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Sphingosine-1-Phosphate and Fingolimod (FTY720) Regulate ICl,swell In HL-1 Cardiac Myocytes via Intracellular Binding And Mitochondrial ROS Production

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SPHINGOSINE-1-PHOSPHATE AND FINGOLIMOD (FTY720) REGULATE $I_{Cl,swell}$ IN HL-1 CARDIAC MYOCYTES VIA INTRACELLULAR BINDING AND MITOCHONDRIAL ROS PRODUCTION

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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List of Abbreviations

Abbreviation	Full Name
4-β-PDB.	4-β-phorbol-12,12-dibutyrate
5-HD.	5-hydroxy-decanoic acid
9-AC.	9-Anthracene carboxylic acid
AEBSF.	4-(2-aminoethyl)benzenesulfonyl fluoride
ACh.	acetylcholine
Ang II.	angiotensin II
APD.	action potential duration
A-SMase.	acid sphingomyelinase
AT1R.	Ang II receptor type 1
AT2R.	Ang II receptor type 2
AVD.	apoptotic volume decrease
BACE1.	beta-Site APP-Cleaving Enzyme 1

CAPP. .....ceramide-activated protein phosphatase

CAY10444 (CAY). …….2-undecyl-thiazolidine-4-carboxylic acid

Cer. .....ceramide

CHF. …..…….....congestive heart failure

CoA… …….....coenzyme A

CERT. …….....ceramide transfer protein

CFTR. .....cystic fibrosis transmembrane conductance regulator

COP II. .....coat protein complex II

C-H2DCFDA-AM. .....6-carboxy-2',7'-dichlorodihydrofluorescein diacetatedi(acetoxy-methyl ester)

CRAC. …….....store-operated calcium 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

ECl. .....chloride equilibrium potential

EGF. .....epidermal growth factor

EGFR. .....epidermal growth factor receptor

ER. .....endoplasmic reticulum

ERK. .....extracellular signal regulated kinase

ET. .....endothelin

ETA. .....endothelin receptor subtype A

ETB. .....endothelin receptor subtype B

ETC. .....electron transport chain

FAK. ..... focal adhesion kinase

FTY720 (FTY)……..……...fingolimod

GIRK. ……G protein-coupled inwardly-rectifying potassium channel

GPCR. .....G protein coupled receptor

HDACs. ……...Histone deacetylases

HDL. ..... high-density lipoprotein

hERG. .....human Ether-à-go-go Related Gene

ICa,Cl.....…….calcium-activated Cl− current

ICl,cAMP. .....cAMP-activated Cl− current

ICl,swell. .....swelling-activated Cl− current

ICl,vol……...volume-sensitive chloride current

If.....hyperpolariztion-activated inward current

IgE. ……..immunoglobulin E

IgG. …….immunoglobulin G

IgG-HRP. …….immunoglobulin G Horseradish peroxidase

I – V.....current – voltage

IL-1α.....interleukin 1α

IP……...ischemic preconditioning

JNK…….....c-jun N terminal kinase

LDL…….....low-density lipoprotein

MAPK..... mitogen-activated protein kinase

MEK……..... mitogen-activated protein kinase kinase

mitoKATP.....mitochondrial ATP-sensitive K+ channel

MnTBAP.....Mn(III) tetra(4-benzoic acid) porphyrin chloride

mPTP. .....mitochondrial permeability transition pore

NADP. .....nicotinamide adenine dinucleotide phoasphate

NADPH. .....reduced form of nicotinamide adenine dinucleotide phoasphate

NDGA……...nordihydroguaiaretic acid

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

PHB2. ……..……prohibitin2

PI-3K. phosphoinositide-3-kinase

PKC. .....protein kinase C

PKC€. ……protein kinase C €

PLC. .....phospholipase C

PLD. .....phospholipase D

PMA. .....phorbol myristate acetate

PP2a. .....protein phosphatase 2a

PP2b. ..... phosphatase calcineurin

PPC. ……...pharmacological preconditionig

PTK. ..... receptor protein tyrosine kinase

PTX. .....pertussis toxin

RTKs. ……receptor tyrosine kinases

ROS. .....……...reactive oxygen species

RVD. ..…….. regulatory volume decrease

S1P. .....sphingosine 1-phosphate

S1PR ……...…….sphingosine 1-phosphate receptor

$S1PR_{1.}$ ……..…….sphingosine 1-phosphate receptor 1

$S1PR_{2.}$ ……..…….sphingosine 1-phosphate receptor 2

$S1PR_{3.}$ ……...sphingosine 1-phosphate receptor 3

$S1PR_4$. ……..sphingosine 1-phosphate receptor 4

$S1PR_5$. ……..sphingosine 1-phosphate receptor 5

SEW2871 (SEW). …….5-[4-phenyl-5-(trifluoromethyl)-2-thienyl]-3-[3-trifluoromethyl)phenyl]-1,2,4-oxadiazole

SITS. …….....4-acetamido-4-isothiocyanatostilbene-2,2-disulfonic acid

SMase. .....sphingomyelinase

SOD. .....superoxide dismutase

SphK. ……..sphingosine kinase

SphK1. .....sphingosine kinase type 1

SphK2. ..... sphingosine kinase type 2

TBS. ……tris buffered saline

TBS-T. …….tris buffered saline plus tween-20

TNFα. .....tumor necrosis factor α

TRAF2. …….TNF receptor-associated factor 2

VAC. ……..……..volume-activated channel

VEGF. ……...Vascular endothelial growth factor

VPC23019 (VPC). ……..…….2-Amino-N-(3-octylphenyl)-3-(phosph-onooxy)-propanamaide

VSOAC. …….……...volume-sensitive organic osmolyte anion channel

VSMCs. .....vascular smooth muscle cells

Abstract

SPHINGOSINE-1-PHOSPHATE AND FINGOLIMOD (FTY720) REGULATE $I_{Cl,swell}$ IN HL-1 CARDIAC MYOCYTES VIA INTRACELLULAR BINDING AND MITOCHONDRIAL ROS PRODUCTION

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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 mechanoelectrical feedback. Persistent activation of $I_{Cl,swell}$ has been observed in models of cardiovascular disease. We previously suggested sphingosine-1-phosphate (S1P) activates volume-sensitive Cl^- current ($I_{Cl,swell}$) by ROS-dependent signaling. S1P and its analog, FTY720 (fingolimod), primarily act via G-protein coupled receptors (S1PR; $S1PR_{1-3}$ in heart), but several intracellular S1P ligands are known. We investigated how these agents regulate $I_{Cl,swell}$. $I_{Cl,swell}$ was elicited by bath S1P (500 nM), FTY720 ($S1PR_{1,3}$ agonist; 10 μM), and SEW2871 ($S1PR_1$ agonist; 10 μM) and was fully inhibited by DCPIB, a specific blocker. These data suggested role of S1PR in activation of $I_{Cl,swell}$. Surprisingly, neither CAY10444 ($S1PR_3$ antagonist; 10 μM) nor VPC23019 ($S1PR_{1,3}$ antagonist; 13 μM) blocked FTY720-induced $I_{Cl,swell}$. Also, gallein a pan Gβγ inhibitor, failed to block the S1P-induced current. Moreover, 100 nM FTY720 applied via the pipette evoked a larger, faster activating current than 10 μM bath FTY720. Similarly, 500 nM S1P gave larger, faster activating $I_{Cl,swell}$ when added to the pipette than when added in the bath. In contrast to FTY720, bath S1P-induced $I_{Cl,swell}$ was blocked by CAY10444, but a 3-fold higher concentration failed to eliminate the response to pipette S1P, and VPC23019 failed to suppress bath and pipette S1P-induced currents. Taken together, inconsistencies in the responses to S1PR agents and the greater sensitivity to pipette than bath S1P and FTY720 support the notion that intracellular ligands rather than sarcolemmal S1PR activated $I_{Cl,swell}$. Next we tested if S1P and FTY720, like osmotic swelling, require both NADPH oxidase and mitochondrial ROS production to evoke $I_{Cl,swell}$. S1P- and FTY720-induced $I_{Cl,swell}$ were blocked by rotenone but were insensitive to gp91ds-tat, suggesting only

mitochondrial ROS production was needed. One possibility is that S1P and FTY720 elicit $I_{Cl,swell}$ by binding to mitochondrial prohibitin-2, an S1P ligand whose knockdown augments mitochondrial ROS productions. These data suggest $I_{Cl,swell}$ may be activated by S1P accumulation in ischemia-reperfusion and CHF. Understanding S1P-signaling that elicits $I_{Cl,swell}$ may provide insight into electrophysiological mechanisms of cardiac pathology and help identify novel targets for therapy.

Chapter 1 Background

1.1. Introduction of $I_{Cl,swell}$

The swelling-activated chloride current ($I_{Cl,swell}$), also referred to as the volume-sensitive chloride current ($I_{Cl,vol}$), volume-activated channel (VAC), or volume-sensitive organic osmolyte anion channel (VSOAC), is present in virtually all cell types and elicits an outwardly rectifying chloride current. $I_{Cl,swell}$ can be differentiated from other chloride channels based on the several biophysical and pharmacological properties. The current is outwardly rectifying under physiological or symmetrical chloride gradient and partially inactivates at positive potentials. Also current is time-independent over the physiologic voltage range and is attenuated by osmotic shrinkage. The permeability sequence is I_≥NO>Br_>Cl_>Asp (Hume *et al.*, 2000;Baumgarten & Clemo, 2003). Although the molecular identity is not yet determined, extensive pharmacological studies have been reported in various tissues. $I_{Cl,swell}$ is inhibited by class of molecules including Cl^-channel blockers, the antioestrogen such as tamoxifen, clomiphen, and nafoxidine at micro molar concentrations; antimalarials such as mefloquine, gossypol,

arachidonic acid, chromones, quinine, and quinidine, other compounds such as 9-Anthracene carboxylic acid (9-AC), nordihydroguaiaretic acid (NDGA), a dual inhibitor of cyclooxygenase and lipoxygenase, 1,9-dideoxyforskolin, the oxalon dye diBA-5-C4, niflumic acid, mibefradil, a T-type Ca^{2+} channel blocker, glibenclamide, a K_{ATP} and CFTR inhibitor, and by NPPB. It is also inhibited in voltage dependent manner at positive potentials by molecules such as calixarenes, DIDS, SITS (Nilius & Droogmans, 2003). The most selective blocker of $I_{Cl,swell}$ identified so far is the indanone compound DCPIB, which works at micromolar concentrations and was characterized in detail for cardiac $I_{Cl,swell}$ (Decher *et al.*, 2001). Recent studies suggest, however, that DCPIB also blocks GIRK channels, although with lower potency (W Deng, R Mahajan, CM Baumgarten, and DE Logothetis, unpublished observation. 2011).

1.1.1. Function of $I_{Cl,swell}$

As the name implicates $I_{Cl,swell}$ is stimulated by an increase in cell volume or osmotic swelling, as well as other stimuli. Swelling-activated chloride channels are ubiquitously present in mammals, in all cell types (Nilius *et al.*, 1994;Okada, 1997) including cardiac cells (Tseng, 1992;Sorota, 1992) and are involved in cell volume regulation and many other functions. The molecular identity remains elusive. However various groups are actively involved in investigating and understanding its role in cell volume regulation, regulatory volume decrease (RVD) during apoptosis (Okada & Maeno, 2001;Shimizu *et al.*, 2004), cell proliferation (Chen *et al.*, 2007), and cell cycle progression and migration (Mao *et al.*, 2009). $I_{Cl,swell}$ is implicated in fluid and electrolyte transport; for instance in ciliary epithelium cells, regulates secretion of aqueous humor (Jacob, 1997). Cell proliferation in many cell types such as T lymphocytes, microglia, astrocytoma cells, endothelial cells, and liver cells is regulated by $I_{Cl,swell}$ (Nilius &

Droogmans, 2003). $I_{Cl,swell}$ is thought to be involved in cell cycle progression as well (Stutzin & Hoffmann, 2006). Additionally $I_{Cl,swell}$ is important in apoptotic volume decrease (AVD), evidenced by decrease in AVD by blocking the channel (Okada & Maeno, 2001). AVD is known to be an upstream mediator of the events during apoptosis (Maeno *et al.*, 2000). Since AVD is regulated by chloride channel, therefore interpretation of $I_{Cl,swell}$ as a regulator of apoptosis seems reasonable. Depletion in cholesterol content activates $I_{Cl,swell}$, under isoosmotic conditions, however the relationship between $I_{Cl,swell}$ and membrane cholesterol content remains unexplored (Stutzin & Hoffmann, 2006). Recent work from our lab suggests that a high cholesterol diet activated $I_{Cl,swell}$, in LDL receptor null control macrophages by increasing mitochondrial ROS production, and that depleting the cholesterol ester by macrophage-specific transgenic over expression of human cholesterol ester hydrolase inhibited $I_{Cl,swell}$ (Deng *et al.*, 2011).

In cardiac physiology the role of chloride channel is still unclear, but it is thought to be important in cell volume regulation (Hoffmann & Dunham, 1995), a modulator of electrical activity and protects against ischemic reperfusion injury (Mulvaney *et al.*, 2000). Furthermore the channel plays diverse role such as altering resting membrane potential and the duration of action potential, hence acting as an important modulator in physiological and patho-physiological condition (Hume *et al.*, 2000). $I_{Cl,swell}$ is known to regulate cardiac electrical activity, cell volume, apoptosis, cell proliferation and probably serves a role in ischemic preconditioning (Baumgarten *et al.*, 2005;Duan *et al.*, 2005;Hume *et al.*, 2000). $I_{Cl,swell}$ is activated by β1 integrin stretch, hydrostatic inflation, also it is found to be activated under isoosmotic environment in various models of cardiac diseases. The molecular identity of this channel remains controversial, as well as the volume sensing mechanism responsible for activation of channel.

1.2. Regulation of $I_{Cl,swell}$

Over the past few years the signaling molecules regulating the channel have been actively studied. Reactive oxygen species (ROS) seem to be an effective modulator of $I_{Cl,swell}$ in cardio-myocytes (Browe & Baumgarten, 2006;Browe & Baumgarten, 2004;Ren *et al.*, 2008) and other cells (Haskew-Layton *et al.*, 2005;Shimizu *et al.*, 2004;Varela *et al.*, 2004).

1.2.1. Indirect evidence of regulation of $I_{Cl,swell}$ by ROS in heart and other tissue

d'Anglemont de Tassigny et al. (2004) looked at the role of a Cl^- current thought to be $I_{Cl,swell}$ and AVD in doxorubicin-induced apoptotic cell death in adult rabbit ventricular cardiomyocytes. The observations included activation of an outwardly rectifying Cl^- current upon exposure to doxorubicin and C_2-ceramide. Also inhibition of chloride channel led to attenuation of programmed cell death by decreasing caspase activity and annexin V labeling (de Tassigny *et al.*, 2008). However, identification of the current as $I_{Cl,swell}$ was lacking. The anion channel inhibitors utilized were not selective for $I_{Cl,swell}$, the doxorubicin- and C_2-ceramide-induced currents were not shown to be volume-sensitive, and outward rectification in symmetrical Cl^- was not verified. Doxorubicin is known as a proapototic agent, which induce oxidative stress in myocytes, but the role of ROS in current activation was not demonstrated. Current activation was blocked by inhibitors of PI3 kinase/AKT (de Tassigny *et al.*, 2008), and we demonstrated activation of PI3 kinase by EGFR upregulated $I_{Cl,swell}$ via ROS in response to osmotic swelling and mechanical stretch (Ren *et al.*, 2008;Browe & Baumgarten, 2006). Xiaoming Wang et al. showed that activation of $I_{Cl,swell}$ during the reperfusion period led to induction of apoptosis in neonatal mouse cardiomyocyte during ischemia-reperfusion. Elicitation of current was shown to be modulated by ROS. They demonstrated ROS production during

period of reperfusion, also application of non selective inhibitors of channel during the period of reperfusion but not ischemia reduced cell death by apoptosis. In addition ROS scavengers prevented apoptosis by reduction of caspase activation (Wang *et al.,* 2005). Additionally they showed apoptosis and Cl^- channel blockers act independent of the Cl^-/HCO3 exchanger. Though direct evidence of ROS modulating chloride channel activation was not shown, it is clear from previous studies in endothelium cells that $I_{Cl,swell}$ is involved in apoptosis and regulated by ROS (Shimizu *et al.,* 2004).

Another group reported the importance of volume regulation via $I_{Cl,swell}$ in ischemic preconditioning (IP). Ischemic preconditioning is known to activate ROS production. Though many controversies were reported, Baines et al. demonstrated production of ROS during ischemic preconditioning and their role in cardioprotection (Baines *et al.,* 1997). Moreover in another study blockade of chloride channel during IP and pharmacological preconditioning (PPC) failed to offer cardioprotection. The players such as angiotensin II, AT receptors, adenosine, PKC which are involved in PPC are known to modulate chloride channel. Batthish et al. showed that chloride channels play an important physiological role in the cardioprotection of IPC and PPC acting downstream of PKC (Batthish *et al.,* 2002;Diaz *et al.,* 2001). From the above studies indirect involvement of ROS can be inferred. More precise experiments are needed to show involvement of ROS as a modualtor of $I_{Cl,swell}$. Many of these study fail to use specific inhibitor of $I_{Cl,swell}$, and therefore, evidence of the role of this channel may be questioned. Additionally linking of various upstream mediators of ROS production and how it modulates $I_{Cl,swell}$ still poses a challenge. Also the source of ROS production upon stimulation by various upstream mediators is also elusive.

1.2.2 Regulation of $I_{Cl,swell}$ by ROS in heart

Recently, reactive oxygen species (ROS) were identified as a downstream effector, and exogenous H_2O_2 elicits $I_{Cl,swell}$ in cardiomyocytes (Ren *et al.*, 2008;Browe & Baumgarten, 2006;Browe & Baumgarten, 2004) and other cells (Haskew-Layton *et al.*, 2005;Shimizu *et al.*, 2004;Varela *et al.*, 2004). Baumgarten and coworkers, demonstrated β1 integrin stretch and osmotic swelling, activating volume sensitive chloride current in cardiac myocytes. Upstream signaling molecules include Src family kinases (Browe & Baumgarten, 2003;Du *et al.*, 2004;Walsh & Zhang, 2005), protein tyrosine kinase (Sorota, 1995), angiotensin II (Ang II) (Browe & Baumgarten, 2004;Ren *et al.*, 2008), epidermal growth factor receptor (EGFR) kinase (Du *et al.*, 2004), and phosphoinositide 3-kinase (PI-3K) (Browe & 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 $I_{Cl,swell}$ (Gong *et al.*, 2004;Duan *et al.*, 1999). Moreover ET-1-induced $I_{Cl,swell}$ was abrogated by selective blockade of ET_A receptor, EGFR kinase and PI-3K, suggesting that ET-1 activates (Deng *et al.*, 2010a). $I_{Cl,swell}$ via a signaling cascade involving ET_A receptor, EGFR kinase and PI-3K. PI-3K is downstream from EGFR kinase because inhibition of PI-3K suppressed EGF-induced $I_{Cl,swell}$. All these signaling cascade indicated H_2O_2 as the most downstream modulator for $I_{Cl,swell}$. Scavenging H_2O_2 with catalase reverses the β-integrin stretch-induced activation of $I_{Cl,swell}$ (Browe & Baumgarten, 2005). Moreover, the SOD mimetic MnTBAP that speeds conversion of superoxide ($O2^{-}\bullet$) to H_2O_2 augments ACh-induced $I_{Cl,swell}$ (Browe & Baumgarten, 2007). $I_{Cl,swell}$ also is activated by exogenous H_2O_2 with an EC50 of ~8 μM (Browe & Baumgarten, 2004;Ren *et al.*, 2008), and the site of action of H_2O_2 appears to be downstream from the volume-sensing mechanism because H_2O_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 $I_{Cl,swell}$ (Browe & Baumgarten, 2004;Browe & Baumgarten, 2007;Ren *et al.*, 2008;Deng & Baumgarten, 2009). However H1V protease inhibitor ritonavir and lopinavir activated $I_{Cl,swell}$ via mitochondrial ROS production that was independent of NADPH oxidase (Deng *et al.*, 2010b). Furthermore SMase-induced $I_{Cl,swell}$, inhibitor of NADPH oxidase partially reduced the S1P-induced Cl^- current and inhibitor of mitochondrial ROS completely abolished SMase induced current (Raucci & Baumgarten, 2009). Although strong evidence is provided for the signaling pathway involved in modulation of the channel, the studies are based on use of pharmacological inhibitors. Though these have been implicated as specific inhibitors, their non-specific effect cannot be ruled out. There also may be additional players in the cascade which are not yet evident. Hence these studies can by no means present a complete understanding of the signaling molecules involved in activation of $I_{Cl,swell}$.

1.3. Importance of sphingolipid signaling in modulation of $I_{Cl,swell}$

Many of the signaling cascades that activate $I_{Cl,swell}$ overlap with those involved in sphingolipid signaling (Hannun & Obeid, 2008;Levade *et al.*, 2001;Spiegel & Milstien, 2002) raising the possibility that certain sphingolipids might regulate $I_{Cl,swell}$. Sphingolipids are important bioactive lipid metabolites which play an important role in cell growth, apoptosis, motility, vasculogenesis, and immune function. Sphingolipid metabolism is altered in several cardiac diseases resulting in their accumulation (Chatterjee *et al.*, 2006;Levade *et al.*, 2001). Extensive studies in multiple tissues have focused on the sphingolipid metabolites sphingosine 1-phosphate (S1P), sphingosine, and ceramide. The maintenance of a balance between ceramide and S1P levels is important in determining the fate of cells (Van Brocklyn & Williams, 2012). Enzymes such as sphingomyelinase and sphingosine kinase (SphK) play a key role in the

production of these metabolites. Sphingosine kinase (SphK)/S1P pathway acts as regulator of various cellular signaling cascades. Recently SphK/S1P was found to have a role in cardiac physiology and pathology including ischemic/reperfusion injury. Alterations in sphingolipid metabolism are implicated in cardiovascular diseases, including congestive heart failure, atherosclerosis, and ischemia/reperfusion injury (Levade *et al.*, 2001;Chatterjee *et al.*, 2006). S1P is known to act as a ligand for G-protein coupled S1P receptors to initiate signaling pathway regulating many cellular functions. Moreover S1P regulates cardiac ion channel activity. Recently our laboratory showed that exogenous and endogenous ceramide elicited $I_{Cl,swell}$ in ventricular myocytes via ROS. Preliminary data suggests, however, that the ceramide metabolite S1P rather than ceramide itself was responsible for activating $I_{Cl,swell}$. Because of the well-established role of G protein-coupled S1P receptors in transducing S1P signaling (Means & Brown, 2009), we hypothesized that SphK/S1P pathway and the three cardiac G protein coupled S1P receptors ($S1PR_{1-3}$) were responsible for triggering activation of $I_{Cl,swell}$ under isosmotic conditions by ROS production by either NADPH oxidase, mitochondria or both. More recently, intracellular S1P binding sites also have been reported in cardiac and other cells (Hait *et al.*, 2013;Maceyka *et al.*, 2012). Among these is prohibitin 2 (PHB2), a mitochondrial inner membrane protein that controls the assembly and function of cytochrome oxidase. This leads to an alternative hypothesis that S1P elicits $I_{Cl,swell}$ via its binding to intracellular target(s) rather than plasmalemmal S1P receptors.

1.4. Sphingolipds

1.4.1. Role of Sphingolipds

Until recently sphingolipids were considered merely as membrane structural components without further function. In the past two decades sphingolipids have been identified as important bioactive lipids that are involved in various functions ranging from pleiotropic effects on protein kinases, regulating the actin cytoskeleton, endocytosis, the cell cycle and apoptosis (Smith *et al.*, 2000). They are involved in forming specialized structures, mediate cell-cell and cell-substratum interactions, modulate the behavior of cellular proteins and receptors, and participate in signal transduction and the activation of downstream cascades. Sphingolipids 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. Amongst sphingolipid metabolites the most prominent are sphingosine 1-Phosphate (S1P), sphingosine, and ceramide. Ceramide mediates many cell-stress responses, including the regulation of apoptosis (Obeid *et al.*, 1993) and cell senescence (Venable *et al.*, 1995) , whereas S1P has crucial roles in cell survival, cell migration and inflammation (Spiegel & Milstien, 2002). The maintenance of a balance between ceramide and S1P levels seems important in determining the fate of cells (Van Brocklyn & Williams, 2012).

1.4.2. Biosynthesis and Metabolism

Sphingolipids are ubiquitous components of the lipid bilayer in all eukaryotic cells. Ceramide, which is a backbone for most of sphingolipids, is a precursor for formation of S1P, and key elements of the pathway regulating S1P production are illustrated in *Figure 1*. Ceramide can be synthesized by either a de novo pathway or a degradative pathway through the hydrolysis of complex lipids, especially sphingomyelin (SM). De novo synthesis of sphingolipids occurs at the cytosolic face of the endoplasmic reticulum (ER). The initiating reaction is the condensation of serine and palmitoyl-CoA catalyzed by serine palmitoyltransferase to form 3-ketosphinganine,

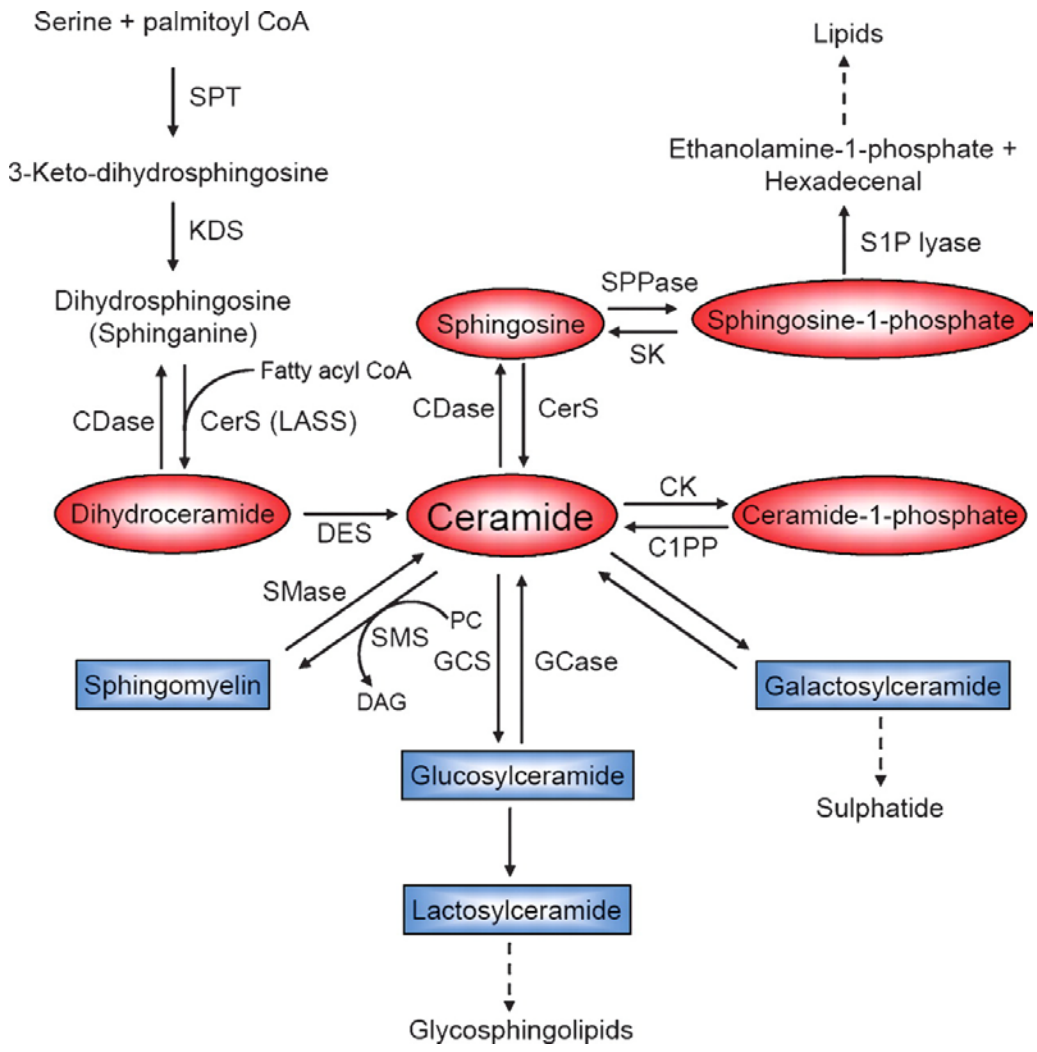

Figure 1: Schematic of S1P metabolism pathway (Bartke & Hannun, 2009). Key abbreviations include sphingomyelinase (SMase), sphingosine kinase (SK).

which is rapidly reduced to dihydrosphingosine and then N-acylated by ceramide synthase to form dihydroceramide. Finally, a trans double bond at C_4–C_5 is introduced by dihydroceramide desaturase, producing ceramide (Bartke & Hannun, 2009). Ceramide is then transported from the ER to Golgi where head groups are added forming sphingolipids. Later, ceramide is acted upon by ceremidase to yield sphingosine, the most common base for other sphingolipids. Finally, SphK catalyzes ATP-dependent phosphorylation of sphingosine to form S1P. S1P is converted back to sphingosine, which can be recycled, by sphingosine phosphatase. Alternatively, S1P can be irreversibly broken down by S1P lyases to hexadecanal and phosphoethanolamine which then can be incorporated into lipids (Spiegel & Milstien, 2003).

