bonferroni post-tests, except in the case of current-voltage relation data, which were compared using two-way ANOVAs with Bonferroni post-tests. Proportion data were compared using the Fisher’s exact test and multiple samples of proportion data were compared using the Chi-square test followed by Fisher’s exact tests. All statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA) and statistical significance was inferred when p < 0.05.

**RESULTS**

**ACCs express TLR-4, CD-14, MD-2 and MyD88**

RT-PCR was performed on RNA extracted from mouse adrenal medullae and mRNA encoding TLR-4, CD-14, MD-2 and MyD88 were detected (Figure 4-1A). Sequencing of the amplicons generated from each primer set confirmed that the PCR products corresponded to TLR-4, CD-14, MD-2 and MyD88. Immunohistochemistry was then performed to investigate TLR-4 localization in cryostat sections of adrenal glands (Figure 4-1B-D) and isolated ACCs (Figure 4-1 E-G). ACCs were identified by their immunoreactivity for TH, the rate limiting enzyme in catecholamine synthesis. TLR-4 immunoreactivity was observed in all TH-immunoreactive ACCs (n = 371). ACCs accounted for 97.5 ± 0.6% of all TLR-4-immunoreactive cells in culture.
Figure 4-1. ACCs express TLR-4 and related signaling molecules. A) RT-PCR detected the expression of TLR-4, CD-14, MD-2 and MyD88 in mouse adrenal medullae. Image is representative of data from the adrenal medullae of four mice. B - D) Double label immunohistochemistry revealing TLR-4 (B) and TH (C) immunoreactivity in an adrenal gland section. An overlay of TH and TLR-4 immunoreactivities is provided in panel D. Images are representative of data from the adrenal glands of four mice. Scale bars correspond to 30 µm. E - F) Z-stack images of TLR-4 (E) and TH (F) immunoreactivity in isolated ACCs. All TH immunoreactive ACCs were also immunoreactive for TLR-4, as can be seen in the overlay of TH and TLR-4 immunoreactivities in panel G. Images are representative of data from three mice. Scale bars correspond to 10 µm.
LPS reduced ACC excitability

Current clamp electrophysiological recordings were performed on control and LPS-treated ACCs to assess the effects of TLR-4 activation on ACC excitability. Similar to previous reports (Lukewich & Lomax, 2011), mouse ACCs had very large input resistances and fired APs in response to small depolarizing current injections (Figure 4-2A). LPS dose-dependently hyperpolarized the resting membrane potential (RMP) of ACCs compared to vehicle-treated, time-matched controls. Statistically significant hyperpolarization of the RMP was observed in ACCs incubated in 10 and 100 µg/mL LPS (Figure 4-2C). The EC50 for the LPS-induced hyperpolarization was 6.6 µg/mL LPS. The LPS-induced hyperpolarization was apparent following 12 and 24 hour incubations in 10 µg/mL LPS (Figure 4-2D). For the remainder of this study, 10 µg/mL LPS was applied to ACCs for 12-24 hours to characterize the effects of TLR-4 activation on ACC function, unless otherwise stated.

Consistent with membrane hyperpolarization, overnight exposure to LPS also reduced ACC excitability (Figure 4-2B). LPS treatment significantly decreased the percentage of ACCs firing spontaneous APs (Figure 4-2E) and reduced the percentage of ACCs discharging anodal break APs (Figure 4-2F). Rheobase was significantly increased in LPS-treated ACCs compared to controls (Figure 4-2G) and the number of APs generated at rheobase was reduced (Figure 4-2H). LPS treatment also significantly enhanced AP amplitude (Figure 4-2I). AP half-width (control, 5.0 ± 0.4 ms, n = 17; LPS, 5.9 ± 1.1 ms, n = 28; p > 0.05), input resistance (control, 4.7 ± 0.4 GΩ, n = 17; LPS, 5.9 ± 0.4 GΩ, n = 28; p > 0.05) and the number of APs fired at twice the rheobase (control, 2.8 ± 0.3, n = 17; LPS, 3.6 ± 0.3, n = 28; p > 0.05) were not significantly different between groups.
Figure 4-2. ACC excitability was reduced by LPS. Current clamp recordings from a control ACC (A) and an ACC incubated in 10 µg/mL LPS overnight (B). Stimulus waveform is provided in inset. C) LPS dose-dependently hyperpolarized ACC RMP. Control, n = 14; 0.01 µg/mL, n = 11; 0.1 µg/mL, n = 7; 1 µg/mL, n = 7; 10 µg/mL, n = 17; 100 µg/mL, n = 10; * p < 0.05 and ** p < 0.01. D) Hyperpolarization of the RMP was observed in ACCs incubated in 10 µg/mL LPS for 12 and 24 hours compared to controls. Control, n = 17; 3 hours, n = 8; 6 hours, n = 10; 12 hours, n = 12; 24 hours, n = 7; * p < 0.05 and ** p < 0.01. The percentage of ACCs exhibiting spontaneous APs (E) and the percentage of ACCs firing anodal break APs (F) were significantly reduced following overnight incubation in 10 µg/mL LPS. Overnight incubation in 10 µg/mL LPS also significantly enhanced rheobase (G), decreased the number of APs generated at rheobase (H) and enhanced AP amplitude (I). For data sets E-I, control, n = 17; LPS, n = 28; * p < 0.05, ** p < 0.01 and *** p < 0.001.
It is possible that changes in cell viability produced by LPS treatment (Vicente et al., 2006) may have contributed to the reduction in ACC excitability that was observed. A trypan blue exclusion assay was therefore performed to determine the viability of ACCs incubated overnight in the presence or absence of LPS. LPS did not affect the percentage of viable ACCs (control, 77.2 ± 1.9%, n = 2472/ N = 4; LPS, 77.1 ± 1.8%, n = 2620/ N = 4; p > 0.05).

**BK Ca\(^{2+}\)-activated K\(^+\) currents were enhanced by LPS**

Alterations in K\(^+\) conductance can affect ACC RMP and excitability (Solaro et al., 1995; Lee et al., 2000; Gullo et al., 2003; Ales et al., 2006; Enyedi & Czirjak, 2010). Voltage-gated K\(^+\) currents, inward rectifier K\(^+\) currents, Ca\(^{2+}\)-activated K\(^+\) currents and currents carried by K\(_{2p}\) channels were therefore measured in control and LPS-treated ACCs. Similar to previous reports (Lukewich & Lomax, 2011), I\(_{K}\) could be recorded in mouse ACCs in response to membrane depolarization, whereas rapidly activating and inactivating K\(^+\) current (I\(_{A}\)) was absent (Figure 4-3A and B). Overnight incubation of ACCs in LPS dose-dependently enhanced I\(_{K}\) compared to controls. I\(_{K}\) was significantly enhanced in ACCs following incubation in 10 and 100 µg/mL LPS (Figure 4-3C), the same concentrations at which effects on RMP were observed. I\(_{K2P}\) was estimated in control and LPS-treated ACCs using the K\(_{2p}\) channel blocker bupivacaine (100 µM). Although I\(_{K2P}\) was present in all control and LPS-treated ACCs at voltages corresponding to the RMP, no significant difference in current amplitude was observed between groups (Figure 4-3D). Consistent with a role for K\(_{2p}\) channels in setting the RMP, bupivacaine application produced a reversible depolarization in ACCs. The magnitude of bupivacaine-induced depolarization was not significantly different between control ACCs and ACCs exposed to LPS (control, 9.1 ± 6.8 mV, n = 3/N = 2;...
LPS, 8.1 ± 2.8 mV, n = 3/N = 2; p > 0.05). I_{KATP} was estimated using glibenclamide (50 μM). I_{KATP} was not detectable in control ACCs or ACCs treated with LPS (data not shown). SK and BK Ca^{2+}-activated K^+ currents were isolated using apamin (500 nM) and iberiotoxin (50 nM), respectively. Apamin-sensitive K^+ currents (I_{SK}) were observed in a subset of control (n = 3 of 5) and LPS-treated (n = 3 of 5) ACCs. In ACCs exhibiting I_{SK}, no significant difference in current amplitude was observed between groups (Figure 4-3E). Iberiotoxin-sensitive K^+ currents (I_{BK}) were detected in all control and LPS-treated ACCs. In the presence of iberiotoxin, peak I_K at +50 mV was reduced by 85.8 ± 1.1% in control ACCs and 86.8 ± 1.3% in LPS-treated ACCs. I_{BK} was significantly enhanced in LPS-treated ACCs compared to controls (Figure 4-3F).

