Effect of sphingosine-1-phosphate on L-type calcium current and Ca²⁺ transient in rat ventricular myocytes

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Abstract

Modulation of Ca^{2+} homeostasis in cardiac myocytes plays a major role in beat-to-beat regulation of heart function. Previously studies suggest that sphingosine 1-phosphate (S1P), a biologically active sphingomyelin metabolite, regulates Ca^{2+} handling in cardiac myocytes, but the underlying mechanism is unclear. Based on our previous studies, in the present study, we tested the hypothesis that S1P-induced functional alteration of intracellular Ca^{2+} handling including the L-type calcium channel current ($\text{I}_{\text{Ca,L}}$) via a signalling pathway involving P21 activated kinase 1 (Pak1). Our results show that, in rat ventricular myocytes, despite that S1P (100 nM) does not affect the basal activity of $\text{I}_{\text{Ca,L}}$ but is able to partially reverse the effect of the β -adrenergic agonist Isopreterenol (ISO, 100 nM) on $\text{I}_{\text{Ca,L}}$. S1P (25 nM) also significantly prevents ISO (5 nM) induced Ca^{2+} waves and diastolic Ca^{2+} release in these cells. Our further molecular characterization demonstrates that Pak1 activity is increased in myocytes treated with S1P (25 nM) compared with those myocytes without treatment of S1P. By immunoprecipitation we demonstrated that Pak1 and protein phosphatase 2A (PP2A) are associated in ventricular tissue indicating their functional interaction. Thus the results indicate that S1P attenuates β -adrenergic stress induced alteration of intracellular Ca^{2+} release and L-type Ca^{2+} channel current at least in part via Pak1-PP2A mediated signalling.

Key words: sphingosine-1-phosphate, S1P; L-type calcium current, $\text{I}_{\text{Ca,L}}$; P21 activated kinase 1, Pak1.

1. Introduction

Sphingosine-1-phosphate (S1P) is a bioactive sphingolipid and has been identified as an important signalling mediator involved in the regulation of a variety of cellular processes such as proliferation, migration, contraction, and intracellular calcium mobilization[1] It has been shown that S1P regulates electrophysiological and contractile properties in cardiac myocytes under both basal and β -adrenergic stress conditions [1], the underlying mechanisms mediating such regulation largely remain elusive. S1P has unique properties as it can act both as an intracellular messenger and also as an intercellular messenger [2-4]. The extracellular effects of S1P are mediated via its interaction with G-protein-coupled receptors. To date, a family of five structurally related receptors with high affinity for S1P has been identified. Tissue expression studies of the S1P1, S1P2, and S1P3 receptors indicates that heart and lung have the highest overall expression of these genes [5, 6], whereas S1P4 and S1P5 receptors are mainly expressed in lymphoid [7] and brain tissues [8], respectively. In recent studies in which mouse ventricular myocytes were used to examine S1P-mediated changes in contractility, S1P treatment dramatically decreased cell shortening and was able to antagonize isoproterenol (ISO) -induced increases in cAMP and positive inotropy[9]. The S1P1 receptor specific agonist, SEW2871, was as efficacious as S1P at antagonizing ISO-induced contractility, and the S1P1,3 receptor antagonist/S1P4 agonist, VPC23019, blocked the negative inotropic effects of SEW2871 which was observed in cells lacking S1P3 receptors[9]. These results therefore suggest that the S1P1 receptor is the predominant receptor mediating the negative inotropic effects of S1P in ventricular myocytes; although the S1P3 receptor may also play a minor role. These results are also corroborated by the findings from the Levkau group indicating that the ability of S1P to inhibit ISO-stimulated contractility is abolished in the cardiac specific S1P1 receptor knockout myocytes [1]. S1P-mediated negative inotropy was also shown to be significantly, but not completely, blocked by the *IK*.Ach inhibitor tertiapin, further substantiating the involvement of *IK*.Ach, a G_i ($\beta\gamma$) regulated ion channel, in S1P1-mediated contractile changes. Thus, S1P1 receptor-mediated negative inotropy may result from other undefined mechanisms. The aims of this study are two-fold: (i) to determine the functional effect of S1P on on $I_{Ca,L}$ and Ca^{2+} transient under basal and β -stimulation states in fresh isolated rat ventricular cells; (ii) to determine the molecular relationship of the S1P receptor, Pak1 and PP2A through co-localization studies.