Although *de novo* synthesis is an important source of sphingosine in cells, much of the sphingosine present under physiological and several pathological conditions is generated by the catabolism of sphingomyelin and other sphingolipids, a process termed as the degradative pathway (Kitatani *et al.*, 2008) . This pathway involves release of ceramide from sphingomyelin by sphingomyelinase, and in turn, ceramide is acted upon by ceramidases to generate sphingosine. Additionally, ceramide is produced through a salvage pathway that begins within acidic cellular compartments including late endosomes and lysosomes (Kitatani *et al.*, 2008). Importantly sphingosine and dihydrosphingosine (sphinganine) are not produced by either the de novo pathway and or catabolism (Strub *et al.*, 2010).

Sphingosine-1 phosphate (S1P) is produced by the action of sphingosine kinases. There are two primary isotypes of sphingosine kinase, denoted as SphK1 and SphK2, that produce S1P in the cytosol, and SphK are stimulated by various growth factors and other molecules. Interestingly, the best studied biological actions of S1P via G protein-coupled S1PR requires inside-out signaling (Takabe *et al.*, 2008). Cytosolic S1P is transported out of the cell via ABC

transporters. Once in the extracellular space, S1P acts as a potent S1PR ligand and activates downstream intracellular signaling cascades in autocrine/paracrine manner (Takabe *et al.*, 2008;Alvarez *et al.*, 2007;Spiegel & Milstien, 2000). In cardiovascular system three G protein-coupled S1P receptors, $S1P_{1-3}$, are expressed (Means & Brown, 2009). $S1P_1$ receptor exclusively couples with G_i, whereas $S1P_2$ $S1P_3$ couple to G_i, G_q, and $G_{12/13.}$

1.4.3. Sphingosine Kinase (SphK)

SphK are lipid kinases that catalyze production of S1P from sphingosine by ATP-dependent phosphorylation. *Figure 2* shows the conversion of sphingosine to S1P and list the various downstream targets and potential functions of both sphingosine and S1P (Spiegel & Milstien, 2002). SphK is a key regulator that determines cell fate. SphK belong to a conserved lipid kinase family containing 5 conserved domains (Kohama *et al.*, 1998). Two mammalian isoforms are known: SphK1 and SphK2. While both isoforms produce S1P, they exhibit different catalytic properties, subcellular locations, tissue distribution, and temporal expression patterns during development, suggesting unique and specific functions. For example, SphK1 and SphK2 have opposing roles in regulation of ceramide biosynthesis (Maceyka *et al.*, 2005). The genes encoding the isoform are located on different chromosomes. SphK1 has a broad tissue distribution with high levels in brain, heart, lungs and spleen. SphK1 displays specificity for the natural trans-isomer of D-erythrosphingosine. The second isoform, SphK2, shares five conserved domains with SphK1 (about 80% similarity and 50% identity), but it has an additional 200 amino acids. SphK2 phosphorylates wider range of sphingolipid base substrate (Hait *et al.*, 2006;Karliner, 2009b).

SphK is stimulated by various external stimuli. Activators include agonist of growth factor receptors, such as PDGF, VEGF, NGF, and EGF, ligands for GPCRs, TGFβ, the

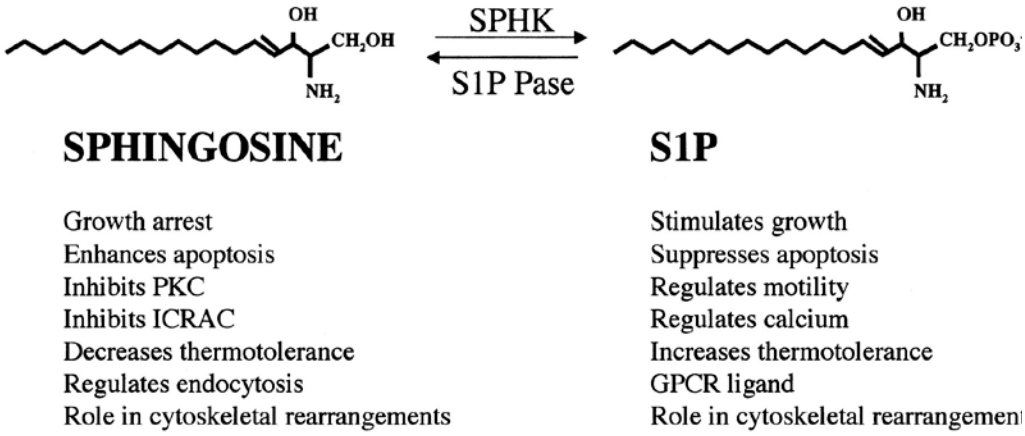

Figure 2: Conversion of D-erythro-sphingosine to S1P by SPHKs, using ATP as the phosphate donor. Various downstream targets and potential functions of both sphingosine and S1P are mentioned (Spiegel & Milstien, 2002).

proinflammatory cytokine TNF-alpha, cross-linking of IgE and IgG receptor, interleukins, estrogen, and activators of PKC_{ε}. A variety of other interacting adaptor protein specifically stimulates SphK1 activation. The growth factors are responsible for translocation of SphK1 to the plasma membrane (Spiegel & Milstien, 2003). Thus, S1P produced by translocation of SphK1 to the plasma membrane has been implicated in transactivation of cell surface S1P receptors (Maceyka *et al.*, 2005). In most cases, the importance of SphK1 activation and concomitant production of S1P in the regulation of many biologically responses was only indirectly inferred by the ability of pan SphK inhibitors to block agonist-induced effects (Hait *et al.*, 2006;Spiegel & Milstien, 2002). In contrast to SphK1, SphK2 is mainly present in intracellular compartments, including the nucleus and mitochondria. Nuclear SphK2 regulates gene transcription at least in part by producing S1P, which acts as an endogenous inhibitor of histone deacetylases (Hait *et al.*, 2009). In mitochondria the action(s) of SphK2 are required for correct assembly of the cytochrome oxidase complex. However, the exact ligand for the mitochondrial targeting signal is not known (Strub *et al.*, 2011).

Relatively little is known about the signaling pathways that regulate SphK2 activity. Studies indicate that pro-survival protein Bcl-x_L activates (Liu *et al.*, 2003) SphK2. There is strong evidence that disordered or altered cell volume regulation is associated with apoptosis (Maeno *et al.*, 2000;Okada & Maeno, 2001). $I_{Cl,swell}$ is known to regulate the apoptotic cell volume (Okada *et al.*, 2006). There is also a link between the apoptotic resistance, offered by survival protein Bcl-2 and the strengthening of RVD capability by upregulation of $I_{Cl,swell}$.(Shen *et al.*, 2002;Lemonnier *et al.*, 2004). Thus S1P produced by the action of SphK2 due to BCl-xl activation could be responsible to modulate the activity of $I_{Cl,swell}$. Similar to SphK1, SphK2 is upregulated by the Erk1/2 pathway. SphK 2 and SphK1 have opposing functions in ceramide

signaling (Spiegel & Milstien, 2003). The differences between the pro-survival and anti-survival effects of SphK are related to the distinct sub-cellular localization and spatially restricted production of S1P (Karliner, 2009b). But these differences are not yet demonstrated in cardiac cells or *in vivo* in any organs.

1.5. Sphingosine-1 Phosphate: Important Signaling Biolipid

Sphingolipids have been extensively studied for the past two decades. The pathways in which sphingolipids work are enigmatic, however, various signaling cascades have been identified. The unprecedented complexities in sphingolipid biochemical interconnections enable cells to execute cellular responses by regulating sphingolipid inter-conversions. Therefore, it is important to determine which sphingolipid is responsible for orchestrating a particular signaling pathway. Amongst several sphingolipids, the S1P/ceramide rheostat plays a critical role in determining the fate of the cell (Van Brocklyn & Williams, 2012). This notion is supported by the fact that S1P and ceramide control the cell-cycle process (Spiegel & Milstien, 2002) and apoptotic (Obeid *et al.*, 1993) pathway, respectively. Thus, S1P and ceramide have antagonistic effect on the cell. However, recently it has been shown that ceramide and S1P can have similar effects in certain circumstances. For example, both ceramide and S1P induced ROS generation in bovine coronary arteries (Zhang *et al.*, 2003;Keller *et al.*, 2006).

After establishing the role of S1P in cell growth (Olivera & Spiegel, 1993;Zhang *et al.*, 1991), studies have aimed at determining the role of SphK in setting the level of S1P in different cellular compartment, the compartment-specific actions of S1P, and more broadly, the signaling cascades triggered by plasmalemmal S1P receptors. The primary step in signal transduction is

inside-out S1P transport and binding to G-protein coupled S1P receptors (S1PR). However the importance and role of potential intracellular S1P binding targets remains unclear.

1.5.1. Inside-out transport of S1P

S1P produced by the SphK can act in a paracrine-autocrine manner and bind 5 S1P receptors ($S1P_{1-5}$) that are expressed in various tissues (Chun *et al.*, 2002;Spiegel & Milstien, 2000), although only $S1P_{1-3}$ have been identified in cardiac myocytes (Means & Brown, 2009). Because S1P is synthesized in the cytosol and binds to G protein-coupled receptors facing the extracellular milieu, this bioactive lipid must be transported from the inside to the outside of cells to act. Inside-out transport of S1P and presumably its analog FTY720-P are mediated via several ATP-binding cassette (ABC) proteins known as ABC transporters, including ABCA1, ABCB1, ABCC1, and ABCG2 (Sato *et al.*, 2007;Nieuwenhuis *et al.*, 2009;Honig *et al.*, 2003;Takabe *et al.*, 2010). In mast cells, for example, the ABCC1 transporter is responsible for S1P export (Mitra *et al.*, 2006). In astrocyte ABCA1 transports S1P out of the cells (Sato *et al.*, 2007). Similarly, S1P secretion from platelets is also mediated by an ABC transporter, ABCA7 (Kobayashi *et al.*, 2006;Anada *et al.*, 2007), and in breast cancer cells, stimulation with estradiol led to S1P release via ABCC1 and ABCG2 transporters (Takabe *et al.*, 2010). However, the involvement of ABC transporters in the secretion of S1P in a number of other systems, including cardiac myocytes, is yet to be determined, and knock out of selected ABC transporters does not fully reproduce the expected phenotype. Recently human Spns2, a putative 12-transmembrane domain protein, was shown to transport S1P, dihydro-S1P, and FTY720-P in CHO cells (Hisano *et al.*, 2011). Interestingly, Spns2 regulates the migration of myocardial precursors during Zebrafish development (Kawahara *et al.*, 2009) by regulating S1P secretion from the extraembryonic yolk syncytial layer, and Spns2 mRNA is found in homogenates of human heart

(Hisano *et al.*, 2011). In addition to its local autocrine/paracrine action, S1P is synthesized (Yatomi *et al.*, 1997) and released from platelets in response to thrombin stimulation (Yatomi *et al.*, 1995) and erythrocytes (Hanel *et al.*, 1993). S1P levels in the extracellular fluids plasma (associated with albumin or lipoproteins (Aoki *et al.*, 2005;Hla, 2005;Murata *et al.*, 2000;Yatomi *et al.*, 2000)) and lymph are generally high, with concentrations reported of 200 nM to 3 μM and up to 500 nM (Hla, 2005), respectively (Kihara & Igarashi, 2008). In our experiments we use 500 nM S1P to match closely with the physiological concentration.

1.5.2. Extracellular targets of S1P signaling

As mentioned above, S1P acts as a ligand for the family of five G protein-coupled receptors, termed $S1PR_{1-5}$, formerly called endothelial differentiation gene (EDG) receptors. These receptors are differentially expressed across tissues and are coupled to different G proteins resulting in diverse signaling by S1P. Amongst the array of responses evoked upon S1P binding to the GPC receptors are Ca^{2+} mobilization, proliferation, cytoskeletal organization and migration, adherence- and tight junction assembly, and morphogenesis (Hla, 2004;Lee *et al.*, 1999). As mentioned previously, to date, five members of the S1PR family have been cloned including S1P1 (EDG-1), S1P2 (EDG- 5), S1P3 (EDG-3), S1P4 (EDG-6), and S1P5 (EDG-8) all of which bind and are activated specifically by S1P and dihydro-S1P (also known as sphinganine 1-phosphate) (Chun *et al.*, 2002), whose structure is identical to that of S1P but lacks the 4,5-*trans* double bond). S1P thus is able to activate and regulate a diverse array of signal transduction pathways in different cell types, depending on the relative abundance of S1PRs and associated G proteins, and elicit a wide range of responses (Spiegel & Milstien, 2000;Pyne & Pyne, 2000). Localization and G protein coupling of each of the S1P receptors and their signaling upon activation will be described in the following sections.

1.5.3. $S1PR_1$

$S1PR_1$, historically known as EDG1, was the first receptor S1PR identified. It is ubiquitously expressed with high levels in brain, lung spleen, cardiovascular system and kidney and is coupled to $G_{i/o}$. Initially $S1PR_1$ was thought to be an orphan GPCR receptor responsible for differentiation of endothelial cells (Brinkmann, 2007). Further studies confirmed $S1PR_1$ possessed high affinity for S1P (*K*d 8.1 nM using radioligand binding assay in HEK293.(Lee *et al.*, 1998) and 13.2 nM estimated by [3H]S1P in Chinese hamster ovary cells transfected with putative S1P receptor (Kon *et al.*, 1999). Genetic deletion of S1P1 in mice indicates a key role in angiogenesis and neurogenesis. The observation that $S1PR_1$ plays a critical role in directed cell motility suggests the underlying mechanism by which S1P acts as a regulator of angiogenesis (Liu *et al.*, 2000). Additionally, $S1PR_1$ plays a role in the regulation of immune cell trafficking, endothelial and epithelial barrier function and integrity (Singleton *et al.*, 2005). Also it has a key role in angiogenesis, vascular maturation, increased vascular integrity (Singleton *et al.*, 2005), and maintaining vascular tone (Sanna *et al.*, 2004). The maintenance of vascular integrity is carried out by downstream activation of Akt and Rac upon $S1PR_1$ activation (Singleton *et al.*, 2005). Additionally, binding of S1P to $S1PR_1$ can transactivate various growth factor receptor tyrosine kinases (RTKs), such as VEGF receptor, EGF receptor, and PDGF receptor (Takabe *et al.*, 2008). Forming a complex with VEGFR, it associates with PKC alpha and ERK1/2 (Bergelin *et al.*, 2010). The signaling triggered by S1PR is summarized in Fig. 3 (Pyne & Pyne, 2000).

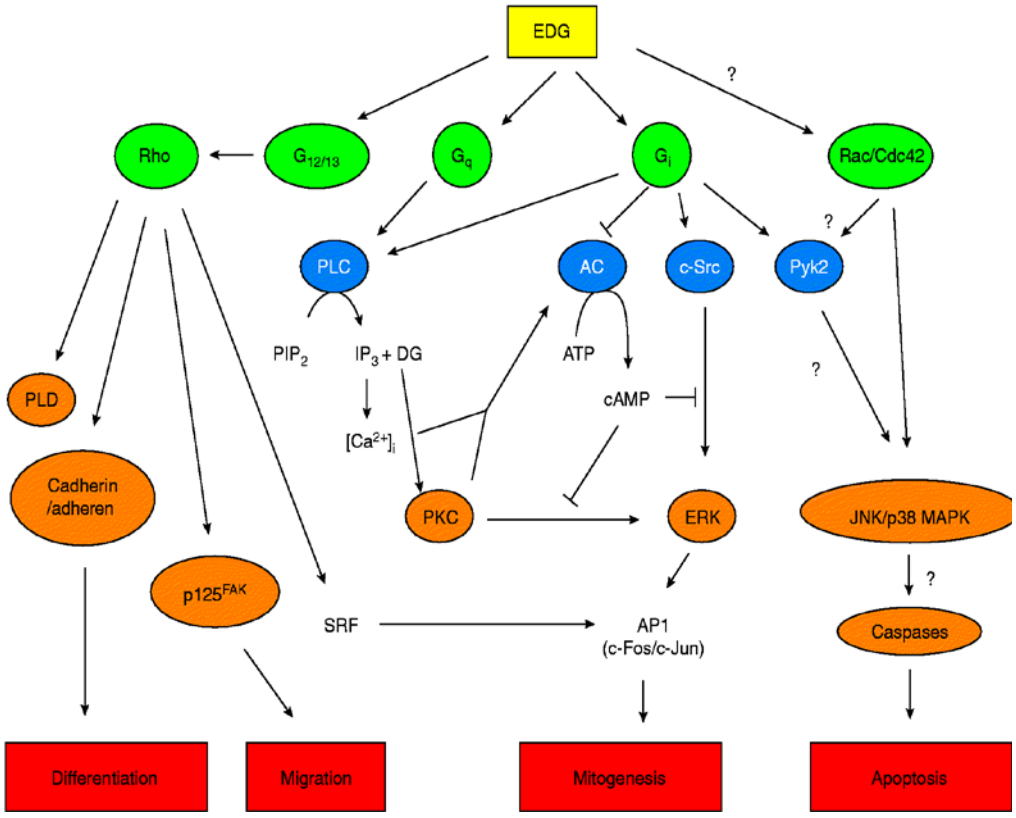

Figure 3: Signaling pathways activated by S1P binding to EDG/S1P receptors regulating mitogenesis, chemotaxis, differentiation and apoptosis. Differential coupling to G-proteins and effectors for $S1PR_{1\text{-}3}$may be apparent for EDG1, EDG3 and EDG5. Abbreviations : AC, adenylate cyclase ; PIP2 , PtdIns(4,5)P2; IP3, Ins(1,4,5)P3 ; DG,diacylglcerol ; AP1, activator protein 1 (Pyne & Pyne, 2000).

$S1PR_1$ is also involved in immune function of cell by modulating lymphocyte recirculation (Strub *et al.*, 2010). This underlies the clinical utility of fingolimod (FTY720; Gilenya®), an immunosupressor recently approved for the treatment of relapsing forms of multiple sclerosis. FTY720 is a sphingosine analog prodrug that is converted to its active form by phosphorylation by SphK and induces lymphopenia (Brinkmann *et al.*, 2002;Mandala *et al.*, 2002).

1.5.4. $S1PR_2$

$S1PR_2$ was originally known as EDG5. Like $S1PR_1$, $S1PR_2$ is also widely distributed and is prominently expressed in vascular smooth muscle cells (Waeber *et al.*, 2004). It is coupled to $G_{i/o}$, G_q, and $G_{12/13,}$ thereby imparting a wide range of actions. In contrast to $S1PR_1$, $S1PR_2$ receptors inhibit migration and proliferation of endothelial and vascular smooth muscle cells, probably because of their unique stimulatory effect on a GTPase-activating protein that inhibits Rac activity (Waeber *et al.*, 2004). $S1PR_2$ also play an essential role in the proper functioning of the auditory and vestibular systems, as $S1PR_2$ knockout mice exhibit deafness (Kono *et al.*, 2007). This S1P receptor is also required for degranulation of mast cells. Moreover, $S1PR_2$ are important for pyramidal neuron development and the regulation of excitability, as their loss causes increased excitability and seizures that can be lethal (MacLennan *et al.*, 2001). $S1PR_2$ activates Rho, PLC, c-Jun, JNK, p38 and inhibits Rac. Additionally, it inhibits tumor angiogenesis and growth (Yester *et al.*, 2011). The affinity of $S1PR_2$ for S1P is (Kd 20–27 nM) (Ishii *et al.*, 2004).

1.5.5. $S1PR_3$

$S1PR_3$, also known as EDG3, is coupled with $G_{i/o}$, G_q, and $G_{12/13}$. Similar to $S1PR_1$, it has a wide range of distribution throughout the body with predominant expression in heart, peri-

vascular smooth muscle cells, lung, spleen, kidney, intestine, diaphragm, and cartilage. The signaling pathway activated are Rho, ERK, PLC, Rac. $S1PR_3$ expression is localized to myocytes and perivascular smooth muscle cells, thus $S1PR_3$ exhibits effects on the cardiovascular system, regulating bradycardia and hypertension (Strub *et al.*, 2010). The rank order of compound potency for bradycardia is more closely aligned with the potency for activation of $S1PR_3$ than the other S1P receptors (Forrest *et al.*, 2004) The S1P3 receptor is the primary receptor coupling to PLC and the activation of this receptor also results in bradycardia (Means & Brown, 2009).

1.5.6. $S1PR_4$/$S1PR_5$

$S1PR_4$ and $S1PR_5$ are also known as EDG6 and EDG8, respectively. As compared to other receptors, the localization of $S1P_{4-5}$ is rather restricted, with predominant expression limited to human leukocytes, Natural Killer cells, airway smooth muscle cells and white matter of CNS tracts. S1P4 is primarily expressed in lymphoid tissues, including the thymus, spleen, bone marrow, appendix, and peripheral leukocytes (Strub *et al.*, 2010) This expression pattern suggests potential roles of S1P4 in the immune system. In vivo roles and functions of S1P4 are still unknown (Ishii *et al.*, 2004). Expression of $S1P_5$ is restricted to specific tissues: brain, spleen, and peripheral blood leukocytes in human and brain, skin, and spleen in rat and mouse (Ishii *et al.*, 2004). $S1PR_5$ is highly expressed in oligodendrocytes suggesting its potential roles in maturation and myelination of oligodendrocytes $S1PR_4$ couples with $G_{i/o}$, G_s, and $G_{12/13}$, whereas $S1PR_5$ couples with $G_{i/o}$ and $G_{12/13}$.

1.5.6. Intracellular targets of S1P

For many years, the myriad of effects of S1P on cellular function have been attributed to downstream signaling elicited by the five well-known and extensively studied Gprotein-coupled

S1P receptors. However, a number of effects of S1P cannot be explained by this receptor family, raising the possibility that S1P also binds to and modulates the function of intracellular targets.. For example, organisms such as Dictyostelium discoideum, Sacharomyces cerevisiae and Arabidopsis thaliana do not express S1PR but display cellular responses to S1P (Yester *et al.*, 2011). Discrepancies are also found in mammalian systems. Dihydro-S1P and S1P bind and activate S1PRs with similar affinities (Chun *et al.,* 2002), but dihydro-S1P, which is rapidly dephosphorylated in the cytosol, does not mimic all of the actions of S1P. For example dihydro-S1P, although a good substrate for $S1PR_1$ in situ, does not cause significant ceramide accumulation or increase apoptosis (Le Stunff *et al.*, 2002) and, does not have any significant cytoprotective effect (Van Brocklyn *et al.*, 1998). For example, S1P, but not dihydro-S1P, protects male germ cells from apoptosis, an effect that was linked to inhibition of NF-κB and activation of Akt (Suomalainen *et al.*, 2005). On the contrary, S1P-phosphonate, which does not bind to S1PRs, still protects Swiss 3T3 fibroblasts cells from apoptosis (Van Brocklyn *et al.*, 1998). Furthermore, microinjection of S1P appears to increase DNA synthesis independent of G proteins (Van Brocklyn *et al.*, 1998).

The identity of intracellular binding sites for S1P is only beginning to emerge. Cytoplasmic and subcellular targets identified to date include histone deacetylases (HDACs) (Hait *et al.*, 2009) this study showed that, HDACs are direct intracellular targets of S1P and link nuclear S1P to epigenetic regulation of gene expression. S1P was shown to be a missing cofactor for the E3 ubiquitin ligase TRAF2 and that TRAF2 was a novel intracellular target of S1P Interestingly, only S1P, and not dihydro-S1P, which lacks the double bond in S1P, was shown to bind to and activate TRAF2 (Alvarez *et al.,* 2010). β-site amyloid precursor protein cleaving enzyme-1 (BACE1) was shown to be another site in neurons to which S1P bound. S1P also

specifically bound to BACE1 in vitro and increased its proteolytic activity, suggesting that cellular S1P directly modulates BACE1 activity (Takasugi *et al.*, 2011). In these studies inhibition or downregulation of SphK1 and SphK2, or overexpression of S1P-degrading enzymes all decreased BACE1 activity and Aβ production (Maceyka *et al.*, 2012). These responses were mediated by intracellular S1P independently of its cell surface G-protein-coupled receptors. Also prohibitin 2 PHB2, a highly conserved protein that regulates mitochondrial assembly and function, was recently shown to bind S1P in vitro and in vivo (Maceyka *et al.*, 2012;Strub *et al.*, 2011;Yester *et al.*, 2011). PHB2 localizes predominantly to the inner mitochondrial membrane where it is thought to form a large, macromolecular complex with PHB1 that is involved in mitochondrial biogenesis and metabolism (Takasugi *et al.*, 2011;rtal-Sanz & Tavernarakis, 2009) By targeting monomeric PHB2 (but not closely related PHB1), S1P is implicated in the regulation of proper assembly and function of cytochrome-c oxidase of the mitochondrial respiratory chain in cardiac myocytes (Strub *et al.*, 2011). Moreover, PHB is reported to reduce mitochondrial free radical production and oxidative stress in brain injury models, perhaps by stabilizing the function of complex I (Zhou *et al.*, 2012). Binding of S1P to PHB2 and its action on mitochondrial function represent a potential novel link between intracellular S1P binding and $I_{Cl,swell}$, an ion channel activated by ROS.

1.6. FTY720, an S1PR agonist

FTY720 is an immunomodulating drug, approved for treating multiple sclerosis. This agent modulates S1PR function and was highly effective in Phase II clinical trials for recurring multiple sclerosis (MS) (Kappos *et al.*, 2006). FTY720 displays structural similarity to S1P and efficacy as an immunosuppressant in models of autoimmune disease and in solid organ

transplantation. However, this sphingosine analog is a prodrug that must be phosphorylated *in vivo* by SphK2 (Zemann *et al.*, 2006) to form its active form, FTY720-phosphate (FTY720-P) (Albert *et al.*, 2005;Brinkmann *et al.*, 2002), that binds to four of the five G protein-coupled S1P receptor subtypes (Brinkmann, 2007). There are differences in the affinity of FTY720-P conformers for S1PRs. (*S*)-FTY720-P, but not (*R*)-FTY720-P or the parent FTY720, acts as a full agonist at $S1PR_1$ (EC_{50} = 0.3 nM), $S1PR_4$ (0.6 nM) and $S1PR_5$ (0.3 nM), with ~10-fold lower affinity for $S1PR_3$ (3.1 nM), but is inactive at $S1P_2$ (>10,000 nM) (Albert *et al.*, 2005;Brinkmann *et al.*, 2002;Mandala *et al.*, 2002). The differing receptor affinities and potencies of FTY720-P and S1P suggest they might induce distinct responses *in vivo*, either by G protein-coupled signaling or byfunctional antagonism upon internalization of S1P receptors (Brinkmann, 2009).