**LPS did not alter voltage-gated Na^+ currents in ACCs**

Given the importance of voltage-gated Na^+ conductance in determining AP discharge patterns, I_{Na} was characterized in control and LPS-treated ACCs. No significant difference in the peak amplitude of I_{Na} was observed between groups (Figure 4-3G). The voltage of half-maximal activation (control, -14.8 ± 1.3 mV, n = 21; LPS, -16.2 ± 0.8 mV, n = 40; p > 0.05) and the slope factor for activation of I_{Na} (control, 3.8 ± 0.1, n = 21; LPS, 4.0 ± 0.1, n = 40; p > 0.05) were not significantly different between control and LPS-treated ACCs.

**Voltage-dependent Ca^{2+} signaling was not affected by LPS treatment**

To assess the effects of LPS on VGCCs, I_{Ca} was measured in control ACCs and ACCs incubated in LPS overnight. Square-pulse depolarizations elicited I_{Ca} in both control (Figure 4-4A) and LPS-treated ACCs. No significant difference in the amplitude of I_{Ca} was observed between groups (Figure 4-4B). The voltage of half-maximal activation (control, -13.3 ± 1.5 mV, n = 13; LPS, -16.2 ± 1.6 mV, n = 13; p > 0.05) and
Figure 4-3. LPS enhanced BK Ca\textsuperscript{2+}-activated K\textsuperscript{+} currents in ACCs. Representative voltage-clamp recordings illustrating I\textsubscript{K} in a control ACC (A) and an ACC incubated overnight in 10 µg/mL LPS (B). Stimulus waveform is shown in inset. C) Current-voltage relations for I\textsubscript{K} in ACCs. I\textsubscript{K} was significantly enhanced in ACCs treated with 10 µg/mL and 100 µg/mL LPS compared to controls. Control, n = 11; 1 µg/mL LPS, n = 5; 10 µg/mL LPS, n = 8; 100 µg/mL LPS, n = 5; ** p < 0.01 and *** p < 0.001. D) Bupivacaine (100 µM)-sensitive I\textsubscript{K2P} current was not significantly different between control and LPS-treated ACCs. Control, n = 4; LPS, n = 5. E) No significant difference in apamin (500 nM)-sensitive I\textsubscript{BK} current was observed between control and LPS-treated ACCs. Control, n = 3; LPS, n = 3. F) I\textsubscript{BK} was significantly enhanced in ACCs incubated in LPS compared to controls. Control, n = 9; LPS, n = 5; *** p < 0.001. G) I\textsubscript{Na} was not affected by 10 µg/mL LPS exposure. Control, n = 21; LPS, n = 40.
the slope factor for activation of $I_{\text{Ca}}$ (control, $6.3 \pm 0.2$, $n = 13$; LPS, $6.3 \pm 0.4$, $n = 13$; $p > 0.05$) were not significantly different between control and LPS-treated ACCs.

Although square-pulse depolarizations provide important information about VGCC function, AP waveforms may provide a more physiological stimulus for voltage-dependent $\text{Ca}^{2+}$ influx in ACCs (Chan et al., 2005). $I_{\text{Ca}}$ was therefore measured in control and LPS-treated ACCs during stimulations with AP waveforms modeled from the native APs fired by each group of cells. To account for the differences in RMP and AP amplitude observed between control and LPS-treated ACCs, control ACCs were held at -52 mV and stimulated with 10 ms AP waveforms peaking at +20 mV, whereas ACCs treated with LPS were held at -63 mV and stimulated with 10 ms AP waveforms with a peak membrane potential of +20 mV. The peak amplitude of $I_{\text{Ca}}$ generated by LPS-treated ACCs in response to AP waveforms was not significantly different from that of controls (Figure 4-4D).

$\text{Ca}^{2+}$ imaging techniques were then used to determine whether intracellular $\text{Ca}^{2+}$ dynamics were affected by LPS treatment. Large, transient elevations in 340/380 were observed in control ACCs following stimulation with high-$K^+$ (Figure 4-4E), similar to previous reports (Lukewich & Lomax, 2011). LPS significantly enhanced baseline 340/380 (control, $0.243 \pm 0.006$, $n = 173/N = 2$; LPS, $0.266 \pm 0.008$, $n = 169/N = 2$; $p < 0.05$). No significant difference in the high-$K^+$-induced peak percentage change in 340/380 from baseline was observed between control ACCs and ACCs treated with LPS (Figure 4-4F).
Figure 4-4. Ca\(^{2+}\) signaling in ACCs was not affected by LPS treatment. A) Voltage-clamp recording illustrating \(I_{\text{Ca}}\) in a control ACC. Stimulus waveform is provided in inset below. B) Current voltage-relations for \(I_{\text{Ca}}\) demonstrating that LPS did not alter \(I_{\text{Ca}}\) in ACCs. Control, \(n = 13\); LPS, \(n = 13\). C) Representative trace of \(I_{\text{Ca}}\) generated in a control ACC by a stimulus waveform modeling the native APs fired by control ACCs. The parameters of the AP waveform are shown in the inset. D) The peak amplitudes of \(I_{\text{Ca}}\) generated by AP waveforms were not significantly different between control ACCs and ACCs incubated in LPS overnight. Control, \(n = 15\); LPS, \(n = 16\). E) Representative Ca\(^{2+}\) imaging tracing from a control ACC stimulated with a high-K\(^+\) saline solution. F) High-K\(^+\)-stimulated elevations in 340/380 were not affected by LPS treatment. Control, \(n = 173/N = 2\); LPS, \(n = 169/N = 2\).
LPS reduced NPY release from ACCs

High-K⁺-stimulated catecholamine and NPY secretion were measured in control and LPS-treated ACCs to determine whether LPS affects neurotransmitter and neuropeptide release. LPS significantly reduced high-K⁺-stimulated NPY secretion from isolated ACCs (Figure 4-5A). In contrast, no significant difference in high-K⁺-stimulated epinephrine (Figure 4-5B) or norepinephrine (Figure 4-5C) release was observed between groups. LPS did not affect the number of high-K⁺-stimulated exocytotic events, or the quantal content, amplitude or half-width of individual secretory spikes, as assessed using carbon fiber amperometry (Figure 4-6).

