SPHINGOLIPID-INDUCED ACTIVATION OF THE VOLUME-SENSITIVE Cl− CURRENT IS MEDIATED BY MITOCHONDRIAL REACTIVE OXYGEN SPECIES

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SPHINGOLIPID-INDUCED ACTIVATION OF THE VOLUME-SENSITIVE Cl− CURRENT IS MEDIATED BY MITOCHONDRIAL REACTIVE OXYGEN SPECIES

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University School of Medicine

by

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<tr>
<td>4-β-PDB</td>
<td>4-β-phorbol-12,12-dibutyrate</td>
</tr>
<tr>
<td>5-HD</td>
<td>5-hydroxy-decanoic acid</td>
</tr>
<tr>
<td>AEBSF</td>
<td>4-(2-aminoethyl)benzenesulfonyl fluoride</td>
</tr>
<tr>
<td>ACh</td>
<td>acetylcholine</td>
</tr>
<tr>
<td>Ang II</td>
<td>angiotensin II</td>
</tr>
<tr>
<td>APD</td>
<td>action potential duration</td>
</tr>
<tr>
<td>A-SMase</td>
<td>acid sphingomyelinase</td>
</tr>
<tr>
<td>AT&lt;sub&gt;1&lt;/sub&gt;R</td>
<td>Ang II receptor type 1</td>
</tr>
<tr>
<td>AT&lt;sub&gt;2&lt;/sub&gt;R</td>
<td>Ang II receptor type 2</td>
</tr>
<tr>
<td>AVD</td>
<td>apoptotic volume decrease</td>
</tr>
<tr>
<td>CAPP</td>
<td>ceramide-activated protein phosphatase</td>
</tr>
<tr>
<td>Cer</td>
<td>ceramide</td>
</tr>
<tr>
<td>CHF</td>
<td>congestive heart failure</td>
</tr>
<tr>
<td>CoA</td>
<td>coenzyme A</td>
</tr>
<tr>
<td>CERT</td>
<td>ceramide transfer protein</td>
</tr>
<tr>
<td>CFTR</td>
<td>cystic fibrosis transmembrane conductance regulator</td>
</tr>
<tr>
<td>COP II</td>
<td>coat protein complex II</td>
</tr>
</tbody>
</table>
C-H₂DCFDA-AM. .......................... 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate-di(acetoxy-methyl ester)

CRAC. ................................................................. calcium-release activated channels

DCPIB. ................. 4-(2-butyl-7-dichloro-2-cyclopentyl-indan-1-on-5-yl) oxobutyric acid

D-erythro-MAPP............. (1S,2R-D-erythro-2-N-myristoylamino)-1-phenyl-1-propanol

DIDS. ............................................. 4,4′-diisothiocyanostilbene-2,2′-disulfonic acids

DMS. .............................................. N,N-dimethyl-D-erythro-sphingosine

DMSO.......................... dimethyl sulfoxide

DPI.......................... diphenyliodonium

EAD. .............................................. early after depolarization

E_{Cl}................................................. chloride equilibrium potential

EGF. .............................................. epidermal growth factor

EGFR. ............................................. epidermal growth factor receptor

ER. .................................................. endoplasmic reticulum

ERK. ............................................. extracellular signal regulated kinase

ET. ................................................. endothelin

ET_{A}............................................. endothelin receptor subtype A

ET_{B}............................................... endothelin receptor subtype B

ETC. ............................................... electron transport chain

FAK. ............................................. focal adhesion kinase

GPCR. ........................................... G protein coupled receptor

HDL. .............................................. high-density lipoprotein
hERG. .......................................................... human Ether-à-go-go Related Gene
I_{Ca,Cl}. .......................................................... calcium-activated Cl\(^-\) current
I_{Cl,cAMP}. .......................................................... cAMP-activated Cl\(^-\) current
I_{Cl,swell}. .......................................................... swelling-activated Cl\(^-\) current
I_f. .............................................................. hyperpolarization-activated inward current
I – V. .............................................................. current – voltage
IL-1\(\beta\). ........................................................ interleukin 1\(\beta\)
JNK. ............................................................ c-jun N terminal kinase
LDL. .............................................................. low-density lipoprotein
MAPK. .......................................................... mitogen-activated protein kinase
MEK. .......................................................... mitogen-activated protein kinase kinase
mitoK\textsubscript{ATP}. ........................................... mitochondrial ATP-sensitive K\(^+\) channel
MnTBAP. ...................................................... Mn(III) tetra(4-benzoic acid) porphyrin chloride
mPTP. .......................................................... mitochondrial permeability transition pore
NADP. .......................................................... nicotinamide adenine dinucleotide phosphate
NADPH. ...................................................... reduced form of nicotinamide adenine dinucleotide phosphate
NMDG. .......................................................... N-methyl-D-glucamine
NOX. ............................................................ NADPH oxidase
N-SMase. ........................................................ neutral sphingomyelinase
PDGF. .......................................................... platelet-derived growth factor
PDGFR. ........................................................ platelet-derived growth factor receptor
PDMP. .......................................................... 1-phenyl-2-decanoylamino-3-morpholino-1-propanol
PI-3K. .......................................................... phosphoinositide-3-kinase
PKC. .......................................................... protein kinase C
PLC. .......................................................... phospholipase C
PLD. .......................................................... phospholipase D
PMA. .......................................................... phorbol myristate acetate
PP2a. .......................................................... protein phosphatase 2a
PP2b. .......................................................... phosphatase calcineurin
PTK. .......................................................... receptor protein tyrosine kinase
PTX. .......................................................... pertussis toxin
ROS. .......................................................... reactive oxygen species
S1P .......................................................... sphingosine-1-phosphate
SMase. .......................................................... sphingomyelinase
SOD. .......................................................... superoxide dismutase
SPHK1. .......................................................... sphingosine kinase type 1
SPHK 2. .......................................................... sphingosine kinase type 2
TNFα. .......................................................... tumor necrosis factor α
VSMCs. .......................................................... vascular smooth muscle cells
Abstract

SPHINGOLIPID-INDUCED ACTIVATION OF THE VOLUME-SENSITIVE Cl− CURRENT IS MEDIATED BY MITOCHONDRIAL REACTIVE OXYGEN SPECIES

By Frank J. Raucci, Jr., Ph.D.

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

Virginia Commonwealth University, 2009

Dissertation Director: Clive M. Baumgarten, Ph.D.
Professor of Physiology and Biophysics, of Internal Medicine (Cardiology), and of Biomedical Engineering

Swelling-activated Cl− current (I_{Cl,swell}) is an outwardly-rectifying current that plays an important role in cardiac electrical activity, cellular volume regulation, apoptosis, and acts as a potential effector of mechano-electrical feedback. Persistent activation of I_{Cl,swell} has been observed in a number of models of cardiovascular disease. Previously we showed that angiotensin II (Ang II), endothelin-1 (ET-1), endothelial growth factor receptor (EGFR), and reactive oxygen species (ROS) produced by NADPH oxidase (NOX) and mitochondria are involved in the activation of I_{Cl,swell} by both osmotic swelling and β1 integrin stretch. Sphingolipid metabolism is modulated in several
cardiopathologies and because sphingolipids are bioactive lipids involved in signaling cascades that overlap significantly with these modulators of $I_{Cl,swell}$, we investigated the role of sphingolipids in the regulation of $I_{Cl,swell}$ in cardiac ventricular myocytes.

Under isoosmotic conditions that isolate anions currents, addition of exogenous, cell permeant C$_2$-ceramide (C$_2$-Cer) elicited an outwardly-rectifying Cl$^-$ current that reversed near the Cl$^-$ equilibrium potential ($E_{Cl}$) in both physiological and symmetrical Cl$^-$ gradients. This current was inhibited by the $I_{Cl,swell}$-specific blockers DCPIB. Dihydro-C$_2$-ceramide (C$_2$-H$_2$Cer), the inactive analogue of C$_2$-Cer, failed to elicit current. These data strongly suggest that the identity of C$_2$-Cer-induced Cl$^-$ current is $I_{Cl,swell}$ and indicate that sphingolipid signaling pathways may be involved.

Bacterial sphingomyelinase (SMase), which converts endogenous sphingomyelin in the outer leaflet of the sarcolemmal membrane to native chain-length ceramides, elicited a DCPIB-sensitive Cl$^-$ current. SMase-induced current is also suppressed by tamoxifen, which under conditions that isolate anion currents is a specific inhibitor of $I_{Cl,swell}$. SMase-induced $I_{Cl,swell}$ was abrogated by ebselen, a membrane permeant glutathione peroxidase mimetic that dismutates H$_2$O$_2$ to H$_2$O. This suggests that ROS are required mediators of SMase-induced activation of $I_{Cl,swell}$. Both NOX and mitochondria are important sources of ROS in cardiomyocytes and both have been implicated in modulating $I_{Cl,swell}$. Blocking NOX with apocynin or the NOX fusion peptide inhibitor gp91ds-tat had no effect on SMase-induced current. However, pretreatment of cardiomyocytes with gp91ds-tat reduced the maximum current amplitude of SMase-induced $I_{Cl,swell}$, indicating that NOX may play a time-dependent role in this mechanism.
By contrast, the mitochondrial Complex I blocker rotenone, which suppresses extra-mitochondrial ROS release by Complex III, completely suppresses SMase-induced $I_{\text{Cl,swell}}$. Additionally, SMase-induced $I_{\text{Cl,swell}}$ is partially inhibited by blockade of mitochondrial $K_{\text{ATP}}$ (mito$K_{\text{ATP}}$) channels with 5-hydroxy-decanoic acid (5-HD). Mito$K_{\text{ATP}}$ channels have been implicated as modulators of mitochondrial ROS release. Thus these data suggest that mitochondrial ROS generation is required for SMase-induced activation of $I_{\text{Cl,swell}}$.

Ceramides are metabolized to form several sphingolipids, including sphingosine-1-phosphate (S1P). We tested whether ceramide metabolites are responsible for eliciting $I_{\text{Cl,swell}}$. Under isosmotic conditions that isolate anion currents, SMase-induced $I_{\text{Cl,swell}}$ was abrogated by blockade of ceramidase, which converts ceramide to sphingosine, with D-erythro-MAPP. SMase-induced $I_{\text{Cl,swell}}$ also was suppressed by inhibition of sphingosine kinase with DL-threo-dihydrosphingosine. These data suggested that the ceramide metabolite S1P is likely to stimulate $I_{\text{Cl,swell}}$. As expected, exogenous S1P elicited an outwardly rectifying $\text{Cl}^-$ current that was fully inhibited by DCPIB. As seen with SMase-induced $I_{\text{Cl,swell}}$, S1P-induced $I_{\text{Cl,swell}}$ was fully inhibited by rotenone. In contrast to results with SMase, S1P-induced current was partially inhibited by blockade of NOX with apocynin. These data indicate that S1P is a necessary component of SMase-induced $I_{\text{Cl,swell}}$ activation and that the action of exogenous S1P involves ROS from both mitochondria and NOX. Importantly, the fact that exogenous C$_2$-ceramide also activates $I_{\text{Cl,swell}}$ even though C$_2$-ceramide may not metabolized to S1P in native cells. Thus, it
seems likely that ceramides can elicit $I_{\text{Cl,swell}}$ via S1P and also by a distinct pathway and that both pathways converge at mitochondrial ROS.

In order to determine the role of ERK in the proposed signaling pathway that regulates $I_{\text{Cl,swell}}$, we examined the effect of ERK inhibitors PD98059 and U0126 on the activation of $I_{\text{Cl,swell}}$. Both of these agents partially inhibited SMase-induced activation of $I_{\text{Cl,swell}}$, indicating SMase acts through both ERK-dependent and ERK-independent signaling pathways.

HL-1 cells are derived from a murine atrial cell line that retains phenotypic characteristics of adult cardiomyocytes. Recently, $I_{\text{Cl,swell}}$ has been observed in HL-1 cells with similar regulatory mechanisms to those seen in native cells. We showed that SMase elicits an outwardly-rectifying, DCPIB-sensitive $\text{Cl}^-$ current that reverses near $E_{\text{Cl}}$ in HL-1 cells. Finally, we confirmed the production of ROS by SMase-induced signaling by flow cytometry in HL-1 cells using the nominally $\text{H}_2\text{O}_2$-selective fluorescent probe C-$\text{H}_2\text{DCFDA-AM}$. Exposure to SMase increased ROS production, as did the positive control $\text{H}_2\text{O}_2$. SMase-induced ROS generation was suppressed by pretreatment with rotenone but was unaffected by pretreatment with gp91ds-tat.

These data indicate that exogenous and endogenous sphingolipids elicit $I_{\text{Cl,swell}}$ in cardiomyocytes by stimulating mitochondrial ROS production. NOX may contribute to the ROS generation, but is not a required step in this mechanism. Sphingolipid signaling is likely to play an important role in stimulating ROS production and activating $I_{\text{Cl,swell}}$ in a number of cardiovascular diseases.
Chapter 1

INTRODUCTION

The volume-sensitive Cl current, $I_{\text{Cl,swell}}$, is elicited in cardiac myocytes by osmotic swelling, hydrostatic inflation, $\beta_1$ integrin stretch, and in several models of cardiac disease. In turn, $I_{\text{Cl,swell}}$ modulates cardiac electrical activity, cell volume, apoptosis, and is implicated in ischemic preconditioning (Baumgarten et al., 2005; Duan et al., 2005; Hume et al., 2000). Regulation of $I_{\text{Cl,swell}}$ is complex and involves a number of signaling pathways. Recently, reactive oxygen species (ROS) were identified as a downstream effector, and exogenous H$_2$O$_2$ elicits $I_{\text{Cl,swell}}$ in cardiomyocytes (Browe and Baumgarten, 2004; Browe and Baumgarten, 2006; Ren et al., 2008) and other cells (Haskew-Layton et al., 2005; Shimizu et al., 2004; Varela et al., 2004).

Many of the signaling cascades that activate $I_{\text{Cl,swell}}$ overlap those involved in sphingolipid signaling (Hannun and Obeid, 2008; Levade et al., 2001; Spiegel et al., 2002), raising the possibility that certain sphingolipids might regulate $I_{\text{Cl,swell}}$. Initially sphingolipids were considered to be structural components of membranes without further function. More recently, sphingolipids, specifically ceramide and sphingosine, were
recognized as bioactive molecules that participate in a number of signaling cascades and mediate apoptosis, mitogenesis, and other cellular processes. Alterations in sphingolipid metabolism are implicated in cardiovascular diseases, including congestive heart failure, atherosclerosis, and ischemia/reperfusion injury (Chatterjee et al., 2006; Levade et al., 2001). Sphingosine kinase, which phosphorylates the ceramide metabolite sphingosine, mediates Ang II-induced PI-3K activation (Mulders et al., 2006) and EGFR up-regulation (Tanimoto et al., 2004b) in vascular smooth muscle cells. Exogenous ceramide elicits ROS production via NADPH oxidase in bovine coronary artery cells (Zhang et al., 2003) and the via mitochondrial electron transport chain in rat liver (Garcia-Ruiz et al., 1997b) and heart (Gudz et al., 1997). Moreover, $I_{\text{Cl,swell}}$ has been postulated to control the ceramide-induced apoptotic volume decrease in cardiomyocytes (d'Anglemont de Tassigny et al., 2004), but effects of ceramide on $I_{\text{Cl,swell}}$ were not assessed. We hypothesize that sphingolipids will activate $I_{\text{Cl,swell}}$ through one or more of these shared signaling pathways. ROS generation in particular is a prime candidate for this activation.

1.1. Sphingolipid biosynthesis and metabolism

Sphingolipids are important components of all eukaryotic cells and are found in the plasma membrane, lysosomes, and Golgi and mitochondrial membranes (Hannun, 1994; Hannun and Obeid, 2008). They are usually classified into two separate groups: sphingophospholipids, which include sphingomyelin, ceramide, sphingosine and the phosphorylated forms of these lipids; and glycosphingolipids, which include gangliosides and others that accumulate in lipid storage diseases such as Neimann-Pick disease.

The sphingoid backbone confers a more asymmetric structure than the
diacylglycerol moiety of phosphatidylcholine (Kolesnick, 2002). The interface region of phosphatidylcholine contains a glycerol backbone and fatty ester linkages. By contrast, the interface region of sphingolipids contains an amide-linked fatty acid, a free hydroxyl group at the third position, and a \textit{trans} double bond between carbons four and five (Auge et al., 2000) (see Figure 1). These differences make the interface region of sphingolipids more polar than their phosphatidylcholine counterparts and, thus, allows for more opportunities for hydrogen bonding and greater interaction with polar solvents, features that may contribute to the unusually potent bioactivity of sphingolipids. In addition, the sphingoid backbone distinguishes sphingolipids from other amphipathic lipids.

A number of enzymes are involved in sphingolipid biosynthesis and metabolism. Ceramide, which forms the backbone of all sphingolipids, is synthesized \textit{de novo} beginning with the condensation of serine and palmitoyl-CoA to form 3-ketosphinganine by serine palmitoyltransferase at the cytosolic face of the endoplasmic reticulum. Reduction of 3-ketosphinganine leads to formation of sphinganine, which is N-acylated with free fatty acids by ceramide synthase to form dihydroceramide. This step is where ceramide acyl chain length of sphingolipid sub-species is established. The bond between carbons four and five of dihydroceramide is then desaturated to form ceramide. Ceramide is subsequently transported to Golgi either via vesicular [coat protein complex II (COP II)-dependent] or and non-vesicular [ceramide transfer protein (CERT)-
Ceramide forms the backbone of all sphingolipids. A majority of ceramide in cells is produced from the action of sphingomyelinases that cleave the phosphocholine group from sphingomyelin. Additionally, ceramide can be synthesized de novo from palmitoyl-CoA, serine, and fatty acids. Ceramide can then be further enzymatically modified by phosphorylation or glycosylation. Ceramide may also be metabolized by ceramidases to sphingosine which in turn is phosphorylated by sphingosine kinases to form the bioactive metabolite sphingosine-1-phosphate.
transport mechanisms (Perry and Ridgway, 2005). Once in the Golgi, ceramide is further processed to form other sphingolipids.