FTY720 has shown to have effects on heart as well. FTY720 slows spontaneous heart rate due to activation of the G protein-gated K^+ channel $I_{K,ACh}$ (GIRK) activation in mouse atrial myocytes (Koyrakh *et al.*, 2005). Although FTY720 is well-tolerated in humans, it produces a transient mild to moderate bradycardia (Schmouder *et al.*, 2006). This bradycardia may be due to FTY720-induced activation of $I_{K,ACh}$ in sino-atrial node, which would slow phase 4 depolarization, shift maximum diastolic potential to a more negative voltage, and shift threshold potential to a more positive voltage. Furthermore, FTY720 prevents ischemia-reperfusion (I/R) injury and I/R-associated arrhythmias. This cardioprotective effect of FTY720 is thought to result from activation of the Pak1 pathway (Egom *et al.*, 2010). Moreover, in diabetic heart up-regulation of $S1PR_1$ by FTY720 contributes to its agonist action, and FTY720 exerts functional antagonism by stimulating translocation of $S1PR_3$ from the plasmalemmal to the kinase C (PKC) (Brinkmann *et al.*, 2002). Furthermore, FTY720, preferentially distributes into HDL and inhibits the development of atherosclerotic lesions in $LDLR^{-/-}$ mice on a cholesterol-rich diet (Nofer *et*

al., 2007). These effect are mediated via S1PR. Although FTY720 did not change HDL or total cholesterol levels or the lipoprotein profile, it protected against the development of atherosclerosis (Nofer *et al.*, 2007). In addition, FTY720 was shown to effectively reduce the progression of atherosclerosis in apolipoprotein E-deficient mice on a high-cholesterol diet (Yin *et al.*, 2012;Tolle *et al.*, 2007).

1.7. Role of S1P in cardiac physiology and pathology

Heart disease is the leading cause of death in the United States. Alteration in lipid metabolism leading to pathological changes in cardiovascular system has been studied considerably, and lipid metabolites with beneficial effects on the heart also have been identified. The sphingolipid metabolites regulate the function of heart by acting as second messengers to activate various signaling pathway, and alterations in sphingolipid metabolism are implicated in many cardiovascular diseases, including congestive heart failure, atherosclerosis, and ischemia/reperfusion injury (Karliner & Brown, 2009).

Sphingolipid, especially ceramide and S1P are known to accumulate in various cardiac diseases (Chatterjee *et al.*, 2006;Levade *et al.*, 2001). Sphingolipids are implicated to have effect on cardiac function at the level of signal transduction. Both SphK1 and SphK2 are expressed in cardiac myocytes (Kohama *et al.*, 1998). Also three of the five known GPC $S1PR_{1-3}$ are expressed in heart. Figure 4 shows the action of the three S1P receptors in heart, with their respective Gprotein coupling and downstream effect on the heart function (Means & Brown, 2009). The differential expression of these receptors may contribute to differential cellular responses (Anliker & Chun, 2004;Ishii *et al.*, 2004).

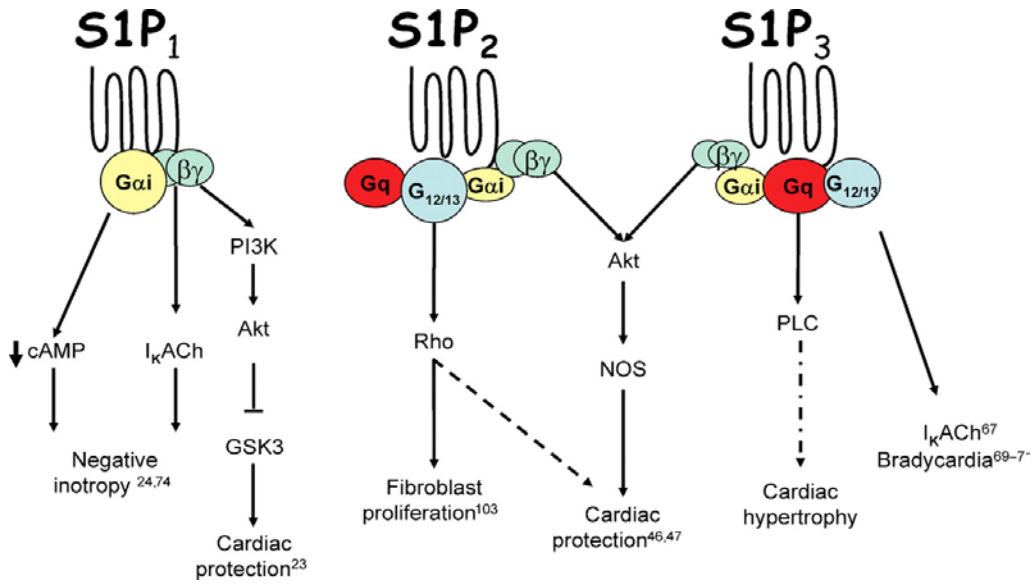

Figure 4: S1P receptor signaling in the heart(Means & Brown, 2009). For cited references, see Means, 2009.

In cardiac myocytes, the expression pattern is: $S1PR_1$ > $S1PR_3$ > $S1PR_2$ (Zhang *et al.*, 2007;Means *et al.*, 2008). The pattern is similar in vascular endothelial cells, but $S1PR_2$ levels are very low: $S1PR_1$ > $S1PR_3$ ≫ $S1PR_2$. In contrast, cardiac fibroblast $S1PR_3$ expression is the highest and $S1PR_2$ expression is minimal, whereas aortic smooth muscle cells have the highest expression of $S1PR_2$ (Means & Brown, 2009;Alewijnse *et al.*, 2004).

The cardiovascular effects of S1P signaling include hypertrophy, cardioprotection (Karliner *et al.*, 2001;Zhang *et al.*, 2007), mobilization of calcium, and effects on ion channels and contractility (Sugiyama *et al.*, 2000a;Sugiyama *et al.*, 2000b). Acting on the fibroblast, S1P mediates migration and proliferation, which are required for fibrosis and important in remodeling (Gellings *et al.*, 2009;Takuwa *et al.*, 2013). Additionally S1P can modulate vascular permeability, angiogenesis, and vascular tone (Chae *et al.*, 2004;Wymann & Schneiter, 2008;Visentin *et al.*, 2006). In the blood, S1P circulates bound to high density lipoprotein (HDL). S1P offers cardio-protective effects by inhibiting endothelial cell apoptosis, converging with the cardioprotective effects of HDL (Chatterjee *et al.*, 2006;Keul *et al.*, 2007). In contrast, pro-atherogenic properties of S1P have also been cited (Keul *et al.*, 2007).

1.7.1. S1P and Cardioprotection

S1P is known to be involved in cardioprotection in both the intact heart and isolated cardiac myocyte models of ischemia and hypoxia (Karliner *et al.*, 2001). Exogenously applied S1P enhanced the nenonatal rat cardiomyocyte survival during hypoxia (Karliner *et al.*, 2001). Similarly, S1P produced intracellularly by SphK in response to FM ganglioside had similar cardioprotection in response to hypoxia (Karliner *et al.*, 2001). Identical effect is observed in adult mouse ventricular myocytes models, and various $S1PR_1$ and $S1PR_3$ agonist and antagonist elicited predictable protective effect from hypoxia. These actions are attributed to activation of the PI3K signaling pathway, leading to activation of Akt and inhibition of GSK-3β (Zhang *et al.*, 2007). Similar to pharmacological approaches, knockout of sphingosine kinase 1 suggested that S1P protects the heart against hypoxic injury during glucose deprivation (Tao *et al.*, 2007).

Identical to studies in isolated cardiac myocytes, S1P was shown to be protective against I/R injury in intact heart. Both exogenous S1P and S1P generated by the stimulation of SphK activity offered cardioprotection against ischemic injury which was measured by cardiac function such as LVDP or creatine kinase release (Vessey *et al.*, 2008). Whereas the protection afforded by exogenous S1P administration was demonstrated to occur through a PKCε independent pathway (based on studies with PKCε knockout mice), protection afforded by intracellularly generated S1P required PKCε (Jin *et al.*, 2007). Levkau's group showed protection of heart against damage from 30 min ischaemia/24 h reperfusion following exogenous S1P administration (Keul *et al.*, 2007). Although it has been argued that $S1PR_1$ mediates cardioprotection against I/R injury (Karliner, 2004), based on studies with the specific $S1PR_1$ agonist SEW2871, however the role of other S1PR cannot be excluded (Tsukada *et al.*, 2007). There have been contradicting data about which receptors are involved in offering

cardioprotection. Means et. al. claims that, $S1PR_2$ and $S1PR_3$-mediated Akt activation protects against in vivo myocardial ischemia-reperfusion injury (Means *et al.*, 2007). This contradicts with Karliner's group which suggests the role of $S1PR_1$ instead.

1.7.2. S1P and Cardiac Electrophysiology

Sphingolipids are known to regulate the activity of several ion channels, both cation and anion channels, found in cardiac myocytes. For example, the voltage-dependent K^+ (K_v) channel, Kv2.1, is activated by the action of SMase D, which splits off choline from sphingomyelin and shifts the voltage-dependence of Kv2.1 activation negative by about ~30 mV; in contrast, SMase C, which removes the negatively charged phosphodiester as well as choline, suppresses Kv2.1 current by 90% in a voltage-independent fashion (Ramu *et al.*, 2006). Both SMase C and D strongly suppress the anion current carried by CFTR (Ramu *et al.*, 2007)). Ceramide a metabolite of sphingolipid, also modulates cardiac ion channels. The HERG (KCNH2) K^+ channel that underlies the rapid component of the delayed rectifier current, I_{Kr}, is down regulated by ceramide, an effect attributed to reduction of surface expression following protein ubiquitylation and lysosomal degradation (Chapman *et al.*, 2005)}. In contrast, membrane permeant C2-ceramide inhibited HERG without altering membrane expression, but HERG inhibition was reversed by antioxidants and the superoxide dismutase mimetic MnTBAP, which converts $O_2^{-\bullet}$ to H_2O_2 (Bai *et al.*, 2007). Findings with C2-ceramide suggested $O_2^{-\bullet}$ was responsible for inhibition of HERG in the experimental model. Also modulaton of gating HERG by C6-cermaide has been attributed by the translocation of the channel within lipid rafts (Ganapathi *et al.*, 2010). Ceramide also suppresses L-type Ca^{2+} channel current in ventricular myocytes (Schreur & Liu, 1997). Furthermore, our laboratory found that ceramide activated $I_{Cl,swell}$ in ventricular myocytes, but this was attributed to the metabolism of ceramide rather than this lipid itself (Raucci &

Baumgarten, 2009;Raucci & Baumgarten, 2010;Raucci *et al.*, 2010). The metabolic product of ceramide, S1P is also known to modulate various ion channel. S1P mediates both chronotropic and inotropic effects in the heart. Very early studies suggested S1P stimulates muscarinic receptor-activated inward rectifier K^+ current ($I_{K,ACh}$). The S1P induced current was inhibited by pertusis toxin suggesting a role of S1PR (Bunemann *et al.*, 1995). Furthermore, a different study in freshly isolated guinea pig, mouse, and human atrial myocytes suggested that S1P-induced $I_{K,ACh}$ activation by the $S1PR_3$ (Himmel *et al.*, 2000). This action is attributed to activation of $S1PR_3$ receptors and, as the antagonist of the receptor suramin, blocked these effects. However suramin is a G protein inhibitor which acts by preventing nucleotide exchange (Beindl *et al.*, 1996;Keul *et al.*, 2007;Nanoff *et al.*, 2002). Thus the receptor subtype cannot be confirmed by the effects of suramin. Also activation of $I_{K,ACh}$ by $S1PR_3$ is thought to explain S1P-induced in bradycardia in mice and humans (Sanna *et al.*, 2004) .However work from Landeen et.al demonstrated that $I_{K,ACh}$ can contribute to the negative inotropy following S1P activation of $S1PR_1$ (perhaps through Giβγ subunits in mice) (Landeen *et al.*, 2008). Moreover, recent work utilizing heterologously expressed receptors, human atrial myocytes, and phase I clinical trial data also argues that $S1PR_1$ rather than $S1PR_3$ is largely responsible for S1P-induced activation of $I_{K,ACh}$ and bradycardia in man and attributes the discrepancy with previous analyses to species-specific effects (Gergely *et al.*, 2012). FTY720, an S1PR agonist, mimics effects of S1P by activating $I_{K,ACh}$ (Koyrakh *et al.*, 2005). S1P slows down the pacemaker activity of the sinoatrial node cells *in vitro* and *in vivo* in majority of models, including human (Ochi *et al.*, 2006;Guo *et al.*, 1999). The chronotropic effects of S1P also are exhibited in mouse ventricular myocytes, where it causes cell shortening (Means *et al.*, 2008). Recently studies in anesthetized and conscious rats administered with two clinically tested S1P agonists, FTY720 or BAF312,

suggest that $S1PR_1$ mediates bradycardia while hypertension is mediated by $S1PR_3$ activation (Fryer *et al.*, 2012).

S1P also antagonizes the effect of isoproterenol-induced increase in cAMP and positive inotropy (Landeen *et al.*, 2008). These studies were done using the pharmacological agents SEW2871 agonist for $S1PR_1$ and VPC23019 antagonist for $S1PR_{1\&3}$, $S1PR_1$ receptor plays a predominant role in mediating the negative inotropic effects in mouse ventricular myocytes (Landeen *et al.*, 2008). However, the role of other the two S1P receptors cannot be ruled out.

Sphingolipids have been implicated in regulation of chloride channel in cardiac muscle, (d'Anglemont de Tassigny *et al.*, 2003). SMaseD induces inhibition of CFTR current, thereby modulating Cl^- channel in chronic inflammatory diseases (Ramu *et al.*, 2007). Furthermore it was demonstrated that ceramide inhibits CFTR-activity of the apical membrane (Ito *et al.*, 2004). In cultured keratocytes isolated from wounded rabbit corneas, S1P activates a lysophosphatidic acid (LPA)-activated Cl^- current that outwardly rectifies with a physiologic Cl^- gradient (Wang *et al.*, 2002). Although the authors claimed the current was the volume-sensitive Cl^- current, they did not demonstrate volume-sensitivity nor provide the pharmacological or biophysical evidence necessary to distinguish between Cl^- channels. In xenopus oocytes, both S1P and LPA activate an oscillatory Ca^{2+}-activated Cl^- current by receptor-dependent release of Ca^{2+} from an IP3-sensitive site (Noh *et al.*, 1998).

1.8. S1P and ROS signaling

Sphingolipid signaling is known to produce ROS in response to various stimuli. S1P generates ROS production in response to elevated transmural pressure in ex vivo model of isolated resistance vessels, from skeletal muscle. The initial elevation in ROS was entirely dependent on the activation of NADPH oxidase,(Keller *et al.*, 2006). Moreover $S1PR_1$ protein

expression is increased in cultured bovine aortic endothelial cells response to exogenous H_2O_2 without an alteration in mRNA levels (Igarashi *et al.,* 2007). Hence there could be a possible feedback response modulating ROS production by S1P receptors. There have been reports of $S1PR_1$ mediated activation of ROS signaling, that regulates the egress of both hematopoietic progenitors and BM stromal cells (Golan *et al.,* 2012). There have been studies where SphK is responsible for NOX production in neutrophils. This NOX production is shown to be Ca^{2+} store depletion-dependent SphK activation (Schenten *et al.,* 2011). Similarly in human leukemia cell line, inhibitor of SphK, DMPS, was responsible for apoptosis, and ROS were the critical regulators of caspase-8-mediated apoptosis in DMPS-treated leukemia cells (Kim *et al.,* 2009).

There is growing evidence that the sphingolipids also play a role in the cellular response to oxidative stress. I/R injury leads to ROS generation and apoptotic cell death, and ceramide generation has been implicated in mediating cell death in response to ROS in a variety of tissues, including liver (Llacuna *et al.,* 2006), brain (Ohtani *et al.,* 2004), and heart (Bielawska *et al.,* 1997). In the heart, I/R rapidly activates neutral sphingomyelinases in a ROS-dependent manner. In cardiomyocytes, there are two primary sources of ROS: NADPH oxidase (NOX) and the mitochondrial electron transport chain (ETC). Evidence shows that S1P can activate ROS production via NOX and increases H_2O_2 production in murine fibroblasts (Catarzi *et al.,* 2011). This response was PI-3K- and PKC-dependent and was similar to PDGF-induced NOX activation, and both $S1PR_1$ and $S1PR_3$ receptors and Gi signaling were involved. During hypoxia and pre-conditioning an increased mitochondrial ROS production is associated with sphingolipids, in experimental models in Fibroblastic L-cells (Lecour *et al.,* 2006). Furthermore, a similar increase in mitochondrial ROS is observed in a heart failure model (Lecour *et al.,* 2006;Llacuna *et al.,* 2006). Studies in SphK1 transgenic mice suggested that chronic activation

of SphK1-S1P signaling results in both pathological cardiac remodeling through ROS mediated by $S1PR_3$ activation and favorable cardioprotective effects (Takuwa *et al.*, 2010).

Furthermore, sphingolipid signaling cascades overlap significantly with modulators of $I_{Cl,swell}$. $I_{Cl,swell}$ is known to be spontaneously activated in various model of heart diseases. Also ROS is the key regulator of $I_{Cl,swell}$. Thus sphingolipid signaling is likely to play an important role in stimulating ROS production and activating $I_{Cl,swell}$ in a number of cardiovascular diseases. Thereby sphingolipid can act as effector molecules mediating cardiac function in pathophysiological conditions.

1.9. HL-1 cells as a potential tool to investigate $I_{Cl,swell}$

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 its contractile, morphological and electrophysiological characteristics (Claycomb *et al.*, 1998). Studies utilizing a variety of genetic, immunohistochemical, electrophysiological, and pharmacological techniques have demonstrated that HL-1 cells possess many of the features of adult atrial cardiomyocytes and are a useful experimental tool. For example, cardiac muscle cell structure and function have been assessed in HL-1 cells (McWhinney *et al.*, 2000), and calcium handling was studied using the three ryanodine receptor (RyR) mutations associated with stress-induced ventricular tachycardia in humans (George *et al.*, 2003). Electrophysiological studies in HL-1 cells characterized the delayed rectifier K^+ current (*I*Kr), (Claycomb *et al.*, 1998;Akhavan *et al.*, 2003), L- and T-type Ca^{2+} currents (Xia *et al.*, 2004), and pacemaker current (Sartiani *et al.*, 2002). HL-1 cells also have been used to study the cardiac response to pathological states, including hypoxia (Nguyen & Claycomb, 1999;Cormier-Regard *et al.*, 1998), ischemia/reperfusion injury (White *et al.*, 2004;Ruiz-Meana *et al.*, 2006), apoptosis (Kim *et al.*,

2003;Carlson *et al.*, 2002), and rapid pacing-induced electrical remodeling (Yang *et al.*, 2005). The rennin-angiotensin system machinery is expressed in HL-1 cardiomyocytes, and exogenous Ang II modulates 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 $I_{Cl,swell}$ 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 *et al.*, 2010c). Furthermore, our preliminary studies showed that S1P activated $I_{Cl,swell}$ in HL-1. Because this cell line would be amenable to molecular approaches initially considered, we decided to define the S1P signaling pathway responsible for the activation of $I_{Cl,swell}$ in the HL-1 cell model.

1.10. The objective of the present study

The objective of this study were the following (1) to determine the signaling mechanisms utilized by S1P to activate $I_{Cl,swell}$ in heart either via GPC-S1PR or via intracellular binding site, and (2) whether S1P activates $I_{Cl,swell}$, a ROS-activated current, by triggering ROS production by NOX, mitochondria or both. This work extends earlier studies in the laboratory demonstrating that SMase C and ceramide activate $I_{Cl,swell}$ (Raucci & Baumgarten, 2009). These studies demonstrated SMase-induced current is also suppressed by DCPIB, which under conditions that isolate anion currents is a specific inhibitor of $I_{Cl,swell}$. SMase-induced $I_{Cl,swell}$ was inhibited by ebselen, a membrane permeant glutathione peroxidase mimetic that breakdowns H_2O_2 to H2O. Thus, the role of ROS was demonstrated as a modulator of SMase-induced activation of $I_{Cl,swell}$. Preliminary data from our laboratory using whole cell patch clamp recording showed that exogenous S1P (500 nM, 10 min) activated outwardly rectifying Cl^- current with a reversal potential near E_{Cl}. This current was fully inhibited by addition of DCPIB (10 μM, 10 - 12 min) in the continued presence of S1P. Sphingosine is converted to S1P by the action of SphK. The

Sphingosine kinase inhibitor DL-*threo*dihydrosphginosine (10 μM, 15 – 20 min) fully inhibited bacterial SMase-induced $I_{Cl,swell.}$ Thus SMase induced production of sphingosine does not get converted to S1P due to the use of inhibitor of SphK. Thus, S1P rather than upstream sphingolipids must be responsible for the observed effects of SMase C and ceramide on $I_{Cl,swell}$. Moreover previously it is demonstrated that SMase-induced Cl^- current is mediated by ROS (Raucci & Baumgarten, 2009). Furthermore, the source of ROS modulating $I_{Cl,swell}$ in response to exogenous S1P application was tested. As found for the SMase-induced Cl^- current, the activation of $I_{Cl,swell}$ by S1P was completely inhibited by rotenone (10 μM, 15 – 20 min) a mitochondrial Complex I inhibitor. However, in contrast to SMase-induced $I_{Cl,swell}$, addition of apocynin (500 μM, 10 min) inhibitor of NADPH oxidase, in the continued presence of S1P partially reduced the S1P-induced Cl^- current. The following aims were addressed in this study SphK/S1P pathway and cardiac G protein coupled S1P receptors ($S1P_{1-3}$) modulate the activity of $I_{Cl,swell}$. S1P receptor signaling modulates $I_{Cl,swell}$ by ROS production via either NADPH oxidase, mitochondria or both. Furthermore the aim of this study was to test if S1P elicits $I_{Cl,swell}$ via its binding to intracellular target(s) or plasmalemmal S1P receptors.

Chapter 2 MATERIALS and METHODS

2.1 Culture of HL-1 cardiomyocytes

HL-1 cardiac myocytes passage 69-89, an immortalized mouse atrial cell line (Claycomb *et al.*, 1998), were used for the studies. Tissue culture dishes were first coated with a gelatin (Becton-Dickinson 214340) (0.02%)/fibronectin (Sigma F1141 comes as 1 mg/ml) (0.5%) mixture (3 mL in 60 x 15 ml mm dishes) and incubated overnight at 37°C. HL-1 cells were cultured in pre-coated dishes using Claycomb medium (Sigma-Aldrich 51800C) supplemented with 10% fetal bovine serum (Sigma-Aldrich, F2442), 100 μg/mL penicillin/streptomycin (Cellgro 30-002-CI), 0.1 mM norepinephrine (Sigma-Aldrich A0937), and 2 mM L-glutamine (Invitrogen/Life Technologies, 25030-081). The cells were kept at 37°C in an atmosphere of 5% CO_2 and 95% air at a relative humidity of approximately 95%. Once the cells reached confluence, they were passaged in a 1:3 split. To split the HL-1 cells, they were briefly rinsed with Dulbecco's phosphate buffered saline without Ca^{2+} or Mg^{2+} (DPBS; Quality Biological QBI14057101) and then incubated with 0.05% trypsin/0.481 mM EDTA-Na_2

(2 mL for 60 x 15 ml10 mm dish; Life Technologies, 25300120 & 25200056, respectively) at 37°C for 2 min until the cells dissociated from the flask surface. Double the amount of soybean trypsin inhibitor (25 mg/100 mL DPBS; Life Technologies, 17075029) was added directly to flask, and then the contents were transferred to a 15 mL centrifuge tube. The cells were centrifuged at 1100 rpm for 5 min, the supernatant was removed by aspiration, and the pellet was gently resuspended in 3 mL of supplemented Claycomb medium and incubated for 1 hour. For culture preparation, the cells were transferred to gelatin/fibronectin-coated dishes. For electrophysiological studies, the cells were spun after an hour incubation, the supernatant media removed and the pellet re-suspended in 5 ml of 1T bath solution.

2.2. Experimental solutions and drugs

Bath and pipette solutions were designed to isolate the Cl^- current. Isosmotic bath solution (1T; 300 mOsm/kg; T, times isosmotic) contained (in mM): 90 *N*-methyl-Dglucamine-Cl, 3 $MgCl_2$, 10 HEPES, 10 glucose, 5 CsCl, 0.5 $CdCl_2$, 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 (0.85T, 255 mOsm/kg) contained 60 mannitol. 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 freezing-point depression.

Stock solutions of D-*erythro*-sphingosine-1 phosphate in methanol (S1P; 1 mM, Cayman), FTY720 in ethanol (FTY; 0.5 or 10 mM, Cayman), SEW2871 in DMSO (SEW; 10 mM, Cayman), VPC23019 in DMSO (VPC; 130 nM or 13 µM, Tocris), CAY10444 or BML-241 in DMSO (CAY; 1 or 10 mM, Tocris), and DCPIB (20 mM; Tocris) in DMSO purged under

argon were frozen (-20°C) in aliquots until use. Gallein (10 mM, Tocris) in DMSO, stored at (−20°C) in alliquots until use, Rotenone (20 mM, Sigma-Aldrich). The NADPH oxidase inhibitor gp91ds-tat, a membrane permeant fusion peptide, was synthesized by the Tufts University Core Facility, and stocks in 150 mM NaCl plus 10 mM acetic acid were frozen (–20°C) in aliquots until use.

2.3. Whole cell patch clamp and electrophysiological recordings

HL-1 cells 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 were 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 – 10 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 3-5 min prior to the start of recording. Voltage clamp protocols and data acquisition were controlled by pClamp 8.2 (Axon). 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 the displayed I-V curves are from the

corresponding current traces. To minimize variability, experiments were designed to use cells as their own controls.

2.3.1. Intracellular application of agents:

In some cases, S1P and FTY720 were delivered via the patch pipette. In these studies, pipettes first were dipped in drug-free solution for 60 s, taking up a ~1 mm column of solution by capillary action. Then, the pipettes were backfilled with drug-containing media, and whole cell recordings were begun within ~2 min. Therefore, the initial traces were obtained under control condition, and the cell served as its own control.

The claim that diffusion of S1P (MW 379.5 Da) and FTY720 (MW 343.9 Da) were slow enough for the cells to serve as its their control is supported by the fact that the current was stable before drug-induced current activation. This assertion is also supported by numerical estimates. Einstein's classic theory of diffusion arising from Brownian motion and the Stokes-Einstein equation consider spherical solute particles that are much larger than their solvent (for detailed discussion, see (Hille, 2001;Crank, 1975)). Their analysis found that the diffusion coefficient, D, is inversely proportional to r^3, where r is the radius of the solute, and that r is proportional to M, the solute's molecular weight. Using glucose (MW 180 Da; observed D = 6.8 $\times$ 10^{-6} cm^2/s; (Finch, 1995).) as an index, the inverse cubic relationship between MW and D suggests that D for S1P and FTY720 should be 5.31 and 5.48 $\times$ 10^{-6} cm^2/s, respectively, similar to the observed D of 5.2 $\times$ 10^{-6} cm^2/s for cellobiose (MW 342 Da) (Finch, 1995). In a 1-dimensional system, as approximated by the nearly parallel sides of the pipette tip, the root mean-square diffusion distance, r_{rms}, equals $(2Dt)^{1/2}$. Thus, the time required for S1P or FTY720 to diffuse ~1 mm through the column of drug-free media is 942 and 912 s, or 15.7 and 15.2 min, respectively. These calculations should be regarded only as estimates, however. First, S1P and

FTY720 are not spherical and may remain associated with their hydrophobic solvent in pipette solution; both will alter D. Second, the height of the column is an estimate, and bulk flow during backfilling the pipette may have reduced the drug-free column height. Third, the time calculated here is for the r_{rms} drug concentration, and the "leading edge" of diffusion is faster. Nevertheless, the calculations suggest that the diffusion time for these agents is likely to be long compared to the time require to make a seal and begin recording, confirming the idea that the cell can be used as its own control.