2. Materials and Methods

2.1 Cell Isolation:

Male rats (3-4 months of age) were killed by cervical dislocation according to the Home Office Guidance on the operation of the Animals (Scientific Procedures) Act 1986 (H.M.S.O.). The heart was rapidly excised, washed in a modified Tyrode medium containing EGTA and heparin and mounted on a Langendorff apparatus for retrograde perfusion via the aorta. Perfusion was initially carried out in a modified Tyrode solution containing (mM): NaCl 136, KCl 5.4, NaHCO₃ 12, Na⁺ pyruvate 1, NaH₂PO₄ 1, MgCl₂ 1, EGTA 0.04, glucose 5; gassed with 95% O₂/5% CO₂ to maintain a pH of 7.4. This was replaced after 2 minutes with a modified Tyrode solution containing 30 mg of collagenase and 10 µL of 0.1 mM CaCl₂ (type II, Worthington Biochemical Corp., Lakewood, NJ, USA), but no EGTA. Additional 20 µL aliquots of 0.1 mM CaCl₂ were added during perfusion. After this enzymatic digestion of the heart, atrial myocytes were isolated by trituration (mechanical disruption using a flame-smoothed glass pipette) and stored at 4 °C in KB medium containing (mM): KCl 70, MgCl₂ 5, K⁺ glutamine 5, taurine 20, EGTA 0.04, succinic acid 5, KH₂PO₄ 20, HEPES 5, glucose 10; pH to 7.2 with KOH.

2.2 Electrophysiology Recording:

Ventricular myocytes were transferred to a small tissue bath on the stage of an inverted microscope on a vibration isolation table and superfused with modified Tyrode containing (mM): NaCl 125, NaHCO₃ 25, KCl 5.4, NaH₂PO₄ 1.2, MgCl₂ 1, CaCl₂ 1.8, glucose 5.5; gassed with 95% O₂/5% CO₂ to maintain a pH of 7.4. Solutions were perfused by gravity and experiments were performed at 36 ± 1 °C. Action potentials were recorded using the perforated patch clamp technique (Axoclamp-2B amplifier, Axon Instruments, USA). Patch pipettes (4–6 MΩ) contained (mM): KCl 150, NaCl 5, MgCl₂ 2, K₂ATP 2, HEPES 5, pH 7.2 with KOH; perforation using amphotericin (Sigma-Aldrich, 250 µg/mL) occurred within a few minutes after seal formation. Electrophysiology data was analysed using pCLAMP software (Axon Instruments).

2.3 Measurements of Ca²⁺ transients

Cells placed on a Grade 1 circular laminin-coated cover slip (Menzel, Glasbearbeitungswerk, Germany) forming the floor of a 1.5 mL perfusion chamber were incubated with 5 µM Fluo-3 AM (Molecular Probes, Leiden, The Netherlands) in K–H buffer (1.2 mM CaCl₂) for 10–20 min in the dark before washout. Cells were scanned using a Leica TCS SP5 confocal scanning laser microscopy system incorporating a Leica 6000 CS inverted microscope with a ×63

water-immersion objective lens (numerical aperture 1.2; confocal aperture 600 μm ; slice thickness 20.5 μm). The Fluo-3 was excited using a 488 nm argon laser, and emission collected between 505 and 550 nm. Series of 250 frames (128 $\times$ 64 pixels/frame) were collected at 25 ms/frame. Images were analysed using custom-made software. Fluorescence, corrected for background signal in regions outside the cells, was measured within defined regions of interest (ROIs; F) and normalized to resting (F₀) values. Peak F/F₀ values were calculated through each time series for each myocyte and a mean peak F/F₀ was calculated for that series. Where indicated, cells were paced at 1 Hz (5 V above threshold of 30–60 V for 2 ms) with two field electrodes. Ca²⁺ transients were measured both from ROIs covering entire cells, as well as localized ROIs.

Myocytes loaded with Fluo-3 AM were selected for the study of spontaneous Ca²⁺ release events (sparks) on the basis of their clear striation pattern, contraction on electrical field stimulation by 5–15% of their resting length, and an absence of spontaneous contractions during a preliminary 1 min observation period. Sparks were recorded in the line scan imaging mode before and after addition of isoproterenol at resting condition along the long axis of the myocyte. Each scan consisted of 512 pixels (=40 μm) scanned at intervals of 1 ms over 5 s. Regions were selected for such imaging with the aid of series of preliminary frame scans obtained at intervals of 25 ms over 5 s from which a presence of spontaneous Ca²⁺ events could be reconstructed. The frequencies (in s⁻¹) of Ca²⁺ sparks were analysed using an in-house custom-made software program. All fluorescence studies were performed at room temperature.