While de novo synthesis is an important source of ceramide in cells, much of the ceramide present under physiological and several pathological conditions is generated by the catabolism of sphingomyelin and other sphingolipids (Kitatani et al., 2008). Hydrolysis of sphingomyelin by enzymes known as sphingomyelin phosphodiesterases or simply sphingomyelinases, yields ceramide and phosphocholine. Additionally, ceramide is produced through a salvage pathway that begins within acidic cellular compartments including late endosomes and lysosomes (Kitatani et al., 2008). Sphingomyelinases are stimulated by TNF-\(\alpha\) (Kim et al., 1991), Fas ligand (Brenner et al., 1998), and oxidative stress (Goldkorn et al., 1998) and the different members of this enzyme family are classified by their pH optima and their cation dependence. Acid sphingomyelinase (A-SMase) is primarily present in lysosomes and has a maximum activity over a pH range of 3.5 – 5.2 (Hannun, 1996; Hannun and Obeid, 2008). There is evidence of a secreted isotype of acid sphingomyelinase (known as secretory sphingomyelinase, or S-SMase) that is Zn\(^{2+}\)-dependent and has been implicated in atherosclerotic development. Neutral sphingomyelinases (N-SMases) are Mg\(^{2+}\)– or Mn\(^{2+}\)–dependent transmembrane enzymes with optimal activity near pH 7.5 (Chatterjee, 1999). Aside from the plasma membrane, N-SMases, along with alkaline sphingomyelinase, have been localized to mitochondria, nuclear membranes, and the endoplasmic reticulum (Merrill, Jr. et al., 1997; Hannun and Obeid, 2008).
Ceramide may be further metabolized by ceramidases to form sphingosine. This is important in the ceramide salvage pathway, where acid ceramidase hydrolyzes the ceramide fatty acids, allowing the resulting sphingosine to leave the lysosome (Kitatani et al., 2008). Sphingosine may then be phosphorylated by sphingosine kinases to form the potent signaling lipid, sphingosine-1-phosphate (S1P). There are two primary isotypes of sphingosine kinase, denoted as SPHK1 and SPHK2 (Spiegel and Milstien, 2002). Although there is significant sequence homology between the two isotypes, they exhibit significantly different properties and distributions. SPHK1 is smaller and is expressed primarily in the cytosol, and the isoforms exhibit significant differences in their kinetics and temporal expression during development (Spiegel and Milstien, 2002). Thus S1P generation and its subsequent signaling effects may show significant cell-type, temporal, and compartmental specificity.

1.2 Sphingolipids in cardiovascular physiology and pathology

Ceramide accumulation is seen in many forms of human cardiovascular disease, including congestive heart failure (CHF), acute myocardial infarction, diabetic cardiomyopathy, atherosclerosis, and ischemia/reperfusion injury (Auge et al., 2000; Chatterjee et al., 2006), and S1P plays a role in cardioprotection, cardiac hypertrophy, and normal cardiac physiology (Karliner, 2009; Means and Brown, 2009). While the role of sphingolipids has been extensively studied, there is still much that is unknown about their underlying physiological and pathophysiological mechanisms.
Sphingolipids have been implicated as mediators of atherogenesis. Sphingomyelin is a component of low-density lipoprotein (LDL) and may enter cells via the LDL receptor pathway. Sphingomyelin accumulates in human atheromas and plasma levels are elevated in patients with coronary artery disease (Jiang et al., 2000). The relative concentration of sphingomyelin relative to other phospholipids is important for determining the level of lipoprotein sphingomyelin hydrolysis and risk of foam cell development. S-SMase, an acid sphingomyelinase found in secretory vesicles near the plasma membrane, has been implicated in the aggregation of LDL on endothelial cells during fatty streak formation (Marathe et al., 1998). S-SMase is also upregulated in patients with chronic heart failure (Doehner et al., 2007). S1P circulates in plasma primarily as a component of high density lipoprotein (HDL), and the ability of S1P to inhibit endothelial cell apoptosis has led some to suggest that this is at least part of the origin of the cardioprotective effects of HDL (Chatterjee et al., 2006; Murata et al., 2000). Other studies indicate, however, that S1P has pro-atherogenic properties (Xu et al., 2004; Chatterjee et al., 2006),

Sphingolipid metabolism and signaling also may be important in ischemia/reperfusion injury. A-SMase activity is induced during transient focal cerebral ischemia, and pharmacological inhibition of A-SMase and knockout of A-SMase in mice are protective against ischemic injury (Yu et al., 2000). During myocardial infarction, platelets likely release S1P during myocardial infarction (Ford, 2002). Both exogenous S1P and GM-1 ganglioside-induced production of intracellular S1P improve cardiac function as measured by left ventricular diastolic pressure or creatine kinase release following global
ischemia/reperfusion (Vessey et al., 2008; Jin et al., 2002). The use of specific S1P receptor sub-type 1 (S1P$_1$) agonist antibodies provides the same level of cardioprotection as exogenous S1P (Zhang et al., 2007b). There is also evidence for S1P$_2$ and S1P$_3$ receptor-mediated cardioprotection, as S1P$_2$, S1P$_3$, and S1P$_{2,3}$ double knockout mice demonstrate a significant increase in infarct size following ischemia (Means et al., 2007).

Additionally, sphingolipids may affect cardiovascular biology through their modulation of cardiac ion channels. Sphingolipids are known to have effects on a number of channel types, including K$^+$, Ca$^{2+}$, and anion channels. These electrophysiological effects are discussed further in the next section.

1.3 The effect of sphingolipids on cardiac electrical activity and ion channels

Sphingolipids are involved in the regulation of a number of K$^+$ channels found in heart (Ramu et al., 2006; Chapman et al., 2005; Bai et al., 2007) and other tissues (Zhang et al., 2002; Wu et al., 2001; Hida et al., 1998; Gulbins et al., 1997; Chik et al., 2001; Bock et al., 2003). Exogenous C$_2$-ceramide and endogenous ceramides that are generated by SMase elicit a time-dependent inhibition of hERG K$^+$ channel function that is precluded by treatment with the superoxide dismutase (SOD) mimic, Mn(III) tetra(4-benzoic acid) porphyrin chloride (MnTBAP) (Bai et al., 2007). Ceramide also induces hERG downregulation and increases lysosomal degradation of activated inwardly rectifying K$^+$ channels that give rise to I$_{K,ACh}$ and contribute to resting membrane potential in cardiomyocytes of a number of species (Means and Brown, 2009). This response is attributed to S1P$_3$ receptor activation because suramin, a putative S1P$_3$
antagonist, attenuates the effect on $I_{K,ACH}$ (Ancellin and Hla, 1999). The S1P$_3$ receptor may play a role in heart rate regulation, evidenced by the observation that S1P$_3$ stimulation induces bradycardia in mice and humans (Sanna et al., 2004; Budde et al., 2002). Outward $K^+$ current attributable to $Ca^{2+}$-activated $K^+$ channels is also inhibited by ceramide in rat pinealocytes (Chik et al., 2001). One possible mechanism for such inhibition is the formation of lipid rafts. Outwardly rectifying n-type $K^+$ channels are inhibited due to clustering of the channels in $C_{16}$-ceramide enriched membrane rafts (Bock et al., 2003). The importance of lipid rafts, which have higher cholesterol and sphingolipid content relative to other membrane domains, in the regulation of the pacemaker current ($I_f$) has also been suggested (Barbuti et al., 2004).

Additional evidence suggests a role for sphingolipids in regulating $Ca^{2+}$ channels. Stimulation of CD95 receptor blocks $Ca^{2+}$ influx in lymphocytes through store-operated calcium channels (CRAC) that is dependent on ceramide generation from A-SMase (Lepple-Wienhues et al., 1999). Reversible CRAC inhibition by exogenous ceramides, but not their inactive analogues, is also observed in lymphocytes. By contrast, there are reports that ceramide increases CRAC current. In Jurkat human T cells, exogenous ceramide increases intracellular calcium through a thapsigargin-sensitive mechanism (Colina et al., 2005b), although there is some evidence to suggest that this is due to the phosphorylation of ceramide to form ceramide-1-phosphate (C1P) rather than a direct effect of ceramide (Colina et al., 2005a).
Sphingolipids have been implicated in the regulation of Cl− channels in skeletal muscle (De Luca et al., 1998), airway epithelium (Ito et al., 2004), cardiac muscle (d'Anglemont de Tassigny et al., 2004), and *Xenopus laevis* oocytes (Souktani et al., 2000). Exogenous, short-chain C2-ceramide prevents the PKC-induced decrease in fast twitch skeletal muscle Cl− conductance due to the phorbol ester, 4-β-phorbol-12,13-dibutyrate (4-β-PDB). The mechanism of C2-ceramide in this system is thought to be through an okadaic acid-sensitive serine-threonine phosphatase that counteracts PKC activity (De Luca et al., 1998). N,N-dimethyl-D-erythro-sphingosine (DMS), a sphingosine kinase inhibitor, also activates a PKC-sensitive, I_{Cl,swell}-like Cl− current in *Xenopus* oocytes that is blocked by phosphatase inhibitor phorbol myristate acetate (PMA) (Souktani et al., 2000). By contrast, PMA had no effect on bacterial SMase-induced inhibition of CFTR Cl− current (Ito et al., 2004). The ceramidase inhibitor N-oleoylethanolamine and the glucosylceramide synthase inhibitor 1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) both potentiate SMase-induced CFTR inhibition, indicating ceramide, and not its metabolites, are responsible for the effect. Ito *et al.* (2004) have suggested that ceramide may interact specifically with the open state of CFTR channels, similar to their interaction with sulfonylureas. This mechanism remains unexplored, however.

1.4 **Sphingolipid-induced signaling**

Sphingolipid signaling has been well-studied over the past couple of decades. Although much is known about which signaling cascades are modulated by
sphingolipids, the pathways involved are complex, and there is still much that is not understood about how these different signaling pathways interact. For example, it has been well documented that the bioactive sphingolipids ceramide and S1P typically display antagonistic effects on cellular processes such as apoptosis. Such data support the concept that signaling is controlled by a ceramide-S1P rheostat, where the relative concentrations of these two signaling molecules determines the dominant signaling pathway. Recent evidence points, however, to the possibility that ceramide and S1P may have similar effects in some instances. For example, both ceramide and S1P induced ROS generation in bovine coronary arteries (Zhang et al., 2003; Keller et al., 2006). The following sections outline the major sphingolipid-induced signaling cascades and their regulatory pathways.

1.4.1 PKC/protein phosphatase signaling cascades

Protein kinase C (PKC) is a family of enzymes that regulate protein function by phosphorylating hydroxyl groups of serine/threonine residues. Protein phosphatases antagonize this process by dephosphorylating the serine/threonine residue. Ceramide stimulates a group of serine/threonine phosphatases known as ceramide-activated protein phosphatases (CAPPs), which includes PP1 and PP2A (Dobrowsky and Hannun, 1993; Hannun and Obeid, 2008). Additionally, ceramide is known to activate specific isoforms of PKC, including PKCζ (Fox et al., 2007; Hannun and Obeid, 2008), and endogenous S1P production induces activation of PKCε (Jin et al., 2002). While the activation of PKCs and CAPPs is well documented, these enzymes do not seem to be essential to all
sphingolipid signaling as several effects of sphingolipids are found to be PKC- and/or CAPP-independent.

1.4.2 MAPK/ERK/JNK cascades

Mitogen-activated protein kinases (MAPK) phosphorylate serine/threonine residues adjacent to proline in target proteins. This super-family of kinases responds to a wide range of mitogenic and stress stimuli in the cardiovascular system and other tissues (Feuerstein and Young, 2000; Muslin, 2008). MAPK fall into three major families: the extracellular-regulated kinases (ERK), the c-jun amino-terminal kinases (JNK), and the p38MAPK (Feuerstein and Young, 2000). ERK primarily respond to mitogenic factors, whereas JNK and p38 MAPK respond to physiological stresses. However, there is considerable overlap as several stimuli activate more than one pathway at a time.

Sphingolipids stimulate signaling cascades regulated by MAPK pathways in cardiomyocytes (Feuerstein and Young, 2000; Sekiguchi et al., 1999) and other cell types (Zaslavsky et al., 2005; Giltiay et al., 2005). Sphinosylphosphorylcholine (SPC), and to a lesser extent S1P, induce a hypertrophic growth response in cardiomyocytes that can be precluded by blockade with pertussis toxin (PTX) or the specific ERK1 inhibitor PD98059 (Sekiguchi et al., 1999). Additionally, the cytoprotective effects of HDL and S1P have been linked to and ERK-dependent mechanism that is completely inhibited by PD98059 in human umbilical vein endothelial cells (Kimura et al., 2001). Inflammatory mediators, specifically IL-1β, activate ERK through a ceramide-dependent manner in hepatocytes that can be mimicked by exogenous bacterial SMase (Giltiay et al., 2005).
However, other groups have reported that ceramide inhibits (Ghosh et al., 2002) or has no effect on (Pru et al., 2003) MAPK pathways. While the mechanism for these differential effects remains unclear, there is some suggestion that ERK activation correlates with ceramide generation via sphingomyelin turnover in the plasma membrane while ERK inhibition correlates with \textit{de novo} ceramide production (Giltiay et al., 2005; Ghosh et al., 2002).

1.4.3. PI-3K-Dependent Cascades

PI-3K are a family of signal transducing enzymes that phosphorylate the three position hydroxyl group of the inositol ring of phosphatidylinositol. The role of PI-3K in sphingolipid signaling cascades is largely unexplored. However, there is evidence that S1P$_1$ and/or S1P$_3$ receptors are mediated through a PI-3K dependent pathway (Zhang et al., 2007b). Additionally, ceramide-induced mitochondrial apoptosis has been shown to stimulate glycogen synthase kinase 3β through a PI-3K-dependent pathway (Lin et al., 2007).

1.4.4. EGFR/PDGFR-mediated cascades

S1P has been identified as a ligand for the epidermal growth factor receptor (EGFR) family of G protein coupled receptors, although they couple to more than one G protein including G$_{i/o}$, G$_q$, and G$_{12/13}$ (Kluk and Hla, 2002). S1P increases tyrosine phosphorylation of EGFR and platelet-derived growth factor β receptors (PDGFβR) that is precluded by the G$_i$ inhibitor PTX in vascular smooth muscle cells (Tanimoto et al.,
This transactivation could also be prevented by blockade of Src activation and ROS generation, and by cholesterol depletion. By contrast, S1P-induced cell detachment in human embryonic kidney cells acts through PDGFR but not EGFR (Zaslavsky et al., 2005). S1P-induced cell detachment was $G_i$- and ERK-dependent and antagonized by the engagement of active state $\beta_1$-integrin. This suggests that S1P acts in an “inside-out” manner involving an endogenous, cell-surface S1P receptor pathway. In fact, studies indicate that PDGFR can be tethered to S1P receptors (Alderton et al., 2001).

### 1.4.5. ROS signaling

Recently, the role of sphingolipids in reactive oxygen species (ROS) generation and signaling has come into focus. In cardiomyocytes, there are two primary sources of ROS: NADPH oxidase (NOX) and the mitochondrial electron transport chain (ETC). The differential effects of sphingolipids on these two modulators of cellular ROS production will be discussed in the following sections.

#### 1.4.5.1. Sphingolipids as mediators of NOX

NOX is a membrane-bound enzyme that utilizes cytoplasmic NADPH and extracellular $O_2$ to generate ROS and is involved in the phagocyte respiratory burst, immune function, cardiac hypertrophy, and atherogenesis. Gathering evidence points to modulation of NOX by sphingolipids. Ceramide increases the levels of GTP-bound Rac, a key step for the translocation of cytosolic components of NOX to the plasma membrane, in rat mesangial and kidney cells (Yi et al., 2004; Yi et al., 2006). This
increase in NOX activity is attenuated with de novo ceramide synthesis inhibitors fumonisin B₁ and myriocin (Yi et al., 2004; Yi et al., 2006), and inhibition of NOX and/or ceramide synthesis is associated with decreased hyperhomocysteine-induced glomerular injury (Yi et al., 2006). Exogenous, short-chain ceramide also induces endothelial dysfunction attributable to NOX activation and subsequent peroxynitrite formation in small bovine coronary arteries (Zhang et al., 2003).

CD95 ligand (CD95L) is a key upstream mediator of apoptosis and ROS production. CD95L induces ceramide generation and p47phox phosphorylation in hepatocytes that is sensitive to blockade of SMase and PKCζ (Reinehr et al., 2005). This process of NOX-derived ROS production is accompanied by upstream activation of Src kinases and JNK. Similarly, hyperosmolarity-induced hepatocyte apoptosis was attenuated by inhibition of SMase and knockdown of A-SMase or p47phox (Reinehr et al., 2006). Recently, S1P was also shown to increase H₂O₂ production in murine fibroblasts through NOX activation (Catarzi et al., 2007). This response was PI-3K- and PKC-dependent and was similar to PDGF-induced NOX activation, suggesting a possible transactivation mechanism between PDGFR and S1P receptors.

1.4.5.2. Sphingolipids as mediators of mitochondrial ROS

Increased mitochondrial ROS production associated with sphingolipids has been observed in experimental models of hypoxia/preconditioning (Lecour et al., 2006a; Lecour et al., 2006b), heart failure (Suematsu et al., 2003), and cardiac apoptosis and remodeling (Falluel-Morel et al., 2004; Suematsu et al., 2003). Mitochondrial
dysfunction is also seen in human dyslipidemias (Vercesi et al., 2007). The role of sphingolipids as modulators of mitochondrial function has been emphasized by the recent cloning of human mitochondrial ceramidase (El Bawab S. et al., 2000) and the localization of certain isoforms of SPHK to mitochondria (Maceyka et al., 2005).

Pretreatment with exogenous, short-chain C₂-ceramide reduces hypoxia-induced lactate dehydrogenase release in rat fibroblasts. This cytoprotective effect is attributed to mitochondrial ROS generation as the ETC Complex I inhibitor rotenone attenuates the response, whereas inhibition of other sources of ROS, including NOX, xanthine oxidase, and nitric oxide synthase (NOS), had no effect (Lecour et al., 2006b). Ceramide preconditioning provides a similar cytoprotective effect in ischemia/reperfusion of rat cardiac cells (Lecour et al., 2006a). There is some evidence that ischemia/reperfusion-induced ceramide generation and mitochondria damage is dependent on a MAPK/JNK signaling cascade, as JNK3-deficient mice are protected from ceramide accumulation and mitochondrial dysfunction (Yu et al., 2007).

Exogenous C₂-ceramide, but not the inactive analogue dihydro-C₂-ceramide, directly inhibits the mitochondrial ETC at the level of Complex III (Gudz et al., 1997). C₂-ceramide-induced increase in H₂O₂ is not attributable to the induction of the mitochondrial permeability transition pore because C₂-ceramide did not induce mitochondrial swelling in isolated rat liver mitochondria (Garcia-Ruiz et al., 1997b). An alternative mechanism for the action of ceramides is their assembly as pores. Both short- and long-chain ceramides form large, high conductance permeation pathways in the outer
mitochondrial membrane that may contribute to the role of mitochondria in apoptosis (Siskind et al., 2006; Siskind et al., 2002; Siskind and Colombini, 2000).

There is also evidence that ceramides of different chain lengths have differential roles in modulating mitochondrial function. $\text{C}_2$-ceramide stimulates cytochrome c oxidase and collapse of the mitochondrial membrane potential in isolated rat heart mitochondria (Di Paola M. et al., 2000). By contrast, long-chain $\text{C}_{16}$-ceramide inhibits cytochrome c oxidase activity and has negligible effect on mitochondrial membrane potential (Di Paola M. et al., 2000). Additionally, $\text{C}_{16}$-ceramide also inhibits formation of the mitochondrial permeability transition pore in isolated rat liver mitochondria (Novgorodov et al., 2008). Obviously, sphingolipid modulation of mitochondria and mitochondrial ROS generation is complex and there are still many open questions regarding the mechanism of this regulation.