The LPS-induced reduction in ACC excitability was mediated by TLR-4

Although LPS is a potent activator of TLR-4, certain LPS preparations can contain contaminants that activate additional receptors (Yang et al., 1999; Ochoa-Cortes et al., 2010). In order to determine whether the LPS-induced reduction in ACC excitability was TLR-4-dependent, we measured the effects of LPS on parameters of excitability in ACCs obtained from wild-type and TLR-4⁻/⁻ mice. Consistent with our findings in CD1 mice (Figure 4-2), overnight incubation of ACCs from wild-type C57BL/6 mice in LPS hyperpolarized the RMP (Figure 4-7A), reduced the proportion of cells exhibiting spontaneous APs (Figure 4-7B), increased rheobase (Figure 4-7C) and decreased the number of APs generated at rheobase (control, 2.2 ± 0.3, n = 10; LPS, 1.5 ± 0.2, n = 13; p < 0.05) compared to untreated cells. However, none of these parameters were affected following overnight incubation of ACCs from TLR-4⁻/⁻ mice in LPS (Figure 4-7).
Figure 4-5. High-K⁺-stimulated NPY release was reduced in LPS-treated ACCs. 
A) Overnight incubation of isolated ACCs in LPS significantly decreased high-K⁺-stimulated NPY release compared to controls. Control, N = 3; LPS, N = 3; * p < 0.05. LPS did not affect high-K⁺-stimulated epinephrine (B) or norepinephrine (C) release. For data sets B and C, control, N = 4; LPS, N = 4.
Figure 4-6. LPS did not affect the number of high-\(K^+\)-evoked secretory events or the quanta of catecholamine released per secretory event in ACCs. A) Representative amperometry tracing illustrating high-\(K^+\)-evoked catecholamine secretion in a control ACC. B) LPS did not significantly affect the number of secretory events stimulated by high-\(K^+\). C) The quanta of catecholamine released per secretory event was not significantly different between control and LPS-treated ACCs. LPS did not affect the amplitude (D) or half-width (E) of the secretory spikes elicited by high-\(K^+\). For data sets B-E, control, n = 20; LPS, n = 18.
Figure 4-7. TLR-4 was required for the LPS-induced reduction in ACC excitability. Overnight incubation of ACCs from wild-type C57BL/6 mice in LPS significantly hyperpolarized the RMP (A), reduced the percentage of cells firing spontaneous APs (B) and increased rheobase (C). LPS did not affect any of these parameters in ACCs from TLR-4\(^{-/-}\) mice. Wild-type control, n = 10; Wild-type LPS, n = 13; TLR4\(^{-/-}\) control, n = 26; TLR-4\(^{-/-}\) LPS, n = 27; ** p < 0.01 and *** p < 0.001 compared to wild-type controls; ## p < 0.01 and ### p < 0.001 compared to LPS-treated TLR-4\(^{-/-}\) ACCs.
The effects of LPS on ACC excitability and $I_K$ were NF-κB-dependent

ACCs were treated with LPS in the presence or absence of SC-514 (20 µM), a selective inhibitor of IKK-2 (Kishore et al., 2003). SC-514 alone did not alter ACC excitability or the magnitude of $I_K$ (data not shown). Co-incubation in SC-514 and LPS abolished the effects of LPS on RMP (Figure 4-8A), the percentage of ACCs firing spontaneous APs (Figure 4-8B), the percentage of ACCs discharging anodal break APs (Figure 4-8C), rheobase (Figure 4-8D), the number of APs generated at rheobase (Figure 4-8E) and $I_K$ (Figure 4-8F).

DISCUSSION

TLR-4 is a pattern recognition receptor that detects MAMPs, including LPS, and signals the presence of micro-organisms in a variety of host tissues. Although the effects of TLR-4 signaling in various immune cell populations are well-characterized, new roles for TLR-4 activation within non-immune cells continue to emerge (Frantz et al., 1999; Iwasaki & Medzhitov, 2004; Yang et al., 2005; Boyd et al., 2006; Barajon et al., 2009; Goethals et al., 2010; Scirocco et al., 2010). Here we show that ACCs express the machinery required for TLR-4 signaling. Activation of TLR-4 resulted in a hyperpolarization of the RMP, a reduction in AP discharge and a decrease in NPY release through an NF-κB-dependent pathway.

Ionic mechanisms underlying the effects of LPS

The hyperpolarization and decrease in excitability produced by LPS strongly suggested that LPS enhances $K^+$ conductances in ACCs. ACCs can express $K_{2P}$ channels, $K_{ATP}$ channels, SK Ca$^{2+}$-activated $K^+$ channels, BK Ca$^{2+}$-activated $K^+$ channels and voltage-gated $K^+$ channels (Marty, 1981; Park, 1994; Thompson & Nurse, 1998; Gullo et al., 2003; Inoue et al., 2008). $K_{2P}$, $K_{ATP}$ and SK channels are unlikely targets of
Figure 4-8. The LPS-induced alterations in ACC function were NF-κB-dependent. Co-incubation of ACCs in LPS and the IKK-2 inhibitor SC-514 (20 µM) prevented the LPS-induced hyperpolarization (A) and reduction in the percentage of ACCs firing spontaneous APs (B) and anodal break APs (C). SC-514 also abrogated the effect of LPS on rheobase (D) and the number of APs fired at rheobase (E). For panels A-E, control, n = 22; LPS, n = 44; LPS + SC-514, n = 26; * p < 0.05 and *** p < 0.001 compared to controls; # p < 0.05, ## p < 0.01 and ### p < 0.001 compared to LPS + SC-514. F) IK was significantly enhanced in LPS-treated ACCs compared to control ACCs and ACCs co-incubated in LPS and SC-514. Control, n = 11; LPS, n = 10; LPS + SC-514, n = 10; ** p < 0.01 and *** p < 0.001 compared to controls; ## p < 0.01 and ### p < 0.001 compared to LPS + SC-514.
TLR-4 activation in ACCs, as $I_{K_{2P}}$, $I_{K_{ATP}}$ and $I_{SK}$ were not significantly different between control and LPS-treated cells. Similar to previous reports (Marty & Neher, 1985; Thompson & Nurse, 1998), $I_{BK}$ accounted for a large proportion of $I_K$ in control ACCs. Importantly, exposure of ACCs to LPS significantly enhanced $I_{BK}$ amplitude. $I_{BK}$ has been shown to regulate ACC excitability through its effects on AP repolarization (Solaro et al., 1995; Marcantoni et al., 2010) and alterations in BK channel activity can have direct effects on the RMP of excitable cells (Ohbuchi et al., 2010; Hristov et al., 2012). The LPS-induced enhancement of $I_{BK}$ may therefore have contributed to the hyperpolarized RMP and reduced excitability observed in ACCs exposed to LPS. This possibility is supported by the observation that the LPS-induced hyperpolarization and enhancement of $I_K$ followed a similar dose-dependence and that NF-κB inhibition prevented the LPS-mediated effects on RMP, excitability and $I_K$. BK Ca$^{2+}$-activated K$^+$ channels have large unitary conductances (Marty, 1981; Neely & Lingle, 1992). Given that mouse ACCs possess large input resistances, the activation of a small number of BK Ca$^{2+}$-activated K$^+$ channels would be expected to produce large alterations in RMP.

**Role of TLR-4 and NF-κB**

LPS can act on diverse cell types to regulate a variety of important functions. The culture system used in the present report did not contain a pure population of ACCs and it is possible that the effects of LPS were indirectly mediated. However, given that TLR-4 expression was observed in all TH immunoreactive ACCs, and that ACCs accounted for greater than 97% of all TLR-4 expressing cells within the culture system, it is unlikely that LPS acting on contaminating cells played a major role in mediating the effects of LPS on ACC function. It should also be noted that some preparations of LPS contain contaminants that may act on receptors other than TLR-4 (Yang et al., 1999;
Our finding that ACCs from TLR-4\(^{-/-}\) mice were not affected by LPS treatment strongly suggests that the hyperpolarization and reduction in ACC excitability were TLR-4-mediated. Basal TLR-4 activation does not appear to modulate ACC excitability, as the electrophysiological properties of ACCs from wild-type and TLR-4\(^{-/-}\) mice were not significantly different. This is in contrast to adrenal cortical cells, where TLR-4 has been shown to tonically regulate corticosterone secretion (Zacharowski et al., 2006).