2.4 Immunocytochemistry, Imaging with Confocal Microscopy, Western Immunoblotting and Co-immunoprecipitation:

Experiments were carried out on ventricular myocytes from 7 rats aged 3-4 months. All immunocytochemistry experiments in this study were carried out using a two-step staining method. This method involves an unlabelled primary antibody that reacts with tissue antigen, and a labelled secondary antibody which reacts with the primary antibody. In addition, the following key steps are included in the protocol: (i) Fixation: Paraformaldehyde was used to help formation of chemical cross links between the cellular proteins; (ii) Triton treatment: after fixation and subsequent wash with PBS, the cell/section was treated with triton, a detergent capable of penetrating liposomal bilayers; (iii) Blocking of non-specific sites: the specimens were treated with a protein solution (bovine serum albumin) prior to staining with antibodies to reduce non-specific antibody binding; (iv) Labelling of antibodies: Once delivered, primary antibody was reconstituted according to the supplier's suggestions. Stock

antibody solution was divided into small volumes and stored at -20°C until use. Before experimenting, primary antibody was diluted in PBS solution containing 1 % BSA according to supplier's suggestion or optimised by titration. The antigen-antibody reaction was allowed to take place at 4°C over night. The secondary antibody was also diluted in 1 % BSA buffer according to the manufacture's protocol. The incubation was kept in dark box at room temperature for 1.5 hour. In order to limit the destruction of fluorescence by light over long time due to the production of molecular oxygen two mounting media that contain anti fading reagent were used to increase the photo stability of the fluorescence. CitiFluor anti fading medium (Agar Scientific, UK) and Vectorshield H-1000 (Vector Laboratories, Burlingame, CA USA). Various primary antibodies (Abs) (IgGs) were used for Immunocytochemistry: rabbit anti-EDG01 (S1P1) against human EDG-1 (S1P1) residue 241–253 (1:100 dilutions, Cayman Chemical, Tyne & Wear, UK); rabbit anti-EDG-3 (S1P3) against human EDG-3 (S1P3) residue 12–25 (1:100 dilutions, Cayman Chemical); rabbit anti-PAK1 (1:1000 dilutions, Cell Signaling Technology, Hitchin, UK); rabbit anti-phospho-Pak1 (T423) (1:1000 dilutions, Cell Signaling Technology, Hitchin, UK); and rabbit anti-Akt and anti-phospho-Akt Thr308 residue (1:1000 dilutions, Cell Signaling Technology).

Generation of protein samples from tissue, as well as Western immunoblotting, co-immunoprecipitation and chemifluorescent detection have been described previously [10].

2.5 Confocal Microscopy and Image processing

A Zeiss LSM5 Pascal Confocal Laser Scanning Microscope (Carl Zeiss Jena, Germany) equipped with HeNe and Argon laser were used in this study. For experiments in which subcellular distribution was the main aim, the microscope and laser setting for each acquisition were adjusted to achieve maximal image quality for any individual cell. For experiments in which fluorescence intensity was used as a measure of protein expression, the imaging protocol was fixed in order to allow quantitative comparisons of relative fluorescence intensity among comparable samples.

2.6 Data Analysis:

All data passed normality and equal variance tests. Analysis was performed using ANOVA, the paired or unpaired Student's *t* test as appropriate (SigmaStat, Systat Software Inc), and significance was accepted at $P < 0.05$. Data are presented as mean $\pm$ SE.