1.5 Cardiac $\text{I}_{\text{Cl,swell}}$ and its regulation

Volume-sensitive $\text{Cl}^-$ current, $\text{I}_{\text{Cl,swell}}$, is elicited in cardiac myocytes by osmotic swelling (Tseng, 1992), hydrostatic inflation (Hagiwara et al., 1992), anionic amphipaths (Tseng, 1992), $\beta 1$ integrin stretch (Baumgarten and Clemo, 2003), and in several models of cardiac disease. In turn, $\text{I}_{\text{Cl,swell}}$ modulates cardiac electrical activity, cell volume, apoptosis, and is implicated in ischemic preconditioning (Baumgarten et al., 2005; Duan et al., 2005; Hume et al., 2000). Regulation of $\text{I}_{\text{Cl,swell}}$ is complex and involves a number of signaling pathways. Recently, reactive oxygen species (ROS) were identified as a downstream effector, and exogenous $\text{H}_2\text{O}_2$ elicits $\text{I}_{\text{Cl,swell}}$ in cardiomyocytes (Browe and
Upstream signaling molecules include Src family kinases (Walsh and Zhang, 2005; Du et al., 2004; Browe and Baumgarten, 2003d), focal adhesion kinase (Browe and Baumgarten, 2003d; Walsh and Zhang, 2005), protein tyrosine kinase (Sorota, 1995), angiotensin II (Ang II) (Ren et al., 2008; Browe and Baumgarten, 2004), epidermal growth factor receptor (EGFR) kinase (Du et al., 2004), and phosphoinositide 3-kinase (PI-3K) (Browe and Baumgarten, 2006; Ren et al., 2008). Protein kinase C (PKC) also is implicated, although its role is controversial because it appears to inhibit (Duan et al., 1999) or activate $I_{\text{Cl,swell}}$ (Gong et al., 2004; Duan et al., 1999). Although the biophysical properties, pharmacology, and many of the regulatory pathways of $I_{\text{Cl,swell}}$ have been extensively studied, the molecular identity of the channel remains elusive.

Several biophysical properties are used to distinguish $I_{\text{Cl,swell}}$ from other cardiac anion currents. $I_{\text{Cl,swell}}$ is outwardly rectifying and reverses near the chloride equilibrium potential ($E_{\text{Cl}}$) in either physiological or symmetrical $\text{Cl}^-$ gradients. The current is time-independent over the physiologic voltage range but partially inactivates at more positive potentials (Duan and Nattel, 1994; Shuba et al., 1996). The time-dependent component can be blocked by addition of Cd$^{2+}$ to the perfusate (Ren and Baumgarten, 2005). $I_{\text{Cl,swell}}$ is independent of cytoplasmic Ca$^{2+}$ (Lemonnier et al., 2002) and the channel is permeant to other anions with the permeability sequence: $\Gamma^- > \text{NO}_3^- > \text{Br}^- > \text{Cl}^- > \text{Asp}^-$ (Hagiwara et al., 1992; Vandenberg et al., 1994).
Sorota (1994) compared block of $I_{Cl,swell}$ in cardiac myocytes by several anion channel inhibitors. $I_{Cl,swell}$ is insensitive to Gd$^{3+}$ (Clemo and Baumgarten, 1997), which is an inhibitor of mechanosensitive cation channels. Although they also inhibit numerous cation channels and transporters, tamoxifen and DIDS (4,4’-diisothiocyanostilbene-2, 2’-disulfonic acid) were found to be useful pharmacological instruments for identifying $I_{Cl,swell}$ under conditions that isolate anion currents (Baumgarten and Clemo, 2003). Decher et al. (2001) demonstrated the highly selective block of $I_{Cl,swell}$ by the ethacrynic acid-derivative DCPIB [4-(2-butyl-6,7-dichloro-2-cyclopentyl-indan-1-on-5-yl) oxobutyric acid]. At 10 $\mu$M, a concentration sufficient to fully block $I_{Cl,swell}$, this agent does not inhibit native CFTR or Ca$^{2+}$-activated Cl$^-$ channels ($I_{Cl,\text{Ca}}$), exogenously expressed CIC-1, CIC-2, CIC-4, CIC-5, and CIC-K1 Cl$^-$ channels, and also has no effect on various K$^+$, Ca$^{2+}$, and Na$^+$ channels (Decher et al., 2001). CIC-3 also is unaffected by DCPIB (F. Lamb, personal communication to C.M. Baumgarten).

$I_{Cl,swell}$ also is implicated in several physiological and pathophysiological processes including cell volume regulation, migration, proliferation, differentiation, apoptosis, and ischemia-reperfusion injury (Baumgarten et al., 2005; Hume et al., 2000; Okada et al., 2009). $I_{Cl,swell}$ affects cardiac electrical activity and may play a role in mechanoelectrical feedback by antagonizing the effects of stretch-activated cation channels (Baumgarten and Clemo, 2003). Since $I_{Cl,swell}$ reverses between the resting membrane potential ($E_m$) and the plateau phase potential in cardiomyocytes, $I_{Cl,swell}$ activation depolarizes $E_m$ and acts to shorten APD (Hiraoka et al., 1998; Decher et al., 2001). This has important implications for arrhythmogenesis, as APD shortening due to
$I_{\text{Cl,swell}}$ activation would tend to dampen cardiac afterdepolarizations and be protective against triggered arrhythmias. Alternatively, shortening of APD may favor reentrant arrhythmias, such as atrial fibrillation, by reducing the minimum length conduction pathways needed to sustain them.

$I_{\text{Cl,swell}}$ may also influence cardiac function and arrhythmogenesis by its effects on cell survival. Cl$^-$ channel inhibitors preclude the preconditioning seen with brief episodes of ischemia and hypoosmotic stress (Diaz and Wilson, 2006). Apoptosis is also important during normal cardiomyocytes development and during ischemia and heart failure, and $I_{\text{Cl,swell}}$ appears to be an important modulator of this process in heart and other cell types. Block of $I_{\text{Cl,swell}}$ prevents doxorubincin-induced apoptosis in cardiomyocytes (d’Anglemont de Tassigny et al., 2004). The link between $I_{\text{Cl,swell}}$ and apoptosis appears to be the apoptotic volume decrease (AVD). AVD is an early required, Cl$^-$ flux-dependent decrease in cell volume that precedes caspase-3 activation, cytochrome c release, and DNA fragmentation (Rasola et al., 1999; Okada and Maeno, 2001). Overexpression of the anti-apoptotic regulating protein Bcl-2 leads to upregualtion of $I_{\text{Cl,swell}}$ in both dog kidney (Shen et al., 2002) and human prostate cancer epithelial (Lemonnier et al., 2004) cells. Additionally, AVD is specifically associated with a ROS-dependent activation of $I_{\text{Cl,swell}}$ in HeLa cells (Shimizu et al., 2004).

H$_2$O$_2$, a long-lived, cell permeable ROS, has been identified as a downstream mediator of $I_{\text{Cl,swell}}$ in cardiac myocytes. Scavenging H$_2$O$_2$ with catalase reverses β1-integrin stretch-induced activation of $I_{\text{Cl,swell}}$ (Browe and Baumgarten, 2004). Moreover, the SOD mimetic MnTBAP that speeds conversion of superoxide (O$_2^{-}$) to H$_2$O$_2$,
augments ACh-induced $I_{\text{Cl,swell}}$ (Browe and Baumgarten, 2007). $I_{\text{Cl,swell}}$ also is activated by exogenous H$_2$O$_2$ with an EC50 of ~8 µM (Ren et al., 2008; Browe and Baumgarten, 2004), and the site of action of H$_2$O$_2$ appears to be downstream from the volume-sensing mechanism because H$_2$O$_2$–induced current is insensitive to hyperosmotic shrinkage (Ren et al., 2008). Potential sources for ROS in cardiomyocytes include NADPH oxidase (NOX) and the mitochondrial electron transport chain (ETC). Both have been implicated in activation of $I_{\text{Cl,swell}}$ (Browe and Baumgarten, 2004; Browe and Baumgarten, 2007; Ren et al., 2008) and will be discussed in detail in the following sections.

1.5.1 NOX-derived ROS and upstream mediators

1.5.1.1. Integrin, FAK, and Src

As $I_{\text{Cl,swell}}$ is a mechanosensitive current, it is important to understand the mechanism of how mechanical forces are sensed and transmitted. Integrins are heterodimeric proteins that provide a physical link between the extracellular matrix, plasma membrane, and the cytoskeleton that are important for signal transduction, cell migration, and a number of other cellular processes (Babbitt et al., 2002; Force et al., 2002). Integrins form cell adhesion complexes and co-localize with a number of signaling molecules, adaptor and regulating proteins, and cell surface receptors. Focal adhesion kinase (FAK) and members of the Src kinase family are protein tyrosine kinases (PTK) and are important components of cell adhesion complexes that mediate integrin-induced mechanotransduction (Shai et al., 2002; Babbitt et al., 2002). Upon membrane stretch, FAK binds to the cytoplasmic domain of β1-intergins and autophosphorylates a
number of tyrosine residues, including Tyr397. The SH2 domain of Src binds then to Tyr397 of FAK and the activated complex recruit a number of signaling molecules including protein kinase C (PKC), phosphoinositide-3 kinase (PI-3K), and protein phosphatases and stimulate a number of downstream signaling cascades (Parsons, 2003).

β1-integrin stretch has been shown to play an important role in cardiac cell growth and remodeling during hypertrophy (Pham et al., 2000). β1-integrins, FAK, and Src are important in the regulation of $I_{Cl,swell}$, and β1-integrin stretch elicits $I_{Cl,swell}$ under isoosmotic conditions (Browe and Baumgarten, 2003d). β1-integrin-induced activation of $I_{Cl,swell}$ is blocked by both genistein, a non-specific PTK inhibitor, and protein phosphatase 2 (PP2), a selective Src inhibitor (Browe and Baumgarten, 2003d). The role of Src in $I_{Cl,swell}$ regulation is complex, however, and varies depending on the stimuli. In contrast to its effect following β1-integrin stretch, PP2 substantially enhances $I_{Cl,swell}$ induced by osmotic swelling in both atrial and ventricular myocytes (Du et al., 2004; Ren and Baumgarten, 2005; Walsh and Zhang, 2005). The mechanisms underlying the differential regulation of $I_{Cl,swell}$ by FAK and Src remains unclear.

1.5.1.2. Ang II and Ang II receptors

Upon mechanical stretch, cardiomyocytes release a number of autocrine/paracrine factors, including angiotensin II (Ang II) and endothelin-1 (ET-1), which bind to their surface receptors and induce a number of downstream signaling cascades (Ruwhof et al., 2001). Additionally, stretch induces Ang II receptor type 1 (AT$_1$R) activation exclusive of Ang II binding, suggesting Ang II may not be required for AT$_1$R-mediated
mechanotransduction (Zou et al., 2004). It is not unexpected then, that Ang II and AT$_1$R play a role in the regulation of $I_{\text{Cl,swell}}$ in cardiomyocytes. This is evidenced by the observations that exogenous Ang II elicits $I_{\text{Cl,swell}}$ under isoosmotic conditions and that competitive AT1R antagonists, losartan and eposartan, inhibit osmotic swelling- and $\beta_1$-integrin stretch-induced $I_{\text{Cl,swell}}$ (Ren and Baumgarten, 2005; Browe and Baumgarten, 2004).

**1.5.1.3. Endothelin-1**

Endothelins (ETs) are a family of 21-amino acid peptides that includes three primary isoforms: ET-1, ET-2, and ET-3. ET-1 is the major isoform responsible for the cardiovascular effects of this protein family (Kedzierski and Yanagisawa, 2001). ET-1 interacts with two G protein coupled receptors subtypes, ET$_A$ and ET$_B$, both of which are heterogeneously expressed in heart (Russell and Molenaar, 2000).

ET-1 elicits $I_{\text{Cl,swell}}$ in atrial cardiomyocytes via activation of ET$_A$ receptors (Deng and Baumgarten, 2009). ET-1-induced $I_{\text{Cl,swell}}$ is abrogated by block of EGFR kinase with AG1478 and inhibition of PI-3K with both wortmannin and LY294002. Additionally, dismutation of $H_2O_2$ with the glutathione peroxidase mimetic ebselen completely attenuates ET-1-induced current (Deng and Baumgarten, 2009). These results suggest that the action of ET-1 in this cascade is upstream of EGFR kinase and PI-3K activation and generation of ROS.
1.5.1.4. EGFR kinase

Epidermal growth factor receptor (EGFR) kinase has been identified as a common element in signaling cascades induced by changes in cell volume (Lezama et al., 2005; Browe and Baumgarten, 2006), TNFα (Tetreault et al., 2008), integrins (Moro et al., 2002), and AT1Rs (Shah and Catt, 2003). EGFRs have also been implicated in the regulation of \( I_{\text{Cl,swell}} \) (Baumgarten, 2006; Du et al., 2004; Ren et al., 2008). Exogenous EGF elicits \( I_{\text{Cl,swell}} \) under isoosmotic conditions and EGFR kinase inhibitors block \( I_{\text{Cl,swell}} \) activation induced by osmotic swelling, \( \beta1 \)-integrin stretch, and Ang II (Ren et al., 2008; Browe and Baumgarten, 2006).

1.5.1.5. PI-3K

PI-3K are a family of signal transducing enzymes that phosphorylate the three position hydroxyl group of the inositol ring of phosphatidylinositol. They are heterodimers comprised of a p110 catalytic subunit and one of a number of different regulatory subunits. In the heart, the predominant isotypes are p110-p85, activated by PTK, and p110-p101, activated by a G protein mechanism (Pretorius et al., 2009; Till et al., 2000). Myocardial PI-3K activation is induced by osmotic swelling (Ren et al., 2008; Tilly et al., 1996), myocardial stretch (Kim et al., 2002), and integrin/FAK stimulation (Franchini et al., 2000). PI-3K is coupled to EGFR and mediates downstream signaling induced by EGFR and AT1R activation (Browe and Baumgarten, 2006; Kippenberger et al., 2005; Ren et al., 2008). In cardiomyocytes, the PI-3K inhibitors wortmannin and LY294002 block \( I_{\text{Cl,swell}} \) activation induced by EGF, \( \beta1 \)-intergrin stretch, and osmotic
swelling (Browe and Baumgarten, 2006; Ren et al., 2008). This suggests that PI-3K is downstream from EGFR, AT₁R, and the volume-sensing mechanism of I_{Cl,swell}.

1.5.1.6. NOX

NADPH oxidase (NOX) is a membrane-bound enzyme that generates ROS from cytosolic NADPH and is involved in the respiratory burst, cardiac hypertrophy, and atherogenesis. NOX2 is the primary isoform expressed in cardiomyocytes, although expression of NOX4 has also been reported (Byrne et al., 2003a). NOX2 is comprised of six subunits: a transmembrane flavocytochrome b_{558} complex consisting of a large gp91^{phox} subunit, a smaller p22^{phox} subunit, cytosolic p47^{phox} and p67^{phox} subunits, and the GTP-binding protein Rac (Bedard and Krause, 2007; Wallach and Segal, 1996). NOX2 becomes functionally active when Rac and the cytosolic subunits translocate from the cytosol to the plasma membrane and assemble with gp91^{phox} and p22^{phox}. This process is mediated by a number of signaling molecules, including PTK such as Src, PKC, and PI-3K (Bedard and Krause, 2007). The fully assembled, functional complex transfers electrons across the plasma membrane, resulting in production of O_{2}− that is then rapidly converted to H_{2}O_{2} spontaneously and through the action of SOD (Bedard and Krause, 2007). NOX stimulation also leads to downstream signaling cascades, including activation of MAPK and ERK1/2 (Bedard and Krause, 2007).

NOX is a required component of I_{Cl,swell} activation induced by β1-intergrin stretch, osmotic swelling, EGF, ET-1 and Ang II. I_{Cl,swell} evoked by these stimuli is completely inhibited by block of NOX with the membrane-permeant fusion peptide gp91ds-tat,
diphenyleneiodonium (DPI), 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), or apocynin (Browe and Baumgarten, 2006; Ren et al., 2008).

1.5.2. Mitochondria-derived ROS and upstream mediators

Mitochondria, through the function of the ETC, are another source of ROS in cardiomyocytes. Increased mitochondrial ROS production has been observed in experimental models of heart failure (Giordano, 2005; Ide et al., 1999), ischemia/reperfusion injury (Vanden Hoek et al., 1998; Giordano, 2005), myocardial infarction (Vanden Hoek et al., 1998; Ide et al., 2001; Giordano, 2005; Byrne et al., 2003b), and cardiac apoptosis and remodeling (Byrne et al., 2003b; Kumar and Jugdutt, 2003). Mitochondrial ROS production at ETC Complex III is seen very early during global ischemia and leads to depletion of cardiolipin from the mitochondrial membrane and loss of cytochrome c (Chen et al., 2008). Additionally, mitochondrial ROS are reported to play a role in the slow force response to myocardial stretch and the positive inotropic effects of Ang II (Caldiz et al., 2007; De Cavanagh et al., 2009).

Acetylcholine (ACh) was also shown to stimulate ROS production via muscarinic receptors (Yao et al., 1999). This process is dependent on Src, PI-3K, and opening of mitochondrial ATP-sensitive K$^+$ (mitoK$\text{ATP}$) channels (Oldenburg et al., 2003). Preliminary data indicate that exogenous ACh elicits $I_{\text{Cl,swell}}$ via a mitochondrial ROS-dependent mechanism. ACh-induced $I_{\text{Cl,swell}}$ is completely inhibited by the mitoK$\text{ATP}$ inhibitor 5-hydroxydecanoic acid (5-HD) and the mitochondrial ETC Complex I inhibitor rotenone (Browe and Baumgarten, 2007). Additionally, it has been shown that certain
stimuli, such as certain HIV protease inhibitors, activate $I_{cIswell}$ via mitochondrial ROS production independent of NOX (Deng et al., 2009).