2.4 Western Blots

2.4.1 Protein extraction for western blots

HL-1 cells were grown to 90% confluence, and the media was removed by aspiration. The dish was then rinsed with 10 mL of ice-cold PBS. Next, ice-cold RIPA cell lysis buffer (200 μL) was added [RIPA lysis buffer: 25 mM TRIS-HCl (pH 7.5), 150 mM NaCl, 1% NP-40 (detergent solution), 1 mM Na_3VO_4, and 1× Protease Inhibitor Cocktail (Sigma-Aldrich; P2714)]. To dislodge and break the cells, RIPA lysis buffer was repeatedly pipetted slowly. The resulting lysate was transferred into 2 ml Eppendorf tube, and was again pipetted 15-times. The cell lysis solution was then sonicated on ice for 10 s to break the cells apart. Next, the lysate was subject to centrifugation at 12,000 × g in a pre-cooled centrifuge for 15 min. The supernatant was immediately transferred to a fresh centrifuge tube, and the pellet was discarded. Protein concentration as determined with a BCA protein assay kit (Thermo Scientific; 23227), and the sample was divided into aliquots and stored at -80^{O}C for further use.

2.4.2 Immunoblotting for S1PRs

Total protein (30 μg) was loaded into 10% Bis-Tris polyacrylamide electrophoresis gels (Life Technologies) and after electrophoresis was subsequently transferred to a nitrocellulose membrane (Life Technologies). After the transfer, membranes were blocked for 2 h at room temperature in blocking buffer [Tris buffered saline plus Tween-20 (TBS-T); 10 mM Tris, 150mM NaCl, 0.1 % Tween-20, pH 7.5 with 10 % instant non-fat dried milk. The membrane was then incubated overnight at 4ºC in the same buffer with antibody, polyclonal rabbit anti-$S1PR_{1-3}$ (1:500; Santa Cruz' sc-25489, sc25491, sc-30024). The following day, membranes were washed four times for 10 min in TBS-T, blocked for 30 min, and subsequently incubated for 1 h in secondary antibodies using goat anti-mouse IgG-HRP (1:10,000; Santa Cruz; sc-2004). After four 10-min washes in TBS-T and two 10-min washes in TBS, immunodetection of S1PR protein was accomplished using an ECL system and X-ray film (exposure, 1-5 min). The following day, the membranes were stripped, blocked for 30 min at room temperature in Tris-buffered saline plus TBS-T with 3% instant non-fat dried milk and incubated for 2.5 h at room temperature in the same buffer with polyclonal, rabbit, anti-cyclophilin A (1:5,000; Millipore, 07-313). Membranes were then washed three-times for 10 min in TBS and subsequently incubated for 1 h in secondary antibodies using goat anti-rabbit IgG-HRP (1:5,000; Rockland, 611-1302). After three 10-min washes in TBS-T and two ten-min washes in TBS, immunodetection of cyclophilin-A protein was accomplished using an ECL system (exposure, 1-5 min).

2.4.3. Densitometry:

Densitometric analysis was used to quantify S1PR protein expression levels by determining the ratio of intensity of each band relative to that of goat anti-rabbit cyclophilin-A, which was used as a loading control. Analysis was done using Quantity One software and the VersaDoc Imaging System (BioRad).

2.5 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 ANOVA was performed followed by a Holm-Sidak test for all pair wise comparison. Non-linear curve fits were done in SigmaPlot 10.0 (Systat) using the Hill equation. In case of comparing the current densities of two independent groups, unpaired t test were used. In all case, $P < 0.05$ was taken as significant.

Chapter 3 Results

3.1. Exogenous sphingosine-1-phosphate activates a Cl^- current resembling $I_{Cl,swell}$ in HL-1 cardiac myocytes

As shown in *Figure 5*, sphingosine-1-phosphate (S1P; 500 μM, 5 – 10 min), a bioactive sphingolipid molecule, activated an outwardly-rectifying Cl^- current with a reversal potential near the Cl^- equilibrium potential (E_{Cl}), −43 mV, under isosmotic conditions. S1P increased current at +60 mV by 3.84 ± 0.87 pA/pF (n = 17, P <0.001), from 1.48 ± 0.18 to 5.32 ± 1.06 pA/pF, and a S1P-induced current was observed in >60% of the cells tested. Addition of DCPIB (10 μM, 5-10 min), a highly selective $I_{Cl,swell}$ blocker (Decher *et al.*, 2001), inhibited the S1P-induced Cl^- current by 113 ± 8% (n = 5, P < 0.001) in the continued presence of S1P, and there was no significant difference between the DCPIB-inhibited and control currents (n = 5). The greater than 100% block of S1P-induced current by DCPIB suggests that there may have been a small amount of basal activation of the current under control condition, as previously reported in adult myocytes (Baumgarten & Clemo, 2003;Sorota, 1992).One of the characteristic property of $I_{Cl,swell}$ is that it is outwardly rectifying under both physiological as well as symmetrical chloride gradients, distinguishing it from other Cl^- currents, such as CFTR, that exhibit linear current

voltage relationships with a symmetrical chloride gradient (Hume *et al.*, 2000). S1P-induced current was outwardly rectifying under symmetrical as well as physiological chloride solutions.

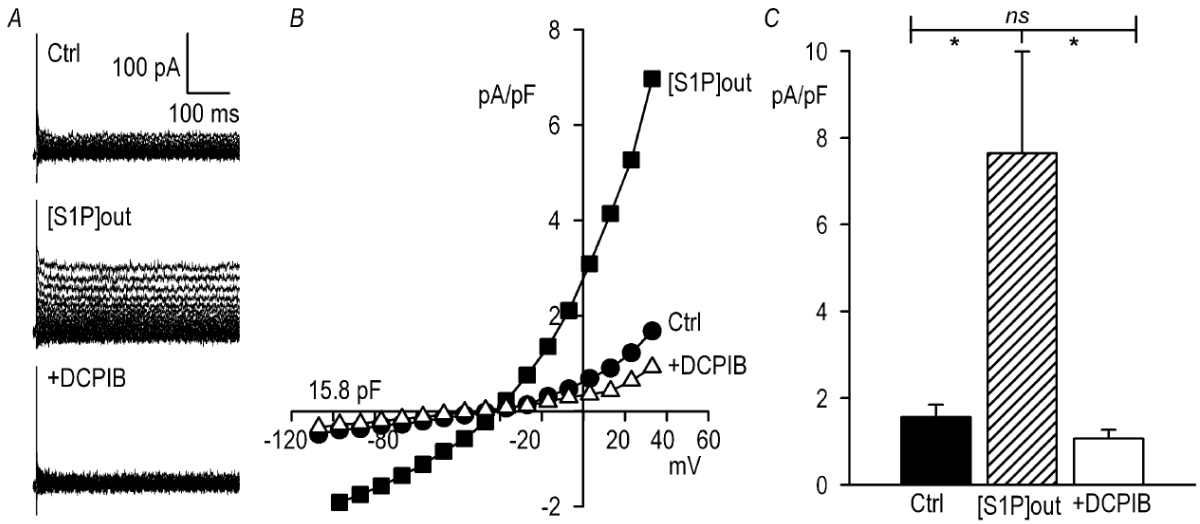

Figure 5: Sphingosine-1 phosphate (S1P) elicited a Cl^- current in HL-1 atrial cardiac myocytes that resembled $I_{Cl,swell}$. (*A*) Families of currents under control conditions (Ctrl), and after treatment with 500 nM [S1P]out for 10 min, and after addition of DCPIB (+DCPIB; 10 μM, 5 min) in the continued presence of S1P. Each cell was used as its own control; holding potential, −60 mV; test potentials, −100 to +60 mV. (*B*) Current-voltage (I-V) relationships for *A*; cell capacitance, 15.8 pF. (*C*) Current densities at +60 mV. S1P increased Cl^- current by 3.84 ± 0.87 pA/pF (n = 14, P < 0.001). The S1P-induced current was inhibited by 113 ± 8% (n = 5, P < 0.001) by the $I_{Cl,swell}$-specific inhibitor DCPIB. One-way repeated measures ANOVA and Holm-Sidak test were used for all pairwise comparison.

Figure. 6 shows the response to exogenous S1P when Cl^- was 100 mM in both bath and pipette solutions (i.e., symmetrical). The Cl^- current elicited by S1P (500 nM) continued to outwardly rectify. Thus, the S1P-induced current showed both outward rectification under physiological and symmetrical Cl^- conditions and block by DCPIB. These features are diagnostic for $I_{Cl,swell}$.

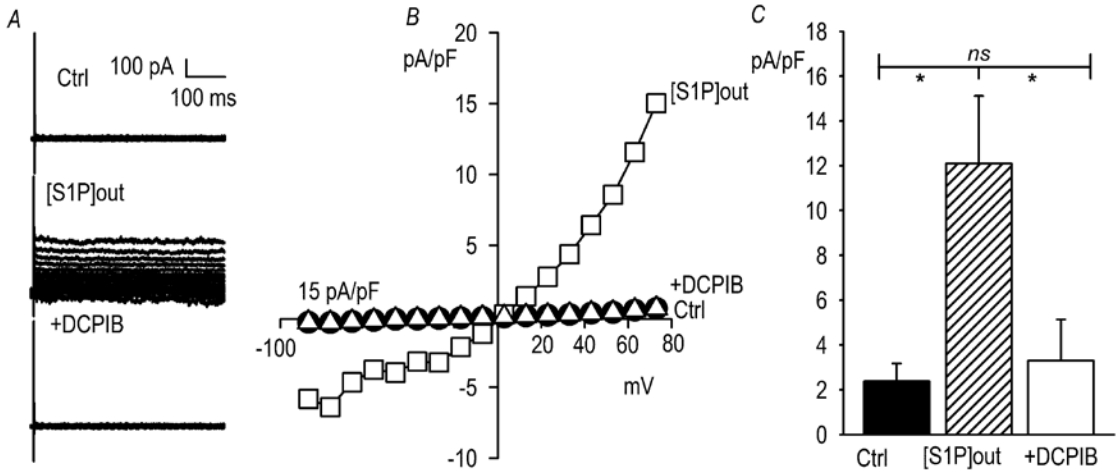

Figure 6: Sphingosine-1-phosphate (S1P) elicited a Cl^- current in HL-1 atrial cardiac myocytes in symmetrical chloride solution. (*A*) Families of currents under control conditions (Ctrl), and after treatment with 500 nM [S1P]out for 10 min, and after addition of DCPIB (+DCPIB; 10 μM, 5 min) in the continued presence of S1P. Cl^- concentrations: bath, 100 mM; pipette, 100 mM. (*B*) I-V relationships for *A*. (*C*) Current densities at +60 mV. S1P increased Cl^- current by 12.01 ± 2.21 pA/pF (n = 4, P < 0.001). The S1P-induced current was inhibited by 104 ± 18% (n = 4, P < 0.001) by DCPIB. The mean current shown in C represents the unpaired comparison of the data. However the percent values of inhibition by DCPIB is derived from paired analysis, where the cell is used as its own control.

3.2. Role of G protein coupled-S1P receptors

Most of the actions of S1P are mediated by its binding to G protein-coupled S1P receptors (Means & Brown, 2009;Karliner & Brown, 2009). S1P is produced inside cells by the action of sphingosine kinases, and it is transported out of cells via ABC cassette binding proteins known as ABC transporters. S1P then acts as a ligand for a family of five G protein-coupled receptors, termed $S1PR_{1-5}$, formerly called endothelial differentiation gene (EDG) receptors. Three ($S1P_{1-3}$) out of the five receptors are present in heart (Means & Brown, 2009).

3.2.1 S1P receptor agonists elicit $I_{Cl,swell}$

Previous work in our laboratory demonstrated that the G protein-coupled angiotensin II AT_1 receptor, the endothelin ET_A receptor, and the epidermal growth factor EGF receptor activate $I_{Cl,swell}$ in response to osmotic swelling and mechanical stretch (Browe & Baumgarten, 2004;Ren *et al.*, 2005;Browe & Baumgarten, 2005;Browe & Baumgarten, 2006). To test the hypothesis that S1P also activated $I_{Cl,swell}$ via G protein coupled receptors, we initially made use of receptor agonists. SEW2871, a small heterocyclic compound, and FTY720, an S1P analog, are agonists of the $S1PR_1$ (Tsukada *et al.*, 2007) and $S1PR_{1\&3}$ (Brinkmann *et al.*, 2002;Brinkmann, 2009), respectively. If $S1P_1$ and/or $S1P_3$ are involved in the activation of $I_{Cl,swell}$, by S1P, these compounds should act as mimetics and also activate the current As shown in *Figure 7*, SEW2871 (SEW; 1 μM, 5 – 10 min), activated an outwardly-rectifying Cl^- current with a reversal potential near E_{Cl} under isosmotic conditions. Current at +60 mV increased by 2.69 ± 0.62 pA/pF (n = 5, P <0.001), from 1.98 ± 0.38 to 4.66 ± 0.1 pA/pF.

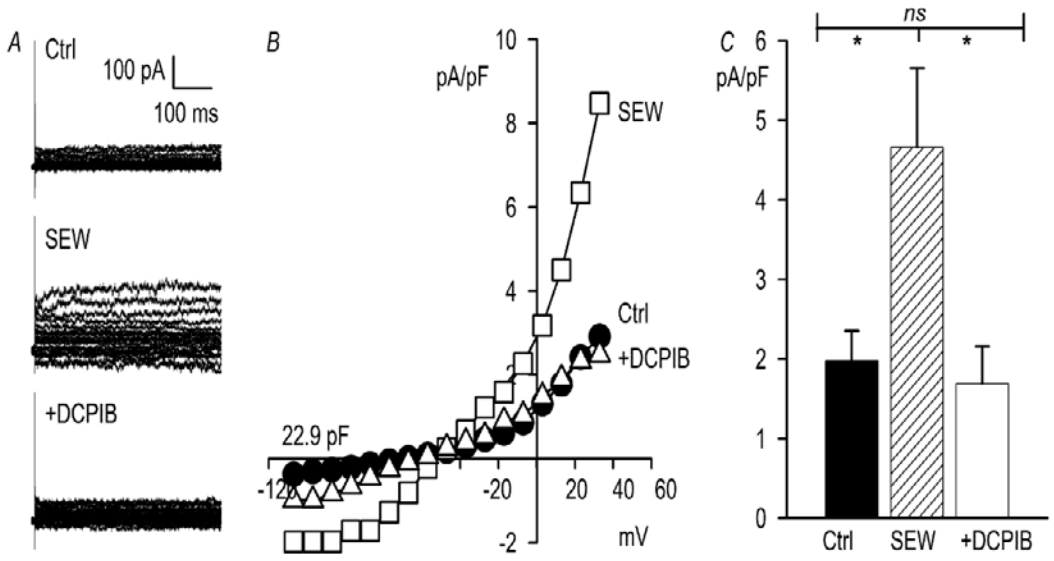

Figure 7 SEW2871, a selective agonist for S1PR$_1$, elicited $I_{Cl,swell}$. (*A*) Families of currents under control conditions (Ctrl), and after treatment with 1 μM exogenous SEW2871 (SEW) for 10 min, and after addition of DCPIB (+DCPIB; 10 μM) in the continued presence of SEW. (*B*) I-V relationships for *A*. (*C*) Current densities at +60 mV. SEW increased Cl^- current from 1.98 ± 0.38 to 4.66 ± 0.1 pA/pF (n = 5, P < 0.001). DCPIB inhibited SEW-induced current by 103 ± 12%.

Addition of DCPIB (10 μM, 5-10 min), a highly selective $I_{Cl,swell}$ blocker, inhibited the SEW-induced Cl^- current by 103 ± 12 % (n = 5, P < 0.001) in the continued presence of SEW, and there was no significant difference between the DCPIB-inhibited and control currents. Because SEW2871 is a selective $S1PR_1$ agonist (Tsukada *et al.*, 2007), these data are consistent with the idea that G protein-coupled S1PR and specifically $S1PR_1$ was responsible for the activation of $I_{Cl,swell}$ by exogenous S1P.

FTY720 (FTY), an $S1PR_{1\&3}$ agonist, is used as an immunosupressor in the treatment of multiple sclerosis (Kappos *et al.*, 2006). Based on the response to exogenous SEW2871, an $S1PR_1$ agonist, we predicted that exogenous FTY720 also would activate $I_{Cl,swell}$. The literature indicates that 100 nM exogenous FTY720 ([FTY]out) is sufficient to activate $S1PR_{1\&3}$ and their downstream signaling targets (Egom *et al.*, 2010;Forrest *et al.*, 2004;Yin *et al.*, 2012), including $I_{K,ACh}$ in cardiac myocytes (Fryer *et al.*, 2012;Koyrakh *et al.*, 2005). As shown in *Figure 8*, however, 100 nM [FTY]out (10 min) failed to activate $I_{Cl,swell}$ under isoosmotic condition. To verify that the channels responsible for $I_{Cl,swell}$ were present, 100 μM H_2O_2, was added to the same cells in the continued presence of FTY. H_2O_2 previously was shown by our lab to activate $I_{Cl,swell}$ under isoosmotic conditions and acts downstream in the signaling cascade (Ren *et al.*, 2008;Deng *et al.*, 2010c). Despite their insensitivity to 100 nM [FTY]out, $I_{Cl,swell}$ in the same cells was activated by 100 μM H_2O_2, confirming the expression of the channels underlying this current. Moreover, increasing the applied concentration 10-fold to 1 μM [FTY]out also failed to significantly evoke $I_{Cl,swell}$ (n = 3, P = 0.338 data not shown).

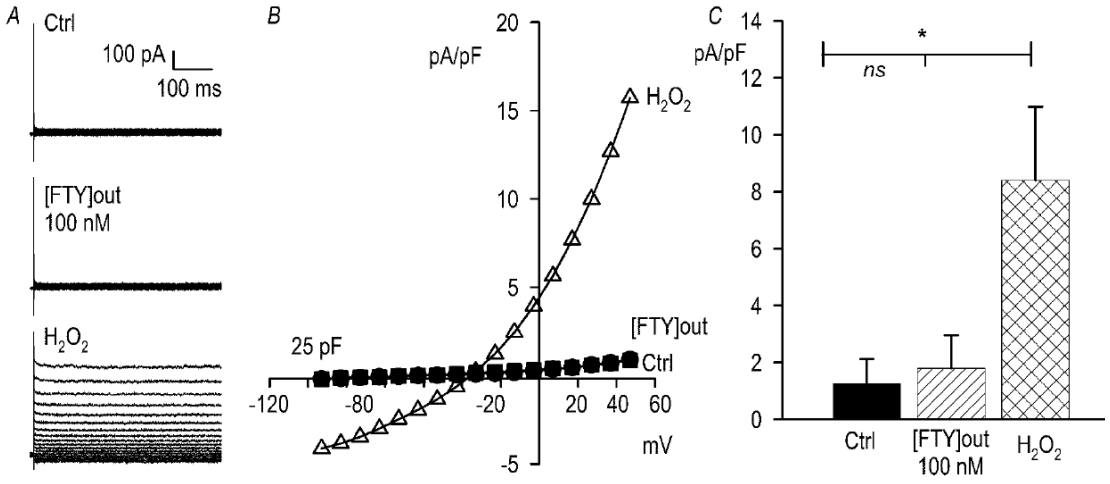

Figure 8: Exogenous FTY720, an agonist for $S1PR_{1\&3}$, failed to activate $I_{Cl,swell}$ (*A*) Families of currents under control conditions (Ctrl), and after treatment with 100 nM [FTY]out for 10 min, and after addition of H_2O_2 (100 μM) in the continued presence of FTY. (*B*) I-V relationships for *A*. (*C*) Current densities at +60 mV. FTY failed to activate significant current under isosmotic conditions. In the same cells, H_2O_2 (100 μM) increased Cl^- current from 1.72 to 5.15 ± 1.71 pA/pF (n = 4, P < 0.001) demonstrating that the $I_{Cl,swell}$ was expressed and could be activated by a downstream effector.

Figure 9 illustrates the response to 10 μM [FTY]out, a 100-fold higher concentration than that nominally required to activate G protein receptor-coupled signaling. At this very high concentration, [FTY]out increased $I_{Cl,swell}$, from 0.93 to 4.30 ± 0.39 pA/pF at + 60 mV (n = 4, P < 0.001), and the FTY-induced current was completely blocked by DCPIB (101 ± 8%). Thus, although exogenous FTY720 was able to elicit $I_{Cl,swell}$, the action of this agent appeared to have an exceptionally low potency compared to other published measures of FTY720-induced $S1P_{1\&3}$ signaling.

FTY720 is a prodrug and must be taken up, phosphorylated by intracellular SphK2 to its active phosphorylated form, FTY720-P, and then exported via an ABC transporter before it can reach plasmalemmal S1PR that face the extracellular environment (Brinkmann, 2007). Once phosphorylated and exported, FTY720-P can bind to and activate all of the G protein-coupled S1PR except $S1PR_2$. This raised the possibility that uptake, phosphorylation or export of FTY720 were defective. When exogenous FTY720 was applied, such defects might substantially lower the concentration of FTY720-P at the cell membrane surface and, thereby, both might significantly reduce the G protein receptor-dependent signaling necessary to evoke $I_{Cl,swell}$ and the potency of exogenous FTY720. If this was the case, exogenous application of FTY720-P should bypass the postulated defects and should potently elicit $I_{Cl,swell}$ under isosmotic conditions, as was observed with both exogenous S1P and the S1PR1 agonist SEW2871. To the contrary, 100 nM [FTY-P]out also failed to significantly activate $I_{Cl,swell}$. (n = 3, P = 0.104 data not shown). Moreover, *Figure 10* shows a comparison of FTY-P 100 nM vs. FTY-P 10 μM. FTY-P (100 nM) activated significantly less current, 1.90 ± 0.34 pA/pF, than FTY-P (10 μM), 4.92 ± 0.97 pA/pF. There was no statistical difference between current activated at Ctrl conditions vs. [FTY-P]out (100 nM)-induced current (not shown). However there was statistical

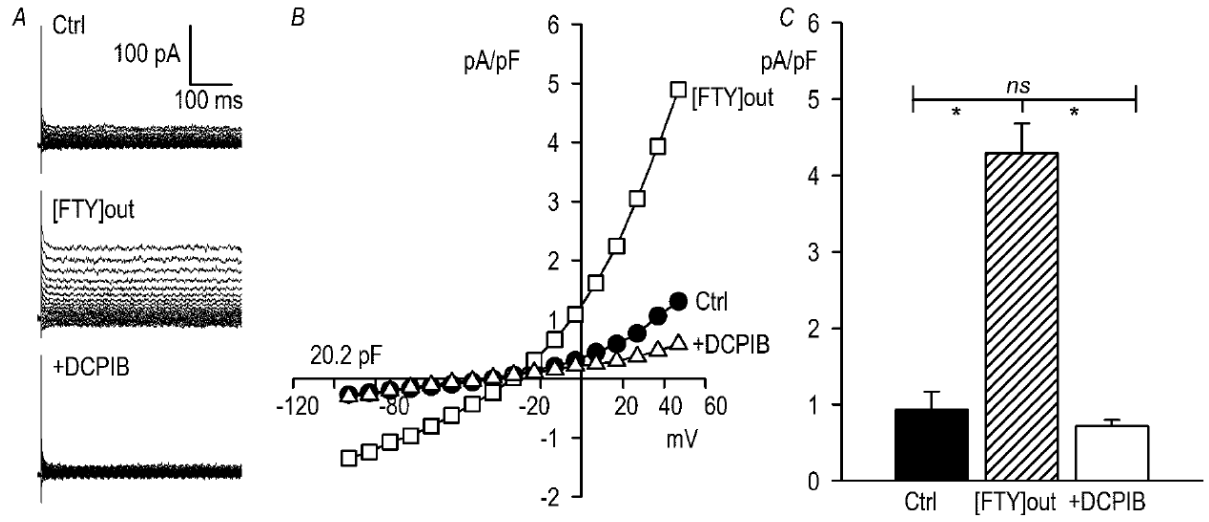

Figure 9: FTY720, a selective agonist for S1PR$_{1\&3}$, elicited $I_{Cl,swell}$. (*A*) Families of currents under control conditions (Ctrl), and after treatment with 10 μM [FTY]out for 10 min, and after addition of DCPIB (+DCPIB; 10 μM) in the continued presence of FTY. (*B*) I-V relationships for *A*. (*C*) Current densities at +60 mV. FTY increased Cl^- current from 0.93 to 4.30 ± 0.39 pA/pF (n = 14, P < 0.001). DCPIB inhibited FTY-induced current by 101 ± 8% (n = 4).

difference between the current activated by [FTY-P]out (10 μM).vs. control as shown in *Figure 13*. where FTY-P (10 μM) increased the current from 1.77 to 4.92 ± 0.61 pA/pF ($n = 5$, $P < 0.001$). This argues that failure of the uptake, phosphorylation of FTY720 or export of FTY720-P are not sufficient to explain the observed insensitivity to FTY720 and FTY720-P.

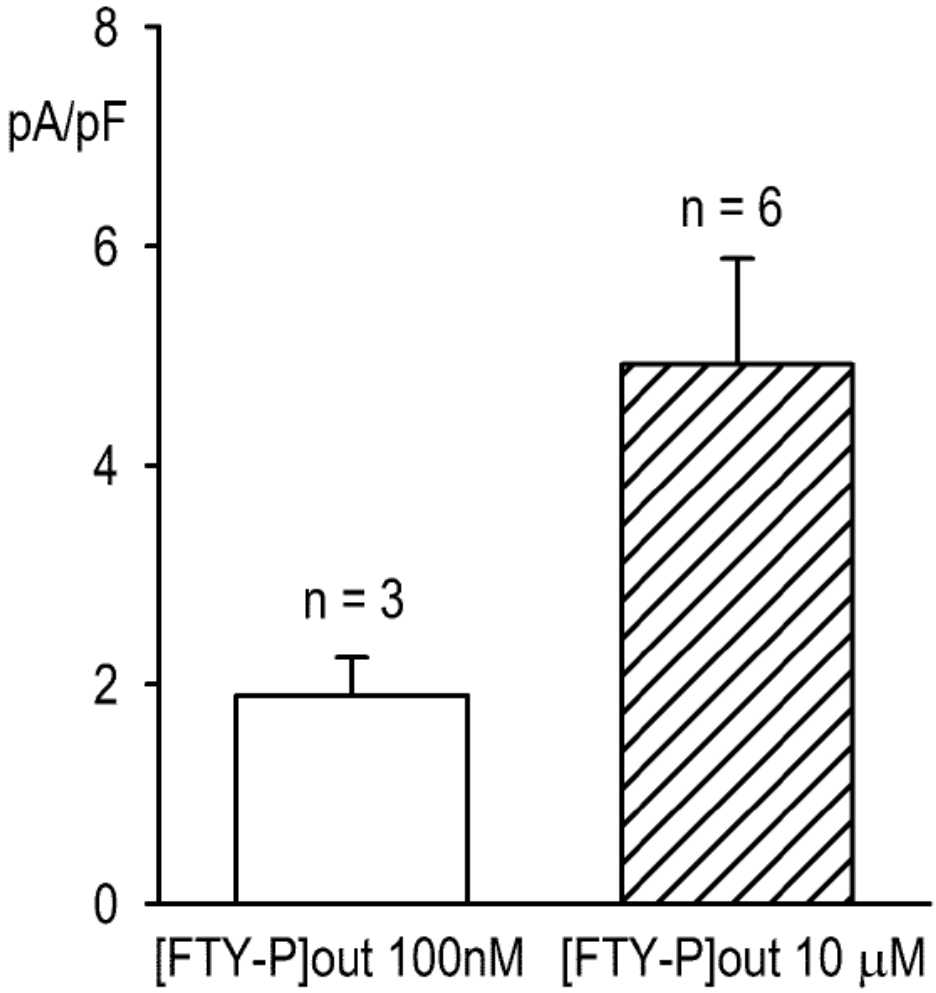

Figure 10: Extracellular FTY-P- (100 nM) vs. FTY-P (10 μM)- induced current. Comparison of $I_{Cl,swell}$ current density with FTY-P (10 μM, 10 min) (n = 5) applied outside of myocytes elicited significantly greater $I_{Cl,swell}$ than FTY-P (100 nM) (n = 5) in the two groups of cells (P = 0.036). FTY-P (100 nM) activated significantly less current, 1.90 ± 0.34 pA/pF, than FTY-P (10 μM), 4.92 ± 0.97 pA/pF. Mann-Whitney Rank Sum Test was utilized for statistical analysis. There was no statistical difference between current activated at Ctrl conditions vs. [FTY-P]out (100 nM)-induced current (not shown). However there was statistical difference between the current activated by [FTY-P]out (10 μM).vs. control as shown in Figure 13. Where FTY-P (10 μM) increased the current from 1.77 to 4.92 ± 0.61 pA/pF (n = 5, P < 0.001).