3. Results

We first determined the effect of S1P (100 nM) on L-type Ca^{2+} current ($I_{\text{Ca,L}}$) in the absence and presence of β -stimulation by perfusion of isoproterenol (ISO, 100 nM). $I_{\text{Ca,L}}$ was activated by 100 ms step depolarization pulse from to 0 mV from a holding potential of -40 mV at a rate of 0.2 Hz. Figure 1 shows the effect 100 nM S1P on $I_{\text{Ca,L}}$ in ventricular myocytes under the basal condition. Basal $I_{\text{Ca,L}}$ in cells in the conditions of absence, presence 100 nM S1P and wash-off was statistically indistinguishable, which indicates that 100 nM S1P has no effect on basal $I_{\text{Ca,L}}$ in these cells. Figure 2 shows the effect of S1P on ISO-stimulation of $I_{\text{Ca,L}}$ in ventricular myocytes. The amplitude of $I_{\text{Ca,L}}$ was significantly increased in the presence of 100 nM ISO, the peak current at 0 mV increased by 82 % from -344 ± 0.07 pA at the basal condition to -625 ± 0.76 pA in the presence of 100 nM ISO ($p < 0.05$, $n = 9$). Addition of 100 nM S1P significantly blunts the effect of ISO on $I_{\text{Ca,L}}$: in 100nM ISO + 100nM S1P cells, the peak current at 0 mV increased by 32% from -344 ± 0.07 pA to -455 ± 1.80 pA ($p < 0.05$, $n = 9$). The difference in response to ISO indicates that ISO-induced increase in $I_{\text{Ca,L}}$ in cells treated with S1P was significantly attenuated.

We subsequently studied the effect of S1P (25nM) on field-stimulated (5nM) Ca^{2+} transients under i) baseline ii) S1P iii) S1P and isoprenaline (ISO) iv) ISO conditions, as illustrated in figure 3. Transients were recorded for 60 seconds after five minutes of perfusion under a specific condition. As shown in figure 3, administration of S1P significantly prevents ISO (5 nM) induced spontaneous Ca^{2+} waves and diastolic Ca^{2+} release in isolated rat ventricular myocytes under physiological conditions.

Our previous studies demonstrated the link of S1P1 receptor signalling and activation of Pak1 and regulation of Pak1 on LTCC and SR Ca^{2+} release (refs [11, 12]). Thus, we hypothesised that S1P regulate that LTCC and Ca^{2+} transient through Pak1 signaling [11]. We examined the effect of S1P on the activity of Pak1, Immunocytochemistry and confocal microscopy were used to detect phosphorylation of Pak1 protein in ventricular myocytes. The specificity of the primary was examined by antigenic peptide. As illustrated in Figure 4, labelling of rat ventricular myocytes with non-phospho-specific Pak1 antibody (indicating total protein, as opposed to 'active' kinase), in the absence of S1P, shows a striated pattern and some nuclear labelling (figure 4A). In the presence of 25 nM S1P, labelling with the same antibody shows more intense nuclear staining (figure 4B). Immunofluorescence signal intensity measurements

for non-phospho-specific Pak1 were higher in ventricular myocytes treated with S1P compared to controls (Figure 4C). We also employed Western blot analysis to determine Pak1 activation with an anti-phospho Thr 423 antibody (Thr-423 phosphorylation being a requirement for activation of Pak1). As shown in Figure 5, there was a significant (16%) increase in the levels of phospho-Pak1 in myocytes treated with 25 nM S1P compared with non-treated myocytes.

Our previous studies indicated that Pak1 regulates LTCC through PP2A. We therefore examined the effect of S1P on Pak1-PP2A activity. As illustrated in Figure 6, double labelling of rat ventricular myocytes with Pak1 and PP2A antibodies shows co-localisation of Pak1 with PP2A when cells were treated with S1P. We further performed immunoprecipitation (IP) to study the relationship between Pak1 and PP2A. IP was run first and then the proteins were resolved by SDS PAGE and stained with Coomassie blue. As shown in figure 7, a 37 kD band (PP2Ac) is the major protein co-precipitated with Pak1 and PP2A-B antibodies. These experiments suggest S1P may blunt the β -adrenergic stimulation via Pak1-PP2A interaction.

4. Discussion

4.1 Major conclusions

In rat cardiac myocytes, S1P does not affect the basal activity of L-type Ca^{2+} channels but partially reverses the effect of the β -adrenergic agonist ISO. This effect is likely to be mediated by the Pak1-PP2A signalling pathway via S1P1 receptor, although other mechanisms cannot be excluded.