1.5.3. Crosstalk between NOX and mitochondria

There are two populations of mitochondria in cardiomyocytes: those found near the sarcolemmal membrane, known as subsarcolemmal mitochondria; and those interspersed between the myofibrils, known as interfibrillar mitochondria. The proximity of subsarcolemmal mitochondria to the plasma membrane raises the possibility of crosstalk between NOX and mitochondria. There is evidence that such an interaction occurs, as Ang II and ET-1 induce ROS via NOX and mitochondria (De Giusti et al., 2008; De Giusti et al., 2009). Inhibition of either NOX or mitoK$_{ATP}$ channels blocked these responses. Additionally, inhibition of NOX assembly or blockade of mitoK$_{ATP}$ channels abrogate the effects of preconditioning, lipid peroxidation, activation of MAPK pathway, and apoptosis in cardiomyocytes (Zhang et al., 2007a; Caldiz et al., 2007; Bedard and Krause, 2007). Block of either source completely ameliorates these responses, indicating that NOX and mitochondria may act in series rather than in parallel. Mitochondrial ROS may be induced by NOX-derived ROS and/or by paracine induction due to ROS from neighboring mitochondria (Aon et al., 2003; Zorov et al., 2006). Recent evidence indicates that mitochondria ROS generation may be downstream of NOX in $I_{cIswell}$ activation due to some stimuli, as the ETC Complex III inhibitor antimycin A and the mitoKATP agonist diazoxide elicit gp91ds-tat-insensitive $I_{cIswell}$ activation (Deng and Baumgarten, 2009).
1.5.4. PKC and ERK1/2

Aside from ROS, regulation of $I_{\text{Cl,swell}}$ by PKC, ERK1/2, and Rho/Rho kinase has been observed. PKC has contrasting effects in different cell types. PKC antagonizes the action of serine/threonine phosphatases and inhibits $I_{\text{Cl,swell}}$ activation in rabbit atrial myocytes (Duan et al., 1995). In normal canine and rabbit cardiomyocytes, $I_{\text{Cl,swell}}$ activation is protein phosphatase 2a (PP2a)-dependent and Ca$^{2+}$–independent (Clemo et al., 1999). In heart failure cells, $I_{\text{Cl,swell}}$ activation is modulated by the Ca$^{2+}$–dependent phosphatase calcineurin (PP2b) (Clemo et al., 2000). By contrast, PKC stimulates $I_{\text{Cl,swell}}$ in canine atrial and murine ventricular cells (Du and Sorota, 1999; Gong et al., 2004). The MAPK pathway has also been implicated in $I_{\text{Cl,swell}}$ regulation. Inhibition of ERK1/2 prevented the persistent activation of $I_{\text{Cl,swell}}$ in heart failure cardiomyocytes (Clemo and Baumgarten, 1999).

1.6 HL-1 cells as a potential tool to investigate $I_{\text{Cl,swell}}$

HL-1 cells are an immortalized murine atrial cell line derived from a primary culture of SV40 large T antigen-induced atrial tumors. This cell line may be repeatedly passaged while maintaining their contractile ability and their morphological and electrophysiological characteristics (Claycomb et al., 1998). HL-1 cells have been used to study cellular signaling (McWhinney et al., 2000), calcium handling (George et al., 2003), and electrophysiological properties (Walker et al., 2007; Akhavan et al., 2003) of cardiomyocytes. They have also been used as a model for various pathological states, including hypoxia (Nguyen and Claycomb, 1999; Cormier-Regard et al., 1998), apoptosis
(Kim et al., 2003; Carlson et al., 2002), ischemia/reperfusion injury (White et al., 2004; Ruiz-Meana et al., 2006; Ruiz-Meana et al., 2003), and rapid pacing-induced electrical remodeling (Yang et al., 2005).

The rennin-angiotensin system machinery is expressed in HL-1 cardiomyocytes and Ang II induced expression of L-type Ca\(^{2+}\) channels via a NOX-dependent pathway (Tsai et al., 2008; Tsai et al., 2007). Moreover, preliminary data from this laboratory demonstrates that \(I_{\text{Cl,swell}}\) is present in HL-1 myocytes and is regulated by ET-1 signaling and by ROS generated by NOX and mitochondria in a similar manner to native cardiomyocytes (Deng and Baumgarten, 2009). Therefore, this cell line is a valuable tool for studying \(I_{\text{Cl,swell}}\).

1.7 Aims of the present study

Based on evidence in that \(I_{\text{Cl,swell}}\) is activated by ROS and ceramides elicit ROS production by NADPH oxidase and mitochondria, we hypothesized that sphingolipids would activate cardiac \(I_{\text{Cl,swell}}\) under isosmotic conditions by a ROS-dependent pathway. To test this hypothesis we used a combination of whole-cell patch clamp to measure ionic currents, flow cytometry to measure ROS production, and tandem mass spectroscopy to measure membrane lipid composition. Five specific aims were addressed. The first aim was to determine if exogenous, membrane-permeant, short-chain synthetic ceramide activates \(I_{\text{Cl,swell}}\) in rabbit ventricular myocytes. The second aim was to determine if endogenous long-chain ceramides activate \(I_{\text{Cl,swell}}\). Bacterial sphingomyelinase (SMase) was used to generate native ceramides, and with the assistance of collaborators, membrane sphingolipid content was measured with tandem mass spectrometry. The third
aim was to examine the role of ROS in the signaling cascade leading to SMase-induced $I_{Cl,swell}$. The fourth aim was to determine the role of ceramide metabolites in the activation of $I_{Cl,swell}$. The fifth aim was to use flow cytometry to determine if SMase-induced ROS production. HL-1 myocytes were used to address this last aim.
Chapter 2
MATERIALS and METHODS

2.1. Rabbits and ventricular myocyte isolation

Ventricular myocytes were isolated from adult New Zealand white rabbits (~3 kg). The animals were first sedated with a combination of acepromazine (1 mg/kg, IM) and xylazine (5 mg/kg, IM). This was followed by ketamine (35 mg/kg, IM) to induce a surgical plan of anesthetic. Prior to sacrifice (approximately 5 min), heparin (1000 U, IV) was administered to the animals via the ear vein to prevent clotting within the coronary arteries. When the animal is non-responsive to toe-pincher and corneal contact, a midline sternotomy is performed and the heart is removed. Following excision, hearts were affixed to a Langendorff perfusion apparatus, where the heart then undergoes retrograde perfusion via the aorta. First, a $\text{Ca}^{2+}$-containing, oxygenated (100% $\text{O}_2$) Tyrode solution was perfused to maintain contraction and clear the coronary vessels of blood. Next, an oxygenated, EGTA-containing $\text{Ca}^{2+}$-free Tyrode solution was perfused in order to chelate any residual $\text{Ca}^{2+}$, as enzymatic digestion is sensitive to free $\text{Ca}^{2+}$ concentration. Then, after a small amount (~40 mL) of nominally $\text{Ca}^{2+}$-free Tyrode
solution was perfused to flush out EGTA, the coronary arteries were perfused with enzyme solution that was recirculated. The heart was digested on the column for 20 – 24 min. and was then removed and minced (~1 mm³). The tissue was placed in modified Kraft-Bruhe (KB) solution and gently agitated by stirring for 3 – 5 min. to release individual myocytes from the tissue. Subsequently, the myocytes were washed in fresh KB solution twice and allowed to quiesce for ~1 hr before the study was begun.

Tyrode solution for cell isolation contained (in mM): 130 NaCl, 5 KCl, 1.8 CaCl₂, 0.4 KH₂PO₄, 3 MgCl₂, 5 HEPES, 15 taurine, 5 creatine, 10 glucose (pH 7.25, adjusted with NaOH). For Ca²⁺-free Tyrode solution, CaCl₂ was replaced with 0.1 mM Na₂-EGTA. Enzyme solution contained 0.45 mg/mL collagenase (CLS 4, Worthington) and 0.015 mg/mL pronase (Type XIV, Sigma-Aldrich) in nominally Ca²⁺-free Tyrode solution without EGTA. Isolated myocytes were washed and stored in a modified KB solution that contained (in mM): 120 K-glutamate, 10 KCl, 10 KH₂PO₄, 1.8 MgSO₄, 0.5 K₂-EGTA, 10 taurine, 10 HEPES, 20 glucose 13.7 mannitol (pH 7.20, adjusted with CsOH). Experiments were conducted within 12 h of myocyte isolation. Cells chosen for experiments were rod-shaped with clear striations, quiescent, and free of membrane blebs or other morphological irregularities.

2.2. Culture of HL-1 cells

HL-1 cardiac myocytes (passage 67 to 81), an immortalized mouse atrial cell line (Claycomb et al., 1998), were also studied. Tissue culture flasks were first coated with a gelatin (0.02%)/fibronectin (0.5%) mixture (2 mL in T25 or 6 mL in T75 flask) and
incubated overnight at 37°C. HL-1 cells were culture in pre-coated flasks using Claycomb medium (JRH Biosciences, Lenexa, KS) supplemented with 10% fetal bovine serum (Sigma-Aldrich), 100 µg/mL penicillin/streptomycin, 0.1 mM norepinephrine (Sigma-Aldrich), and 2 mM L-glutamine (Invitrogen, Carlsbad, CA). The cells were kept at 37°C in an atmosphere of 5% CO₂ and 95% air at a relative humidity of approximately 95%. Once the cells reached confluence, they were passaged in a 1:2 split. To split the HL-1 cells, they were briefly rinsed with DPBS and then incubated with 0.05% trypsin/EDTA (3 mL for T25 or 6 mL for T75 flask) at 37°C for 3 – 5 min until the cells dissociated from the flask surface. An equal amount of soybean trypsin inhibitor (25 mg/100 mL PBS) was added directly to the flask and then the contents were transferred to a 15 mL centrifuge tube. The cells were centrifuged at 1100 rpm for 5 min and the supernatant was removed by aspiration and the pellet was gently suspended in 3 mL of supplemented Claycomb medium. For culture preparation, the cells were transferred to gelatin/fibronectin-coated flasks. For electrophysiological studies, the cells were transferred to a 15 mL centrifuge tube.

2.3. Experimental solutions and drugs

Bath and pipette solutions were designed to isolated Cl⁻ current. Isosmotic bath solution (1T; 300 mOsm/kg; T, times isosmotic) contained (in mM) : 90 N-methyl-D-glucamine-Cl, 3 MgCl₂, 10 HEPES, 10 glucose, 5 CsCl, 0.5 CdCl₂, 70 mannitol (pH 7.4, adjusted CsOH). Hyperosmotic bath solution (1.5T, 450 mOsm/kg) had the same composition except for an additional 150 mM mannitol, and hypoosmotic bath solution
(0.7T, 230 mOsm/kg) contained no mannitol. For experiments with SMase, 6 mM MgCl\(_2\) was added to augment enzymatic activity, with mannitol concentrations adjusted accordingly. Pipette solution contained (in mM): 110 Cs-Aspartate, 20 TEA-Cl, 5 Mg-ATP, 0.1 Tris-GTP, 0.15 CaCl\(_2\), 8 Cs\(_2\)-EGTA, 10 HEPES (pH 7.1, adjusted with CsOH). To make symmetrical Cl\(^-\) pipette solution, 82 mM CsCl replaced an equal amount of Cs-Aspartate. Osmolarity was verified by measurement of freezing-point depression.

Stock solutions of D-erythro-C\(_2\)Ceramide (C\(_2\)-Cer; 5 mM; Biomol), D-erythro-dihydro-C\(_2\)Ceramide (C\(_2\)-H\(_2\)Cer; 5 mM; Biomol), ebselen (15 mM, Calbiochem), rotenone (20 mM; Sigma-Aldrich), apocynin (500 mM; Sigma-Aldrich), PD98059 (10 mM; Calbiochem), U0126 (1 mM; Biomol), diphenyliodonium (DPI, 60 mM; Sigma-Aldrich), and DCPIB (20 mM; Tocris) in DMSO and tamoxifen (20 mM; Sigma-Aldrich), 5-hydroxy-decanic acid (5-HD, 500 mM; MP Biochemicals), DL-threo-dihydrosphingosine (10 mM; Cayman), and (1S,2R-D-erythro-2-N-myristoylamino)-1-phenyl-1-propanol (D-erythro-MAPP, 10 mM; Cayman) in ethanol were frozen (−20°C) in aliquots until use. The NOX inhibitor gp91ds-tat was synthesized by the Tufts University Core Facility. Peptide stocks of gp91ds-tat were made in 150 mM NaCl plus 10 mM acetic acid and frozen (−20°C) in aliquots until use. Stock solutions of Mg\(^{2+}\)-dependent, neutral bacterial sphingomyelinase, also known as SMase C, from B. cereus (50 U/mL, in H\(_2\)O; Sigma-Aldrich) were stored in aliquots at 4°C until use.
Although it is not present physiologically, application of exogenous synthetic short-chain C2-Cer was employed because it is membrane permeant and is adequately soluble in the serum-free experimental solutions without forming micelles. On the other hand, bacterial SMase generates native long-chain ceramides from membrane sphingomyelinase and may better represent ceramide accumulation in physiologic and pathophysiologic settings.

2.4. Whole cell patch clamp and electrophysiological recordings

Ventricular myocytes were scattered on a glass-bottomed chamber and placed on the stage of an inverted light microscope (Nikon) with Hoffman modulation optics, and a high resolution video camera (CCD72; Dage-MTI) was used to visualize individual cells. Cells were suprafused with bath solution at 2–3 mL/min, and recordings were made at room temperature (22–23°C). Pipettes were pulled from 7740 thin-walled borosilicate capillary tubing (Sutter) and fire polished to a final tip diameter of approximately 3 µm with a resistance in bath solution of 2 – 4 MΩ. Membrane currents were recorded in the whole-cell configuration using an Axopatch 200B amplifier and Digidata 1322A data acquisition system (Axon). A 3-M KCl agar bridge served as the ground electrode. Seal resistances of 2 – 20 GΩ typically were obtained, and membrane capacitance routinely was measured. All membrane potential data were corrected for the measured liquid junction potential, and myocytes were dialyzed with pipette solution for 8 – 10 min prior to the start of recording. Voltage clamp protocols and data acquisition were controlled by pClamp 8.2. Successive 500-ms voltage steps were implemented from a holding
potential of −60 mV to test potentials ranging from −100 to +60 mV in +10 mV increments. Membrane currents were low-pass filtered at 2 kHz and digitized at 5 kHz. Representative current traces were low-pass filtered at 500 Hz for presentation, and displayed I-V curves are from the corresponding current traces. To minimize variability, experiments were designed to use cells as their own controls.

2.5 ROS detection by flow cytometry

ROS production was assessed in HL-1 cells rather than freshly isolated myocytes, as damaged myocytes are inevitably included in freshly isolated preparations. ROS was detected with C-H$_2$DCFDA-AM [6-carboxy-2',7';-dichlorodihydro-fluorescein diacetate di(acetoxy-methyl ester)] (Invitrogen), which is converted to carboxy-H$_2$DCF, a non-fluorescent derivative, by intracellular esterases. Carboxy-H$_2$DCF remains in the cytoplasm and is oxidized to fluorescent carboxy-DCF by intracellular ROS. Confluent HL-1 cells were incubated with C-H$_2$DCFDA-AM (2.5 or 5 µM) for 30 min at 37°C, washed twice with DPBS, and isolated using the splitting procedure described previously. Single myocyte suspensions in isoosmotic bath solution were analyzed using a EPICS XL cytometer (Beckman Coulter). The geometric means of the gated fluorescence distributions (excitation: 485 nm; emission: 520 nm) were calculated using the EXPO32 software (Beckman Coulter). After gating, 20,000 to 40,000 cells contributed to each fluorescence distribution. Fluorescence histograms (1024 bins) were exported to Excel (Microsoft) and plotted in SigmaPlot (Systat) after filtering (5 point running average, replotted in 512 bins). This gives plots that were comparable to those displayed with the commonly used but ill-defined smoothing function in EXPO32.
2.6 Tandem mass spectrometry

Ventricular myocytes were subjected to the relevant treatment, and harvested in 1T bath solution as described above. An aliquot of cells were taken for standardization (total protein). Lipids were extracted from 500 µL of the remaining cells as described by Wijesinghe et al. (2009) and Merrill et al. (2005) with slight modifications. Briefly, 2 mL of methanol and 1 mL of chloroform was added to 500 µL of the cell suspension, together with an internal standard containing 500 pmol of the following: d$_{17:1}$ sphingosine, sphinganine, sphingosine-1-phosphate and sphinganine-1-phosphate, d$_{18:1/12:0}$ ceramide, ceramide-1-phosphate, sphingomyelin and glucosylceramide. The mixture was sonicated and incubated at 48°C overnight. The following day, extracts were subjected to base hydrolysis for 2 hr at 37°C using 150 µL of 1.0 M methanolic KOH. Following base hydrolysis the extract was completely neutralized by the addition of glacial acetic acid. The neutralization was confirmed with pH paper. Half of the extract was dried down and brought up in reversed phase sample buffer (60%A:40%B).

To the remainder of the extract, 1 mL of chloroform and 2 mL of water were added, and the lower phase was transferred to another tube, dried down and brought up in normal phase sample buffer (98%A:2%B). Sphingosine, sphinganine, sphingosine-1-phosphate sphinganine-1-phosphate and ceramide-1-phosphate were quantified via reversed phase HPLC ESI-MS/MS using a Discovery C18 column attached to a Shimadzu HPLC (20AD series) and subjected to mass spectrometric analysis using a 4000 Q-Trap (Applied Biosystems) as described (Wijesinghe et al., 2009). Ceramides, sphingomyelins and monohexosyl ceramides were quantified via normal phase HPLC ESI-MS/MS using
an amino column (Sigma) as described (Merrill, Jr. et al., 2005).