3.2.2 Inconsistencies in block of S1P- and FTY720-induced activation of $I_{Cl,swell}$ by nominally specific S1P receptor antagonists.

The ability of exogenous S1P and a high concentration of exogenous FTY720 to activate $I_{Cl,swell}$ under isosmotic conditions raised the possibility that S1PR are involved in signaling pathway that evoked channel activation but could not simply explain the low potency of FTY720 nor the insensitivity to FTY720-P. To further explore the role of S1PR, we determined whether well-defined SIPR antagonists had their expected effects on S1P- and FTY720-induced $I_{Cl,swell}$ activation. Exogenous S1P is expected to stimulate all three cardiac S1PR ($S1PR_{1-3}$), whereas exogenous FTY720 is expected to stimulate only $S1PR_{1\&3}$.

First, we examined the action of CAY10444 (CAY), a selective antagonist for $S1PR_3$ in the presence of [S1P]out. CAY10444 is a selective antagonist of S1P binding to the S1PR3 receptor. For example, CAY strongly suppresses reduced $S1PR_3$-dependent S1P-induced calcium increase in HeLa cells (Pyne & Pyne, 2011). Also CAY inhibits, increase in intracellular calcium mediated by P2 receptor or α1A-adrenoceptor stimulation in CHO cells and reduces α1A-adrenoceptor stimulated contraction of mesenteric artery by acting on $S1PR_3$.(Jongsma *et al.*, 2006). As seen in *Figure 11*, in the presence of 500 nM extracellular S1P, CAY (10 μM, 10 min) inhibited the S1P-induced current by 90 ± 10%. These data are consistent with the notion that S1P elicits $I_{Cl,swell}$ by acting a ligand for $S1PR_3$ and, thereby, triggering a signaling pathway that is responsible for fully activating the current. On the other hand, we expect that S1P also will have activated $S1PR_1$, and previously we showed that an $S1PR_1$ agonist, SEW, activated $I_{Cl,swell}$. Such effects via $S1PR_1$, should not have been abrogated by a CAY, a selective $S1PR_3$ antagonist.

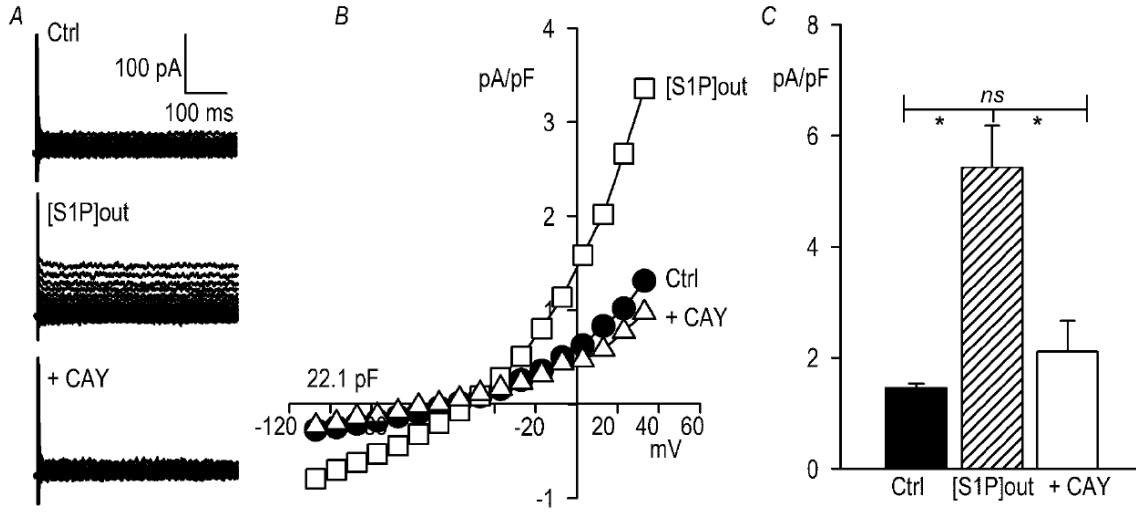

Figure 11: CAY10444, a selective antagonist for $S1PR_3$, blocked S1P-induced $I_{Cl,swell}$. (*A*) Families of currents under control conditions (Ctrl), and after treatment with 500 nM [S1P]out for 10 min, and after addition of CAY10444 (+CAY; 10 μM) in the continued presence of extracellular S1P. (*B*) I-V relationships for *A*. (*C*) Current densities at +60 mV. S1P increased the current from 1.46 to 5.43 ± 0.67 pA/pF (n = 5, P < 0.001). S1P-induced current was blocked 90 ± 10% by CAY, and current after block was not significantly different than the Ctrl.

To further test whether $S1PR_1$ and/or $S1PR_3$ modulate $I_{Cl,swell}$, we utilized VPC23019 (VPC), an antagonist for $S1PR_{1\&3}$ (Davis *et al.*, 2005). Contrary to the expectation, VPC (13 μM, 10 min) failed to block the S1P-induced current. *Figure 12* shows that [S1P]out-activated outwardly rectifying chloride current under isoosmotic conditions and that VPC had no effect on $I_{Cl,swell}$ in the continued presence of S1P. Nevertheless, the S1P-induced current was completely blocked by DCPIB in the presence of VPC, confirming that the current activated by S1P was $I_{Cl,swell}$.

The next step was to test the role of these antagonists in the presence of the agonist $S1PR_{1\&3}$, FTY720. CAY which blocked the [S1P]out-induced current, failed to block the current activated by FTY720. These inconsistencies could be attributed to the conformational change imparted by FTY on the receptors and therefore inability of CAY to block the current. Nonetheless other site of action of FTY could not be excluded.

Next we tested whether the action of antagonists on FTY720-P induced current was consistent with S1PR actions. As seen in *Figure 13*, FTY720-P (10 μM, 10 min) activated $I_{Cl,swell}$ under isoosmotic condition. CAY10444 (10 μM, 10 min), a selective antagonist for $S1PR_3$, failed to block the FTY-elicited current despite the fact that CAY10444 fully inhibited the S1P-induced current (see Fig. 11). The FTY-P-induced current was completely blocked by DCPIB in the presence of CAY, however, showing it was $I_{Cl,swell}$. In addition, we tested whether the $S1PR_{1\&3}$ antagonist. VPC23019 (13 μM, 10 min) affected the response to extracellular FTY (*Figure 14*). As seen with CAY (in response to [FTY-P]out) (Fig. 13), VPC also failed to inhibit the [FTY]out induced current.

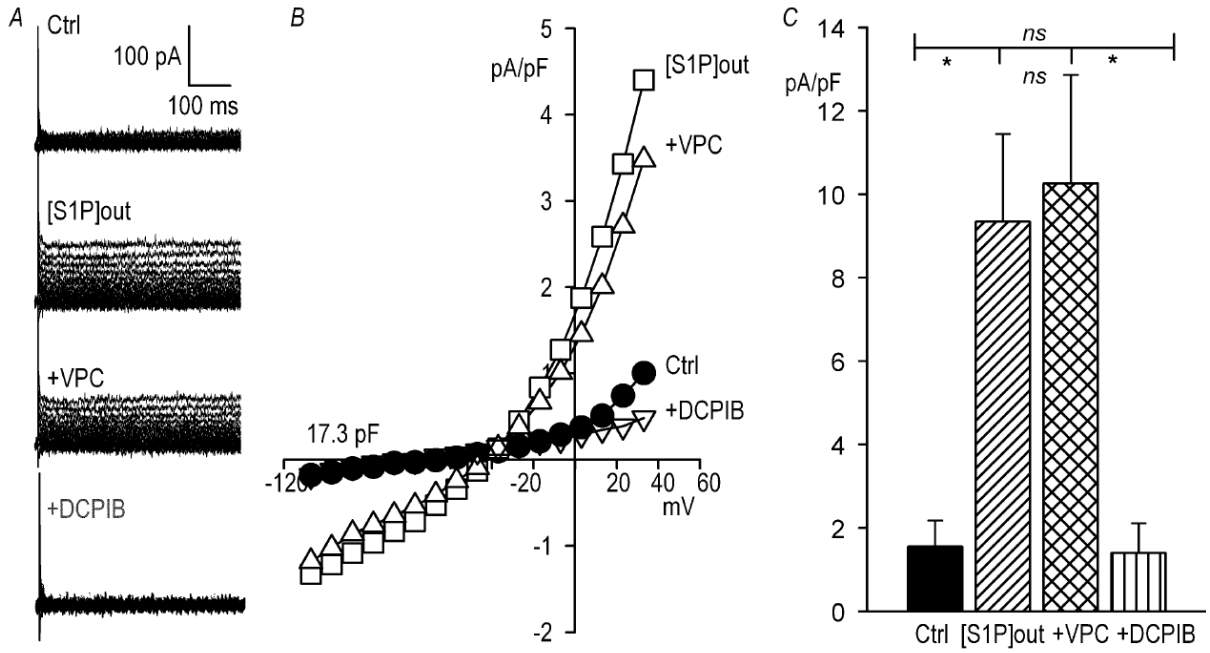

Figure 12: VPC23019, a selective antagonist for $S1PR_{1\&3}$, failed to block S1P-induced $I_{Cl,swell}$ (*A*) Families of currents under control conditions (Ctrl), and after treatment with 500 nM [S1P]out for 10 min, and after addition of VPC23019 (+VPC; 13 μM) in the continued presence of extracellular S1P. (*B*) I-V relationships for *A*. (*C*) Current densities at +60 mV. S1P increased the current from 1.55 to 9.34 ± 1.48 pA/pF (n = 4, P < 0.001). VPC failed to block S1P-induced current. S1P-indcued current was inhibited 102 ± 11%, by DCPIB.

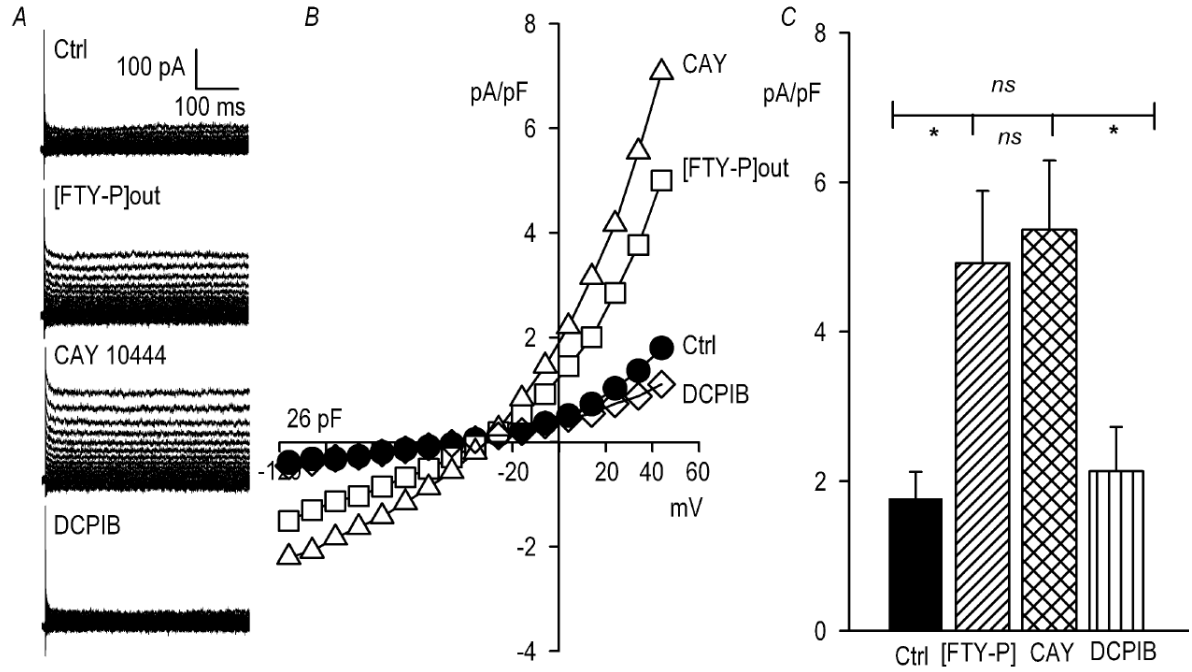

Figure 13: CAY10444, a selective antagonist for $S1PR_3$, failed to block [FTY-P]out-induced $I_{Cl,swell}$. (*A*) Families of currents under control conditions (Ctrl), and after treatment with 10 µM [FTY-P]out for 10 min, and after addition of CAY10444 (+CAY; 10 µM) in the continued presence of FTY-P. (*B*) I-V relationships for *A*. (*C*) Current densities at +60 mV. FTY-P increased the current from 1.77 to 4.92 ± 0.61 pA/pF (n = 5, P < 0.001). CAY failed to block FTY-P-induced current, whereas the FTY-P-induced current was inhibited 81 ± 16%, by DCPIB.

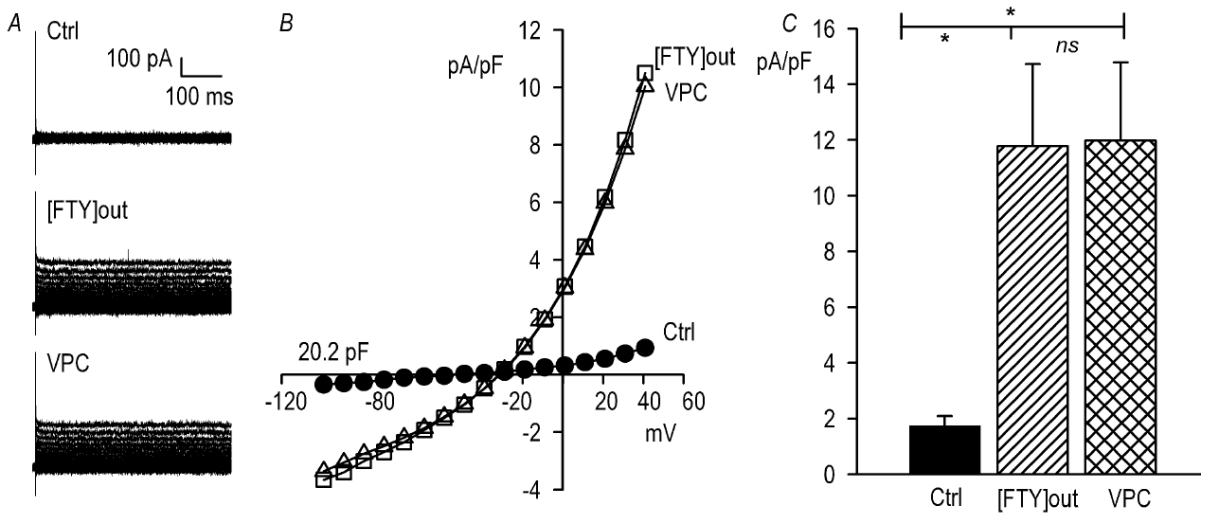

Figure 14: VPC23019, a selective antagonist for S1PR$_1$&$_3$, failed to block [FTY]out-induced $I_{Cl,swell}$. (*A*) Families of currents under control conditions (Ctrl), and after treatment with 10 μM [FTY]out for 10 min, and after addition of VPC23019 (+VPC; 10 μM) in the continued presence of FTY. (*B*) I-V relationships for *A*. (*C*) Current densities at +60 mV. FTY increased the current from 1.70 to 13.73 ± 2.64 pA/pF (n = 4, P < 0.001). VPC failed to block FTY-induced current.

Taken together, the effects of putative S1PR agonists and antagonists cannot be explained by their nominal actions on S1PR and instead raise the possibility that the target(s) of S1P, FTY720, SEW and CAY that modulate $I_{Cl,swell}$ are not the G protein-coupled S1P receptor family but rather distinct binding site(s). Alternatively, it has been proposed that S1PR might exist in different conformational states that regulate the affinity, potency, or efficacy of S1PR and thereby explain the unusual pharmacology of receptor ligands that could otherwise be interpreted as “off-target” effects (Pyne & Pyne, 2011). We have not tested the possibility that S1PR exist in alternative conformational states in HL-1 cardiomyocytes.

3.3. Intracellular vs. Extracellular S1P-induced current

Most of the biological effects of S1P are explained by its action as a ligand for plasmalemmal G protein-coupled S1PR and the resulting activation of downstream signaling pathways. More recently, however, various intracellular binding targets for S1P have been recognized (Maceyka *et al.*, 2012;Strub *et al.*, 2011;Yester *et al.*, 2011). Based on the observed inconsistencies in the regulation of $I_{Cl,swell}$ by nominally S1PR selective agonists and antagonists, we considered the possibility that intracellular target(s) of S1P and its mimetics were responsible in modulating $I_{Cl,swell}$. To test this idea, we introduced S1P inside ([S1P]in). HL-1 myocytes via open tip of the patch pipette. Because the pipette contains a much larger volume than that of the myocyte, it effectively dialyze the cell, and as found for other mobile species, we expect that S1P in the pipette and cytosol will equilibrate As before, selected S1PR antagonists were utilized after exposure to S1P. Failure of S1PR antagonists to suppress current activation by [S1P]in would add support to the idea that S1P acted at intracellular targets rather than at extracellular S1PR after ABC transporter-mediated export.

Figure 15 illustrates the results of applying 500 nM S1P inside the cell via the patch pipette. Because the first ~1 mm of the pipette tip was filled with S1P-free media and diffusion over this distance is slow (see Methods), control current was recorded immediately after breaking into the cell, and then, the current elicited by [S1P]in was obtained. [S1P]in activated an outwardly rectifying Cl^- current that reversed near E_{Cl}. Addition of CAY (10 or 30 μM) to the bathing media during continued dialysis of the cytosol did not inhibit the [S1P]in-induced current. However, DCPIB blocked 94 ± 5% of the [S1P]in-induced current, implying the current was $I_{Cl,swell}$.

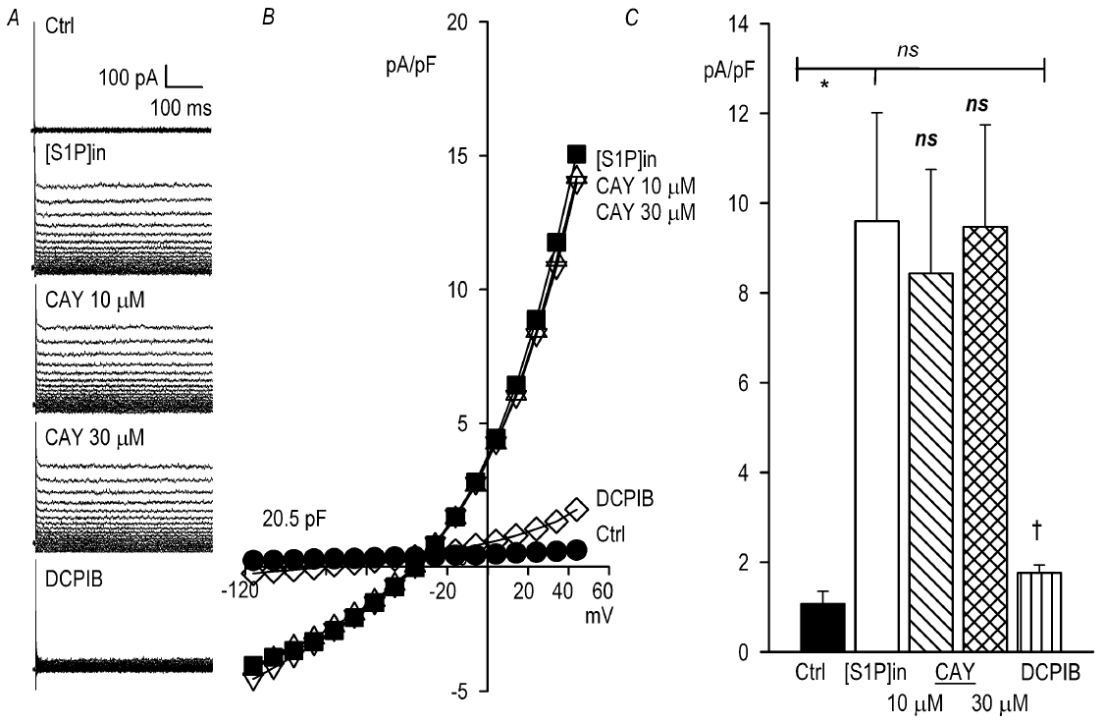

Figure 15: CAY10444, a selective antagonist for $S1PR_3$, failed to block [S1P]in-induced $I_{Cl,swell}$. (*A*) Families of currents under control conditions (Ctrl), and after treatment with 500 nM [S1P]in for 10 min, and after addition of CAY10444 (+CAY; 10 or 30 μM) in the continued presence of [S1P]in. (*B*) I-V relationships for *A*. (Ctrl; filled circle, [S1P]in; open square, CAY 10 μM; open triangle, CAY 30 μM; inverted open triangle, DCPIB; filled diamond.) (*C*) Current densities at +60 mV. [S1P]in increased the current from 1.20 to 10.33 ± 1.75 pA/pF (n = 4, P < 0.001). The [S1P]in-induced current was not significantly blocked by 10 or 30 μM CAY (ns), but was blocked by DCPIB by 94.18 ± 5.20% (P < 0.001). Insensitivity of [S1P]in-induced current contrasts with its block of [S1P]out-induced current (see Fig. 10). DCPIB blocked current was significantly different from [S1P]in-induced current (†), but not different than control condition.

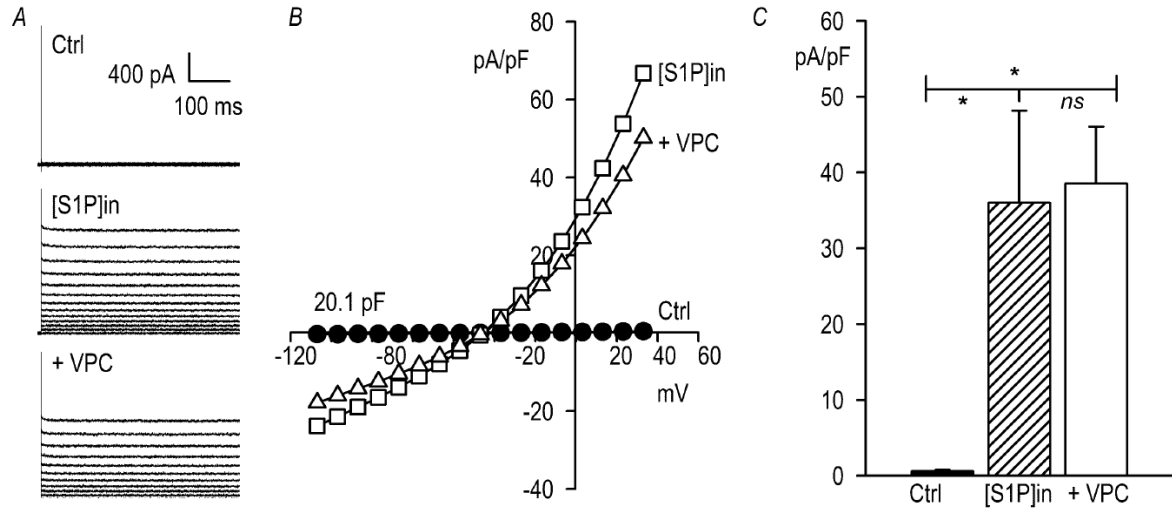

Figure 16: VPC23019, a selective antagonist for $S1PR_{1\&3}$, failed to inhibit [S1P]in-induced $I_{Cl,swell}$. (*A*) Families of currents under control conditions (Ctrl), and after treatment with 500 nM [S1P]in for 10 min, and after addition of VPC23019 (+VPC; 13 μM) in the continued presence of S1P. (*B*) I-V relationships for *A*. (*C*) Current densities at +60 mV. S1P increased the current from 0.60 to 35.99 ± 11.99 pA/pF (n = 4, P < 0.001). VPC failed to block [S1P]in-induced current (ns).

The response to intracellular S1P application was contrary to findings with its extracellular application, where 10 μM CAY fully blocked the [S1P]out-induced current (see Fig. 11). The observation that CAY blocked the response to extracellular but not intracellular S1P excludes the possibility that pipette S1P acts on S1PR after its inside-out transport but is consistent with the notion that S1P elicits $I_{Cl,swell}$ by acting on an intracellular target. Additionally, VPC23019, an $S1PR_{1\&3}$ antagonist, was unsuccessful in blocking the [S1P]in-induced $I_{Cl,swell}$ (Fig. 16). This parallels the insensitivity of $I_{Cl,swell}$ to VPC when S1P was applied in the bathing media (see Fig. 14). Nevertheless, these data are also inconsistent with activation of $I_{Cl,swell}$ via inside-out S1P transport and S1PR signaling.

Compared to [S1P]out, [S1P]in activated a higher $I_{Cl,swell}$ current density (pA/pF) when both were applied at 500 nM. *Figure 17A* shows that the S1P-induced current was 6.86 ± 1.27 pA/pF for [S1P]out and 18.47 ± 4.42 pA/pF for [S1P]in (n = 14 each group, unpaired t test P = 0.018). The greater response to [S1P]in than [S1P]out is contrary to expectations for S1P binding to extracellular facing S1PR. S1P applied via the patch pipette would need to equilibrate with the cytosol and then undergo inside-out transport to reach its presumed target, G protein-coupled S1PR. Therefore, one would predict a reduced response to [S1P]in or, if equilibration and inside-out transport were very efficient, equal responses.