4.2 Expression of S1P receptors in cardiac tissues

In our previous study, we showed that S1P1, S1P2 and S1P3 transcripts and proteins are expressed in rat cardiac tissues and myocytes and that the S1P1 receptor is the dominant isoform in this tissue [13]. To date the S1P receptor subtypes that are found to play a functional role in the cardiovascular system are the S1P1, S1P2 and S1P3 receptors. Cardiac expression of S1P receptors has been studied in mice, rats and humans. The murine heart expresses transcripts for S1P1, S1P2 and S1P3 but not S1P4 and S1P5 receptors [5]. While S1P1, S1P2 and S1P3 receptor mRNA expression also dominates in rat heart [14, 15], RT-PCR analysis additionally detected the presence of transcripts for S1P5 (but not S1P4) receptors in rat atrial and ventricular myocytes [14]. That said, if the overall expression of S1P5 receptors in the heart is low and/or restricted to certain areas, its signal might evade detection in whole tissue lysates. Localization of S1P1, S1P2 and S1P3 receptors within the

human heart was studied by Mazurais et al. [16]. While expression of S1P2 and S1P3 receptors was lower than that of S1P1 receptors, all three subtypes exhibited a similar distribution pattern. Thus, they were present in left and right ventricle, septum and atrium. Himmel et al. had also detected transcripts for these three subtypes of S1P receptors in human atrial cardiomyocytes [17].

4. 3 Functional effect of S1P on L-type calcium current in rat cardiac myocytes

We have demonstrated that, in rat cardiac myocytes, S1P does not affect the basal activity of L-type Ca^{2+} channels but partially reverses the effect of the β -adrenergic agonist ISO on this channel. Our data also showed S1P significantly prevents ISO induced Ca^{2+} waves and diastolic Ca^{2+} release in isolated rat ventricular myocytes under physiological conditions.

Numerous studies have indicated that S1P can alter intracellular ion concentrations in the heart by regulating the activity of various ion channels in the membranes of the sarcoplasmic reticulum and/or in the cell membrane. The influence of S1P on calcium signalling in cardiomyocytes has not been studied in detail, but there are indications that S1P can modulate intracellular Ca^{2+} levels in myocytes [15]. It has been reported that S1P can increase free intracellular Ca^{2+} concentrations in cardiomyocytes from adult [18, 19] and neonatal rats [15]. The observed S1P-induced alterations in free intracellular Ca^{2+} concentration could result from alterations in intracellular Ca^{2+} handling and/or from alterations in the influx of extracellular Ca^{2+} . In rabbit isolated sinoatrial node cells, S1P did not affect the basal activity of L-type Ca^{2+} channels but reversed the effects of the β -adrenergic agonist isoprenaline [20]. This is in well line with our results: in rat cardiomyocytes, S1P does not affect the basal activity of L-type Ca^{2+} channels but reversed the effect of the β -adrenergic agonist ISO on this channel.

4. 4 Co-localization between Pak1 and PP2A

The final objective of this study was to explore some aspects of the signalling pathways of S1P effects in adult rat ventricular myocytes. Our data shows co-localization of Pak1 and PP2A in S1P treated cells. We have also shown that S1P may downregulate ryanodine receptors as well as upregulating L-type Ca^{2+} channels, which may lead to a neutral effect on calcium handling. We show a significant increase in the levels of phospho-Pak1, by 16%, in myocytes treated with 25 nM S1P for 15 minutes compared with non-treated myocytes. Our IP experiments demonstrated that a 37 kD band (PP2Ac) is the major protein co-precipitated with Pak1 and PP2A-B antibodies.

Binding of S1P to its receptors activates different signalling pathways via heterotrimeric G proteins. Fundamental differences in signalling through S1P receptors relate primarily to variations in G-protein coupling. S1P1 couples exclusively via Gi, whereas S1P2 and S1P3 have been shown to couple to either Gi, Gq or G12/13 [21]. The activation of the same second messenger by two receptors does not necessarily imply the same outcome. It is true that there is some redundancy in the system, but the receptors do not have the same “power” in activating a particular second messenger. For example S1P1 null lack support for the blood vessels, which eventually rupture. This receptor is vital for survival and cannot be compensated for by other S1P receptors even if all these receptors are linked to Gi [22]. While S1P1 seems to be the strongest modulator of adenylate cyclase and possibly Rac, S1P2 drives Rho activation and S1P3 is a stronger inducer of the PLC/ IP3/ Ca²⁺ pathway [23-25]. Our prior studies showed S1P1 to be the main S1P receptor isoform in rat ventricular myocytes. Therefore, the effects of S1P seem likely to be linked mainly to Gi-mediated reduction in cAMP production/accumulation in these cells. It follows that S1P may antagonize ISO-mediated activation of adenylyl cyclase through activation of Gi, resulting in the inhibition of cAMP accumulation and prevention of cAMP-mediated calcium increase. Our work suggests that Pak1-PP2A-mediated dephosphorylation of ICaL also contributes to the observed effects of S1P, though it remains to be determined whether other mechanisms and/or proteins contribute to these observed effects and signalling pathways.