NF-κB is a ubiquitous transcription factor that is activated by a variety of stimuli and can modulate the expression of numerous target genes. In the present study, NF-κB was found to participate in the LPS-induced alterations in ACC function, since inhibition of IKK-2 with SC-514 prevented the hyperpolarization, reduction in excitability and enhancement of \(I_K\) produced by LPS exposure. The slow onset of the LPS-induced hyperpolarization was also consistent with a role for NF-κB in mediating this effect. Similar to our findings, LPS-induced NF-κB activation hyperpolarizes the RMP of vascular smooth muscle cells and dorsal root ganglion (DRG) neurons, and chronic exposure to LPS reduces hippocampal CA1 pyramidal neuron excitability (Hellstrom et al., 2005; Shi et al., 2010; Ochoa-Cortes et al., 2010). Basal NF-κB activation also appears to regulate the excitability of certain neuronal populations, as selective deletion of IKK within DRG neurons increases AP discharge (Bockhart et al., 2009). Given that IKK inhibition with SC-514 on its own did not affect any of the electrophysiological parameters assessed in ACCs, it appears that basal NF-κB activity does not play a prominent role in determining RMP, excitability or \(I_K\) in these cells.
LPS-induced inhibition of NPY release

In the present study, LPS inhibited stimulus-evoked NPY secretion from ACCs without significantly affecting catecholamine release. Although catecholamines and neuropeptides are co-packaged into large dense-core vesicles (LDCVs) (Winkler & Westhead, 1980; Whim, 2006), differential secretion of neurotransmitters and neuropeptides can be achieved by ACCs. At low stimulation frequencies resembling basal preganglionic sympathetic tone, LDCVs transiently fuse with the plasma membrane and generate narrow secretory pores that readily allow catecholamines to be released, while retaining larger less-soluble neuropeptides. In contrast, during high frequency stimulation, LDCVs undergo complete fusion with the plasma membrane and release all of their vesicular contents (Fulop et al., 2005; Fulop & Smith, 2006). It is therefore possible that LPS shifted the mode of exocytosis from full fusion to kiss and run, thereby restricting NPY release. Altered NPY expression, synthesis, processing or storage may also have contributed to the LPS-induced reduction in NPY secretion.

Potential significance

Inflammatory disorders that promote bacterial translocation into the circulation have been associated with changes in ACC function (Qi et al., 1991; Zhou & Jones, 1993; Lukewich & Lomax, 2011). Although ACCs are responsive to a variety of cytokines released during inflammation (Tachikawa et al., 1997; Morita et al., 2004; Ait-Ali et al., 2004), our results suggest that direct actions of LPS on ACCs may also contribute to the functional alterations that are observed. Sepsis is a severe systemic inflammatory disorder characterized by a rapid and sustained enhancement of catecholamine secretion from ACCs (Jones & Romano, 1989; Qi et al., 1991; Zhou & Jones, 1993). Although patients with sepsis often exhibit prolonged elevations in
circulating LPS (Brandtzaeg et al., 1989; Guidet et al., 1994), given that LPS did not alter catecholamine secretion or Ca\textsuperscript{2+} signaling, it is unlikely that the direct effects of LPS on ACCs contribute to the alterations in ACC function that are observed during this condition. IBD is also associated with aberrant TLR-4 signaling and translocation of enteric microbes across the compromised gut mucosal barrier (Fukata & Abreu, 2007; Ungaro et al., 2009). ACCs from mice with colitis exhibit a hyperpolarized RMP and enhanced I\textsubscript{K} (Lukewich & Lomax, 2011), similar to that seen in the present study. Thus changes in ACC function that have been reported during animal models of IBD may be at least partly mediated through TLR-4 activation.

Similar to ACCs, incubation of macrophages in LPS dose-dependently enhances I\textsubscript{BK} (Blunck et al., 2001; Seydel et al., 2001). The increase in K\textsuperscript{+} efflux through BK channels produced by LPS treatment is thought to stimulate cytokine secretion within these cells, as BK channel inhibitors reduce LPS-induced NF-κB activation and decrease tumor necrosis factor (TNF)-α and interleukin (IL)-8 secretion (Blunck et al., 2001; Seydel et al., 2001; Papavlassopoulos et al., 2006). In addition to catecholamines and neuropeptides, ACCs can also produce cytokines, including IL-1\textalpha, IL-1β, IL-6 and TNF-α (Schultzberg et al., 1989; Andersson et al., 1992; Schultzberg et al., 1995; Gadient et al., 1995; Call et al., 2000). Incubation of isolated ACCs in LPS dose-dependently enhances TNF-α and IL-6 secretion (Call et al., 2000). Given that a similar dose-dependence was observed for the LPS-induced enhancement of K\textsuperscript{+} conductance in the present study, LPS may stimulate cytokine secretion in ACCs through an increase in I\textsubscript{BK}. The role of BK channels and RMP in cytokine secretion from ACCs should therefore be investigated in future studies.
In conclusion, our data suggest that TLR-4 is expressed by ACCs and its activation by LPS reduces ACC excitability and NPY release downstream of NF-κB activation (Figure 4-9). Our findings highlight the possibility that direct microbe-ACC interactions may influence the neuroendocrine response to infection. Given that NPY can act as an immunomodulator (Nair et al., 1993; Bedoui et al., 2008; Chandrasekharan et al., 2008), altered NPY secretion may have important effects on the immune response against Gram-negative bacteria.

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Figure 4-9. Schematic model depicting the LPS-induced reduction in NPY release from ACCs. Our findings suggest that LPS activates TLR-4 expressed by ACCs, which promotes IKK-2-dependent NF-κB translocation to the nucleus. NF-κB activation hyperpolarizes the RMP and reduces ACC excitability through an enhancement of I_{BK}. The LPS-induced reduction in ACC excitability may favour the secretion of catecholamines alone over the co-secretion of catecholamines and NPY, thereby resulting in decreased NPY release. Modulation of NPY expression, synthesis, processing or storage may also contribute to the reduction in NPY release that is observed following LPS exposure.
Chapter 5: GENERAL DISCUSSION
ACC function is dynamically regulated during inflammation and infection. Throughout this thesis, the direct effects of GI inflammation, systemic inflammation and MAMPs on ACC function were thoroughly characterized. These studies revealed that GI inflammation hyperpolarizes ACC RMP, enhances $I_K$ and increases $I_{Na}$, without significantly affecting overall ACC excitability. Importantly, $I_{Ca}$ is inhibited during two immunologically distinct models of acute colitis and these effects persist during successive bouts of GI inflammation. Systemic inflammation also directly affects ACC function, albeit in the opposite direction. Sepsis enhances the number of stimulus-evoked secretory events in ACCs through an increase in CICR. This effect occurs during the endotoxemia and CLP models of sepsis, and is mediated by circulating factors produced during systemic inflammation. A similar increase in Ca$^{2+}$ signaling is also produced in postganglionic sympathetic neurons during animal models of sepsis. Furthermore, these studies have shown that ACCs possess the ability to directly detect the MAMP, LPS, in the absence of an inflammatory response. LPS activates TLR-4 expressed by ACCs to selectively enhance $I_{BK}$, hyperpolarize the RMP and reduce ACC excitability. These effects are dose- and time-dependent, and are mediated by the transcription factor, NF-$\kappa$B. Importantly, LPS reduces NPY release from ACCs and may therefore allow the SNS to relay information regarding the presence of invading microorganisms to additional cells.