It might be argued that [S1P]in or [S1P]out altered the ability of the cell to generate $I_{Cl,swell}$ and, therefore current density, by a mechanism unrelated to binding the target responsible for S1P-induced current activation. To rule out this possibility, *Figure 17B* compares the responses of a subset of cells that underwent osmotic swelling (0.85T) in the presence of 500 nM [S1P]in or [S1P]out. In both cases, the current after osmotic swelling plus S1P was much larger than that after S1P alone (n = 5 for each group, $P < 0.001$ for each group) but was not

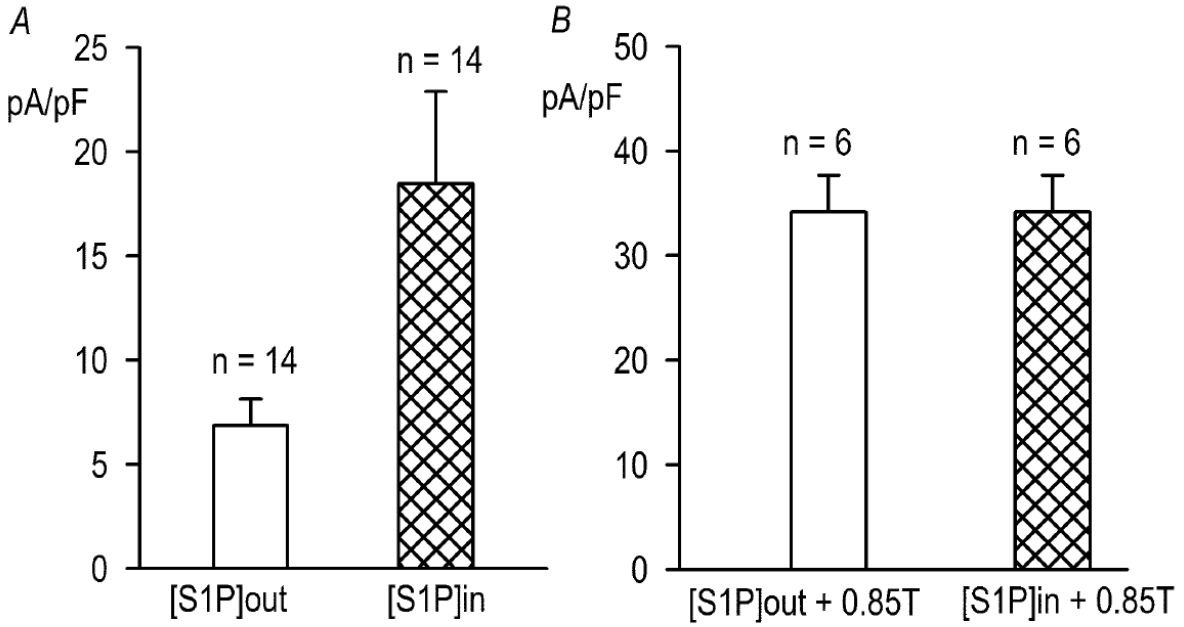

Figure 17: Intracellular vs. extracellular S1P and osmotic swelling-induced current.(*A*) Comparison of $I_{Cl,swell}$ current density with S1P (500 nM, 10 min) applied inside or outside of myocytes. [S1P]in (*n* = 14) elicited significantly greater $I_{Cl,swell}$ than [S1P]out (*n* = 14) in the two groups of cells (*P* = 0.018). [S1P]out (500 nM) activated significantly less current, 6.85± 1.27 pA/pF, than [S1P]in, 16.55 ±4.42pA/pF, at same S1P concentration (*B*) Comparison of $I_{Cl,swell}$ activated by osmotic swelling in 0.85T bath solution in the presence of S1P (subset of myocytes) in *A*. Currents after osmotic swelling were not significantly different (*n* = 6, for each group, *ns*). Note, change in scale for current density.

significantly affected by the location of S1P application (*P* = ns). This suggests that the number of functional $I_{Cl,swell}$ channels and the components of signaling cascade that are activated by osmotic swelling were not differentially affected by intracellular versus extracellular S1P application. The very high $I_{Cl,swell}$ current density after osmotic swelling recorded here is consistent with previous observations by our laboratory in HL-1 cardiomyocytes (Deng *et al.*, 2010c).

In addition to evoking a higher $I_{Cl,swell}$ current densities, [S1P]in activated the current with faster kinetics than [SIP]out, depicted in *Figure 18.* Typically, there was a lag of 3-4 min after switching the bath solution using a rapid flow system before $I_{Cl,swell}$ activation by [S1P]out was apparent. With [S1P]in inside the pipette, the lag before $I_{Cl,swell}$ activation began was only 1-2 min. Similar differences in activation kinetics were observed in n = 6 [S1P]in and n = 10 [S1P]out experiments. Furthermore the dose response relationship of [S1P]in as seen in *Fig 19* exhibits an half maximal current at 240 nM with Hill coefficient of 1.68. To avoid the variability from different passage number of cells, the dose response relationship was done in a subset of cells (cells grown on the same plate) for concentrations (30 nM, 100 nm, and 500 nM) [S1P]in. Based on the above results, this suggests S1P is more potent when applied inside the pipette vs. the extracellular application in the bath.

The differences in activation kinetics and current density are opposite to that expected if S1P elicited $I_{Cl,swell}$ via binding to G protein-coupled S1PR facing the bath solution. Bulk flow of the bath solution should result in the rapid delivery of S1P at its final concentration to S1PR. In contrast, S1P delivered via the patch pipette needs to be exported via the ABC transporters and then act in autocrine/paracrine manner on the plasmalemmal G protein-coupled S1PR. To the extent exported S1P diffuses across the unstirred layer at the plasmalemmal surface, it will be

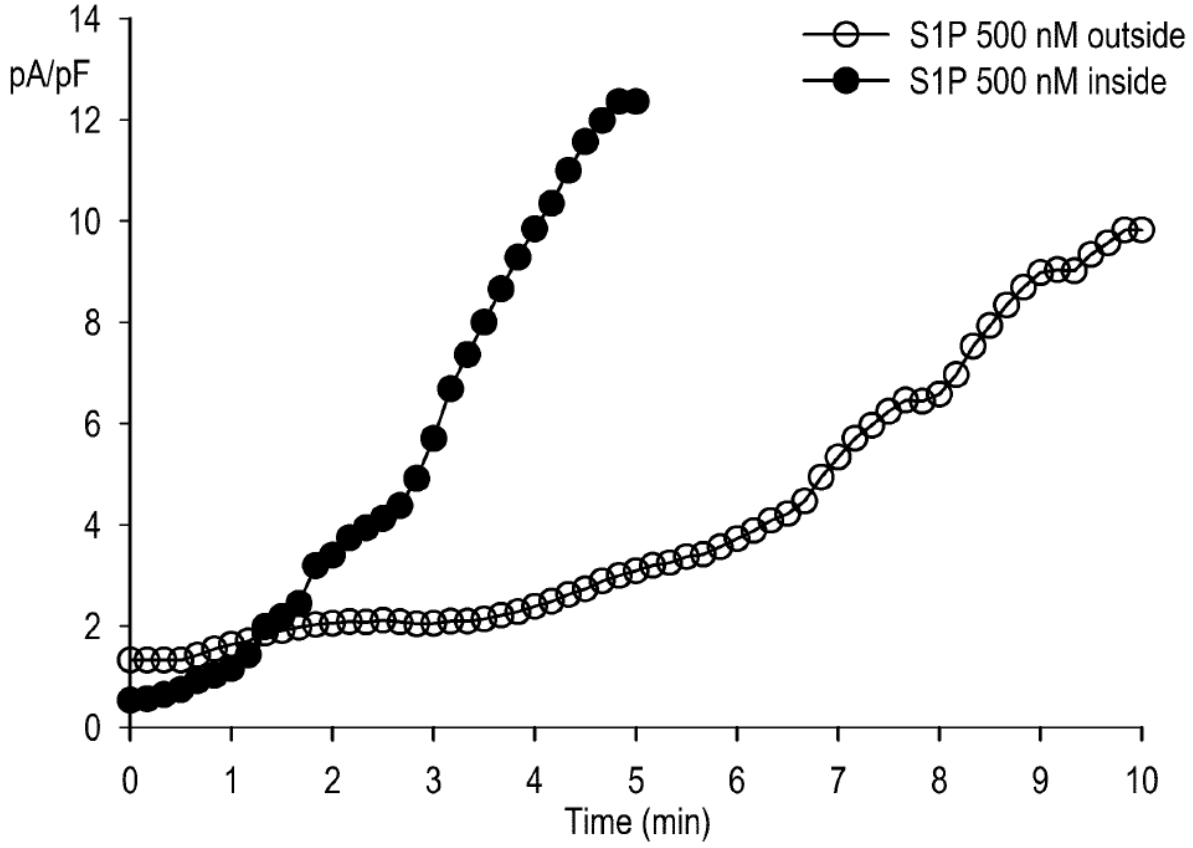

Figure 18: Representative time course of activation of $I_{Cl,swell}$ by [S1P]out and [S1P]in. Intracellular application of S1P acted more rapidly and elicited a larger $I_{Cl,swell}$ than extracellular application of S1P (500 nM for both). This is contrary to expectations if inside-out transport of S1P and S1PR activation is required to evoke $I_{Cl,swell}$ but is consistent with intracellular site(s) of action. Similar differences in kinetics were noted in n = 10 [S1P]in and n = 6 [S1P]out experiments.

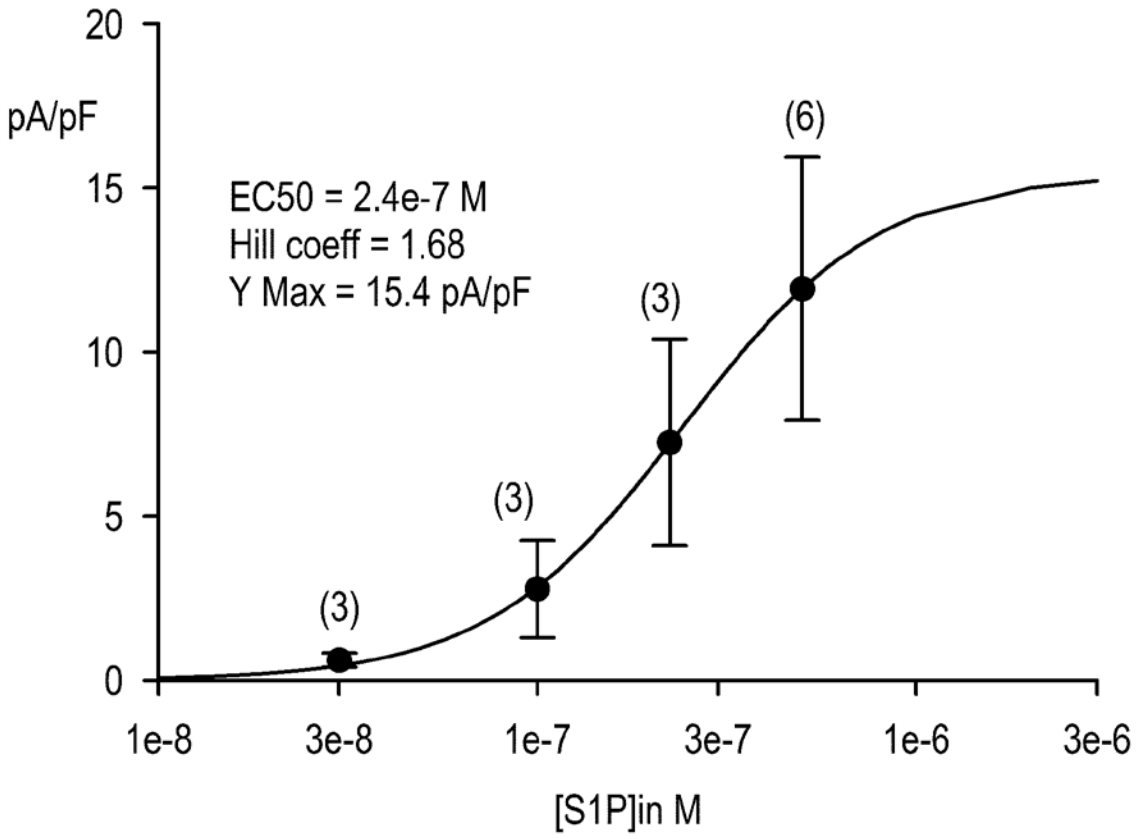

Figure 19: Concentration-response relationship for [S1P]in. Current elicited by [S1P]in was measured at 30 (*n* = 3), 100 (*n* = 3), 225 (n = 3) and 500 nM (*n* = 6). Data were fit (solid line) with a sigmoidal curve with an EC_{50} of 240 nM, Hill coefficient of 1.68, and maximum current density of 15.4 pA/pF. The dose response relationship was done in a subset of cells (from same plate) for concentration (30 nM, 100 nm, and 500 nM).

washed away by bulk flow of S1P-free bath solution. This suggests that inside-out export of S1P during [S1P]in application will result in a lower S1P concentration at the receptors that achieved during [S1P]out application of the same concentration of S1P. Thus, if binding to plasmalemmal S1PR was responsible for the activation of $I_{Cl,swell}$, it seems likely that the current activated by 500 nM [S1P]out would have faster activation kinetics and greater current density than that elicited by 500 nM [S1P]in.

To directly investigate the participation of G protein-coupled S1PR the activation of $I_{Cl,swell}$, we tested whether gallein, a pan Gβγ inhibitor. gallein, a cell-permeable xanthene compound that binds to Gβγ with high affinity (K_d ~400 nM derived from a surface plasma resonance binding study using $G\beta_1\gamma_2$) and blocks Gβγ-dependent cellular activities (Lehmann *et al.*, 2008), affected the response to [S1P]out. The [S1P]out-induced current was not inhibited by gallein As seen in *Figure 20*, gallein (10 μM, 10 min) did not inhibit the [S1P]out-induced current. However, DCPIB blocked 115 ± 10% of the S1P-induced current verifying it was $I_{Cl,swell}$. These data argue that G protein-coupled receptors probably are not involved for the activation of $I_{Cl,swell}$ by S1P but are consistent with the idea that S1P acts on intracellular binding site(s) and initiates a signaling pathway ultimately responsible for activating $I_{Cl,swell}$.

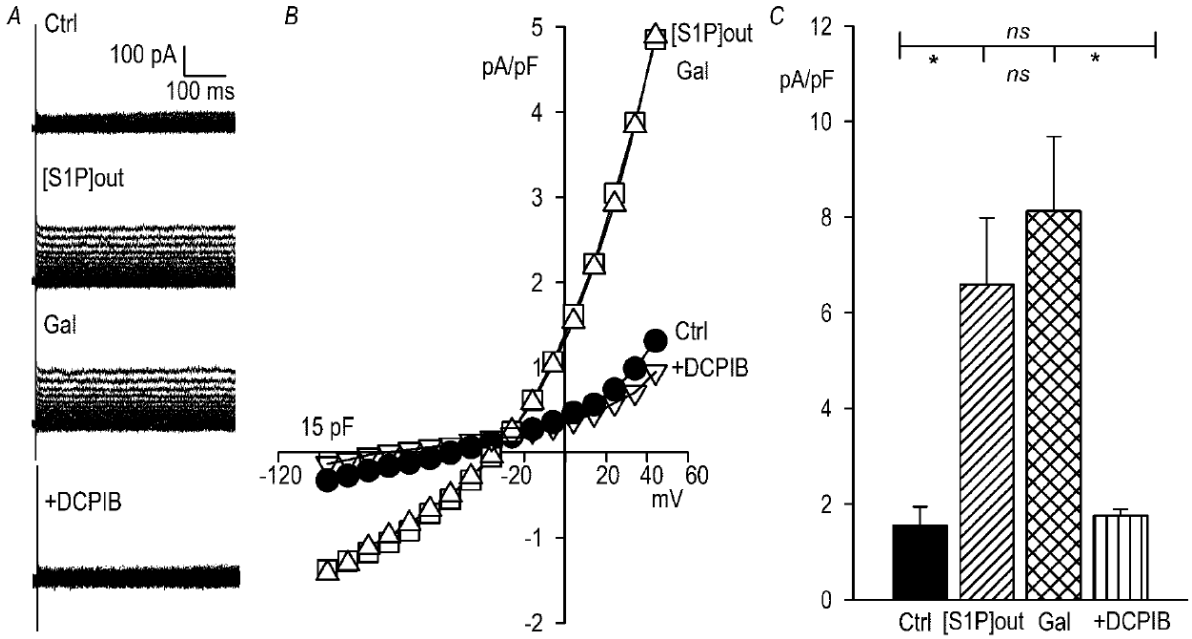

Figure 20: Gallein, a pan Gβγ inhibitor, failed to suppress [S1P]out-induced $I_{Cl,swell}$. (*A*) Families of currents under control conditions (Ctrl), and after treatment with 500 nM [S1P]out for 10 min, and after addition of gallein (+Gal; 10 μM, 10 min) in the continued presence of S1P. (*B*) I-V relationships for *A*. (*C*) Current densities at +60 mV. S1P increased the current from 1.55 to 6.59 ± 1.0 pA/pF (n = 5, P < 0.001). Gal failed to block [S1P]out-induced current, but it was blocked by 115 ± 10% by DCPIB.

3.4. Intracellular vs. Extracellular FTY720

FTY is identified as an agonist for plasmalemmal G protein-coupled $S1PR_{1\&3}$ and activates downstream signaling pathway at a concentration of 100 nM (Hofmann *et al.*, 2009). However, we observed that a 100-fold higher concentration was required to activate $I_{Cl,swell}$ (see Fig.10). Even the phosphorylated form, FTY720-P, required a 100-fold higher concentration that what is known to activate the S1PR (Hofmann *et al.*, 2009) (see Fig.10 & 13). In addition, the S1PR antagonists VPC and CAY failed to block the [FTY]out-induced current (see Fig.13 & 14). Taken together, these data suggested that FTY720 acts on intracellular target(s) similar to S1P.

To test the possibility that an intracellular target for FTY720 was responsible for eliciting $I_{Cl,swell}$, we introduced FTY720 inside the patch pipette ([FTY]in). *Figure 21* compares the response of 100 nM [FTY]in to a 100-fold greater concentration, 10 μM, applied in bath solution ([FTY]out). Although [FTY]in was at a 100-fold lower concentration, it evoked a 2-fold greater current (n = 20 for each group, unpaired t test P = 0.010). Furthermore, the kinetics of activation of $I_{Cl,swell}$ was much faster with [FTY]in than [FTY]out, as depicted in *Figure 22.* Activation of current was not obvious for more than 4 min after applying FTY via the bath solution. With FTY inside the pipette, the lag before current activation was about 1 min, and a much larger current as turned on. Thus, the greater potency and faster time course of activation with [FTY]in than [FTY]out was even more striking than for [S1P]in and [S1P]out. These data argue that FTY is likely to elicit $I_{Cl,swell}$ by binding to intracellular targets rather than to plasmalemmal S1PR.

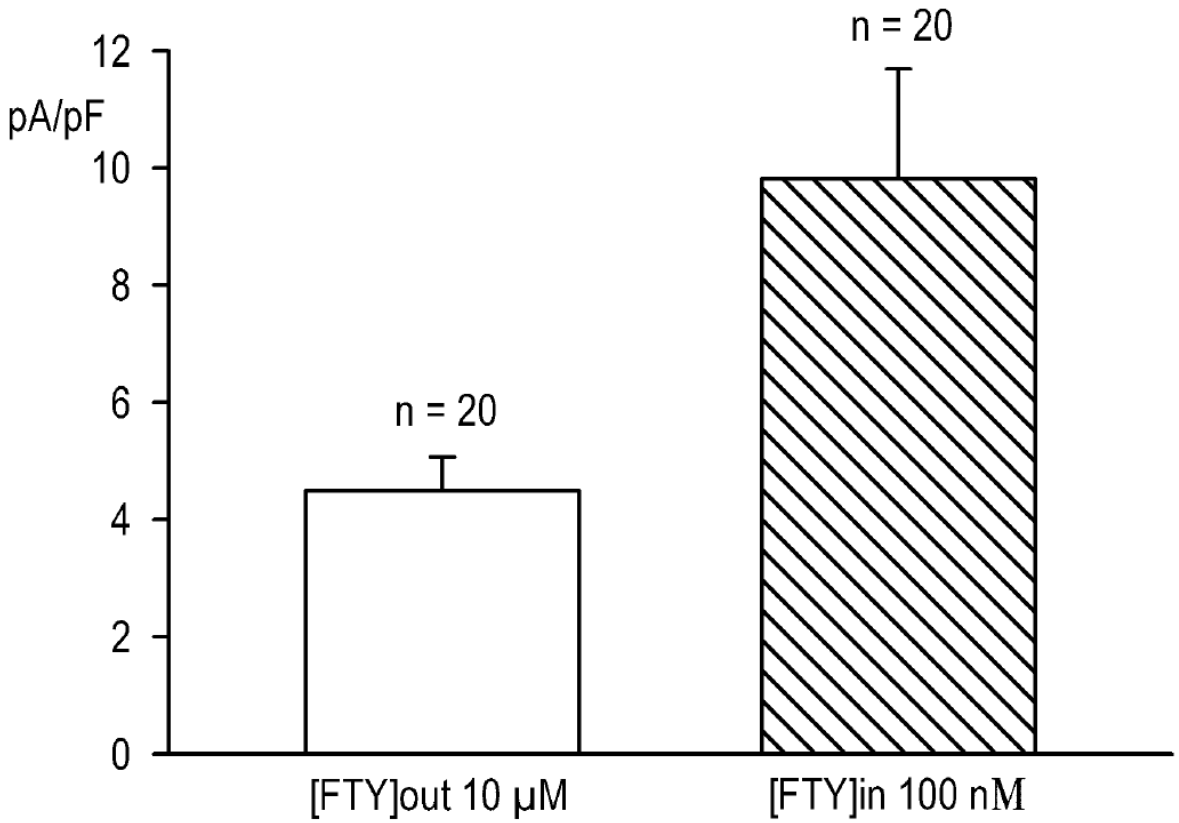

Figure 21: Intracellular application of FTY activates a larger $I_{Cl,swell}$ than extracellular application. (A) Comparison of current densities with 10 μM FTY (10 min) added to the bath solution ([FTY]out) and a 100-fold lower concentration, 100 nM (5 min), included in the pipette solution ([FTY]in). [FTY]in elicited a significantly larger current (*n for each* = 20, *P* = 0.010, unpaired *t*-test). As noted in section 3.2.1, 100 nM and 1 μM [FTY]out failed to evoke significant activation of $I_{Cl,swell}$.

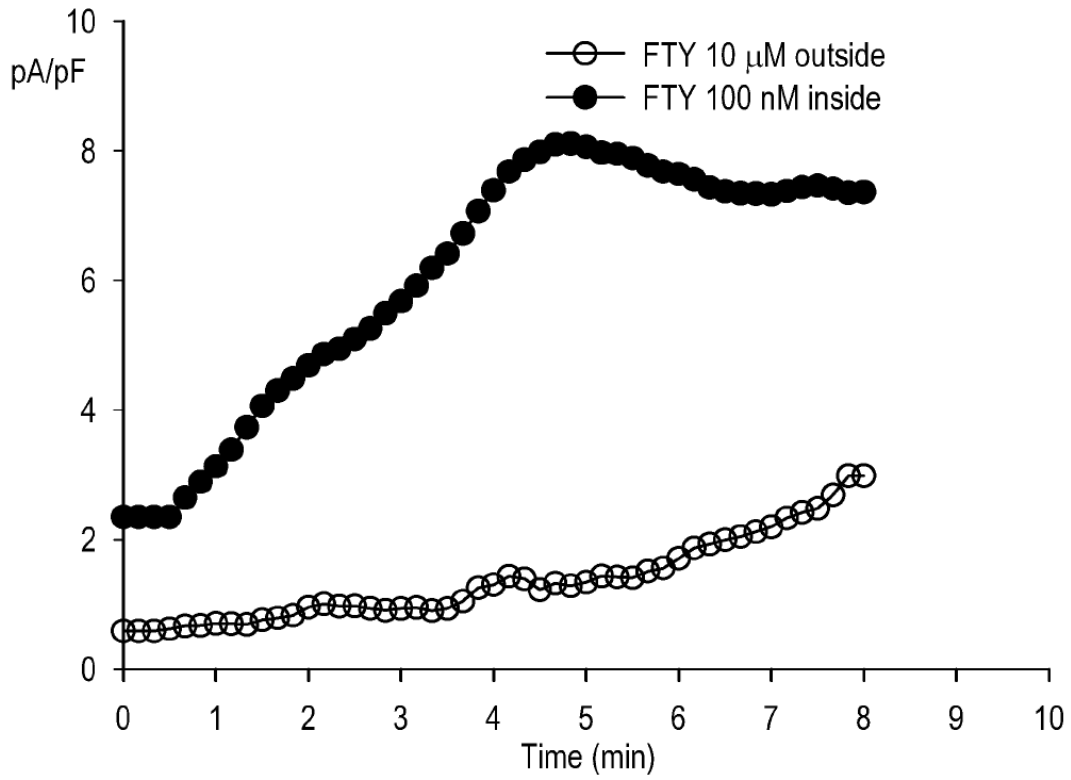

Figure 22: Representative time course of activation of $I_{Cl,swell}$ by [FTY]out and [FTY]in. Inclusion of 100 nM FTY in the patch pipette ([FTY]in) caused activation of $I_{Cl,swell}$ to begin within 1 min of recording, whereas 10 μM FTY in the bath solution ([FTY]out) turned on a smaller current more slowly. Similar results were observed with n = 12 [FTY]in and n = 8 [FTY]out experiments.

To test whether intracellular FTY was phosphorylated in the cytosol, underwent inside-out transport, and then bound to plasmalemmal S1PR, we used S1PR antagonists. As shown in *Figure 23*, [FTY]in (100 nM, 10 min) strongly activated $I_{Cl,swell}$, but CAY did not inhibit the [FTY]in-induced current at either 10 or 30 µM. Nevertheless, the [FTY]in-induced current was blocked by 100 ± 21% by DCPIB, implying the current was $I_{Cl,swell}$. Furthermore, the $S1PR_{1\&3}$ antagonist VPC23019 (13 µM, 10 min) also was unable to block the [FTY]in-induced current, as shown in *Figure 24*. Thus neither CAY nor VPC23019 suppressed the response to [FTY]in.

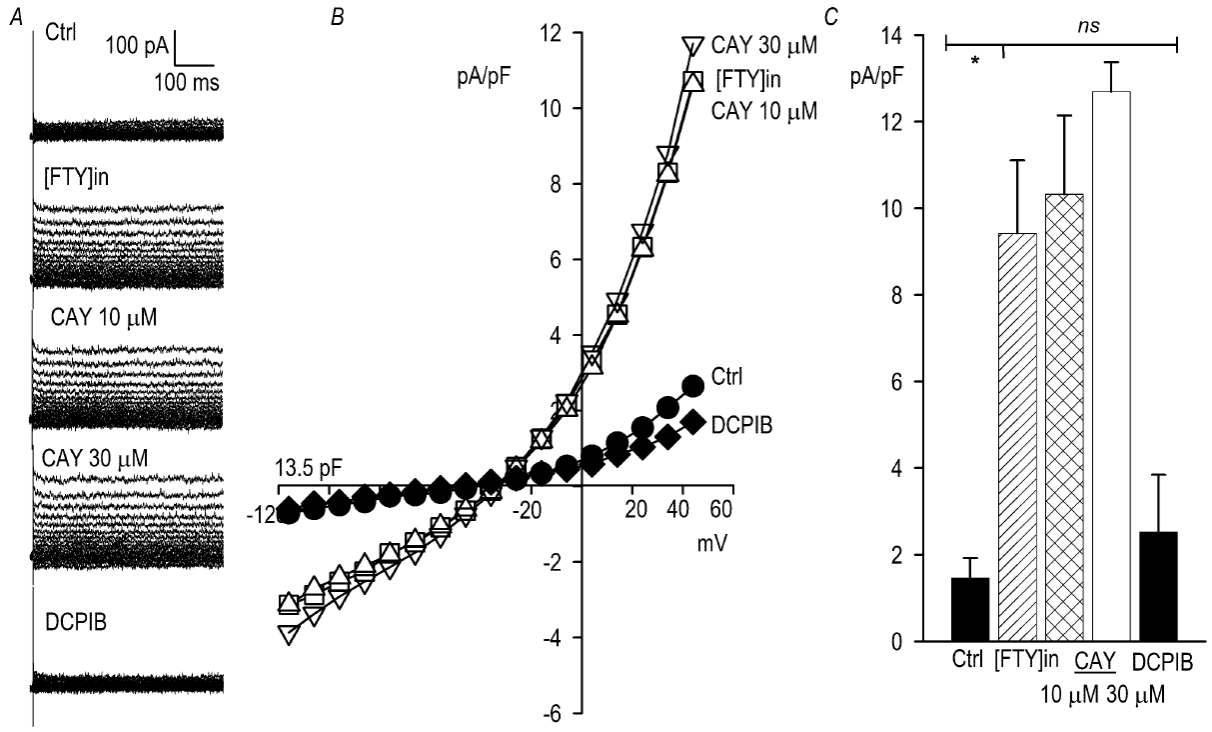

Figure 23: CAY10444, a selective antagonist for $S1PR_3$, failed to block [FTY]in-induced $I_{Cl,swell}$. (*A*) Families of currents under control conditions (Ctrl), and after treatment with 100 nM [FTY]in for 10 min, and after addition of CAY10444 (+CAY; 10 or 30 μM) in the continued presence of [FTY]in. (*B*) I-V relationships for *A*. (Ctrl; filled circle, [FTY]in; open squares, CAY 10 μM; open triangle, CAY 30 μM; inverted open triangle, DCPIB; filled diamond.)(*C*) Current densities at +60 mV. [FTY]in increased the current from 1.46 to 9.42 ± 2.44 pA/pF (n = 4, P < 0.001). Whereas CAY failed to inhibit the FTY-induced current, DCPIB reduced the current by 100 ± 21%.(P < 0.001).