Paks are a highly conserved family of serine/threonine protein kinases [26].

Nearly all eukaryote genomes contain one or more Pak genes [27]. Pak family members regulate cellular proliferation, differentiation, transformation, and survival. They also play important roles in cytoskeletal rearrangement during cell migration. In mammals, Paks can be grouped into two subfamilies: group A (PAK1, 2, and 3) can be activated by small GTPases such as Rac-GTP or Cdc42-GTP binding [26]. Group B (PAK4, 5, and 6) can interact with Cdc42-GTP but are not activated by this binding. In addition, PAK1, 2, and 3 can be activated by Rac/Cdc42-independent mechanisms such as by caspase-mediated cleavage, membrane recruitment via adaptor proteins, and sphingolipids [26]. Guo et al. [28] demonstrated that an activated form of Rac1 GTPase—but not the related Rho or Cdc42 GTPases— stimulates transmembrane but not soluble guanylyl cyclase (GC) activity in transfected cells. Guo et al. also showed that PAK directly increases the activity of purified GC. This new Rac/PAK/GC/cGMP pathway postulated by the authors could provide a general mechanism for various types of signalling receptors to increase the cellular concentrations of cGMP. Like cAMP and Ca²⁺, cGMP is a ubiquitous second messenger mediating cellular responses to

various exogenous and endogenous signalling molecules. Since cGMP regulates physiological processes by activating protein kinases, gating specific ion channels and modulating cellular cyclic nucleotide concentrations through phosphodiesterases [29], this brings up another potential mechanism underlying S1P effects. Cyclic Nucleotide-Gated Channels (CNG) are a family of voltage-gated cation channels expressed in a variety of cells. Hyperpolarization-activated cyclic nucleotide-gated (HCN) channels are a specific subfamily of CNG channels. These channels are expressed in brain and heart and are modulated by both direct binding of cyclic nucleotides and hyperpolarization of the plasma membrane [30, 31]. The principal family of CNG channels regulates influx of Na^+ and Ca^{2+} into cells and is comprised of tetrameric proteins that are directly "opened" by cyclic nucleotides [32]. In contrast to HCN, CNG proteins display very weak voltage sensitivity and consist of a heterotetrameric assemblage of α and β -subunits [32]. Cyclic GMP-Dependent Protein Kinases (PKG) represent the principal intracellular mediator of cGMP signals. Two PKG genes, coding for PKG type I and type II, have been identified in mammals [33, 34]. In general, type I and II are expressed in different cell types. The type I has been detected at high concentrations (above $0.1 \mu\text{mol/L}$) in all types of smooth muscle cells (SMCs) including vascular SMCs and in platelets [35]. Lower levels are present in vascular endothelium and cardiomyocytes [35]. Previous studies suggested that nitric oxide (NO)/cGMP contribute to the regulation of cardiac function and remodelling [36]. The combined analysis of conventional and cardiomyocyte-specific PKG type I knockout mice demonstrated that PKG type I mediates the negative inotropic effect of NO/cGMP in the juvenile as well as in the adult murine heart [37]. However, the NO/cGMP/PKG type I pathway does not appear to be involved in the negative inotropic action of acetylcholine [37]. This again brings up another potential mechanism that could explain S1P effects.

In summary, as illustrated in Figure Figure 8 [38], these S1P/Pak1 effects on Ca^{2+} homeostasis complement previously established actions upon PP2A and the resulting balance between kinase and phosphatase activity in controlling cardiac ion channel activity and rhythmic Ca^{2+} cycling.

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Conflict of interest: On behalf of all authors, the corresponding author states that there is no conflict of interest.

Figure Legends

Figure 1. L-type Ca^{2+} current ($I_{\text{Ca,L}}$) was recorded from ventricular cells in the presence of 100 nM S1P for 2 min. Current was recorded by 100 ms step depolarization pulse from -40 mV to 0 mV from holding potential of -40 mV (left). There is no statically significant difference in amplitude of $I_{\text{Ca,L}}$, in control and presence of 100 nM S1P and wash-off conditions in these cells (n=8).