The SNS is part of an integrative network that regulates the inflammatory response to injury and infection. Throughout this discussion, the significance of altered ACC function during GI inflammation, systemic inflammation and exposure to LPS will be described in the context of this integrative network. These explanations will draw upon the known effects of endogenously released catecholamines and NPY on
inflammation and immune cell function. Future directions in the field of sympathetic-immune interactions will also be highlighted.

NEGATIVE FEEDBACK CONTROL OF INFLAMMATION

Inflammation is a homeostatic response to injury and infection that functions to neutralize invading micro-organisms and repair damaged tissue. As with all homeostatic responses, inflammation is regulated by negative feedback mechanisms that ensure that the inflammatory response is appropriate for the inciting stimulus and that the response subsides once the homeostatic set point is re-established. Negative feedback is provided by anti-inflammatory mediators that are released by activated immune cells, as well as components of the nervous system, including the hypothalamic pituitary adrenal (HPA) axis, the PNS and the SNS.

The HPA axis reduces the severity of inflammation through the release of glucocorticoids from adrenal cortical cells. Glucocorticoids inhibit immune cell proliferation, antigen presentation and cytokine secretion (Wahl et al., 1975; Snyder & Unanue, 1982). They also favour the development of Th2 T helper cell-mediated humoral immune responses over Th1 T helper cell-mediated cellular immunity (Ramirez et al., 1996).

The PNS also provides anti-inflammatory effects through the activation of NACHRs expressed by innate immune cells. Activation of α7 NACHRs inhibits LPS-stimulated TNF-α, IL-1β, high mobility group box 1 (HMGB1) and IL-6 secretion in macrophages (Borovikova et al., 2000; Wang et al., 2003; Wang et al., 2004). Certain macrophage populations also express non-α7 NACHRs that can reduce bacterial clearance and inhibit cytokine release in the presence of acetylcholine (Matsunaga et al., 2001).
With respect to the SNS, catecholamines released from postganglionic sympathetic neurons and ACCs produce anti-inflammatory effects through the activation of β-ARs. β-AR activation enhances anti-inflammatory IL-10 secretion and decreases proinflammatory TNF-α production in LPS-stimulated macrophages (Spengler et al., 1994; Ignatowski & Spengler, 1995; Ignatowski et al., 1996; Suberville et al., 1996). Catecholamines also inhibit macrophage phagocytosis and NO production (Abrass et al., 1985; Sigola & Zinyama, 2000; Zinyama et al., 2001). Similarly, the generation of reactive oxygen species by neutrophils is inhibited by β-AR agonists (Weiss et al., 1996; Barnett et al., 1997). Furthermore, β-AR activation inhibits dendritic cell migration and antigen presentation, and favours the development of Th2 T helper cell-mediated humoral responses (Borger et al., 1998; Panina-Bordignon et al., 1997; Sanders et al., 1997; Ramer-Quinn et al., 1997; Maestroni & Mazzola, 2003; Maestroni, 2002; Seiffert et al., 2002). It is important to mention, however, that catecholamines can also provide proinflammatory effects in certain immune cell populations through the activation of α-ARs (Spengler et al., 1990; Ignatowski et al., 1996; Maestroni, 2000).

**The Sympathetic-Immune Network**

The immune system communicates with the SNS through several mechanisms. Vagal afferent neurons, DRG afferent neurons, baroreceptors and chemoreceptors activate sympathetic reflexes during local and systemic inflammation. Circulating cytokines communicate with central autonomic structures via circumventricular organs, and endothelial cells and perivascular macrophages of the cerebral vasculature. Cytokines can also cross the blood brain barrier through carrier mediated transport. Furthermore, circulating cytokines can readily enter the adrenal medulla and
sympathetic ganglia to alter ACC and postganglionic sympathetic neuron activity and function.

Evidence suggests that the vagus nerve plays an important role in activating the SNS during inflammation. Peripherally produced cytokines can activate local vagal afferent neurons, which relay this information to the sensory nuclei of the solitary tract (NST) (Watkins et al., 1995; Niijima, 1996; Gordon, 2000). During more severe inflammatory responses, cytokines can directly access the dorsal vagal complex and sensitize vagal afferent varicosities within this region (Rogers et al., 2006; Rogers & Hermann, 2012). Electrical stimulation of vagal afferent neurons stimulates epinephrine secretion from ACCs (Vida et al., 2011a). Activation of vagal afferent neurons also initiates vago-vagal reflexes, including the ‘cholinergic anti-inflammatory pathway,’ which stimulates norepinephrine secretion from postganglionic sympathetic neurons innervating the spleen (Borovikova et al., 2000; Huston et al., 2006; Rosas-Ballina et al., 2008; Vida et al., 2011a). Although the SNS has been shown to participate in the cholinergic anti-inflammatory pathway, the mechanism underlying increased norepinephrine release within the spleen remains controversial (Rosas-Ballina et al., 2008; Bratton et al., 2012).

DRG afferent neurons participate in spinal and supraspinal sympathetic reflexes (Coote & Downman, 1966; Coote et al., 1969). During inflammation, DRG afferent neurons detect changes in temperature, stretch and osmolarity, and relay this information to the SNS. DRG neurons can also directly detect cytokines and other mediators that are released during inflammation (Kidd & Urban, 2001; Ibeakanma & Vanner, 2010). Additional sensory input to the SNS is provided by baroreceptors and chemoreceptors that detect changes in mean arterial pressure and blood composition, respectively.
Systemic inflammation can produce profound hypotension and global tissue hypoxia, both of which can increase SNS activity (Dellinger et al., 2008). Chemoreceptors can also directly detect circulating cytokines and activate central sympathetic reflexes during inflammation (Shu et al., 2007).

Inflammation can also affect SNS output through direct and indirect actions on central autonomic structures. Circulating cytokines can cross the blood brain barrier through carrier mediated transport (Banks et al., 1991; Plotkin et al., 1996). Regions of the brain that are devoid of a blood brain barrier, known as circumventricular organs, can also relay information regarding the presence of circulating cytokines to central autonomic structures (see (Roth et al., 2004)). In addition, cytokines can indirectly communicate with central neurons by stimulating the production of PGs by endothelial cells and perivascular macrophages within the cerebral vasculature (Blais & Rivest, 2001; Schiltz & Sawchenko, 2002). PGs subsequently activate central autonomic structures, such as the rostroventrolateral medulla and paraventricular nucleus, to increase sympathetic drive during inflammation (Zhang et al., 2003; Yu et al., 2010).

Sympathetic ganglia and the adrenal medullae are peripheral structures that do not possess a blood-brain-barrier. Circulating cytokines can readily enter these structures across fenestrated capillaries to directly interact with postganglionic sympathetic neurons and ACCs (Ryan et al., 1975). Adrenal cortical cells and immune cells residing within the adrenal medulla can also locally release inflammatory mediators (Hume et al., 1984; Gonzalez-Hernandez et al., 1994; Judd, 1998). Several cytokines have been shown to regulate the functional properties of ACCs and postganglionic sympathetic neurons and likely play an important role in the sympathetic response to inflammation.
(Hurst & Collins, 1993; Tachikawa et al., 1997; Morita et al., 2004; Ait-Ali et al., 2004; Motagally et al., 2009a).