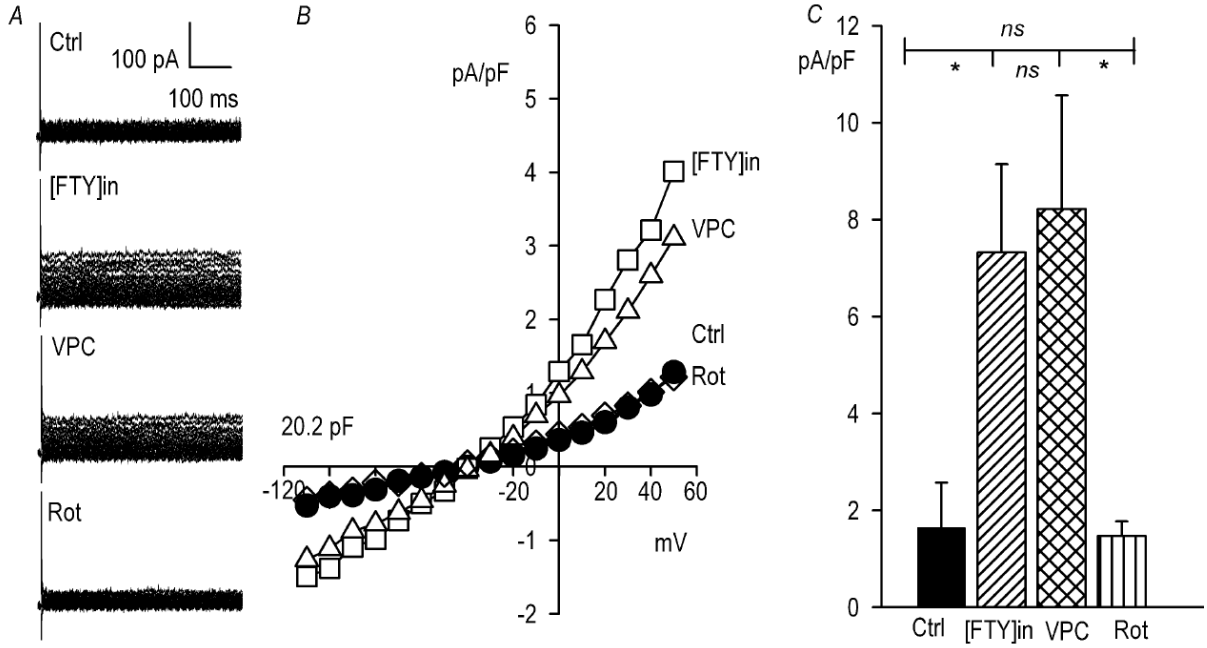

Figure 24: VPC23019, a selective antagonist for S1PR1&3, failed to inhibit [FTY]in-induced $I_{Cl,swell}$. (A) Families of currents under control conditions (Ctrl), and after treatment with 100 nM [FTY]in for 10 min, after addition of VPC23019 (+VPC; 13 μM, 10 min) in the continued presence of FTY, and after addition of rotenone (Rot; 10 μM, 20 min), a mitochondrial complex I inhibitor. (B) I-V relationships for *A*. (C) Current densities at +60 mV. [FTY]in increased the current from 1.65 ± to 7.32 ± 0.89 pA/pF (n = 4, P < 0.001). VPC failed to block [S1P]in-induced current, but rotenone (Rot)blocked FTY-induced current by 109 ± 12% (P < 0.001).

3.5. Western Blot Analysis

As mentioned previously S1PR are differentially expressed in different tissues, and studies in heart show that only $S1PR_1$, $S1PR_2$ and $S1PR_3$ are expressed (Zhang *et al.*, 2007;Means *et al.*, 2008). Hl-1 cells are an immortalized mouse atrial cell line. As discussed above, studies utilizing a variety of genetic, immunohistochemical, electrophysiological, and pharmacological techniques have demonstrated that HL-1 cells possess many of the features of adult atrial cardiomyocytes and are a useful experimental tool. However S1P receptors are not reported to date in this cell line. Given the inconsistencies in the action of the S1PR antagonist and lower potency of FTY720, we verified by immunoblotting that $S1PR_{1-3}$ proteins were expressed in HL-1 cardiomyocytes, a point not previously established in the literature. *Figure 25* shows the expression of $S1PR_{1-3}$ in HL-1 cardiomyocyte whole cell lysate and cyclophilin A used as control. On WB, the reaction products were seen with a molecular mass of about 42–47 kDa in the whole cell lysate, which was consistent with the findings of S1PRs. Total protein (30 μg) was loaded into 10% Bis-Tris polyacrylamide electrophoresis gels. Antibodies to S1PR1-3 were used at a dilution of 1:500 (S1P1, rabbit polyclonal IgG Ab sc-25489; S1P2, rabbit polyclonal IgG Ab sc25491; S1P3, rabbit polyclonal IgG Ab sc-30024; Santa Cruz), and for cyclophilin A, at a dilution of 1:5,000 (rabbit polyclonal IgG Ab 07-313; Millipore). Detail of the western blot procedure is given in the Methods section. Various controls were implemented for this study including loading control to ensure equal loading of protein in each lane; cyclophilin A was used as the house keeping protein. Some extra bands were observed in addition to the band for $S1PR_1$. One of the possible reasons for the multiple bands is non-specific binding of primary and secondary antibody. To rule out this possibility following controls were done. "No primary" control, the primary antibody was not added to one strip of membrane, and

only secondary antibody was added. The "no primary" indicates if any non specific binding or false positives bands due to non specific binding of the secondary antibody. This was done by using antibody dilution buffer containing no primary antibody. The secondary antibody was incubated on the sample in the same way as usual. Under these condition no bands were observed (data not shown), suggesting that there were no unspecific binding. One other control involved lowering the quantity of primary antibody concentration and increasing the number of washes. These intervention still showed extra band in addition to the band for $S1PR_1$ (data not shown). The other possibility for extra band is presence of post-translational modifications with higher molecular weight proteins. This would require investigating the possibility of phosphorylation, glycosylation, acetylation, methylation or myristylation. For the immunoblotting studies we utilized polyclonal antibody, the polyclonal antibodies recognize several epitopes, and thereby show several bands.

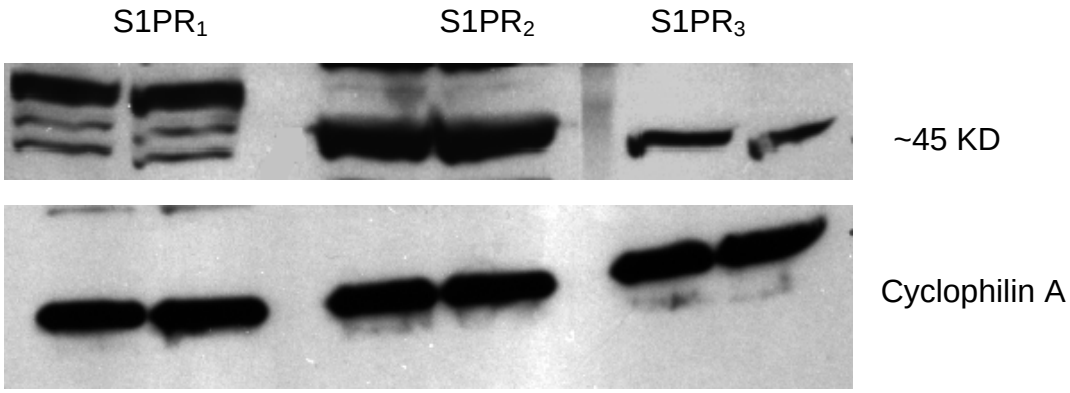

Figure 25: Identification of $S1PR_{1-3}$ protein expression in HL-1 myocytes. Representative Western blots for expression of $S1PR_{1-3}$ and cyclophilin A were obtained from cell lysates under control condition. Antibodies to $S1PR_{1-3}$ were used at a dilution of 1:500 ($S1P_1$, rabbit polyclonal IgG Ab sc-25489; $S1P_2$, rabbit polyclonal IgG Ab sc25491; $S1P_3$, rabbit polyclonal IgG Ab sc-30024; Santa Cruz'), and for cyclophilin A, at a dilution of 1:5,000 (rabbit polyclonal IgG Ab 07-313; Millipore). Blots were done on 10% Bis-Tris polyacrylamide gels. For quantitative analysis, densities of the protein and for $S1PR_{1-3}$ were normalized by their respective cyclophilin densities.

3.6. ROS mediates S1P and FTY720-induced activation of $I_{Cl,swell}$

Previously the laboratory demonstrated that ROS are necessary downstream modulators in the regulation of $I_{Cl,swell}$ by stretch, osmotic swelling, and other agents such as HIV protease inhibitors and ceramide (Browe & Baumgarten, 2004;Browe & Baumgarten, 2006;Ren *et al.*, 2008;Deng *et al.*, 2010b;Raucci & Baumgarten, 2009). Osmotic swelling triggers $I_{Cl,swell}$ via a signaling cascade that requires ROS production by NADPH oxidase followed by mitochondrial ROS production. In contrast, HIV protease inhibitors and, for example, inhibition of the mitochondrial electron transport chain at complex III by antimycin A, which causes ROS leakage to the cytosol, activate $I_{Cl,swell}$ independent of NADPH oxidase. Therefore, we examined the role and source of ROS in S1P and FTY720-induced activation of $I_{Cl,swell}$.

As shown in *Figure 24*, 100 nM [FTY]in activated $I_{Cl,swell}$ but VPC23019 failed to block the current. We next tested whether mitochondrial ROS production was required by blocking the electron transport chain at complex I with rotenone. Rotenone precludes electron transport to ROS leakage from complex III (Chen *et al.*, 2003), and our laboratory previously showed that rotenone inhibits activation of $I_{Cl,swell}$ by swelling, HIV protease inhibitors, antimycin A, and other interventions. Rotenone (10 μM, 20 min) blocked the FTY-induced current by 109 ± 12% (n = 4, P < 0.001) This suggests that [FTY]in triggers $I_{Cl,swell}$ by stimulating mitochondrial ROS production and its leakage to the cytosol. To test whether ROS production by NOX was an upstream to mito ROS production, as found for swelling, stretch, and the G protein-coupled AT_1 and ET_A receptors for angiotensin II and endothelin, respectively. And also to rule out the possibility that the presence of VPC affected the result for rotenone. In the presence of [FTY]in, we tested gp91ds-tat followed by rotenone. As seen in *Figure 27*, In the continued presence of FTY in the patch pipette, addition of gp91ds-tat (500 nM, 5 min) did not significantly affect

$I_{Cl,swell}$, but in contrast, addition of rotenone (10 μM, 20 min) abolished 86 ± 5 % of the current (n = 4, $P < 0.001$). Although the studies illustrated in *Figures 24* and *27* demonstrate that 100 nM FTY720 applied inside the myocyte activate $I_{Cl,swell}$ by augmenting mitochondrial ROS production, presumably from complex III, independent of NADPH oxidase ROS production, it is unclear whether the physiological effector, S1P, acts by the same mechanism. *Figure 26* shows the effect of gp91ds-tat and rotenone, inhibitors of NADPH oxidase and mitochondrial ROS production, respectively, on the response to [S1P]in. First, myocytes were exposed to [S1P]in (500 nM, 5 min) to activate $I_{Cl,swell}$. In the continued presence of S1P in the patch pipette, addition of gp91ds-tat (500 nM, 5 min) did not significantly affect $I_{Cl,swell}$, but in contrast, addition of rotenone (10 μM, 20 min) abolished 96 ± 5 %. of the current (n = 5, $P < 0.001$) as seen in *Figure 26*. These data suggest that ROS production from mitochondria, independent of NADPH oxidase, is sufficient to activate S1P- and FTY720-induced $I_{Cl,swell}$.

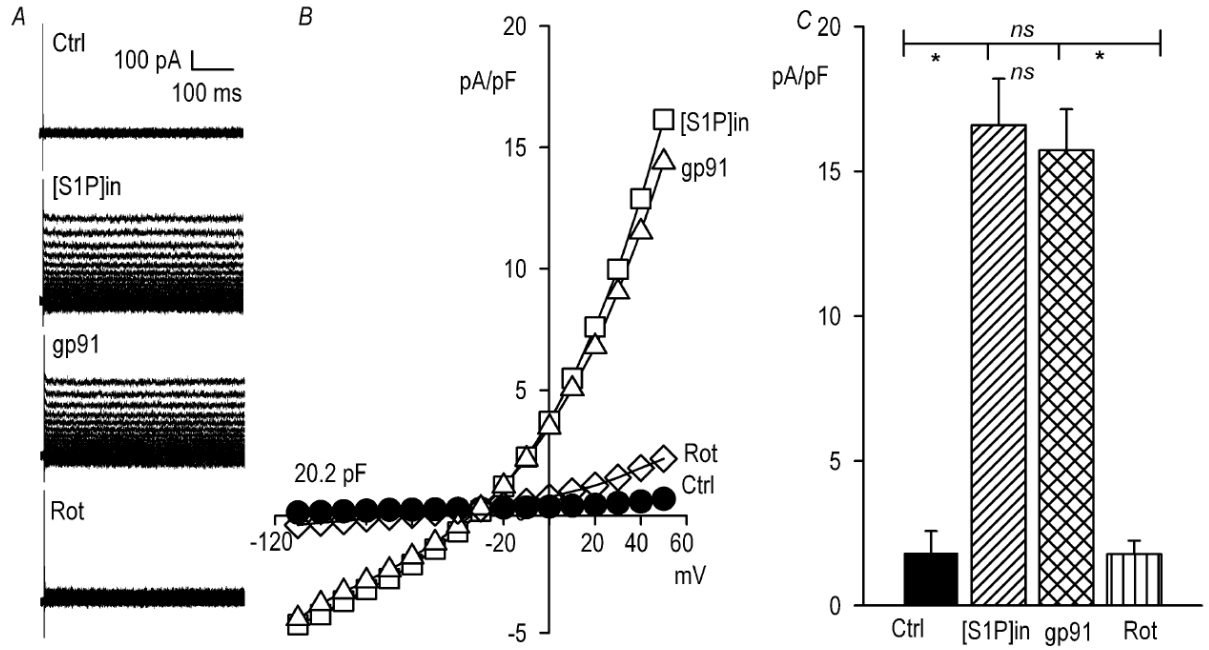

Figure 26: S1P activated $I_{Cl,swell}$ via mitochondrial ROS and, contrary to $I_{Cl,swell}$ evoked by swelling or stretch, did not require ROS production by NADPH oxidase. $I_{Cl,swell}$ elicited by pipette S1P (500 nM, 5 min) was blocked by suppressing mitochondrial complex I with rotenone (10 μM, 20 min), but not by gp91ds-tat (500 nM, 5 min), a fusion peptide inhibitor of NADPH oxidase. (*A*) Families of currents before and after treatment with S1P (500 nM, 5 min) and, then, after addition of gp91ds-tat and rotenone; (*B*) I-V relationships for *A;* (*C*) Current densities at +60 mV after S1P exposure and addition of gp91ds-tat and then rotenone in the continued presence of S1P (n = 5, P < 0.001). In contrast, the laboratory previously showed that osmotic swelling- and stretch-induced $I_{Cl,swell}$, which signal via AT_1, ET_a and EGF receptors, are fully blocked by both gp91ds-tat and rotenone, and mitochondrial ROS production is downstream from NADPH oxidase ROS production. In contrast, S1P-induced $I_{Cl,swell}$, was not inhibited by gp91ds-tat but still was fully blocked by rotenone.

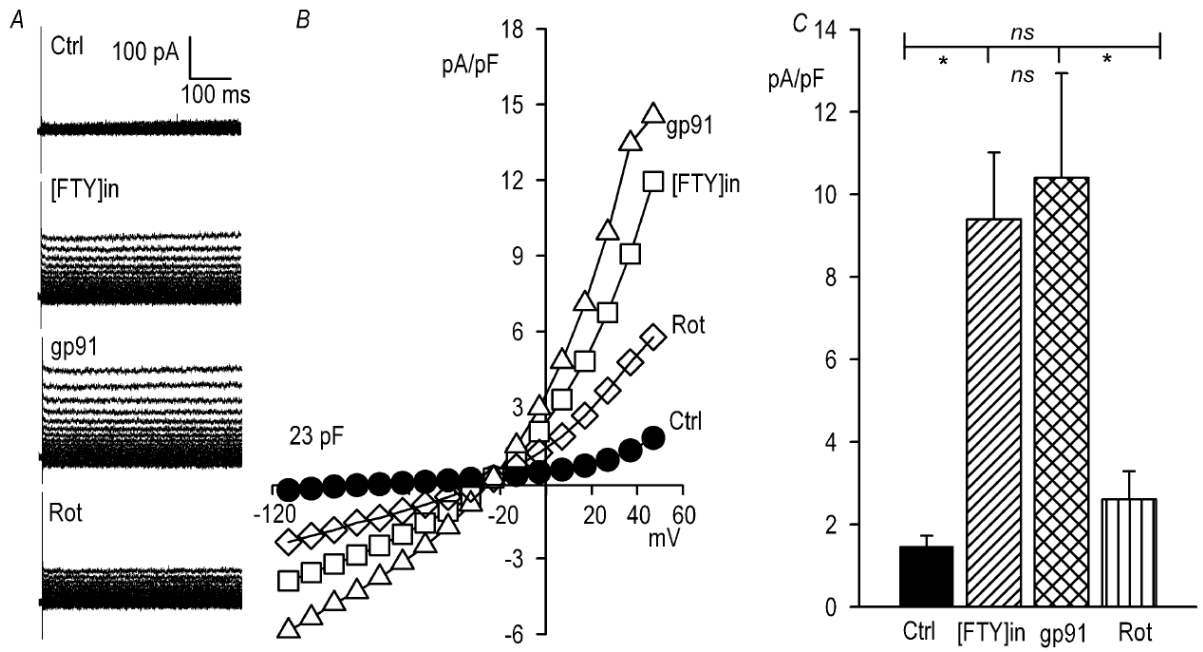

Figure 27: Intracellular FTY720 activated $I_{Cl,swell}$ via mitochondrial ROS and, contrary to $I_{Cl,swell}$ evoked by swelling or stretch, did not require ROS production by NADPH oxidase. $I_{Cl,swell}$ elicited by pipette (100 nM, 5 min) FTY was blocked by suppressing mitochondrial complex I with rotenone (10 μM, 20 min) but not by gp91ds-tat (500 nM, 5 min), an inhibitor of NADPH oxidase. (*A*) Families of currents before and after treatment with FTY720 and after addition of gp91ds-tat and rotenone. (*B*) I-V relationships for *A*. (*C*) Current densities at +60 mV after FTY exposure and addition of gp91ds-tat (n = 5, P < 0.01), and rotenone (n = 4, P < 0.001) in the continued presence of FTY.

Chapter 4 Discussion

This study demonstrated for the first time that both S1P and FTY720, a prodrug mimetic of S1P and agonist for $S1PR_{1\&3}$, activates a Cl^- current with the biophysical and pharmacological properties of $I_{Cl,swell}$ in immortalized HL-1 atrial myocytes. Furthermore, the selective $S1PR_1$ agonist SEW2871 also elicited $I_{Cl,swell}$. Rather than directly interacting with the channel, S1P and FTY720 evoke $I_{Cl,swell}$ by augmenting mitochondrial ROS production. S1P- and FTY720-induced currents were fully suppressed by rotenone, a blocker of the mitochondrial electron transport chain at complex I, suggesting ROS generated by the Q_o cite of complex III was responsible. Contrary to the signaling underlying activation of $I_{Cl,swell}$ in cardiomyocytes in response to osmotic swelling, integrin stretch, angiotensin II, endothelin, and epithelial growth factor, ROS production by NADPH oxidase was not a required upstream effector. Moreover, based on inconsistencies in the responses to S1P agonists and antagonists and differences in the potency and kinetics of $I_{Cl,swell}$ activation by intracellular and extracellular S1P, FTY720, and FTY-P, we propose that activation of mitochondrial ROS production and $I_{Cl,swell}$ by these agents is due to binding to an intracellular ligand rather than to the well-known G protein-coupled S1PR that are expressed in cardiomyocytes, including HL-1 cells. Nevertheless, we cannot rigorously exclude a

role of S1PR in modulating the current. The proposed signaling pathway responsible for the activation of $I_{Cl,swell}$ by S1P and FTY720 is shown in *Figure 28*.

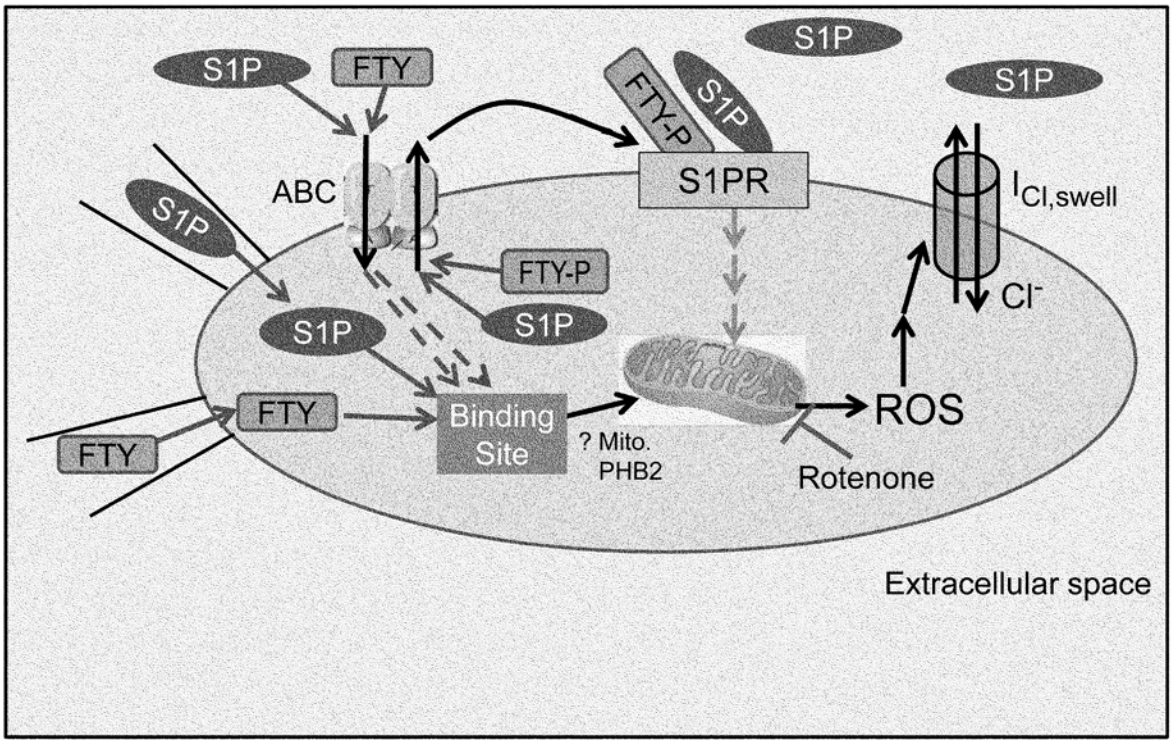

Figure 28: Proposed model of $I_{Cl,swell}$ regulation by S1P and FTY720. S1P and FTY720 binding to intracellular site(s), perhaps mitochondrial prohibitin 2 (PHB2), triggers mitochondrial ROS production independent of NADPH oxidase and, thereby, activates $I_{Cl,swell}$. This model can explain the observed greater potency and faster kinetics of $I_{Cl,swell}$ activation by S1P and FTY720 delivered to the cytosol via the patch pipette than via the bath solution, insensitivity to FTY720-P, and apparent inconsistencies in the actions of well-described S1PR agonists and antagonists. S1P and FTY applied extracellularly can reach their intracellular ligands after uptake by ABC transporters (ABC). It seems likely that at least some nominally S1PR-specific agonists and antagonists also bind to intracellular S1P ligands that modulate $I_{Cl,swell}$ with selectivity and affinities that have not yet been characterized. The possibility that S1PR also can modulate $I_{Cl,swell}$ cannot be rigorously excluded, however.

4.1 Properties of S1P-, FTY720-, and SEW2871-induced Cl^- current

Sarcolemmal Cl^- channels expressed in the heart include the cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel (I_{CFTR}), Ca^{2+}-activated Cl^- channels ($I_{Cl,Ca}$), and $I_{Cl,swell}$ (Hume *et al.*, 2000), as well as an inwardly rectifying Cl^- current attributed to ClC-2 (Duan, 2009). In the present study, we showed that S1P, FTY720, FTY720-P, and SEW2871 activated an outwardly rectifying Cl^- current with properties that distinguish $I_{Cl,swell}$ from other cardiac Cl^- channels. We demonstrated that the Cl^- current elicited by all four of these S1PR agonists was blocked by DCPIB. This ethacrynic acid derivative is the most selective anion channel inhibitor identified to date; DCPIB fully suppresses $I_{Cl,swell}$ at 10 μM, the concentration used here, whereas CFTR, Ca^{2+}-activated Cl^- channels, and cardiac cation currents are insensitive to this agent (Decher *et al.*, 2001). Another fundamental distinguishing characteristic of $I_{Cl,swell}$ is its outward rectification with both physiological and symmetrical Cl^- gradients (Hume *et al.*, 2000;Baumgarten & Clemo, 2003;Sorota, 1994). In contrast, although I_{CFTR} and $I_{Cl,Ca}$ outwardly rectify with a physiological Cl^- gradient, their I-V relationships are linear in symmetrical Cl^- (Hume *et al.*, 2000), and the ClC2 Cl^- current inwardly rectifies in physiological Cl^- (Duan, 2009). Thus, outward rectification of the S1P- and FTY720-induced current with both physiological and symmetrical Cl^- gradients is strong biophysical evidence supporting the conclusion; the current is $I_{Cl,swell}$. In addition, as previously demonstrated for $I_{Cl,swell}$ (Deng *et al.*, 2010c;Baumgarten *et al.*, 2011), the S1P- and FTY720-induced currents were triggered by mitochondrial ROS production, a regulatory pathway not reported for other cardiac anion channels. Finally, we focus here only on how these agents modulate Cl^- channels because the experimental pipette and bath solutions were designed to suppress cation currents and the observed currents reversed at the expected Cl^- equilibrium potential. Thus, the present

study demonstrated that S1P and its analogue FTY720 elicit $I_{Cl,swell}$ under isosmotic conditions via production of mitochondrial ROS. Nevertheless, the molecular identity of $I_{Cl,swell}$ remains elusive, and the mechanisms of $I_{Cl,swell}$ inhibition by DCPIB is not understood.