2.7 Statistics

Summary patch clamp data are reported as mean ± SEM; \( n \) denotes the number of cells. Mean currents are expressed as current density (pA/pF) to account for differences in myocyte surface membrane area, and selected paired comparisons are expressed as a percentage or as the intervention-induced difference current. Statistical analysis was executed using SigmaStat 3.11 (Systat). For multiple comparisons, a One Way or a One Way Repeated Measures Analysis of Variance was performed, followed by a Student-Newman-Keuls test. In both cases, \( P < 0.05 \) was taken as significant. Geometric means of fluorescence histogram data were not normally distributed and were analyzed using Kruskal-Wallis One Way Analysis of Variance on Ranks followed by comparison to background fluorescence by Dunn's method. Non-linear curve fits were done in SigmaPlot 10.0 (Systat).
Chapter 3

RESULTS

3.1. Exogenous ceramide activates a Cl\(^{-}\) current resembling \(I_{\text{Cl,swell}}\)

As shown in Figure 2, C\(_2\)-ceramide (C\(_2\)-Cer; 2 \(\mu\)M, 10 – 12 min), a membrane-permeant, short-chain ceramide analogue, activated an outwardly-rectifying Cl\(^{-}\) current with a reversal potential near the Cl\(^{-}\) equilibrium potential (\(E_{\text{Cl}}\)), \(-43\) mV. Current at +60 mV increased by 0.70 \(\pm\) 0.09 pA/pF (\(n = 15, P < 0.001\)), from 0.87 \(\pm\) 0.13 to 1.57 \(\pm\) 0.22 pA/pF, and a C\(_2\)-Cer-induced current was observed in >90\% of the cells tested. Addition of DCPIB (10 \(\mu\)M, 12–15 min), a highly selective \(I_{\text{Cl,swell}}\) blocker, inhibited the C\(_2\)-Cer-induced Cl\(^{-}\) current by 76 \(\pm\) 8 \% (\(n = 6, P < 0.001\)) in the continued presence of C\(_2\)-Cer, and there was no significant difference between the DCPIB-inhibited and control currents (\(P = 0.24\)). Furthermore, the C\(_2\)-Cer-induced current was steeply concentration dependent with an EC\(_{50}\) of 0.5 \(\mu\)M and Hill coefficient of 5. The physiological range for native ceramide in many cell types is 1 – 5 \(\mu\)M, (Hannun, 1996) although local concentrations under some conditions may be greater. (Hannun and Obeid, 2008) Because C\(_2\)-Cer is a short-chain synthetic ceramide, its concentration dependence may
Figure 2: C\textsubscript{2}-ceramide (C\textsubscript{2}-Cer) elicited a Cl\textsuperscript{−} current in rabbit ventricular myocytes that resembled I\textsubscript{Cl,swell}. (A) Families of currents under control conditions (Ctrl), and after treatment with 2 \muM C\textsubscript{2}-Cer for 10 min, and after addition of DCPiB (+DCPiB; 10 \muM) in the continued presence of C\textsubscript{2}-Cer. Holding potential, −60 mV; test potentials, −100 to +60 mV. (B) Current-voltage (I-V) relationships for A. (C) Normalized currents at +60 mV. C\textsubscript{2}-Cer increased Cl\textsuperscript{−} current by 0.70 ± 0.09 pA/pF (n = 14, P < 0.001). The C\textsubscript{2}-Cer-induced current was inhibited by 76 ± 8% (n = 6, P < 0.001) by the I\textsubscript{Cl,swell}-specific inhibitor DCPiB, and the remaining current was not significantly different than control (P = 0.24). (D) Concentration-response relationship for 0.2 (n = 3), 0.6 (n = 3), 2 (n = 15) and 20 \muM (n = 4) C\textsubscript{2}-Cer. Data were fit (solid line) with an EC\textsubscript{50} of 0.5 \muM and a Hill coefficient of 5.
not match that of native ceramides. No change in membrane capacitance was observed in individual cells treated with C$_2$-Cer (data not shown). Under control conditions, the background current usually displayed modest outward rectification, and its amplitude at +60 mV varied somewhat from cell-to-cell. Such variation has been noted previously and likely reflects partial activation of I$_{Cl,swell}$ under control conditions.

Outward rectification in symmetrical Cl$^-$ solutions is a characteristic of I$_{Cl,swell}$ that distinguishes it from several other Cl$^-$ currents, including CFTR and Ca$^{2+}$-activated Cl$^-$ currents. (Hume et al., 2000) Under symmetrical Cl$^-$ conditions (Figure 3), C$_2$-Cer (2 µM, 10–12 min) elicited current that outwardly rectified and reversed at 0 mV. At +60 mV, C$_2$-Cer increased current density by 1.30 ± 0.32 pA/pF ($n=6$, $P<0.01$), from 1.03 ± 0.23 to 2.33 ± 0.52 pA/pF. Taken together, outward rectification in both physiological and symmetrical Cl$^-$ solutions and block by DCPIB are diagnostic for I$_{Cl,swell}$.

Alterations in membrane curvature due to asymmetric insertion of amphipathic molecules into the outer or inner leaflets of the plasma membrane are known to mimic changes in cell volume by altering membrane tension and shape and can activate I$_{Cl,swell}$ (Tseng, 1992). To exclude the possibility that C$_2$-Cer activated I$_{Cl,swell}$ via alteration of membrane curvature or other non-specific mechanisms, we used dihydro-C$_2$-ceramide (C$_2$-H$_2$Cer), an analogue of C$_2$-Cer that is inactive in ceramide signaling (Bielawska et al., 1993) but should exert similar mechanical effects on membranes. As depicted in Figure 4, C$_2$-H$_2$Cer failed to activate current above control ($n=6$, $P=0.94$). In order to
Figure 3: C₂-Cer (2 µM, 10 min) activated outwardly-rectifying Cl⁻ current in symmetrical Cl⁻. (A) Families of currents in symmetrical Cl⁻, and (B) I-V relationships. C₂-Cer-induced current reversed near 0 mV. (C) Current densities at +60 mV. C₂-Cer-induced current was 1.30 ± 0.35 pA/pF (n = 6, P < 0.01). Outward rectification in symmetrical Cl⁻ and block by DCPIB (Figure 1) indicate that C₂-Cer activated I_{Cl,swell} in ventricular myocytes.
Figure 4: C₂-dihydroceramide (C₂-H₂Cer), a metabolically inactive analogue of C₂-Cer, did not alter membrane current but C₂-Cer elicited $I_{\text{Cl,swell}}$ in the same cell. (A) Typical currents at +60 mV. (B) Exposure to inactive C₂-H₂Cer (2 μM, 10 min) failed to elicit Cl⁻ current (−4 ± 5%, n = 6, ns), whereas C₂-Cer (2 μM, 10 min) subsequently stimulated current in the same cell (n = 4, P < 0.01). Activation by C₂-Cer but not C₂-H₂Cer indicates that C₂-Cer elicited $I_{\text{Cl,swell}}$ via its normal signaling cascade rather than by a non-specific mechanism.
verify that $I_{\text{Cl,swell}}$ was present in cells that were unresponsive to $C_2$-$H_2$Cer, $C_2$-Cer was then added. In the same cells, $C_2$-Cer evoked $I_{\text{Cl,swell}}$ ($n = 4$, $P < 0.01$). Activation by $C_2$-Cer but not $C_2$-$H_2$Cer suggests that $I_{\text{Cl,swell}}$ was elicited via a ceramide signaling pathway rather than by a non-specific mechanism.

### 3.2 Endogenous ceramide generation is sufficient to activate $I_{\text{Cl,swell}}$

Bacterial SMase is a neutral, Mg$^{2+}$-dependent enzyme that acts specifically at the plasma membrane to convert sphingomyelin to long-chain ceramides that are native to the cell. Bacterial SMase (0.03 U/mL, 15–18 min), like exogenous $C_2$-Cer, evoked an outwardly rectifying $\text{Cl}^{-}$ current in >90% of the cells tested, and the current at +60 mV increased by $1.01 \pm 0.05$ pA/pF ($n = 75$, $P < 0.001$), from $1.22 \pm 0.07$ to $2.23 \pm 0.10$ pA/pF (Figure 5A,B). The SMase-induced current was reversible with 20 min of washout in control bath solution in each of the cells tested ($n = 3$, $P < 0.05$) (Figure 5C). No change in membrane capacitance was observed in individual cells treated with bacterial SMase (data not shown).

Two blockers of $I_{\text{Cl,swell}}$ inhibited bacterial SMase-induced current. DCPIB suppressed 78 ± 6 % (10 µM, $n = 7$, $P < 0.01$) of the current, and the remaining current was not significantly different than that in control ($P = 0.90$) (Figure 5B). Increasing the concentration of DCPIB to 30 µM did not reduce the SMase-induced current further (81 ± 6 %; $n = 4$, $P < 0.01$ vs control; $ns$ vs 10 µM DCPIB). Tamoxifen (10 µM, 5 – 8 min) also was effective in blocking the SMase-induced $\text{Cl}^{-}$ current (Figure 6); it decreased
Figure 5: Bacterial sphingomyelinase (SMase) reversibly activated $I_{\text{Cl,swell}}$. (A) I-V relationships for $\text{Cl}^-$ current elicited by SMase (0.03 U/mL, 15 – 18 min) and inhibition by DCPIB (10 µM). (B) SMase increased $\text{Cl}^-$ current by $1.1 \pm 0.1 \text{ pA/pF}$ at +60 mV ($n = 30$). DCPIB (10 or 30 µM) suppressed $78 \pm 6\%$ ($n = 7$) or $81 \pm 6\%$ ($n = 4$), respectively ($P < 0.01$ for both). (C) Effect of SMase reversed on washout (18 – 20 min, $n = 3$, $P < 0.05$). (D,E) Exposure to SMase (20 min) generated endogenous long-chain ceramides from sarcolemmal sphingomyelins. Most medium- and long-chain ceramides were significantly increased at the expense of the corresponding chain-lengths of sphingomyelin ($n = 6$; *, $P < 0.05$). Each lipid species was compared separately using a 3-way ANOVA based on two separate experimental data sets, each analyzed in triplicate.
current by 116 ± 16 % at +60 mV (n = 5, P < 0.01). Block of SMase-induced current by DCPIB and tamoxifen confirm its attribution to $I_{Cl,swell}$. As expected, bacterial SMase increased myocyte ceramides and decreased sphingomyelins (Figure 5 D,E). Tandem mass spectrometry demonstrated that SMase treatment significantly increased the concentrations of 8 of 11 ceramide chain-lengths examined, with C16-, C18:1-, C20:0-, and C22:0-ceramide demonstrating the most dramatic increases in total lipid normalized by cell protein. The corresponding sphingomyelins showed the largest fraction decreases.

The volume-sensitivity of $Cl^-$ current elicited by bacterial SMase was tested by exposure to hyperosmotic (1.5T) bathing solution in the continued presence of SMase (Figure 7). Cell shrinkage under these conditions for 15 min inhibited SMase-induced current by 43 ± 8% (n = 6, P < 0.02), from 1.88 ± 0.20 to 1.41 ± 0.14 pA/pF at +60 mV (Figure 7B). In contrast, we previously showed 1.5T fully suppresses $I_{Cl,swell}$ elicited by upstream signaling molecules (Clemo et al., 1999). SMase-induced current in 1.5T bath solution remained, however, significantly greater than control (n = 6, P < 0.02). Partial inhibition by hyperosmotic cell shrinkage indicates that activation of SMase-induced current had both volume-sensitive and volume-independent components.
Figure 6: Tamoxifen (Tam) inhibited SMase-induced $I_{\text{Cl,swell}}$. (A) Families of currents before and after treatment with SMase (0.03 U/mL, 15 – 18 min) and then after addition of Tam (10 µM). (B) I-V relationships. (C) Tam fully blocks SMase-induced $\mathrm{Cl}^-$ current (116 ± 16%, $n = 5$, $P < 0.01$). Tamoxifen selectively blocks $I_{\text{Cl,swell}}$ under conditions that isolate $\mathrm{Cl}^-$ currents, confirming identification of SMase-induced current by DCPIB.
Figure 7: Osmotic shrinkage partially inhibited SMase-induced $I_{\text{Cl,swell}}$. (A) I-V relationships before (1T Ctrl) and after (1T+SMase) exposure to SMase (0.03 U/mL, 18 min) in isosmotic bath solution, and then, after shrinking the same cell in hyperosmotic bath solution containing SMase (1.5T+SMase; 0.03 U/mL, 15 min). (B) Current densities at +60 mV before and after treatment with SMase in 1T and 1.5T bath solutions. Cell shrinkage in 1.5T partially inhibited the SMase-induced Cl$^-$ current ($43 \pm 8\%$, $n = 6$, $P < 0.02$). This suggested that SMase elicits $I_{\text{Cl,swell}}$ via volume-dependent and volume-independent pathways.
3.3. Differences in time course of activation due to exogenous and endogenous ceramide

Figure 8 compares the time course of activation of $I_{Cl,swell}$ by C$_2$-Cer and bacterial SMase. The time course of C$_2$-Cer-induced difference current was fit by a single exponential function with a time constant of 6.4 ± 1.6 min ($R^2 = 0.93$, $n = 11$), equivalent to a $t_{1/2}$ of 4.8 ± 1.2 min. In contrast, SMase-induced difference current was fit by a sigmoid function with a $t_{1/2}$ of 9.3 ± 0.6 min ($R^2 = 0.99$, $n = 10$). The magnitude of the current elicited at +60 mV by C$_2$-Cer, bacterial SMase, and osmotic swelling (i.e., test – control) also were compared. The C$_2$-Cer-induced current (0.70 ± 0.09 pA/pF; $n = 14$) was significantly different than that evoked by bacterial SMase (1.01 ± 0.05 pA/pF; $n = 75$, $P < 0.02$) or by hypoosmotic cell swelling in 0.7T bath solution (1.22 ± 0.17 pA/pF; data not shown; $n = 6$, $P < 0.05$), whereas the SMase- and swelling-induced currents were indistinguishable ($P = 0.244$).

3.4. ROS mediate bacterial SMase-induced activation of $I_{Cl,swell}$

Previously we demonstrated that H$_2$O$_2$ is a downstream mediator of $I_{Cl,swell}$ activation and exogenous H$_2$O$_2$ elicits $I_{Cl,swell}$ even under hyperosmotic conditions (Browe and Baumgarten, 2004; Ren et al., 2008). As shown in Figure 9, ebselen (20 µM, 5 min), a cell-permeable glutathione peroxidase mimetic that converts H$_2$O$_2$ to H$_2$O, inhibited the SMase-induced Cl$^-$ current by 124 ± 39% ($n = 5$, $P < 0.01$) from 2.46 ± 0.43 pA/pF to 1.56 ± 0.33 pA/pF at +60 mV. There was no difference in Cl$^-$ currents under control
Figure 8: Time course of activation of $I_{Cl,swell}$ by C$_2$-Cer and bacterial SMase. C$_2$-Cer data were fit by an exponential function with time constant of $6.4 \pm 1.6$ min ($R^2 = 0.98$, $n = 11$), equivalent to a $t_{1/2}$ of $4.8 \pm 1.2$ min. SMase data were fit by a sigmoid function with a $t_{1/2} = 9.3 \pm 0.6$ min ($R^2 = 0.99$, $n = 10$). Soluble C$_2$-Cer may reach the site of activation of $I_{Cl,swell}$ more quickly than long-chain endogenous ceramides that first must be produced by SMase. Alternatively, ceramides with different chain lengths may activate different sites in the signaling cascade.
Figure 9: The bacterial SMase-induced Cl\(^-\) current was completely inhibited by ebselen. (A) Families of currents before and after treatment with SMase (0.03 U/mL, 15 – 18 min) and then after addition of ebselen (20 \(\mu\)M, 5 min). (B) I-V relationships. (C) Ebselen, a glutathione peroxidase mimetic that scavenges H\(_2\)O\(_2\), fully blocks SMase-induced Cl\(^-\) current, reducing the current density from 2.46 ± 0.43 pA/pF to 1.56 ± 0.33 pA/pF at +60 mV. These data suggest the SMase-induced Cl\(^-\) current is mediated by H\(_2\)O\(_2\), a downstream mediator of \(I_{\text{Cl,swell}}\) (Browe and Baumgarten, 2004; Ren et al., 2008).
conditions (1.59 ± 0.55 pA/pF) and after addition of ebselen ($P < 0.87$). This demonstrates that the SMase-induced Cl\textsuperscript{−} current is mediated by ROS and is consistent with $I_{\text{Cl,swell}}$ activation by exogenous H\textsubscript{2}O\textsubscript{2}.

Once it was established that ROS, most likely H\textsubscript{2}O\textsubscript{2}, mediates SMase-induced $I_{\text{Cl,swell}}$ activation, the next step was to determine the source of ROS in this process. One of the primary sources of ROS generation in cardiomyocytes is the membrane-bound enzyme NOX. It was previously demonstrated that inhibition of NOX abrogates hypoosmotic swelling- and β1 integrin stretch-induced $I_{\text{Cl,swell}}$ (Browe and Baumgarten, 2004; Ren et al., 2008). As shown in Figure 10AB, the NOX inhibitor apocynin (500 µM, 10 min) failed to inhibit SMase-induced $I_{\text{Cl,swell}}$ activation ($n = 9$, $P = 0.22$ vs SMase). Additionally, administration of the NOX-specific, membrane-permeant fusion peptide gp91ds-tat (500 nM, 10 min) also failed to inhibit $I_{\text{Cl,swell}}$ that was activated by SMase ($n = 4$, $P = 0.85$ vs SMase) (Fig 10C). By contrast, diphenyliodonium (DPI, 60 µM, (20 – 25 min) fully inhibited SMase-induced current, from 2.01 ± 0.16 pA/pF to 1.24 ± 0.20 pA/pF at +60 mV ($n = 4$, $P = 0.71$ vs control). Previously, apocynin, gp91ds-tat, and DPI where shown to inhibit NOX-dependent $I_{\text{Cl,swell}}$ activation by osmotic swelling or β1 integrin stretch of ventricular myocytes within 5 min of administration. The failure of apocynin and gp91ds-tat to block SMase-induced current and the slow kinetics of block by DPI suggest that DPI may not be acting at NOX in this instance.
Figure 10: Specific NOX inhibitors did not block SMase-induced $I_{\text{Cl,swell}}$. (A) I-V relationships for $\text{Cl}^-$ current elicited by bacterial SMase (0.03 U/mL, 15-18 min) and after addition of apocynin (500 µM, 10 min) or DPI (60 µM, 20 – 25 min). (B) Apocynin (Apo) failed to inhibit SMase-induced $\text{Cl}^-$ current while DPI abrogated $\text{Cl}^-$ current at +60 mV. (C) Gp91ds-tat (500 nM, 10 min), the cell-permeant fusion peptide inhibitor of NOX, also failed to inhibit bacterial SMase-induced activation of $I_{\text{Cl,swell}}$. 
DPI is a flavoprotein inhibitor that suppresses NOX activity by dissociating an electron from NOX and forming a radical complex that covalently binds to and inhibits the NOX protein (O’Donnell et al., 1993). DPI also inhibits other flavinoid compounds, including mitochondrial ETC Complex I (Lambert et al., 2008). To test the idea that mitochondrial ROS production was responsible for SMase-induced $I_{Cl,swell}$, we used rotenone (10 µM, 20 min), which inhibits the transfer of electrons from iron-sulfur centers of Complex I to ubiquinone (Degli-Esposti M., 1998).

As seen in Figure 11, rotenone abrogated SMase-induced $I_{Cl,swell}$. Exposure to SMase increased current from $1.17 \pm 0.24 \text{ pA/pF}$ to $2.22 \pm 0.47 \text{ pA/pF}$, and addition of rotenone decreased the current to $1.06 \pm 0.11 \text{ pA/pF}$ ($n = 5$, $P = 0.74$ vs control). Taken together, suppression of SMase-induced $I_{Cl,swell}$ by rotenone and DPI is consistent with the idea that mitochondrial ROS generation is a required element of SMase-induced $I_{Cl,swell}$ activation.