Figure 2. Representative current traces demonstrating the effects of 100 nM S1P on the response of L-type Ca^{2+} current to 100nM ISO. 100 ms steps depolarisations were applied from -40 mV to 0mV from holding potential of -40 mV (n=9). Addition of 100 nM S1P significantly blunts the effect of ISO on $I_{\text{Ca,L}}$.

Figure 3. A- Ca^{2+} transients in field-stimulated cardiac myocytes under i) baseline ii) sphingosine-1-phosphahte (S1P) iii) S1P and isoprenaline (ISO) iv) ISO conditions. Administration of S1P shows significant reduction in β -adrenergic stress conditions induced by ISO.. B-Effect of S1P (25nM), ISO (5nM) and S1P+ISO on spontaneous cytosolic calcium signals during regular field-stimulated pacing. Quantification was based on the total number of irregular calcium transients divided by the total number of calcium transients measured in 60 seconds.

Calcium-dependent fluorescence (Fluo 4-AM) transients were recorded from isolated rat ventricular myocytes. Transients were illuminated and recorded at 490nm. Measurements were recorded for 60 seconds after five minute of solvent perfusion. Data are presented as mean $\pm$ S.E.M. * indicates $P < 0.05$.

Figure 4. Effect of S1P on non-phospho-specific Pak1 expression. A-Confocal images of isolated non-treated rat ventricular myocytes labelled for non-phospho-specific Pak1. a) Labelling of a representative rat ventricular myocyte under control conditions with the non-phospho-specific Pak1 antibody (Biocompare) shows a striated pattern and nuclear labelling. b) Labelling of a representative rat ventricular myocyte under control conditions, following omission of the primary antibody, shows no discernable pattern of labelling. B- Confocal

images of isolated, S1P-treated rat ventricular myocytes labelled for non-phospho-specific Pak1. a) Labelling of a representative rat ventricular myocyte treated with S1P shows more diffuse pattern and more intense nuclear labelling. b) Labelling of a representative rat ventricular myocyte after exposure to S1P, following omission of the primary antibody, shows no discernable pattern of labelling. C-Density of labelling of non-phospho-specific Pak1 in control and treated ventricular myocytes. Immunofluorescence signal intensity measurements for non-phospho-specific Pak1 were higher in ventricular myocytes treated with S1P compared to the control. Scale bars, 20 μ m. * p <0.05, n=18.

Figure 5. A-Representative Western blot to show Phospho-Pak1 and Phospho-Akt in isolated rat cardiac myocytes treated with 25nM of S1P. Ventricular cardiac myocytes were exposed for 15 min. B- Quantification of Pak1 and Phospho-Pak1 (Thr 423) proteins in these cells.

Figure 6. Effect of S1P on Pak1-PP2A expression. A-Confocal images of isolated rat ventricular myocytes without S1P show co-localisation of Pak1 with PP2A. B-Confocal images of isolated S1P-treated rat ventricular myocytes show co-localisation of Pak1 with PP2A. In both A and B, a) Pak1 antibody labelling (from Spring Bioscience), b) PP2A antibody (from Exalpa Biologicals) labelling. c) Co-localisation of Pak1 and PP2A. C - Higher magnification of A & Bc. D-Density of labelling of Pak1-PP2A in control and S1P-treated ventricular myocytes. Immunofluorescence signal intensity measurements for Pak1-PP2A were higher in ventricular myocytes treated with S1P compared to the control. Scale bars, 20 μ m. * p <0.05, n=24.

Figure 7. Immunoprecipitation (IP) in rat ventricular muscle homogenate. Lane 1, Pak1 antibody. Lane 2, PP2A B alpha antibody. Coomassie blue staining.

Figure 8. Hypothesis for S1P inhibition of ISO stress induced abnormal Ca^{2+} events in rat ventricular myocytes [38]. Protein kinase PKA and phosphatase PP2A are associated with key Ca^{2+} handling and regulatory proteins, which in turn are linked to upstream signaling cascades. A balance of protein kinase and phosphatase activities is required to maintain normal cardiac functions. Pak1 also regulates hypertrophic signaling and gene expression of SERCA2a through other signaling pathways by activating its downstream effectors (e.g., JNK). (NCX: Na^{+} - Ca^{2+} exchanger, PMCA: Plasma membrane Ca^{2+} ATPase, JNK: c-Jun N-terminal Kinase).

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