Each of these pathways provides an opportunity for signal integration and enables the sympathetic-immune network to provide dynamic responses to injury and infection. The multiple pathways through which the immune system regulates SNS function also highlight the importance of this reflex in animal survival. A similar complex network exists between the HPA axis and the immune system, and also serves to down-regulate the immune response (Besedovsky et al., 1986; Berkenbosch et al., 1987; Bernton et al., 1987; Roh et al., 1987; Tsagarakis et al., 1989; Liu et al., 2011).

Direct Detection of Micro-organisms

Recent studies have provided compelling evidence that non-immune cells can directly detect and respond to invading micro-organisms in the absence of inflammation (Wadachi & Hargreaves, 2006; Tang et al., 2007; Barajon et al., 2009; Ochoa-Cortes et al., 2010; Goethals et al., 2010). In the context of the sympathetic-immune network, LPS has been shown to directly activate DRG and vagal afferent neurons (Ochoa-Cortes et al., 2010; Riley et al., 2013). In chapter 4 of this thesis, LPS was also shown to regulate ACC function through the activation of TLR-4. LPS reduced ACC excitability and inhibited NPY release. Similar to catecholamines, NPY promotes a variety of anti-inflammatory effects. NPY decreases monocyte infiltration into inflamed tissues, inhibits TNF-α production in macrophages and favours the development of Th2 T helper cell mediated humoral responses (Levite, 1998; Kawamura et al., 1998; Bedoui et al., 2003; Nave et al., 2004; Macia et al., 2012). Importantly, NPY inhibits macrophage and neutrophil phagocytosis, and decreases the ability of natural killer cells to destroy virally infected cells (Nair et al., 1993; Stanojevic et al., 2007; Bedoui et al., 2008). NPY
therefore impairs the destruction of invading micro-organisms by reducing immune cell recruitment to sites of infection and inhibiting the uptake of microbes into immune cells. The reduction in NPY release produced by TLR-4 activation in ACCs may therefore enhance the clearance of Gram-negative bacteria from the body. It is important to mention, however, that NPY possesses direct antimicrobial effects and can increase the generation of reactive oxygen species in macrophages and neutrophils (Dimitrijevic et al., 2005; Stanojevic et al., 2007; Bedoui et al., 2008; El Karim et al., 2008). A similar reduction in anti-inflammatory glucocorticoid release is observed in adrenal cortical cells of the HPA axis following chronic incubation in LPS and may serve a related function (Liu et al., 2011).

**POTENTIAL CONSEQUENCES OF ALTERED ADRENAL CHROMAFFIN CELL FUNCTION DURING INFLAMMATION**

Despite numerous protective mechanisms that exist throughout the body, damaging systemic inflammatory responses and chronic inflammatory conditions can still occur in susceptible individuals. Once initiated, these inflammatory disorders can produce persistent alterations in the immune system-nervous system network that often have important consequences on disease progression. In the following sections, the consequences of altered ACC function during animal models of IBD and sepsis are described in the context of the pathological processes involved in each of these disorders.

**Inflammatory Bowel Disease**

Catecholamines have been shown to alter the functional properties of several classes of immune cells implicated in the pathogenesis of IBD. Furthermore, stress, which contains a large sympathetic component, can affect disease severity and has been
associated with the reactivation of GI inflammation (Robertson et al., 1989; Gulpinar et al., 2004; Saunders et al., 2006). It is therefore likely that the SNS plays an important role during IBD. Indeed, in patients with active ulcerative colitis, the administration of clonidine, an $\alpha_2$-AR agonist, reduces clinical symptoms and improves endoscopic damage scores. These effects have been attributed to an $\alpha_2$-AR-mediated inhibition of catecholamine release from the SNS (Lechin et al., 1985; Furlan et al., 2006). However, the direct activation of $\alpha_2$-ARs expressed by immune cells may also have important consequences on the severity of IBD (Spengler et al., 1990; Ignatowski et al., 1996).

The role of the SNS during IBD has also been studied using acute and chronic models of colitis. 6-hydroxydopamine is an isomer of norepinephrine that destroys postganglionic sympathetic varicosities, while leaving adrenal catecholamine levels intact (Thoenen & Tranzer, 1968; Mueller et al., 1969; Kolibal-Pegher et al., 1994). Ablation of postganglionic sympathetic neurons with 6-hydroxydopamine prior to the induction of acute TNBS- or DSS-colitis reduces the severity of colonic inflammation (McCafferty et al., 1997; Straub et al., 2008). However, when 6-hydroxydopamine is administered during the chronic phase of colitis in IL-10$^{-/-}$ mice or chronic DSS-treated mice, the severity of colonic inflammation is significantly enhanced (Straub et al., 2008). These results suggest that during the initial stages of inflammation, norepinephrine release from postganglionic sympathetic neurons exacerbates the inflammatory response, whereas at more chronic time points, norepinephrine release improves disease severity.

Low concentrations of norepinephrine preferentially activate $\alpha$-ARs over $\beta$-ARs. Acute GI inflammation inhibits norepinephrine secretion from postganglionic sympathetic neurons and enhances $\alpha$-AR expression within the GI tract (Swain et al., 2007).
Norepinephrine released during acute colitis may therefore preferentially activate proinflammatory $\alpha$-ARs expressed by immune cells and worsen disease severity. In support of this possibility, $\alpha_2$-AR antagonists decrease histological severity, reduce MPO activity, and inhibit TNF-$\alpha$ and IL-1$\beta$ production during acute TNBS- and DSS-induced colitis (Bai et al., 2009). During chronic colitis, our laboratory has shown that the sympathetic innervation of the colon is enhanced. This may restore or even increase norepinephrine levels within the GI tract during chronic colitis and enhance anti-inflammatory $\beta$-AR activation.

In chapter 2, colitis was shown to inhibit $I_{Ca}$ in ACCs, which would be expected to decrease catecholamine secretion. Although the effects of norepinephrine released from postganglionic sympathetic neurons during GI inflammation have been well-characterized, our knowledge of the role of catecholamines secreted by ACCs during colitis is currently limited. Additional studies are therefore required to determine the effects of systemically secreted catecholamines on GI inflammation.

**Sepsis**

Sepsis increases the firing frequencies of preganglionic sympathetic neurons innervating postganglionic sympathetic neurons and ACCs (Mills, 1990; Tkacs & Strack, 1995). In chapter 3, sepsis was also shown to directly enhance the secretory capacity of ACCs through an increase in ER $Ca^{2+}$ release. A similar increase in $Ca^{2+}$ signaling was observed in postganglionic sympathetic neurons of the superior mesenteric ganglion. The direct effects of sepsis on ACC and postganglionic sympathetic neuron function would be expected to amplify the secretory response produced by the increased activity of preganglionic sympathetic neurons. Indeed, a 5-
fold increase in plasma epinephrine concentrations was observed following 6 hours of endotoxemia. This is likely to be an underestimate of the increase in catecholamine release that is produced, as superoxide anions generated during sepsis can directly inactivate catecholamines (Macarthur et al., 2000).

Catecholamine secretion from ACCs and postganglionic sympathetic neurons provides protective cardiovascular and immunomodulatory effects during sepsis. Inhibition of preganglionic sympathetic neuron activation produces more severe hypotension and increases the mortality rate of endotoxemia (Spink et al., 1966; Jones et al., 1994; Poon et al., 2006). Similarly, inhibition of ACC catecholamine secretion through adrenalectomy, adrenal demedullation, adrenal denervation or ligation of the adrenal vein increases the severity of hypotension and decreases animal survival during endotoxemia (Spink et al., 1966; Falk et al., 1983; McKechnie et al., 1985; Zhou & Jones, 1993; Jones et al., 1994). In addition, adrenalectomy increases the mortality rate of the CLP model of sepsis (Bosmann et al., 2013).