Failure of apocynin and gp91ds-tat to inhibit $I_{Cl,swell}$ that already was activated by SMase argues that ongoing production of ROS by NOX is not required to maintain current activation. Alternatively, NOX might serve as trigger that is only transiently required to elicit mitochondrial ROS production. Ceramide and other sphingolipids species are reported to stimulate NOX activity in vascular smooth muscle (Keller et al., 2006; Won and Singh, 2006; Zhang et al., 2003), and there is evidence for crosstalk between NOX and mitochondria (Aon et al., 2003; Zorov et al., 2006).
Figure 11: Block of mitochondrial ETC Complex I by rotenone completely inhibits SMase-induced $I_{\text{Cl,swell}}$. (A) Families of currents before and after treatment with SMase (0.03 U/mL, 15 – 18 min) and then after addition of rotenone (10 µM, 20 min). (B) I-V relationships. (C) Rotenone, a specific inhibitor of Complex I of the mitochondrial ETC, fully blocks SMase-induced Cl$^-$ current, reducing the current density from 2.22 ± 0.47 pA/pF to 1.06 ± 0.11 pA/pF ($n = 5$, $P < 0.01$). These data suggest the mitochondrial ROS production is a required step in SMase-induced activation of $I_{\text{Cl,swell}}$. 
Figure 12: Pretreatment with gp91ds-tat reduced the magnitude of SMase-induced $I_{\text{Cl,swell}}$. (A) Families of currents for cells pretreated with gp91ds-tat (Ctrl) and following addition of bacterial SMase (0.03 U/mL, 18 min). (B) I-V relationships. (C) SMase elicited $I_{\text{Cl,swell}}$ in cardiomyocytes pretreated with gp91ds-tat (500 nM, 20 min). However, the magnitude of this activation was significantly smaller than that seen in non-pretreated cells ($0.60 \pm 0.2 \text{ pA/pF, } n = 7$ vs $1.01 \pm 0.05 \text{ pA/pF, } n = 75; P < 0.03$).
In order to examine whether or not NOX might serve as a trigger, cardiomyocytes were pretreated with gp91ds-tat (500 nM) for 20 min prior to addition of SMase (0.03 U/mL, 20 min) in the continued presence of gp91ds-tat. As seen in Figure 12, SMase still elicited a significant increase in current, from 1.27 ± 0.16 pA/pF to 1.87 ± 0.34 pA/pF at +60 mV (n = 7, P < 0.03). However, the magnitude of SMase-induced current after pretreatment with gp91ds-tat shown here was significantly lower than that in the absence of gp91ds-tat (0.60 ± 0.20 pA/pF, n = 7 vs 1.01 ± 0.05 pA/pF, n = 75; P < 0.03). This suggests that while SMase-induced mitochondrial ROS production may be sufficient to maintain activation of $I_{Cl,swell}$, there may be crosstalk between NOX and mitochondria in the initiation of the process.

3.5. **Downstream metabolites of ceramide are involved in $I_{Cl,swell}$ activation**

The addition of bacterial SMase converts sphingomyelins to ceramides and phosphocholine. However, ceramides may be further modified to downstream metabolites, namely sphingosine and S1P, which are known to be potent signaling molecules (see Figure 1). Our next step was to explore the possibility that such ceramide metabolites activate $I_{Cl,swell}$.

Ceramide is converted to sphingosine through the action of ceramidases. As shown in Figure 13, D-erythro-MAPP (10 μM, 15 – 18 min), a ceramide analogue that inhibits native ceramidase activity (Bielawska et al., 1996), fully blocked bacterial SMase-induced $Cl^-$ current. SMase increased current from 0.62 ± 0.19 pA/pF to 1.45 ±
Figure 13: D-erythro-MAPP (D-e-MAPP), a ceramidase inhibitor that blocks conversion of ceramide to sphingosine, completely inhibited SMase-induced Cl⁻ current. (A) Families of currents under control conditions (Ctrl), after treatment with bacterial SMase (0.03 U/mL, 18 min), and after addition of D-e-MAPP in the continued presence of SMase. (B) I–V relationships for A. (C) D-e-MAPP (10 µM, 15 – 18 min) abrogated bacterial SMase-induced Cl⁻ current at +60 mV. SMase increased current from 0.62 ± 0.19 pA/pF to 1.45 ± 0.42 pA/pF, and D-e-MAPP reduced it to 0.69 ± 0.25 pA/pF (n = 4, P < 0.01).
0.42 pA/pF and D-erythro-MAPP reduced it to 0.69 ± 0.25 pA/pF at +60 mV ($n = 4$, $P < 0.01$), a 92 ±7 % block of the SMase-induced current. There was no difference between control current density and that following treatment with D-erythro-MAPP ($P = 0.70$). This implies that sphingosine or its metabolites must be involved in the SMase-induced activation of $I_{\text{Cl,swell}}$.

Sphingosine can be phosphorylated by sphingosine kinases to form the bioactive lipid S1P. As seen in Figure 14, the sphingosine kinase inhibitor DL-threo-dihydrosphingosine (10 µM, 15 – 20 min) fully inhibited bacterial SMase-induced $I_{\text{Cl,swell}}$. Exposure to SMase increased the current from 1.12 ± 0.14 pA/pF to 2.46 ± 0.36 pA/pF at +60 mV, and DL-threo-dihydrosphingosine reduced it to 1.35 ± 0.17 pA/pF at +60 mV ($n = 6$, $P < 0.001$) in the continued presence of SMase, a 82 ± 5% block. There was no difference between control current density and that following treatment with DL-threo-dihydrosphingosine ($P = 0.31$). Taken together, these data implicate S1P as a modulator of $I_{\text{Cl,swell}}$.

In order to verify that S1P is indeed responsible for $I_{\text{Cl,swell}}$ activation in this system, our next step was to see if exogenous S1P would elicit $I_{\text{Cl,swell}}$. S1P (500 nM, 10 min) activated an outwardly-rectifying Cl$^{-}$ current with a reversal potential near $E_{\text{Cl}}$. Current at +60 mV increased from 0.92 ± 0.19 pA/pF to 2.11 ± 0.13 pA/pF ($n = 4$, $P < 0.02$). As seen in Figure 15, this current was fully inhibited by addition of DCPiB (10 µM, 10 - 12 min) in the continued presence of S1P ($n = 4$, $P = 0.67$ vs control). As found for the SMase-induced Cl$^{-}$ current, the activation of $I_{\text{Cl,swell}}$ by S1P was completely
**Figure 14:** DL-threo-dihydrosphingosine (DL-3-DHS), a sphingosine kinase inhibitor that blocks phosphorylation of sphingosine to S1P, completely inhibited SMase-induced CF current. (A) Families of currents under control conditions (Ctrl), treatment with bacterial SMase (0.03 U/mL, 18 min), and following addition of DL-3-DHS (10 µM, 15 – 20 min) in the continued presence of SMase. (B) I–V relationships for A. (C) Bacterial SMase increased current from 1.12 ± 0.14 pA/pF to 2.46 ± 0.36 pA/pF. DL-3-DHS decreased current to 1.35 ± 0.17 pA/pF (n = 6; P < 0.001 vs SMase, ns vs Ctrl).
Figure 15: Exogenous S1P elicited DCPIB-sensitive Cl\textsuperscript{−} current. (A) Families of currents under control conditions (Ctrl), after treatment with exogenous S1P (500 nM, 10 min), and following addition of DCPIB (10 µM, 10 min) in the continued presence of S1P. (B) I-V relationships for A. (C) Exogenous S1P induced an outwardly-rectifying Cl\textsuperscript{−} current that reversed near E\textsubscript{Cl} and was fully attenuated by DCPIB (n = 4, P <0.02); current at +60 mV under control conditions was 0.88 ± 0.10 pA/pF, after exposure to S1P was 1.84 ± 0.16 pA/pF, and after addition of DCPIB was 0.96 ± 0.14 pA/pF.
inhibited by rotenone (10 µM, 15 – 20 min). S1P increased current at +60 mV from 0.88 ± 0.1 pA/pF to 1.84 ± 0.16 pA/pF and rotenone reduced it to 0.96 ± 0.14 pA/pF (n = 7, P < 0.001) (**Figure 16B**). This amounts to a 94 ± 9% block of the S1P-induced current. Interestingly, in contrast to SMase-induced I_{Cl,swell}, addition of apocynin (500 µM, 10 min) in the continued presence of S1P partially reduced the S1P-induced Cl⁻ current by 56 ± 5%, from 1.64 ± 0.21 pA/pF to 1.23 ± 0.20 pA/pF at +60 mV (n = 4, P < 0.01) (**Figure 16A**).

### 3.6. MAPK and mitoK_{ATP} channels are involved in SMase-induced I_{Cl,swell} activation

Opening of mitoK_{ATP} channels is thought to modulate mitochondrial O₂⁻• production by reducing the inner membrane potential gradient and thereby increasing ETC flux to intermediates that leak electrons to O₂ (Hanley and Daut, 2005). 5-hydroxydeconate (5-HD; 500 µM, 18 – 20 min), a mitoK_{ATP} channel antagonist, inhibited SMase-induced I_{Cl,swell} by 59 ± 5% at +60 mV; current under control conditions was 0.77 ± 0.06 pA/pF, SMase increased current to 1.51 ± 0.18 pA/pF, and 5-HD reduced it to 1.07 ± 0.11 pA/pF (n = 4, P < 0.01) (**Figure 17**). Block of SMase-induced current by 5-HD was not complete, however (P < 0.02 vs Ctrl). These data implicate mitoK_{ATP} channel activation by SMase as an intermediate in the activation of I_{Cl,swell}.

MAPKs, specifically ERK 1/2, are involved in regulation of I_{Cl,swell} during heart failure (Clemo and Baumgarten, 1999) and sphingolipid-mediated signaling cascades
**Figure 16:** S1P-induced Cl$^-$ current is partially inhibited by apocynin and fully inhibited by rotenone. (A) I-V relationships for currents under control conditions (Ctrl), after treatment with S1P (500 nM, 10 min), and after addition of apocynin (+Apo; 500 µM, 10 min) in continued presence of S1P. (B) I-V relationships for block by rotenone (+Rot; 10 µM, 15-20 min); other conditions as in A. (C) Apocynin inhibited 56 ± 5% of S1P-induced $I_{Cl,swell}$ ($n = 4$, $P < 0.01$) and the remaining current was significantly greater than control ($P < 0.01$), whereas rotenone fully abolished the S1P-induced current ($n = 7$, $P < 0.001$).
Figure 17: Block of mitoK<sub>ATP</sub> channels partially inhibited SMase-induced I<sub>Cl,swell</sub>. (A) I-V relationships for currents under control conditions (Ctrl), after treatment with bacterial SMase (0.03 U/mL, 15 - 18 min), and after addition of the mitoK<sub>ATP</sub> blocker, 5-HD (500 µM, 18 - 20 min) in continued presence of SMase. (B) 5-HD inhibited SMase-induced current by 59 ± 5 % (n = 4, P < 0.01 vs SMase, P < 0.02 vs Ctrl).
Figure 18: ERK is involved in SMase-induced $I_{\text{Cl,swell}}$. (A) I-V relationships for currents under control conditions (Ctrl), after treatment with bacterial SMase (0.03 U/mL, 15 - 18 min), and after addition of the ERK1/2 blocker U0126 (5 µM, 15 min) in the continued presence of SMase. (B) U0126 inhibited SMase-induced current by 66 ± 5% ($n = 4$, $P < 0.01$ vs SMase, $P < 0.02$ vs Ctrl). (C) The ERK1 blocker PD98059 inhibited SMase-induced current by 65 ± 8% ($n = 5$, $P < 0.01$ vs SMase, $P < 0.02$ vs Ctrl).
In order to determine the role of ERK in the signaling pathway that regulates SMase-induced $I_{\text{Cl,swell}}$, we examined the effects of ERK inhibitors on this current. As illustrated in Figure 18AB, the ERK 1/2 blocker U0126 (5 µM, 15 min) reduced SMase-induced Cl$^-$ current by 66 ± 5%. SMase increased current at +60 mV from 0.82 ± 0.13 pA/pF to 1.64 ± 0.10 pA/pF, and U0126 in the continued presence of SMase reduced it to 1.13 ± 0.05 pA/pF ($n = 4$, $P < 0.01$). Nevertheless, current after block by U0126 remained significantly greater than under control conditions ($P < 0.02$).

The actions of the ERK 1-specific inhibitor PD98059 (10 µM, 15 min) were similar. PD98059 blocked 65 ± 8% of the SMase-induced current at +60 mV; SMase increased the current from 1.19 ± 0.27 pA/pF to 1.95 ± 0.39 pA/pF and it was reduced to 1.44 ± 0.30 pA/pF by PD08059 in the continued presence of SMase ($n = 5$, $P < 0.01$) (Figure 18C). Current after block by PD98059 remained significantly greater than that under control conditions ($P < 0.02$). In contrast to the partial block of $I_{\text{Cl,swell}}$ observed here with both U0126 and PD98059, the same concentrations of MAPK inhibitors previously were found to fully inhibit $I_{\text{Cl,swell}}$ elicited by EGF (Deng and Baumgarten, 2009). These data suggest that the activation of $I_{\text{Cl,swell}}$ by SMase involves both ERK-dependent and ERK-independent pathways.

### 3.7 SMase-induced ROS production in HL-1 Cardiomyocytes

The electrophysiological evidence presented so far suggested that ceramides activate $I_{\text{Cl,swell}}$ by eliciting ROS production, but the evidence for ROS production is
indirect. To more directly support this hypothesis, we measured ROS production with a fluorescent probe and flow cytometry. These studies were undertaken in the HL-1 cardiomyocytes cell line for technical reasons. HL-1 cells are an immortalized murine atrial cell line that has been used to study the behavior of ion channels (Fox et al., 2005; Fukuda et al., 2005), mitochondria (Vassilopoulos and Papazafiri, 2005; Ruiz-Meana et al., 2006; Ruiz-Meana et al., 2003), and other physiologic and pathophysiologic properties (Claycomb et al., 1998) of cardiac myocytes. Preparations of HL-1 cells are more uniform than freshly dissociated native cardiac myocytes, which unavoidably include damaged myocytes that may produce ROS. Before undertaking the ROS measurements, it also was necessary to demonstrate that ceramides activated $I_{\text{Cl,swell}}$ in HL-1 cells. Unpublished data from the Baumgarten group indicate that $I_{\text{Cl,swell}}$ is present in HL-1 cells at high current density and is regulated by several other signaling pathways previously characterized in native atrial and ventricular myocytes.

We used bacterial SMase to determine whether ceramides activate a $\text{Cl}^-$ current in HL-1 cells. As shown in Figure 19, bacterial SMase (0.03 U/mL, 15-18 min) elicited an outwardly-rectifying $\text{Cl}^-$ current with a reversal potential near $E_{\text{Cl}}$. At +60 mV, $\text{Cl}^-$ was $1.75 \pm 0.35$ pA/pF under control conditions and increased to $15.54 \pm 5.17$ pA/pF ($n = 7$, $P < 0.01$) after exposure to SMase. The SMase-induced current was fully inhibited by addition of DCPIB (10 $\mu$M, 10 min) in the continued presence of bacterial SMase ($n = 5$, $P < 0.01$), and the current remaining after block by DCPIB was not significantly different than the control current ($P = 0.58$ vs Ctrl). These data suggest that SMase evoked $I_{\text{Cl,swell}}$ in HL-1 cardiomyocytes.
The effect of bacterial SMase on ROS production in HL-1 cardiomyocytes was determined by flow cytometry using the fluorophore C-H$_2$DCFDA-AM, which primarily detects H$_2$O$_2$. *Figure 20* shows log fluorescence histograms from a single experiment. The geometric means of the histograms were normalized by background fluorescence, and summary data are depicted in *Figure 21*. Measurements of ROS production were fully consistent with expectations based on the idea that I$_{Cl,swell}$ is a ROS sensor. Bacterial SMase (10 µM, 40 min) significantly increased ROS production 4.4-fold (median) from the background levels. This primarily was due to mitochondrial ROS production rather than NADPH oxidase. Bacterial SMase-induced ROS production was suppressed to 1.52-fold (median) from background levels by pretreatment (30 min) with mitochondrial ETC blocker rotenone (10 µM) but was unaffected by pretreatment with the NADPH oxidase blocker gp91ds-tat (500 nM). In comparison, fluorescence elicited by H$_2$O$_2$ (100 mM, 15 min) was increased 5.2-fold (median) above background (*Figures 20B & 21*). Negligible responses were obtained from HL-1 myocytes in the absence of fluorophore (no drug) with or without addition of SMase (*Figure 20A*), indicating that neither the HL-1 myocytes nor the bacterial SMase itself contributed to the signal attributed to ROS.
Figure 19: Bacterial SMase induced a DCPIB-sensitive Cl⁻ current in HL-1 cardiomyocytes. (A) I-V relationships for currents under control conditions (Ctrl), after treatment with bacterial SMase (0.03 U/mL, 15 – 18 min), and after addition of DCPIB (10 µM, 10 min) in the continued presence of SMase. (B) SMase induced an outwardly-rectifying, DCPIB-sensitive Cl⁻ current that reversed near E_Cl (n = 7, P < 0.01). Currents observed after addition of DCPIB were not statistically different from control (P = 0.59).
Figure 20: Measurement of ROS with C-H$_2$DCFDA-AM by flow cytometry of HL-1 cells. Typical log florescence (F) histograms from a single typical experiment. (A) Negative controls in absence of fluorescent probe without (no drug) or with bacterial SMase (0.03 U/mL, 40 min). (B) In fluorophore-loaded myocytes, background fluorescence (Ctrl) and response to H$_2$O$_2$ (100 µM, 15 min) and bacterial SMase (0.03 U/mL, 40 min) reflect ROS production. (C) Pretreatment with rotenone (Rot+SMase; 10 µM, 40 min) suppressed ROS production to control levels. Pretreatment with gp91ds-tat failed to appreciably reduce SMase-induced ROS. Geometric means of F are shown in parenthesis.
Figure 21: SMase activated ROS production via mitochondrial ROS in HL-1 myocytes. Geometric means of histograms normalized by background fluorescence (F/F₀). Bacterial SMase (0.03 U/mL µM, 30 min; n = 5, for each) significantly increased ROS production, which was suppressed by pretreatment with Rot (10 µM, 40 min; n = 4) but not by pretreatment with gp91ds-tat (500 nM, 40 min; n = 4). H₂O₂ (100 µM, 15 min; n = 4) was positive control. Data were analyzed by Kruskal-Wallis One Way Analysis of Variance on Rank compared to control fluorescence by Dunn’s Method. Box plots show 25, 50 and 75 percentile; dotted line, background florescence.
This study demonstrated for the first time that both exogenous C$_2$-Cer, a membrane-permeant short chain ceramide, and endogenous, long-chain ceramides generated by bacterial SMase activated a Cl$^-$ current with the biophysical and pharmacological properties of I$_{\text{Cl,swell}}$ in rabbit ventricular myocytes. Furthermore, endogenous ceramides generated by bacterial SMase appear to act by eliciting the production of S1P, and exogenous S1P also elicited a Cl$^-$ current with features of I$_{\text{Cl,swell}}$. The slow time course of current activation by these sphingolipids, the involvement of ROS, and suppression of I$_{\text{Cl,swell}}$ by inhibitors of ceramide metabolism suggests the participation of a signaling cascade rather than direct interaction of all of these sphingolipids with the channel.