Sphingolipids have been shown to exert effects on cardiac ion channel function previously. For example, S1P mediates both a chronotropic as well as inotropic effects on the heart. S1P stimulates the muscarinic receptor-activated inward rectifier K^+ current, $I_{K,ACh}$, and this is attributed to the activation of $S1PR_3$ receptors, as the antagonist of the receptor suramin, blocked these effects (Alewijnse *et al.*, 2004;Himmel *et al.*, 2000;Peters & Alewijnse, 2007). However suramin is a G protein inhibitor which acts by preventing nucleotide exchange rather than $S1PR_3$ antagonist (Beindl *et al.*, 1996;Nanoff *et al.*, 2002;Yester *et al.*, 2011). In addition, sphingolipids were implicated in the regulation of a Cl^- channel in cardiac muscle, but the channel responsible was not identified (d'Anglemont de Tassigny *et al.*, 2003). Previously our laboratory demonstrated that exposure to neutral Mg^{2+}-dependent SMase C, which generates ceramide and phosphocholine by breaking down sarcolemmal sphingomyelinase, and exogenous C_2-ceramide both activate $I_{Cl,swell}$ in ventricular myocytes and suggested the response was due to S1P production and involved ROS (Raucci & Baumgarten, 2010). However, neither the signaling cascade activated by these sphingolipids nor the source of ROS were identified. CFTR also is regulated by sphingolipids. SMase D, which generates ceramide-1-P and choline, causes inhibition of CFTR current in whole oocytes injected with the CFTR cDNAs (Ramu *et al.*, 2007), and ceramide inhibits CFTR-activity of the apical membrane of Calu-3 cells (Ito *et al.*, 2004). Work on Kv channels showed gating is modified by direct interactions between membrane lipids and channel proteins (Ramu *et al.*, 2006;Milescu *et al.*, 2007). In addition, prolonged exposure to either C2-ceramide and SMase (>10 h) downregulates the hERG channel

by mechanisms involving ROS and ubiquitin-mediated degradation (Bai *et al.*, 2007;Chapman *et al.*, 2005).

Although it is clear that S1PR agonists turned on $I_{Cl,swell}$, they did not fully activate the current. It previously was observed that osmotic swelling in 0.7T (T; times-isosmotic bath solution) gives full activation of $I_{Cl,swell}$ in adult rabbit ventricular myocytes (Clemo *et al.*, 1999), and 0.8T produces full activation in HL-1 cells myocytes (Deng *et al.*, 2010c). We found, however, the magnitude of the current elicited by extracellular (~6.85 pA/pF) or intracellular S1P (~16.55 pA/pF) was much smaller than that observed after osmotically swelling HL-1 myocytes with 0.85T bathing media (35-40 pA/pF). These differences may reflect a more modest stimulation of myocyte ROS production compared to that achieved with osmotic swelling or the participation of different signaling pathway. We did not directly measure HL-1 cell ROS production in the present studies.

4.2 Do G protein-coupled S1PR elicit $I_{Cl,swell}$?

S1P acts as a ligand for a family of five G protein-coupled receptors, $S1PR_{1-5}$, which formerly were called endothelial differentiation gene (EDG) receptors (Chun *et al.*, 2002). S1PR are differentially expressed in different tissues, and studies in heart show that only $S1PR_1$, $S1PR_2$ and $S1PR_3$ are expressed (Zhang *et al.*, 2007;Means *et al.*, 2008). The binding of S1P and FTY720-P to G protein-coupled receptors and the resultant downstream signaling pathways are thought to be largely responsible for the observed cardiac effects of both molecules (Means & Brown, 2009;Brinkmann *et al.*, 2002); cf.,(Pyne & Pyne, 2011;Strub *et al.*, 2010;Strub *et al.*, 2011). We verified by immunoblotting that $S1PR_{1-3}$ proteins also were expressed in HL-1 cardiomyocytes, a point not previously established in the literature.

In addition to S1P, several other S1PR ligands turned on $I_{Cl,swell}$ in HL-1 cardiomyocytes. We observed that $I_{Cl,swell}$ was activated by SEW2871, a novel selective agonist for $S1PR_1$ that is structurally unrelated to S1P (Sanna *et al.*, 2004). Unlike S1P and FTY720, SEW2871 is a small heterocyclic lipophilic molecule that does not have a charged head-group. Activation of $I_{Cl,swell}$ by a selective $S1PR_1$ agonist lead to the prediction that FTY720, a sphingosine analog and $S1PR_{1\&3}$ agonist (Brinkmann, 2007) also would elicit $I_{Cl,swell}$. To the contrary, addition of 100 nM FTY720 to the bathing media was ineffective, but exogenous H_2O_2 elicited $I_{Cl,swell}$ in the same myocytes providing a positive control. Increasing the bath concentration of FTY720 to 1 μM also failed to stimulate the current, but 10 μM was found to activate $I_{Cl,swell}$. This FTY720 concentration is 100-fold greater than the 100 nM normally sufficient to fully activate $S1PR_{1\&3}$ (Koyrakh *et al.*, 2005).

FTY720 is a prodrug that must be imported into cells by an ABC transporter, phosphorylated *in situ* by SphK2 (Zemann *et al.*, 2006) to yield its active form, FTY720-P (Albert *et al.*, 2005;Brinkmann *et al.*, 2002), and then exported before it can bind to G protein-coupled S1PR at the extracellular face of the cell membrane (Brinkmann, 2007). To exclude the possibility that defects in one or more of required steps explained the insensitivity to FTY720, we directly challenged the S1PR in HL-1 myocytes by adding FTY720-P to the bathing solution. Whereas 100 nM FTY720-P failed to activate current, $I_{Cl,swell}$ was turned on by 10 μM FTY720-P. Thus, like FTY720, the active phosphorylated form, FTY720-P, also had much lower than expected potency. In contrast, previous studies established (*S*)-FTY720-P [but not (*R*)-FTY720-P or parent FTY720] acts as a full agonist at $S1PR_1$ (0.3 nM), $S1PR_4$ (0.6 nM; not found in myocytes) and $S1PR_5$ (0.3 nM; not found in myocytes) and with approximately a 10-fold lower potency at $S1PR_3$ (3.1 nM), but it has no activity at $S1P_2$ (>10,000 nM) (Albert *et al.*,

2005;Brinkmann *et al.*, 2002;Mandala *et al.*, 2002). Therefore, 100 nM FTY720-P added to the bath should have fully activated both $S1PR_{1\&3}$ even if 100 nM and 1 μM FTY720 failed to generate and export sufficient FTY720-P in the present experimental system. Thus, although S1P and SEW2871 activated current at bath concentrations consistent with $S1PR_1$ signaling, the exceptionally low potency of $S1PR_{1\&3}$ agonists FTY720 and FTY720-P argue against the idea that S1PR trigger $I_{Cl,swell}$.

Studies with the S1PR antagonists CAY10444 (also known as, BML-241) and VPC23019 also raised doubts regarding the role of S1PR. CAY10444 is a selective and effective $S1PR_3$ antagonist and in multiple cell line suppresses a variety of downstream effects (Waeber & Salomone, 2011). For example, 10 μM CAY10444 inhibits the prosurvival effect of HDL in a mouse cardiomyocyte hypoxia-reoxygenation model interfering with the PI3K-AKT signaling pathway, an effect attributed to block of $_{S1PR3}$ (Tao *et al.*, 2010). We observed a striking inconsistency with the action of CAY10444. First, the positive response to SEW2871, a selective $S1PR_1$ agonist, suggests CAY10444 should fail to inhibit current. To the contrary, bath S1P-induced current was fully blocked by 10 μM CAY10444, but bath FTY720-induced current was not affected, even after raising the CAY10444 concentration 3-fold. One might argue that full current activation can be evoked by either $S1PR_1$ or $S1PR_3$ signaling and that the failure of even high concentrations of CAY10444 to block the action of FTY720 was due to an inability to compete with the high bath FTY720 concentration (10 μM) needed to elicit the current. On the other hand, VPC23019, a potent $S1PR_{1\&3}$ antagonist, failed to suppress $I_{Cl,swell}$ activated by both bath S1P (500 nM) and bath FTY720 (10 μM). If, in fact, stimulation of either $S1PR_1$ or $S1PR_3$ signaling was sufficient to elicit $I_{Cl,swell}$, VPC23109 (13 μM) should have blocked the response to 500 nM S1P; its K_i for displacing ^{32}P-S1P is 7.86 and 5.98 for $S1PR_1$ and $S1PR_3$, respectively, in

HEK293T cells (Davis *et al.*, 2005). To confirm the presence of $I_{Cl,swell}$ in the antagonist-insensitive myocytes, we demonstrated that DCPIB suppress the current in the same cells. Taken together, the response to these agonists and antagonists was inconsistent with the idea that S1P and FTY720 elicit $I_{Cl,swell}$ simply by binding to G protein-coupled S1PR and evoking their signaling pathways.

It is difficult, however, to rigorously exclude the possibility that "off target effects" and complex pharmacology might contribute to the discrepancy (Pyne & Pyne, 2011). In addition, VPC23019 is a phosphorylated lipid and is a potential substrate for lipid phosphate ectophosphatases (Pyne *et al.*, 2005). Therefore, degradation of VPC23019 might have prevented its inhibitory action at S1P receptors. We did not test whether VPC23019 was hydrolyzed in the present experimental system. There also are reports of the internalization of the S1PR in response to the agonist (Gergely *et al.*, 2012). If internalized S1PR are inaccessible to antagonists but continue to signal and support the activation of $I_{Cl,swell}$, receptor internalization might explain the failure of certain antagonist to block the response to agonists acting on S1PR.

Signaling by heterotrimeric G proteins requires GDP/GTP exchange and the dissociation its α and βγ subunits upon ligand binding to the G protein-coupled receptor. The process is terminated by the hydrolysis of Gα-bound GTP by small GTPases, and then, the heterotrimeric complex reassembles. Therefore, S1PR-induced G protein signaling can be blocked by interrupting the G protein cycle or downstream pathways. We found, however, that 10 μM gallein, a pan βγ inhibitor failed to suppress the response to bath S1P. On the other hand, gallein binding to βγ (K_d, 422 nM; IC_{50}, 241 nM) suppresses G-protein signaling-induced PI-3K and RAC1 activation and ROS production in HL6 cells and primary human neutrophils (Lehmann *et al.*, 2008). This argues against the involvement of G protein-coupled S1PR in ROS-dependent

activation of $I_{Cl,swell}$. Nevertheless, to confirm the conclusion that G protein signaling does not play a role in $I_{Cl,swell}$ activation by S1PR agonists, it would be useful to suppress signaling with additional small molecule inhibitors or molecular methods.

4.3 Intracellular targets of S1P : Potential role in activating $I_{Cl,swell}$.

Rather than acting at S1PR, an alternative possibility is that S1P, FTY720, its phosphorylated analogue FTY720-P, and other S1PR agonists such as SEW activate $I_{Cl,swell}$ by binding to intracellular ligands. Recently identified intracellular targets include HDACs, E3 ubiquitin ligases, and prohibitin-2 (PHB2). One of the study showed that, HDACs are direct intracellular targets of S1P and link nuclear S1P to epigenetic regulation of gene expression (Hait *et al.*, 2009). S1P was shown to be a missing cofactor for the E3 ubiquitin ligase TRAF2 and that TRAF2 was a novel intracellular target of S1P. Interestingly, only S1P, and not dihydro-S1P, which lacks the double bond in S1P, was shown to bind and activates TRAF2.(Alvarez *et al.*, 2010). β-site amyloid precursor protein cleaving enzyme-1 (BACE1) was shown to be another site in neurons to which S1P binds. S1P also specifically bound to BACE1 in vitro and increased its proteolytic activity, suggesting that cellular S1P directly modulates BACE1 activity (Takasugi *et al.*, 2011). In these studies inhibition or downregulation of SphK1 and SphK2, or overexpression of S1P-degrading enzymes all decreased BACE1 activity and Aβ production (Maceyka *et al.*, 2012). These responses were mediated by intracellular S1P independently of its cell surface G-protein-coupled receptors. Also prohibitin 2 PHB2, a highly conserved protein that regulates mitochondrial assembly and function, was recently shown to bind S1P in vitro and in vivo (Maceyka *et al.*, 2012;Strub *et al.*, 2011;Yester *et al.*, 2011). PHB2 localizes predominantly to the inner mitochondrial membrane where it is thought to form a large, macromolecular complex with PHB1 that is involved in mitochondrial biogenesis and

metabolism (rtal-Sanz & Tavernarakis, 2009;Takasugi *et al.*, 2011) By targeting monomeric PHB2 (but not closely related PHB1), S1P is implicated in the regulation of proper assembly and function of cytochrome-c oxidase of the mitochondrial respiratory chain in cardiac myocytes (Strub *et al.*, 2011). Moreover, PHB is reported to reduce mitochondrial free radical production and oxidative stress in brain injury models, perhaps by stabilizing the function of complex I (Zhou *et al.*, 2012). Binding of S1P to PHB2 and its action on mitochondrial function represent a potential novel link between intracellular S1P binding and $I_{Cl,swell}$, an ion channel activated by ROS.

Several lines of evidence support the possibility that $I_{Cl,swell}$ is activated by intracellular targets rather than extracellular-facing G protein-coupled S1PR. Intracellular application of both S1P and FTY720 elicited larger amplitude currents with a faster time course of activation and greater potency than extracellular application. In the case of FTY720, the difference in potency was more than 100-fold. These findings are consistent with the notion that S1P and FTY720 delivered directly to the cytosol via the patch pipette reach their presumed intracellular site of action more rapidly and at higher concentration than when the same agents are delivered via suprafusion by bath solution. Inclusion in the patch pipette avoids the need for outside-in transport via ABC transporters and, perhaps, phosphorylation of FTY720 to FTY720-P by SphK2. As a result, the cytoplasmic concentration of ligand is likely to increase more rapidly with intra- than extracellular exposure, and if the access of ligand to its intracellular target is rate limiting, faster current activation is expected, as was observed.

The idea that S1P and FTY720 act on intracellular binding sites also may explain the inconsistent responses of nominally specific S1PR inhibitors. The ability of these agents to inhibit binding of S1P and FTY720 or FTY720-P to intracellular ligands has not been

characterized. Nevertheless, our results argue that the putative intracellular binding site is not effectively blocked by CAY1044 and VPC23109 or these S1PR antagonists may fail to reach intracellular targets. Competitive inhibitors of agonist binding to S1PR presumably possess structural features that mimic those of S1P. This raises the possibility that S1PR antagonists might compete for binding sites on ABC transporters. Thus, for example, CAY10444 might block the action of 500 nM extracellular S1P on $I_{Cl,swell}$ by preventing its import, but not suppress the response to intracellular S1P because CAY10444 fails to recognize or reach the intracellular S1P binding site. On the other hand, CAY10444 also failed to prevent activation of $I_{Cl,swell}$ by 10 μM extracellular FTY720, which must be imported and phosphorylated. The requirements for uptake of S1P and FTY720 might be different. Alternatively, CAY10444 might be ineffective in competing with a high concentration (10 μM) of FTY720 but still block the uptake of a lower concentration (500 nM) S1P by the same transport mechanism. Of particular interest, CAY10444 and VPC29019 both were ineffective in blocking the response to both S1P and FTY720 applied via the patch pipette. Taken together, these data suggest that that the S1PR inhibitors failed to recognize or failed to gain access to the intracellular ligand that triggers $I_{Cl,swell}$ activation.

An intracellular ligand model also must explain the ability of SEW2871 to elicit $I_{Cl,swell}$. This small molecule heterocyclic $S1PR_1$ agonist is uncharged and lipophilic; its octanol:water partition coefficient calculated by XLogP3-AA is 6.6 (see Pubchem Compound http://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?cid=4077460#x27 accessed 30 March 2013). This suggests SEW2871 will partition into the plasma membrane, but distribution into the cytoplasm has not been studied.

4.4 ROS mediated activation of $I_{Cl,swell}$.

In the present studies we found that intracellular application of 500 nM S1P or 100 nM FTY720-P elicited $I_{Cl,swell}$ that was insensitive to inhibition of NADPH oxidase by the fusion peptide inhibitor gp91ds-tat but was fully suppressed by inhibition of mitochondrial electron transport by rotenone in the continued presence of S1P or FTY720-P. These data argue that S1P and FTY720-P augment mitochondrial ROS production and thereby activate $I_{Cl,swell}$ by signaling that is independent of NADPH oxidase.

In contrast to this conclusion, previous work by our laboratory established that ROS produced by both NADPH oxidase and mitochondria are essential for eliciting $I_{Cl,swell}$ in cardiomyocytes in response to multiple stimuli (Ren *et al.*, 2008;Deng & Baumgarten, 2009;Browe & Baumgarten, 2007;Browe & Baumgarten, 2006;Browe & Baumgarten, 2004). Blockade of NADPH oxidase by gp91ds-tat, apocynin or other agents is sufficient to inhibit $I_{Cl,swell}$ activated by Ang II binding AT_1 receptors, ET-1 to ET_A receptors, EGF to EGFR, integrin stretch, and osmotic swelling. Nevertheless, these stimuli also increase mitochondrial ROS production in series with NADPH oxidase, and rotenone-sensitive mitochondrial ROS production is a required upstream effector for current activation (Deng *et al.*, 2010b;Deng *et al.*, 2009). On the other hand, as found in the present study, interventions that directly affect mitochondrial function are capable of eliciting $I_{Cl,swell}$ via mitochondrial ROS production (blocked by rotenone) that is independent of NADPH oxidase (insensitive to gp91ds-tat and apocynin) (Deng *et al.*, 2010b;Deng *et al.*, 2009;Raucci Jr. & Baumgarten, 2010). Among these interventions are: antimycin A, which blocks the electron transport chain distal to the complex III Q_o site; diazoxide, which is thought to act by opening mitochondrial K_{ATP} channels; and the HIV protease inhibitors ritonavir and lopinavir. In parallel studies with these agents, ROS

production was measured in intact HL-1 cardiomyocytes using C-H_2DCFDA-AM fluorescence and was fully suppressed by rotenone but not gp91ds-tat. Moreover, effects on mitochondrial membrane potential ($\Delta\psi_m$) were confirmed with JC-1 in some cases.

Our findings that S1P and FTY720-P bind to an intracellular ligand, rather than S1PR, and thereby elicit $I_{Cl,swell}$ via mitochondrial ROS production S1PR raises an important question that we have not addressed experimentally: What is the intracellular binding site responsible for augmenting mitochondrial ROS production? One possibility is Phb2. a highly conserved protein that regulates mitochondrial assembly and function, was recently shown to bind S1P in vitro and in vivo (MacLennan *et al.*, 2001;Strub *et al.*, 2011;Yester *et al.*, 2011). PHB2 has been shown to act as a chaperone in the assembly of subunits of mitochondrial respiratory chain complexes (Nijtmans *et al.*, 2002). Moreover, PHB is reported to reduce mitochondrial free radical production and oxidative stress in brain injury models, perhaps by stabilizing the function of complex I (Zhou *et al.*, 2012). Also, PHB1 downregulation in endothelial cells increases mitochondrial ROS production and promotes a senescent phenotype (Schleicher *et al.*, 2008). Binding of S1P to PHB2 and its action on mitochondrial function represent a potential novel link between intracellular S1P binding and $I_{Cl,swell}$, an ion channel activated by ROS.

It is unclear whether S1P and FTY720-P will augment mitochondrial ROS production independent of NADPH oxidase via an intracellular ligand rather than S1PR and thereby stimulate $I_{Cl,swell}$ in cells from other tissues. S1P was recently found to increase in H_2O_2 production in murine NIH3T3 fibroblasts through NADPH oxidase activation (Catarzi *et al.*, 2011). This response was PI-3K- and PKC dependent, was similar to PDGF-induced NADPH oxidase activation, and was mediated via Gi signaling by $S1PR_1$ and $S1PR_3$. The others did not

consider whether mitochondrial ROS production was upregulated by these interventions nor whether $I_{Cl,swell}$ was activated

4.5. Implications

$I_{Cl,swell}$ is known to regulate cardiac action potential duration (Kawata *et al.*, 1974;Vandenberg *et al.*, 1997;EHARA & HASEGAWA, 1983) and cell volume regulation (Okada & Maeno, 2001;Shimizu *et al.*, 2004;Hoffmann & Dunham, 1995), and the current is persistently turned on under isosmotic conditions in models of dilated cardiomyopathy (Clemo *et al.*, 1998); (Baumgarten *et al.*, 2005). Moreover, $I_{Cl,swell}$ also has been implicated in apoptotic or regulatory volume decrease (Rasola *et al.*, 1999;Okada & Maeno, 2001) that precedes apoptotic cell death. To the extent that S1P is elevated during cardiac disease, its effect on $I_{Cl,swell}$ may contribute to the observed responses. Sphingolipid metabolites are known to regulate the function of heart by acting as second messengers to activate various signaling pathway (Karliner & Brown, 2009). Ceramide and S1P accumulate in various cardiac diseases (Chatterjee *et al.*, 2006;Levade *et al.*, 2001). Furthermore, the sphingomyelin/ceramide pathway is activated *in vivo* during ischemia/reperfusion and heart failure (Karliner, 2009a;Means & Brown, 2009), and the oxidation of sphingolipids is implicated in atherosclerotic plaque formation (Holland & Summers, 2008). Also during hypoxia and pre-conditioning an increased mitochondrial ROS production is associated with sphingolipids in experimental models (Lecour *et al.*, 2006). Furthermore, a similar increase in mitochondrial ROS is observed in heart failure model (Sawyer & Colucci, 2000). In the present study, we demonstrate the evidence that , S1P and FTY720-induced current is independent of ROS production from NADPH oxidase, and require only ROS production from mitochondria. In addition, one needs to consider the consequences of mitochondrial ROS production on other ion channels, transporters and signaling pathways. ROS

have multiple targets (for review, see (Thannickal & Fanburg, 2000;D'Autreaux & Toledano, 2007)), and one or more of these may contribute to the effects of S1P and FTY720-P in the heart and other tissues. S1P has long been known to offer cardioprotection during ischemia-reperfusion injury (Karliner, 2009a;Karliner, 2009b). Multiple factors may contribute. First, interventions that stimulate mitochondrial ROS production can be protective in ischemia-reperfusion models (Pain *et al.,* 2000;Vanden Hoek *et al.,* 1998;Carroll *et al.,* 2001;Halestrap *et al.,* 2004). Nevertheless, the effects activation of $I_{Cl,swell}$ by S1P, including regulation of cell volume and action potential duration, may also contribute. In particular, we would expect activation of $I_{Cl,swell}$ and the outwardly rectifying current it generates would lessen the occurrence of ventricular arrhythmias associated with action potential prolongation. On the other hand, abbreviation of action potential duration shortens the minimum length of the conducting pathway required to sustain re-entrant tachyarrhythmia's that can lead to fibrillation. The above predictions of the effects of S1P and FTY720-P on arrhythmogenesis based on their modulation of $I_{Cl,swell}$ are likely to be too simplistic, however. As already noted, ROS production by these agents has multiple targets and the response of all the targets must be integrated to fully appreciate the consequences for cardiac function.

4.6 Future Directions

One of the important direction is to elucidate role of Sphingosine kinase (SphK) in production of S1P and modulation of $I_{Cl,swell}$ SphK are lipid kinase that catalyzes production of S1P from sphingosine by ATP dependent phosphorylation. SphK is stimulated by a variety of agonist. Two mammalian cardiac isoforms are known SphK1 and SphK2. The two isoforms show different temporal, spatial, and tissue distribution. Though they are responsible for production of S1P, due to differences in their expression, S1P produced by action of different

isoforms might play different roles and may be involved in different signaling pathway. Both the isoforms are expressed in heart. In contrast to SphK1, SphK2 is mainly present in intracellular compartments, including the nucleus and mitochondria. Nuclear SphK2 regulates gene transcription at least in part by producing S1P, which acts as an endogenous inhibitor of histone deacetylases (Hait *et al.*, 2009). In mitochondria the action(s) of SphK are required for correct assembly of the cytochrome oxidase complex. However, the exact ligand for the mitochondrial targeting signal is not known (Strub *et al.*, 2011). Thus it would be important to understand how S1P produced by each isoform affect the modulation of $I_{Cl,swell}$. In order to test this hypothesis, we could provide an enzyme which stimulates S1P production by the action of kinase on sphingosine. SMase acts on sphingomyelin to produce ceramide, which in turn is converted to sphingosine. Sphingosine, upon the action of SphK is converted to S1P. Pharmacological blockers of SphK could be used to determine the role of SphK and specifically which isoform is involved in modulation of $I_{Cl,swell}$. An experiment design would include the use of SMase in control bath solutions. Subsequently a SphK inhibitor N,N-dimethylsphingosine (DMS) and D,Lthreo- dihydrosphingosine (DHS), which inhibits both SphK1 and SphK2 could be utilized in presence of SMase. Failure to see SMase induced activation of current would demonstrate either SphK1or SphK2 or both kinases were required in formation of S1P and thereby in regulation of $I_{Cl,swell}$. Furthermore to test which isoform is important (2R,3S,4E)-N-methyl-5-(4'-pentylphenyl)-2-aminopent-4-ene-1,3-diol, designated SK1-I (BML-258), a potent, water-soluble, isoenzyme-specific inhibitor of SphK1 (Paugh *et al.*, 2008) applied in the presence of SMase. Attenuation of current from control would suggest that SphK1 is the required isoform for S1P production in cardiac myocytes and thereby modulates $I_{Cl,swell}$. On the other hand failure to inhibit SMase induced current precludes the role of SphK1 would suggest SphK2 as the required

isoform. Moreover ABC294640 selective inhibitor of SphK 2 (Becker *et al.*, 1992) be utilized in the presence of SMase to confirm the role of SphK2. If the SMase induced current is inhibited then it would confirm the role of SphK2 in S1P production in heart. Thus the aforementioned experiments would help identify the isoform(s) required for S1P production in heart. Similar experiments could be done in presence of ceramide and sphingosine instead of SMase. Ceramide and Sphingosine are downstream metabolites of SMase, observation of similar behavior with their use will support the metabolic pathway in production of S1P as suggested and corroborate the role of SphK in modulating $I_{Cl,swell}$.

Genetic approach also would be promising in determining the role of SphK and identifying the specific isoform involved in modulation of $I_{Cl,swell}$. Knockout mice for SphK ($SphK1^{-/-}$), SphK2 ($SphK2^{-/-}$) can be used to characterize the role of SphK in regulating $I_{Cl,swell}$. If both the isoforms modulate $I_{Cl,swell}$, then the aim would be to identify, if there are difference in the regulation of $I_{Cl,swell}$ by a specific isoform. To do these experiments isolated cardiac myocytes from knockout mice and wild type mice of the same strain would be used. Whole cell patch clamp will be used to record the current. SMase will be applied to the cells and the current recorded under isosmotic solutions. Failure to see SMase induced current in cells from SphK1 knockout mice as compared to wild type mice would suggest SphK1 to be a required isoform for formation of S1P and thereby activation of $I_{Cl,swell.}$ On the other hand failure to observe SMase induced current in SphK2 knockout mice as compared with wild type mice would implicate SphK2 as the isoform necessary for generation of S1P and modulation of current. As a positive control exogenous S1P and intracellular S1P to be utilized in both the knockout mice as well as wild type mice. The activation of current by exogenous S1P in all three types of mice would

implicate that indeed lack of particular iso-forms of SphK in the K/O mice fails to generate S1P upon SMase treatment and hence unable to activate the current.

In the present study, we observed that, some intracellular target for S1P is responsible for activating the current rather than the GPCS1PR. Hence the next daunting question is to identify the intracellular binding site for S1P.

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