Norepinephrine secreted by postganglionic sympathetic neurons is also beneficial during sepsis. Guanethidine treatment, which depletes vesicular norepinephrine in sympathetic varicosities, and ablation of the celiac plexus have both been shown to increase the mortality rate of Gram-negative bacterial sepsis (McKechnie et al., 1985; Solomon et al., 2003). It is important to note, however, that sympathetic nerve ablation with 6-hydroxydopamine decreases the mortality rate associated with endotoxemia in rabbits (Bolton & Atuk, 1978), suggesting that endogenous catecholamine secretion from postganglionic sympathetic neurons may be detrimental under certain experimental conditions.
The consequences of AR activation by endogenously released catecholamines have also been assessed during sepsis. Patients with septic shock that are homozygous for a β2-AR gene polymorphism that accelerates receptor desensitization exhibit a greater degree of organ dysfunction and higher mortality rates than patients without this polymorphism (Nakada et al., 2010). Similarly, mice that lack β1- and β2-ARs exhibit higher mortality rates and more severe systemic inflammatory responses during endotoxemia than wild-type mice (Walker-Brown & Roberts, 2009). Non-selective inhibition of β-ARs also enhances TNF-α production and inhibits IL-10 release during endotoxemia (Elenkov et al., 1995; Suberville et al., 1996). A similar detrimental effect of β-AR inhibition has been observed during the CLP model of sepsis (Schmitz et al., 2007). However, selective β1-AR antagonists reduce the mortality rate of endotoxemia and increase the median survival time during CLP (Ackland et al., 2010). Furthermore, β1-AR antagonists reduce serum TNF-α and IL-6 levels during endotoxemia (Hagiwara et al., 2009; Ackland et al., 2010). Taken together, these studies suggest that while β1-ARs promote detrimental proinflammatory effects during sepsis, β-AR activation as a whole is anti-inflammatory and improves sepsis outcomes. It is therefore likely that the positive effects of β2-ARs outweigh the negative consequences of β1-AR activation during sepsis.

Activation of α2-ARs by endogenous catecholamines also modulates sepsis severity. Inhibition of α2-ARs has been shown to reduce TNF-α production and increase IL-10 release during endotoxemia (Elenkov et al., 1995; Szelenyi et al., 2000). Similarly, the selective α2A-AR antagonist, BRL-44408, decreases serum TNF-α and IL-6 levels, and increases animal survival during the CLP model of sepsis (Miksa et al., 2009; Zhang et al., 2010). α2-AR activation has been shown to increase TNF-α secretion.
from Kupffer cells of the liver and promote hepatocellular dysfunction during sepsis (Yang et al., 2001; Miksa et al., 2009). As a result, α<sub>2A</sub>-AR antagonists may improve sepsis outcomes by inhibiting cytokine secretion from Kupffer cells and reducing hepatocyte damage. However, α<sub>2A</sub>-ARs are also highly expressed by postganglionic sympathetic varicosities, where they promote autoinhibition of catecholamine release (Brede et al., 2003). α<sub>2A</sub>-AR antagonists would therefore be expected to increase norepinephrine release from postganglionic sympathetic neurons and activate additional AR subtypes. In support of this possibility, ganglionic blockade and β-AR antagonists prevent the decrease in TNF-α release that is produced by α<sub>2</sub>-AR antagonists during endotoxemia (Elenkov et al., 1995).

The endogenous sympathetic response to sepsis is often insufficient to sustain tissue perfusion and many patients progress to severe sepsis or septic shock. During septic shock, patients exhibit severe hypotension that is refractory to fluid resuscitation and are therefore routinely administered exogenous vasopressors and positive inotropic agents to maintain mean arterial pressure at an acceptable level. Catecholamines and selective AR agonists are the mainstay treatment for patients with septic shock (Dellinger et al., 2008). Exogenous catecholamines have been shown to increase mean arterial pressure, elevate cardiac index, enhance global O<sub>2</sub> delivery and improve survival rates in patients with septic shock (Mackenzie et al., 1991; Marik & Mohedin, 1994; Martin et al., 2000; Dubin et al., 2009; Jhanji et al., 2009). However, these vasoactive agents are often given as large intravenous bolus doses and are accompanied by various side effects (Dunser et al., 2009; Schmittinger et al., 2013).

Preganglionic sympathetic neurons are activated during sepsis in an attempt to restore homeostasis. A more effective therapeutic strategy than bolus injections of
catecholamines may therefore be to selectively amplify the secretory capacity of ACCs and postganglionic sympathetic neurons, such that a greater amount of catecholamine is released only when the SNS is endogenously activated. This would retain the regulatory mechanisms that normally control SNS function, and allow ACCs and postganglionic sympathetic neurons innervating specific target tissues to be selectively activated when necessary. The observation that sera from endotoxemic and CLP mice produce similar alterations in ACC function as sepsis in vivo suggests that an endogenously released circulating mediator promotes the increased Ca\(^{2+}\) signaling that is observed during sepsis. Future studies should therefore be performed to identify this mediator, as it may prove to provide therapeutically valuable effects during sepsis. Insight into the direct modulation of catecholamine secretion from ACCs also has clinical applicability to pheochromocytoma, a condition characterized by extensive catecholamine release from tumors comprised of ACCs.

**Differential Effects of Systemic and Gastrointestinal Inflammation**

Systemic and GI inflammation produce opposite effects on ACC Ca\(^{2+}\) signaling through two distinct mechanisms: colitis inhibits I\(_{Ca}\), whereas sepsis enhances ER Ca\(^{2+}\) release. The dichotomy of these two responses may result from fundamental differences in the nature of inflammation produced during colitis and sepsis. Animal models of sepsis are associated with large elevations in circulating inflammatory mediators which are likely to directly affect ACC function (Fairchild et al., 2009). Indeed, sera from septic mice enhance ACC Ca\(^{2+}\) signaling, suggesting that circulating factors play an important role in promoting increased catecholamine release during sepsis. Although cytokines that are produced within the GI tract during colitis can also spill over into the systemic circulation, the concentrations of circulating cytokines produced during animal
models of IBD are much lower than those observed during sepsis (Fairchild et al., 2009; Alex et al., 2009). The differential effects of systemic and GI inflammation on ACC function may therefore be promoted by different concentrations of cytokines interacting with ACCs. In support of this possibility, micromolar concentrations of PGE$_2$ have been shown to increase catecholamine secretion from bovine ACCs, whereas nanomolar concentrations of this inflammatory mediator inhibit catecholamine release (Karaplis et al., 1989). Although chronic incubation in PGE$_2$ did not alter mouse ACC Ca$^{2+}$ signaling in the present report, a similar concentration-dependent effect may exist for other cytokines.

The differential effects of GI and systemic inflammation on ACC Ca$^{2+}$ signaling may also arise from the different complement of circulating inflammatory mediators that ACCs are exposed to during each of these conditions (Fairchild et al., 2009; Alex et al., 2009). Furthermore, colitis likely promotes the preferential activation of vagal and DRG afferent neurons innervating the GI tract, whereas sepsis produces inflammation in several visceral structures innervated by these neurons. Differences in the activation profiles of vagal and DRG afferent neurons may also have important consequences on ACC function during these inflammatory disorders through activity-dependent changes.