### 4.1. Characteristics of ceramide-induced Cl$^-$ current

The primary Cl$^-$ channels present in heart are I$_{\text{Cl,swell}}$, the cystic fibrosis transmembrane conductance regulator (CFTR) Cl$^-$ channel, and Ca$^{2+}$-activated Cl$^-$ channels (I$_{\text{Cl,Ca}}$) (Hume et al., 2000). C$_2$-Cer- and bacterial SMase-induced currents
reversed near $E_{Cl}$, exhibited outward rectification in physiological and symmetrical $Cl^{-}$ gradients, and were partially inhibited by hyperosmotic cell shrinkage. These features closely matched those of $I_{Cl,swell}$ described previously (Baumgarten et al., 2005; Hume et al., 2000; Sorota, 1994; Tseng, 1992). Additionally, the block of $C_{2}$-Cer- and SMase-induced $Cl^{-}$ current by selective blockers, DCPIB and tamoxifen, strongly implicated $I_{Cl,swell}$. $I_{Cl,ca}$ and $I_{CFTR}$ are insensitive to block by DCPIB (Decher et al., 2001), and tamoxifen selectively inhibits $I_{Cl,swell}$, and not the outwardly-rectifying $I_{CFTR}$, under conditions that isolate anion currents (Vandenberg et al., 1994). It is unclear why DCPIB gave apparently incomplete inhibition ($76 \pm 8\%$) whereas tamoxifen completely abrogated the current. In contrast, 10-µM DCPIB fully inhibits $I_{Cl,swell}$ elicited by hypoosmotic cell swelling in cardiomyocytes (Decher et al., 2001). Because the molecular identity of $I_{Cl,swell}$ remains elusive, the mechanisms of $I_{Cl,swell}$ inhibition by these two agents is not well-understood, and they may act at different sites. ROS have been shown to activate $I_{Cl,swell}$ (Varela et al., 2004; Shimizu et al., 2004; Ren et al., 2008; Haskew-Layton et al., 2005; Browe and Baumgarten, 2006; Browe and Baumgarten, 2004). Tamoxifen may suppress $I_{Cl,swell}$ by acting as a ROS scavenger and by inhibiting Complex I of the mitochondria electron transport chain (Moreira et al., 2006), preventing ROS from spilling over into the cytoplasm. No information is available on the mechanism of block of $I_{Cl,swell}$ by DCPIB, an ethacrynic acid derivative.

Effects of sphingolipids on sarcolemmal channel function have been explored only recently. Prolonged (>10 h) $C_{2}$-Cer and bacterial SMase exposure down regulates HERG $K^{+}$ channels through a pathway involving ROS production (Chapman et al., 2005;
Bai et al., 2007), and CFTR Cl$^-$ current is inhibited by both more rapidly (<60 min) (Ito et al., 2004). These effects appear to be PKA- and PKC-independent. d'Anglemont de Tassigny et al. (2004) found that $I_{\text{Cl,swell}}$ is a required participant of the apoptotic volume decrease in cardiomyocytes and hypothesized that $I_{\text{Cl,swell}}$ is activated in C2-Cer-induced apoptosis. Although outwardly rectifying Cl$^-$ currents were observed during doxorubicin-induced apoptosis, these authors – in contrast to the present study – did not establish a link between ceramide and $I_{\text{Cl,swell}}$ activation.

Modification of direct interactions between membrane lipids and channel proteins has been invoked to explain altered gating of Kv channels (Ramu et al., 2006; Milescu et al., 2007) and inhibition of CFTR (Ramu et al., 2007) after treatment with SMase D. SMase D is distinct from bacterial SMase (SMase C) and depletes membrane sphingomyelin without stimulating ceramide signaling; hydrolysis of sphingomyelin by SMase D is at the opposite side of the phosphate group, producing choline and ceramide-1-phosphate rather than the phosphocholine and ceramide generated by bacterial SMase. Such depletion of membrane lipids is not likely to explain the present results, however. C2-Cer and bacterial SMase both activated $I_{\text{Cl,swell}}$, whereas C2-Cer will favor, if anything, an increase in sphingolipids rather than their depletion. Nevertheless, a contribution of lipid-protein interactions to the response observed here cannot be rigorously excluded.

Swelling in 0.7T gives nearly full activation of $I_{\text{Cl,swell}}$ in ventricular myocytes (Clemo et al., 1999), and the magnitude of the current elicited by bacterial SMase and hypoosmotic swelling were not distinguishable. In contrast, C2-Cer evoked a significantly smaller current (~70% of SMase- and 60% of 0.7T-induced currents) that
activated more rapidly, and increasing C2-Cer from 2 to 20 μM did not elicit additional current. These differences may reflect, in part, that C2-Cer must permeate the sarcolemma to reach its target(s) and that SMase first must hydrolyze sarcolemmal sphingomyelin to native long-chain ceramides, which also must reach their downstream target(s). The Hill coefficient of the dose-response relationship for C2-Cer-induced \( I_{Cl,swell} \) is greater than one, suggesting cooperativity and that the effect of C2-Cer is likely to be due to induction and amplification of a signaling cascade rather than direct one-to-one ligand binding. It also is possible that synthetic short-chain and native long-chain ceramides work via distinct pathways or differ in their efficacy to stimulate processes causing \( I_{Cl,swell} \) activation. Our observation that hyperosmotic cell shrinkage in 1.5T only partially inhibits SMase-induced current may suggest that endogenous long-chain ceramides act at multiple sites and that one is downstream from the site controlled by hyperosmotic shrinkage. Insensitivity of \( I_{Cl,swell} \) to osmotic shrinkage when the current is elicited by a downstream effector is not unique. We previously showed that \( H_2O_2 \)-induced \( I_{Cl,swell} \) is insensitive to hyperosmotic shrinkage in 1.5T bathing solution (Ren et al., 2008).

The lack of an effect of metabolically inactive C2-H2Cer lends support to the hypothesis that both C2-Cer and endogenous ceramides generated by bacterial SMase act via one or more ceramide signaling cascades rather than through a non-specific mechanism (Bielawska et al., 1993). Furthermore, the slow time course of \( I_{Cl,swell} \) activation by C2-Cer and SMase is consistent with a process that involves signaling rather
than direct activation due to interaction with a binding site on the outer face of the channel.

4.2 **ROS are Required for Bacterial SMase-induced Activation of $I_{\text{Cl,swell}}$**

In cardiomyocytes, ROS produced by NADPH oxidase (Browe and Baumgarten, 2004; Browe and Baumgarten, 2006; Ren et al., 2008) and mitochondria (Browe and Baumgarten, 2005) are essential downstream effectors of $I_{\text{Cl,swell}}$ activation by osmotic swelling, integrin stretch, and growth factors, and ROS elicits $I_{\text{Cl,swell}}$ in other tissues (Varela et al., 2004; Shimizu et al., 2004). Ceramides also produce ROS. For example, apoptosis triggered by ceramide is accompanied by mitochondrial ROS production (Feuerstein and Young, 2000; Hannun, 1996; Levade et al., 2001). Regulation of ROS production by mitochondria is complex, however. Mitochondria can be both the source and target of ROS, and ROS can have either pro- or anti-apoptotic effects. Additionally, ceramide is involved in NADPH oxidase activation in rat mesangial and bovine coronary artery smooth muscle cells (Yi et al., 2004; Zhang et al., 2003).

$H_2O_2$ is a membrane permeant, relatively long-lived ROS that interacts with several signaling molecules and ion channels (Lambeth, 2004). Exogenous $H_2O_2$ elicits $I_{\text{Cl,swell}}$ in rabbit ventricular myocytes (Browe and Baumgarten, 2004; Ren et al., 2008), HeLa cells (Varela et al., 2004), and rat hepatoma cells (Shimizu et al., 2004). Dismutation of $H_2O_2$ with catalase inhibits activation of $I_{\text{Cl,swell}}$ induced by intergrin stretch and osmotic swelling (Browe and Baumgarten, 2004; Ren et al., 2008). Consistent with these observations, bacterial SMase-induced $I_{\text{Cl,swell}}$ activation was completely inhibited by the glutathione peroxidase mimetic ebselen. Combined with
previous evidence that $I_{Cl,swell}$ is activated by exogenous $H_2O_2$, this suggests that SMase-induced $I_{Cl,swell}$ is mediated by $H_2O_2$ and its signaling intermediates. However, as ebselen also reacts with peroxynitrite and phospholipid and cholesterol ester hydroperoxidases (Schewe, 1995), we cannot exclude that other ROS may be involved.

The mechanism of $H_2O_2$-mediated regulation of $I_{Cl,swell}$ remains unknown. Evidence suggests that that $H_2O_2$ must act a site distal to the volume-sensing mechanism as $H_2O_2$-induced $I_{Cl,swell}$ activation is not reversed by hyperosmotic cell shrinkage (Ren et al., 2008). It is possible that direct modification of the channel by ROS modulates the properties of $I_{Cl,swell}$. Although the molecular identity of $I_{Cl,swell}$ is unknown, $H_2O_2$ has been shown to directly modify other cardiac ion channels as well as the redox-sensitive signaling cascades that regulate them (Zima and Blatter, 2006). Such redox-sensitive signaling cascades have recently been implicated in modulation of $I_{Cl,swell}$ in rat hepatoma cells through the activation of PLCγ1 and subsequent $Ca^{2+}$ mobilization (Varela et al., 2007). A $Ca^{2+}$-dependent mechanism for $I_{Cl,swell}$ activation by $H_2O_2$ is difficult to reconcile with our observation in cardiac myocytes that $I_{Cl,swell}$ is observed in $Ca^{2+}$-free bath containing EGTA. In fact, Varela et al. (2007) demonstrated that while $H_2O_2$-induced current was suppressed with IP$_3$ receptor and generic PLC inhibition, reduction of free $Ca^{2+}$ did not alter the magnitude of $I_{Cl,swell}$ in their system. Other studies support the idea that an increase of $Ca^{2+}$ is not required to activate $I_{Cl,swell}$. For example, release of $Ca^{2+}$ by store-operated calcium channels actually inhibits $I_{Cl,swell}$ (Zholos et al., 2005; Lemonnier et al., 2002).

4.3 **Bacterial SMase Activates $I_{Cl,swell}$ via ROS from Mitochondria but not NOX**
As mentioned previously, ROS produced by both NOX and mitochondria are essential mediators of $I_{\text{Cl,swell}}$ activation in cardiomyocytes by several stimuli (Browe and Baumgarten, 2004; Browe and Baumgarten, 2006; Browe and Baumgarten, 2007; Deng et al., 2009; Ren et al., 2008). NOX transfers electrons from intracellular NADPH to form extracellular $O_2^{-\bullet}$, which in turn is rapidly dismutated to $H_2O_2$ spontaneously or via extracellular superoxide dismutase (Bedard and Krause, 2007). Previous studies from our laboratory showed that blockade of NOX inhibits $I_{\text{Cl,swell}}$ activation induced by Ang II, ET-1, EGF, integrin stretch, and osmotic swelling. Sphingolipids have also been implicated as mediators of NOX. Ceramide increases the levels of GTP-bound Rac, thus stimulating translocation of the cytosolic components of NOX to the plasma membrane in mesangial cells (Yi et al., 2004). Additionally, CD95L-induced $p47^{\text{phox}}$ phosphorylation in hepatocytes is sensitive to blockade of SMase (Reinehr et al., 2005) and ceramide induced NOX activation in small coronary arteries is associated with translocation of $p47^{\text{phox}}$ to the plasma membrane (Zhang et al., 2003). Exogenous C$_2$-Cer also increases NOX activity in bovine coronary arteries (Zhang et al., 2003).

However, in the present study we found that bacterial SMase-induced $I_{\text{Cl,swell}}$ activation was insensitive to block of NOX with either apocynin or gp91ds-tat. Interestingly, pretreatment of ventricular cardiomyocytes with gp91ds-tat modestly decreased the magnitude of SMase-induced activation of $I_{\text{Cl,swell}}$ (Figure 12). This may indicate that SMase induces ROS production via NOX acts as a trigger, but that this source of ROS is not required to maintain $I_{\text{Cl,swell}}$ in its activated state. Apocynin prevents the migration of $p47^{\text{phox}}$ to the plasma membrane, whereas the fusion peptide gp91ds-tat
blocks the p47<sub>phox</sub> docking site on the cytoplasmic complex of NOX (Bedard and Krause, 2007). Both are intended to inhibit NOX2, as this is the primary isotype of NOX found in the heart; they should not inhibit NOX4 because p47<sub>phox</sub> is not a component of NOX4, an isoform that is constitutively active rather than being regulated by assembly of a cytoplasmic complex (Byrne et al., 2003a). Nevertheless, we cannot rigorously exclude the possibility that bacterial SMase is leading to ROS production through the activation of the low concentration NOX4 that is present.

Preliminary data indicate that mitochondrial ROS production is involved in the regulation of I<sub>Cl,swell</sub> by ACh and HIV protease inhibitors in cardiomyocytes (Browe and Baumgarten, 2007; Deng et al., 2009). Ceramide also induces mitochondrial ROS production in cardiomyocytes and other cell types (Di Paola M. et al., 2000; Garcia-Ruiz et al., 1997b; Gudz et al., 1997). Mitochondrial ETC Complexes I and III are the primary redox centers responsible for electron leak and O<sub>2</sub>−• generation in mitochondria (Chen et al., 2008; Vanden Hoek et al., 1998). O<sub>2</sub>−• generated by Complex I is released into the mitochondrial matrix and degraded by the matrix anti-oxidant system, which includes Mn<sup>2+</sup>-dependent SOD, catalase, and glutathione peroxidase (Chen et al., 2008; Chen et al., 2007). By contrast, Complex III releases O<sub>2</sub>−• on both sides of the inner mitochondrial membrane and plays a central role in cytoplasmic ROS generation by mitochondria (Muller et al., 2004). The role of mitochondrial ROS in the activation of I<sub>Cl,swell</sub> by bacterial SMase was supported by the observation that selective inhibition of Complex I with rotenone, abrogated I<sub>Cl,swell</sub>. Block of Complex I at a distal binding site
by rotenone will abolish electron transport to and $O_2^{-*}$ production by Complex III, whereas, if anything, it will favor $O_2^{-*}$ production by Complex I.

Although inhibitors of ROS production by mitochondria and NADPH oxidase and $H_2O_2$ scavengers, such as ebselen, are widely used tools with well-understood actions, the electrophysiological evidence supporting the role of ROS in $I_{Cl,swell}$ activation is inherently indirect because ROS are not measured. In contrast, flow cytometry of HL-1 cardiac myocytes that were loaded with C-$H_2$DCFDA-AM, a fluorophore that detects $H_2O_2$, provided direct support for this idea. Pretreatment with rotenone largely prevented SMase-induced ROS production in HL-1 cardiomyocytes, whereas pretreatment with gp91ds-tat had no effect on SMase-induced ROS production in HL-1 cells (Figures 20-21). These fluorescence data support the conclusion drawn from electrophysiological data showing activation of $I_{Cl,swell}$ was reversed on suppressing mitochondrial ROS production but was not reversed on suppressing ROS produced by NADPH oxidase (Figures 10-11).

At first glance, however, one aspect of the data appears inconsistent: pretreatment of native ventricular myocytes with gp91ds-tat slightly reduced the magnitude of SMase-induced $I_{Cl,swell}$ activation (Figure 12). As noted above, this raised the possibility that NOX ROS production can act as a trigger or modulator that speeds mitochondrial ROS production and the activation of $I_{Cl,swell}$. The flow cytometry data also suggested mitochondrial production was not the only source of ROS stimulated SMase. Although rotenone significantly suppressed ROS production, it remained at a level significantly higher than control (Figures 20-21). It also must be recognized that a 1:1 correspondence
between flow cytometry and electrophysiological estimates of ROS production is not expected. Variations in the relationship may arise from kinetics, localization, and sensitivity to ROS production. Because scavenging ROS suppresses $I_{Cl,swell}$ after its activation, $I_{Cl,swell}$ must reflect ROS production throughout its period of activation, although the amount of ROS needed to fully activate current is unknown and the relationship is likely to be non-linear and perhaps time dependent. Moreover, because ROS are highly reactive, the ROS that activates $I_{Cl,swell}$ must be spatially localized. In contrast, ROS is detected in flow cytometry studies by converting a non-fluorescent molecule to one that is fluorescent. Fluorophores integrate ROS production over time, and therefore, a pretreatment paradigm must be used to study interventions that suppress ROS production. Flow cytometry will detect ROS from all cellular sources, even those distant from $I_{Cl,swell}$ such as interfibrillar mitochondria. Finally, the maximum fluorescence depends upon the amount of non-fluorescent probe trapped in the cell. The observation that SMase increased fluorescence to a level indistinguishable from that observed with 100-µM $H_2O_2$ might imply that all of the probe was converted to its fluorescent form by both interventions. In such a situation, SMase-induced fluorescence might not be reduced even if ROS production was partially inhibited by gp91ds-tat.
4.4 Ceramide Metabolites are Required for SMase-induced $I_{Cl,swell}$ Activation

An important aspect of sphingolipid signaling is the ceramide/S1P rheostat (Hannun and Obeid, 2008). Ceramide and its metabolite, S1P, are potent bioactive lipids that act as second messengers, often with opposing effects on signaling and ultimately the fate of the cell. Ceramide tends to activate pathways leading to apoptosis, senescence, and differentiation. By contrast, S1P is involved in proliferation, mitogenesis, migration, and tends to inhibit apoptosis (Hannun, 1994; Hannun and Obeid, 2008). This raises the possibility that the relative ratio of these sphingolipids, rather than their concentrations, may be critical for controlling $I_{Cl,swell}$. Ceramide and S1P are involved in modulation of cytosolic ROS generation due to a number of stimuli, including TNFα, ischemia/reperfusion, and changes in transmural pressure (Di Paola M. et al., 2000; Garcia-Ruiz et al., 1997b; Ide et al., 2001; Keller et al., 2006) and may also modulate $I_{Cl,swell}$, at least in part, by this pathway.

Despite the fact that ceramide and S1P often have opposing actions, S1P appears to have paradoxical roles in some systems. S1P has both anti- and pro-atherogenic properties (Chatterjee, 1998; Chatterjee et al., 2006; Murata et al., 2000; Xu et al., 2004). Additionally, both ceramide and S1P both increase ROS production in cardiomyocytes, vascular smooth muscle cells, and other tissues (Lecour et al., 2006b; Suematsu et al., 2003; Takuwa et al., 2009; Zhang et al., 2003). Exogenous S1P stimulates $H_2O_2$-production in thyroid cells, although the source of this ROS was not investigated (Okajima et al., 1997). Increased transmural pressure induces SPHK1 activity and
subsequent ROS generation in vascular smooth muscle cells, at least in part due to activation of NOX (Keller et al., 2006).