**FUTURE DIRECTIONS**

The present thesis provided comprehensive characterizations of the alterations in ACC function that occur during inflammation and infection, as well as detailed mechanistic insight into the underlying processes involved. Nevertheless, additional studies will be required to further extend these findings and to solidify their importance in the context of health and disease.
Colitis was shown to inhibit $I_{Ca}$ in ACCs. Although this would be expected to decrease catecholamine release from ACCs, additional studies should measure plasma catecholamine concentrations and assess the secretory capacity of ACCs during colitis to directly test this possibility. The effects of catecholamines released by ACCs on disease severity and cytokine release during colitis should also be investigated, as information in this area is currently lacking. This can be achieved by performing adrenal demedullation or adrenal denervation prior to the induction of colitis to inhibit catecholamine release from ACCs. Future studies should also determine whether circulating factors mediate the colitis-induced alterations in ACC function by comparing $Ca^{2+}$ signaling in naïve ACCs incubated overnight in sera from control mice and mice with colitis.

Sepsis was shown to enhance stimulus-evoked $Ca^{2+}$ signaling and catecholamine secretion in ACCs through an increase in ER $Ca^{2+}$ release. A similar increase in $Ca^{2+}$ signaling was also observed in postganglionic sympathetic neurons during the endotoxemia and CLP models of sepsis. This observation sets the foundation for future studies assessing the secretory capacity of postganglionic sympathetic neurons during sepsis and investigating the mechanism responsible for the enhanced $Ca^{2+}$ signaling that is observed. Another important area of investigation that should be addressed in future studies is the role of circulating mediators in promoting the sepsis-induced increase in ACC $Ca^{2+}$ signaling. Although serum from septic mice was found to produce similar increases in ACC $Ca^{2+}$ signaling as sepsis in vivo, initial attempts to identify the specific causative factor were unsuccessful. Serum is a complex mixture of several factors, which makes it difficult to determine the identity of an unknown mediator of interest. In order to obtain additional information about the nature of the causative agent in our sepsis serum samples, techniques such as boiling, size exchange chromatography,
reverse phase high performance liquid chromatography and mass spectrometry should be performed in future studies.

Although this thesis focused on the characterization of TLR-4 signaling in ACCs, preliminary experiments from our laboratory suggest that ACCs express additional TLRs, including TLR-1, TLR-2, TLR-3 and TLR-6 (Figure 5-1). Each TLR detects a specific set of MAMPs and activates NF-κB to regulate gene transcription (see (Iwasaki & Medzhitov, 2004)). Future studies should address whether these additional TLRs are also functional within ACCs and whether they promote similar alterations in ACC excitability and NPY release.

Isolated ACCs were used in essentially all of the studies performed throughout this thesis. Isolated ACCs are ideal for performing electrophysiological, electrochemical and Ca	extsuperscript{2+} imaging experiments. However, ACCs in vivo exist in clusters that are electrically coupled through gap junctions, which can have important consequences on Ca	extsuperscript{2+} signaling and catecholamine release (Martin et al., 2001). It is therefore important that future studies confirm the principle findings of the present work using adrenal slices or alternative preparations that preserve the cell-cell interactions within the adrenal medulla. Given that these techniques do not require overnight culture, any additional effects of sepsis or colitis that are lost during the culture period will readily be identified during these studies.

ACCs are often used as a model system for investigating neurotransmitter release within the nervous system. ACCs synthesize and secrete IL-1β, TNF-α and IL-6 and could therefore be used to explore the mechanisms of cytokine release in neurons (Andersson et al., 1992; Call et al., 2000). In innate immune cells, cytokines are typically synthesized following cell activation and packaged into vesicles that are
Figure 5-1. ACCs express several TLRs. RT-PCR detected the expression of TLR-1, TLR-2, TLR-3, TLR-4, and TLR-6 in mouse adrenal medullae. PCR was performed at an annealing temperature of 60°C for 35 cycles followed by a final elongation at 72°C for 10 min. Image is representative of data from the adrenal medullae of one mouse.
constitutively released. Certain cytokines, such as TNF-α, can also be stored in granules and immediately released in response to specific stimuli. In addition, cytokines, such as IL-1β, are not packaged into vesicles, but rather are released through specific membrane transporters (see (Lacy & Stow, 2011)). To acquire additional information regarding cytokine secretion in ACCs, double-label immunohistochemistry could be performed in control and LPS-treated ACCs for LDCV markers, such as synaptobrevin, and TNF-α, IL-1β or IL-6. These studies will reveal whether cytokines are present within ACC LDCVs at rest and during an immune challenge.

If cytokines are present within LDCVs, real-time monitoring of these vesicles can be performed using green fluorescent protein-tagged cytokines and total internal reflection fluorescence (TIRF) microscopy. TIRF microscopy is an imaging technique that can be used to track LDCVs containing fluorescent molecules that are located within 100 nm of the plasma membrane. During exocytosis, the fluorescently labelled molecules are released from LDCVs and TIRF enables the visualization of this release process (see (Steyer & Almers, 2001)). This technique could therefore be used to confirm that ACCs secrete cytokines through exocytosis and identify stimuli that induce cytokine release in ACCs, such as high-K⁺, acetylcholine, PACAP or LPS.

**CONCLUSIONS**

Although the immune system and nervous system were originally thought to operate independently, it is now clear that these supersystems undergo dynamic, reciprocal interactions during health and disease. Cytokines can regulate neuronal excitability, Ca²⁺ signaling and neurotransmitter release and, in turn, neurotransmitters can modulate immune cell activation, phagocytosis and cytokine secretion. Neurons can
also release cytokines and immune cells can secrete neurotransmitters, thereby providing an additional level of interaction between these two systems.

Interactions between the immune system and nervous system function to restore homeostasis following injury and infection through the co-ordinated activities of several classes of immune cells and neuronal populations. The SNS is an integral component of the immune system-nervous system network. Catecholamine secretion from the SNS is stimulated during inflammation through central reflex loops, the activation of circumventricular organs and central autonomic structures by circulating inflammatory mediators, and the direct actions of cytokines on ACCs and postganglionic sympathetic neurons. The present thesis extended our knowledge of the immune system-nervous system network by demonstrating that ACCs possess the ability to directly detect LPS. This may enable ACCs to signal the presence of Gram-negative bacterial infections to additional cells through a decrease in NPY release. The present thesis also demonstrated that GI and systemic inflammation produce opposite effects on ACC Ca\(^{2+}\) signaling. These effects appear to be mediated by different pathways, as sepsis enhances ER Ca\(^{2+}\) release, whereas colitis inhibits I\(_{\text{Ca}}\). The enhanced Ca\(^{2+}\) signaling and catecholamine secretion observed in ACCs during sepsis is a compensatory response that functions to counteract the severe systemic inflammation and vascular abnormalities associated with this condition. Although our understanding of the physiological significance of catecholamines derived from ACCs during the course of colitis is currently limited, the fact that colitis alters ACC function warrants further investigation in this area.

The most effective immune response is one that can rapidly eliminate the inciting agent while causing minimal collateral tissue damage. However, during inflammatory diseases, such as sepsis and IBD, the immune response becomes dysregulated and
begins to promote excessive tissue damage that can severely impact the patient’s quality of life and even threaten their survival. As our knowledge of the immune system-nervous system network continues to evolve, it is apparent that under normal conditions multiple levels of integration exist between these systems to match the severity of the inflammatory response with the intensity of the inciting stimulus. By determining which components of the nervous system are particularly important during specific inflammatory conditions and identifying strategies to modulate their activity, we may eventually develop the ability to effectively utilize the immunomodulatory capabilities of the nervous system to treat debilitating inflammatory disorders.
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