Consistent with a role for ROS, we observed that exogenous S1P activates $I_{\text{Cl,swell}}$ in ventricular cardiomyocytes. S1P-induced activation of $I_{\text{Cl,swell}}$ was fully blocked by rotenone and partially suppressed by apocynin, implicating both mitochondria and NOX, as discussed above for SMase. Moreover, SMase-induced activation of $I_{\text{Cl,swell}}$ is also dependent on endogenous generation of S1P. Both inhibition of ceramidase with D-\textit{erythro}-MAPP and SPHK with DL-\textit{threo}-dihydrosphingosine abrogated SMase-induced $I_{\text{Cl,swell}}$. D-\textit{erythro}-MAPP prevents the hydrolysis of the acyl chains of ceramide leading to an increase in ceramide and depletion of sphingosine and S1P. Therefore, block of SMase-induced $I_{\text{Cl,swell}}$ by D-\textit{erythro}-MAPP indicates that a SMase-induced increase in the concentration of native ceramides cannot be the primary effector of $I_{\text{Cl,swell}}$. Moreover, inhibition of SPHK specifically depletes cells of S1P. Together these data strongly argue that S1P rather than native ceramide is the essential signaling element in SMase-induced activation of $I_{\text{Cl,swell}}$.

As mentioned previously, there are two primary isotypes of SPHK with different kinetic properties, cellular locations, and temporal expression (Spiegel and Milstien, 2002). Recent evidence suggests that SPHK1 and SPHK2 have antagonizing effects in the regulation of ceramide biosynthesis and mitochondrial Ca$^{2+}$ (Maceyka et al., 2005). Whereas DL-\textit{threo}-dihydrosphingosine is a potent inhibitor of overall SPHK activity (Buehrer and Bell, 1992), the isotype specificity of this blocker is unknown. Recently, SPHK isotype-specific inhibitors have been identified (Takabe et al., 2008); these agents
may represent important pharmacological tools for determining the role of differential activation of SPHK isoforms in ROS generation and $I_{\text{Cl,swell}}$ activation. It remains unclear whether exogenous S1P-induced activation of $I_{\text{Cl,swell}}$ depends on intracellular S1P binding sites or involves G protein-coupled receptors that recognize S1P as a ligand (Maceyka et al., 2005; Means and Brown, 2009). Bacterial SMase-induced S1P production likely makes use of endogenous sphingolipid trafficking mechanisms, whereas exogenous S1P may act at additional sites. This may explain the discrepancy that SMase-induced $I_{\text{Cl,swell}}$ is insensitive to NOX inhibition while exogenous S1P-induced $I_{\text{Cl,swell}}$ is partially inhibited by NOX block.

There are five members of this family, termed S1P1-5. These subtypes are differentially expressed in various tissues and may be differentially expressed in distinct cellular compartments. S1P1 is the primary subtype expressed in cardiomyocytes, but S1P3 and S1P2 are also present at lower levels (Means and Brown, 2009). S1P1 receptor activation stimulates ERK, eNOS, Rac, PLC, and PI-3K signaling pathways and their affinity for S1P is augmented by $H_2O_2$ (Takabe et al., 2008; Igarashi et al., 2007). Exogenous $H_2O_2$ also increases S1P1 receptor expression (Igarashi et al., 2007). This may identify a potential node for crosstalk between S1P receptors and ROS in cardiovascular responses. Moreover, S1P receptors may serve as a potential feedback mechanism for regulating intracellular ROS. Although the current study does not address the role of specific S1P receptors in activation of $I_{\text{Cl,swell}}$, it is a fertile area for future investigation.
ERK and mitoKATP Channels are Involved in SMase-induced $I_{\text{Cl,swell}}$

ERK signaling is important in cardiac hypertrophy and cardioprotection (Muslin, 2008; Heineke and Molkentin, 2006) and is activated by mechanical stretch (Caldiz et al., 2007), Ang II (Ruf et al., 2002; Fischer et al., 1998), EGF (Kodama et al., 2002), and $H_2O_2$ (Duquesnes et al., 2009; Fukuzawa et al., 2002). In the context of sphingolipid signaling, ceramide primarily activates the JNK cascade of MAPKs, whereas S1P activates the ERK pathway (Spiegel and Milstien, 2002; Hannun and Obeid, 2008). $I_{\text{Cl,swell}}$ elicited by EGF and ET-2 is fully inhibited by ERK blockade (Du and Sorota, 2000). By contrast, both the ERK1/2 blocker PD98059 and the ERK 1 blocker U0126 only partially inhibit bacterial SMase-induced $I_{\text{Cl,swell}}$. This indicates that SMase activates $I_{\text{Cl,swell}}$ via a complex mechanism with both ERK-dependent and ERK-independent components. The partial dependence of bacterial SMase-induced $I_{\text{Cl,swell}}$ on ERK activation is consistent with the observation that S1P is a required intermediate in this pathway. The regulation of $I_{\text{Cl,swell}}$ by ERK is complicated and, as the molecular identity of the channel responsible for $I_{\text{Cl,swell}}$ is unknown, it is unclear if ERK acts via direct phosphorylation of channel residues or by activating other signaling molecules involved in regulating $I_{\text{Cl,swell}}$.

The regulation of $I_{\text{Cl,swell}}$ has recently been tied to the activation of mitoKATP channels. Mitochondrial ROS production is stimulated by opening of mitoKATP channels (Andrukhiv et al., 2006). Additionally, glibenclamide, a blocker of mitochondrial and sarcolemmal $K_{\text{ATP}}$ channels, inhibits cardiac $I_{\text{Cl,swell}}$ (Yamazaki and Hume, 1997; Sakaguchi et al., 1997) and blockade of mitoKATP channels with 5-HD abrogates $I_{\text{Cl,swell}}$. 
elicited by ACh (Browe and Baumgarten, 2007). Sphingolipids have also been implicated in the regulation of $K_{\text{ATP}}$ channels. The glycosphingolipid C16:0 sulfatide reduces the sensitivity of mitochondrial $K_{\text{ATP}}$ channels to ATP inhibition, leading to increased channel activity in rat pancreatic $\beta$ cells (Buschard et al., 2006). In the present study, bacterial SMase-induced $I_{\text{Cl,swell}}$ was partially inhibited by 5-HD. Although this is generally accepted as strong evidence of involvement of mito$K_{\text{ATP}}$ channels, there are certain alternatives that must be considered (Ardehali and O'Rourke, 2005; Hanley and Daut, 2005). 5-HD is a substituted medium-chain fatty acid that is metabolized by acyl-CoA synthase. 5-HD-CoA or one of its metabolites may interfere with $\beta$-oxidation of fatty acids and disturb mitochondrial metabolism (Ardehali and O'Rourke, 2005; Hanley and Daut, 2005). $I_{\text{Cl,swell}}$ was elicited in pipette solution which contained ATP and Cs$^+$ replacing $K^+$ and lacked substrate. While Cs$^+$ is a known sarcolemmal $K_{\text{ATP}}$ channel blocker, it does not block mito$K_{\text{ATP}}$ channels (Mironova et al., 1997). In view of these issues, we cannot rigorously exclude the possibility that inhibition of SMase-induced $I_{\text{Cl,swell}}$ by 5-HD is independent of its ability to block mito$K_{\text{ATP}}$ channels.

4.6 Proposed Mechanism for Sphingolipid-induced $I_{\text{Cl,swell}}$ Activation

A proposed model that accounts for both our present and previous studies is shown in Figure 22. Pretreatment with gp91ds-tat reduced maximum current elicited with SMase while apocynin and gp91ds-tat failed to inhibit current following SMase-induced activation of $I_{\text{Cl,swell}}$. The superoxide produced by NOX rapidly undergoes dimutation to $H_2O_2$, which is a distal mediator of $I_{\text{Cl,swell}}$ activation in response to a
number of stimuli (Browe and Baumgarten, 2004; Browe and Baumgarten, 2006; Ren et al., 2008). Consistent with previous observations, SMase-induced $I_{\text{Cl,swell}}$ was abolished by membrane-permeant glutathione peroxidase mimetic ebselen. Low levels of ROS produced by NOX might induce additional ROS production by mitochondria, a process of signal amplification. In agreement with this scheme, $I_{\text{Cl,swell}}$ evoked by bacterial SMase and exogenous S1P was abrogated by selective blockade of mitochondrial ROS production. Moreover, ERK inhibitors U0126 and PD98059 partially inhibit SMase-induced $I_{\text{Cl,swell}}$, as does hyperosmotic shrinkage. This indicates that sphingolipid activation is occurring through at least two parallel pathways.

In Figure 22, we outline the proposed mechanism of sphingolipid activation of $I_{\text{Cl,swell}}$. Exogenous bacterial SMase converts sphingomyelin in the plasma membrane to ceramide. Ceramide is then hydrolyzed by ceramidase to form sphingosine which in turn is phosphorylated by sphingosine kinase to form S1P. S1P is downstream of ceramide in the activation of $I_{\text{Cl,swell}}$ as block of ceramidase with D-erythro-MAPP and sphingosine kinase with DL-threo-dihydrosphingosine completely inhibits SMase-induced current. S1P leads to the production of ROS, a downstream mediator of $I_{\text{Cl,swell}}$, evidenced by the complete block of SMase-induced $I_{\text{Cl,swell}}$ with the glutathione peroxidase mimetic ebselen.

Exogenous S1P acts through at least two parallel pathways that converge at the level of the mitochondria to produce ROS and activate $I_{\text{Cl,swell}}$. SMase-induced $I_{\text{Cl,swell}}$ is
Figure 22: Proposed mechanism of sphingolipid induced activation of $I_{\text{Cl,swell}}$.
Bacterial SMase activates $I_{\text{Cl,swell}}$ through S1P-dependent ROS generation. Both endogenous and exogenous S1P stimulate mitochondrial ROS production through an unknown mechanism. Exogenous S1P stimulates both NOX and mitochondria; either directly or through interaction with G protein coupled S1P receptors. Exogenous C$_2$-Cer, but not C$_2$-H$_2$Cer, also interacts with both NOX and mitochondrial ETC at the level of Complex III. Scavenging of ROS abrogates SMase-induced $I_{\text{Cl,swell}}$, indicating ROS are a downstream effector of $I_{\text{Cl,swell}}$ in this system. Solid arrows indicate stimulatory pathways. Dotted lines indicate potential pathways. Red indicates inhibition and X indicates no action.
completely blocked by the Complex I inhibitor rotenone, as is S1P-induced $I_{Cl,swell}$. Exogenous S1P is also partially inhibited by apocynin, indicating at least part of the S1P-induced ROS production is due to stimulation of NOX. It is unclear, however, if this is a direct effect of S1P or is mediated through S1P receptors in the sarcolemmal membrane.

Exogenous C$_2$-Cer, but not the inactive analogue C$_2$-H$_2$Cer, also activates $I_{Cl,swell}$. Previous studies implicate NOX (Zhang et al., 2003) and mitochondrial Complex III (Gudz et al., 1997) as sources of C$_2$-Cer-induced ROS production. Although the source of ROS was not verified, it is likely that C$_2$-Cer induces ROS generation via both mechanisms in a similar manner to that seen with exogenous S1P.

4.7 Implications

$I_{Cl,swell}$ is persistently activated in models of dilated cardiomyopathy (Baumgarten et al., 2005) and is involved in the apoptotic volume decrease (AVD) (Rasola et al., 1999; Okada and Maeno, 2001) that precedes apoptotic cell death in normal development, ischemia, or heart failure. The sphingomyelin/ceramide pathway is activated in vivo during ischemia/reperfusion (Bielawska et al., 1997; Chatterjee et al., 2006; Gulbins and Li, 2006) and heart failure (Gulbins and Li, 2006; Doehner et al., 2007; Chatterjee et al., 2006), and the oxidation of sphingolipids is implicated in atherosclerotic plaque formation (Auge et al., 2000). The data presented here shows a link between intracardiac ceramide accumulation and $I_{Cl,swell}$ activation that may be important for understanding these cardiovascular disease states. Because $I_{Cl,swell}$ outwardly rectifies, its activation promotes shortening of action potential duration (APD) and tends to depolarize resting
membrane potential, $E_m$. This may be beneficial in heart failure, where the APD is prolonged due to downregulation of repolarizing $K^+$ currents. On the other hand, decreasing APD reduces the minimum length of the conduction pathway needed to sustain re-entrant tachyarrhythmias and could be a contributor to the development of fibrillation.

4.8. Future Directions

There are a number of directions that warrant further study to provide a greater understanding of sphingolipid regulation of $I_{\text{Cl,swell}}$ through ROS production. Ang II and the acute activation of AT$_1$ receptors are involved in activation of $I_{\text{Cl,swell}}$ induced by integrin stretch and osmotic swelling (Browe and Baumgarten, 2004). Ang II has also been implicated in modulation of ceramide production in vascular smooth muscle cells primarily through the action of AT$_2$ receptors (Berry et al., 2001). In this study ceramide accumulation was seen after long periods (1 – 10 h) of exposure to Ang II (Berry et al., 2001). In contrast, we have preliminary evidence that short (10 min) exposure to 5 nM Ang II alters the sphingolipid profile of ventricular cardiomyocytes (Figure 23). At this concentration and treatment duration, Ang II activates $I_{\text{Cl,swell}}$ (Ren et al., 2008). Because of the central role of Ang II in cardiovascular disease, the potential role of sphingolipid signaling in Ang II-induced $I_{\text{Cl,swell}}$ activation is worth investigating.

Sphingolipids are important mediators of apoptosis and have been implicated in initiation of mitochondrial permeability transition pore (mitoPTP) formation, a vital step in the progression of apoptosis (Hannun and Obeid, 2008; Hannun, 1996). Although the
**Figure 23:** Preliminary tandem mass spectrometry data indicate Ang II induced alterations in sphingolipid metabolism. (A) Most medium- and long-chain sphingomyelins were significantly increased in response to 10 min exposure to Ang II (10 nM) ($n=3$; *, $P < 0.05$). (B) No significant change was detected in ceramide levels. Signal strength was not sufficient to detect S1P levels in these experiments. Each lipid species was compared separately using a 3-way ANOVA based on single experimental data set, analyzed in triplicate.
mechanism is not fully understood, ceramide and sphingosine form large conductance channels in the mitochondrial outer membrane that have been postulated to contribute to mitoPTP opening (Siskind et al., 2002; Siskind et al., 2006). $I_{\text{Cl,swell}}$ is also implicated in apoptosis and is necessary for the AVD (Okada et al., 2009; d'Anglemont de Tassigny et al., 2004). As mitoPTP opening would increase ROS permeability of the mitochondrial membranes by forming a ROS-permeant pathway bridging the inner and outer mitochondrial membranes, it may represent a potential area for crosstalk between sphingolipids and $I_{\text{Cl,swell}}$. Use of mitoPMP blockers such as bongkrekic acid or cyclosporin A would help to identify this as a mechanism for modulation SMase-induced $I_{\text{Cl,swell}}$ activation. Additionally, SMase-D could be used to deplete the membrane of sphingomyelin and exogenous ceramide or sphingosine could be added in the perfusate. If mitoPMP induction and ROS release is due to ceramide or sphingosine channel formation, a bongkrekic acid-sensitive activation of $I_{\text{Cl,swell}}$ would be observed under these conditions.

Lipid-protein interactions are significant determinants of protein function. There are two principle methods for lipids and proteins to interact: through direct, species-specific interactions and through indirect, membrane property alterations (Andersen and Koeppe, 2007). As sphingolipids play an important role in the structural properties of membranes, the potential for SMase-induced $I_{\text{Cl,swell}}$ to be mediated through membrane alterations exists. Sphingolipids, along with cholesterol, are the primary components of the localized membrane domains known as lipid rafts. They are also found in high concentrations in the U-shaped membrane invaginations called caveolae (Dobrowsky,
Lipid rafts and caveolae have been implicated as key participants in signaling and ion channel regulation. Detergents such as Triton X-100 and methyl-beta cyclodextrin, a cholesterol ligand, disrupt lipid rafts and caveolae and thus are useful tools for studying their importance in signaling and ion channel function. Use of such detergents in our system would allow the role of these lipid platforms in SMase-induced activation of \( I_{\text{Cl,swell}} \). It is also possible that the generation of \( \text{H}_2\text{O}_2 \) near the sarcolemmal membrane is leading to peroxidation of membrane lipids. This may lead to modification of the membrane properties and thus perturbation of channel function. As the channel identity is unknown, this is one potential way ROS is mediating \( I_{\text{Cl,swell}} \).

As alluded to previously, investigation of the role of S1P receptors and potentially differential effects of sphingolipids of different chain lengths in this system is worth exploration. There is evidence that the length of ceramide acyl chains is a determinant of the differential effect of ceramide on signaling and membrane properties (Wijesinghe et al., 2005; Tang et al., 2007; Sot et al., 2005). Quantifying the absolute and relative changes in sphingolipid metabolism due to stimuli such as osmotic swelling using tandem mass spectrometry may help illuminate the role of various ceramide sub-species. The differential expression and activity of S1P receptors contributes an additional layer of complexity and specificity for sphingolipid signaling. Specific inhibitors of SPHK1 and certain S1P receptors have been developed. While this pharmacopeia is incomplete, it does provide tools that may allow us to probe the role of S1P receptors and localized S1P production in SMase-induced ROS production and \( I_{\text{Cl,swell}} \) activation.
$I_{\text{Cl,swell}}$ plays an obvious role in volume regulation, however the volume-sensing mechanism is not well understood. We have previously shown that ROS are downstream of the volume-sensing mechanism as $I_{\text{Cl,swell}}$ elicited by $H_2O_2$ is insensitive to hyperosmotic cell shrinkage (Ren et al., 2008). $I_{\text{Cl,swell}}$ may more accurately be described as a stress sensor, responding to $H_2O_2$ induced by a number of different stress stimuli. As ceramide and S1P both elicit $I_{\text{Cl,swell}}$ through ROS generation, cell fate may be determined by the total amount of ROS present. Below a certain threshold, ROS induce stress response mechanisms, including $I_{\text{Cl,swell}}$, that attempt to compensate for the perturbation. Above this threshold, the stress response mechanisms may become overwhelmed and begin the apoptotic process. This potentially explains the similar effects of S1P and ceramide in $I_{\text{Cl,swell}}$ activation in cardiomyocytes. As demonstrated in this study, both induce ROS but the relative concentrations produced may differ significantly. This could be addressed by using quantitative methods to determine the amounts of ROS produced by each sphingolipid species. Additionally, ROS generation may be localized to different compartments for the different species. Techniques such as confocal microscopy may be useful in addressing this question. The observation that sphingolipids and ROS are both involved in cardiac preconditioning in ischemia/reperfusion injury lends support to the hypothesis that lower concentrations of ROS may have beneficial effects by “priming” the stress response machinery for cell survival. Determination of the dose response relationship of $I_{\text{Cl,swell}}$ would be an important step in testing this hypothesis.
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ABSTRACTS


