

S1P₁ Receptor Localization Confers Selectivity for G_i-mediated cAMP and Contractile Responses*[§]

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Adult mouse ventricular myocytes express S1P₁, S1P₂, and S1P₃ receptors. S1P activates Akt and ERK in adult mouse ventricular myocytes through a pertussis toxin-sensitive (G_{i/o}-mediated) pathway. Akt and ERK activation by S1P are reduced ~30% in S1P₃ and 60% in S1P₂ receptor knock-out myocytes. With combined S1P_{2,3} receptor deletion, activation of Akt is abolished and ERK activation is reduced by nearly 90%. Thus the S1P₁ receptor, while present in S1P_{2,3} receptor knock-out myocytes, is unable to mediate Akt or ERK activation. In contrast, S1P induces pertussis toxin-sensitive inhibition of isoproterenol-stimulated cAMP accumulation in both WT and S1P_{2,3} receptor knock-out myocytes demonstrating that the S1P₁ receptor can functionally couple to G_i. An S1P₁ receptor selective agonist, SEW2871, also decreased cAMP accumulation but failed to activate ERK or Akt. To determine whether localization of the S1P₁ receptor mediates this signaling specificity, methyl- β -cyclodextrin (M β CD) treatment was used to disrupt caveolae. The S1P₁ receptor was concentrated in caveolar fractions, and associated with caveolin-3 and this localization was disrupted by M β CD. S1P-mediated activation of ERK or Akt was not diminished but inhibition of cAMP accumulation by S1P and SEW2871 was abolished by M β CD treatment. S1P inhibits the positive inotropic response to isoproterenol and this response is also mediated through the S1P₁ receptor and lost following caveolar disruption. Thus localization of S1P₁ receptors to caveolae is required for the ability of this receptor to inhibit adenylyl cyclase and contractility but compromises receptor coupling to Akt and ERK.

The lysophospholipid, sphingosine 1-phosphate (S1P),² regulates numerous cellular responses. These responses are mediated through binding of S1P to its cognate cell surface G pro-

tein-coupled receptors. There are currently five receptors for which S1P is the high affinity ligand. Upon binding S1P, these receptors activate a variety of heterotrimeric G-proteins of the G_v, G_q, and G_{12,13} families to generate second messengers that elicit cellular responses including survival, proliferation, migration, angiogenesis, and actin cytoskeletal rearrangements (1–3). S1P₁, S1P₂, and S1P₃ receptors are expressed in numerous tissues and have been extensively studied, whereas S1P₄ and S1P₅ receptor expression is limited to a few select tissues and their function is less clearly understood.

The S1P₁ receptor has been shown to signal exclusively through the heterotrimeric G-protein, G_i (4). This conclusion is supported by binding assays with [³⁵S]GTP γ S demonstrating that in Sf9 and human embryonic kidney cells expressing the S1P₁ receptor, S1P promotes the exchange of GDP for GTP only on G_i but not on G_s, G_q, G₁₂, or G₁₃ proteins (4). S1P₁ receptors expressed in human embryonic kidney, Sf9, and COS cells have also been shown to activate ERK (4–6) and to inhibit forskolin-stimulated cAMP accumulation (6–8) through pertussis toxin (PTX)-sensitive pathways, corroborating the role of G_i as a mediator of signaling downstream of the S1P₁ receptor.

Whereas the S1P₁ receptor couples exclusively to G_i proteins, the S1P₂ and S1P₃ receptors are more promiscuous, coupling to the G_v, G_q, and G_{12/13} families of heterotrimeric G-proteins. Coupling of S1P₂ and S1P₃ receptors to these G-proteins has been confirmed by GTP γ S binding assays (4). Analysis of the signaling pathways downstream of these receptors, which includes activation of phospholipase C and Rho also implicates G_v, G_q, and G_{12/13} in mediating the effects of the S1P₂ and S1P₃ receptors (4, 7, 9–11).

Heterologous expression systems have, as described above, identified the G-proteins and pathways through which the S1P receptors can signal, but far less is known about which pathways are activated by endogenous receptors. The lack of subtype-specific agonists and antagonists, as well as poor receptor antibodies, has made it difficult to study the function of each endogenous receptor subtype. Thus, the use of mice in which individual S1P receptors have been deleted provides a powerful tool for investigating the signaling downstream of discrete S1P receptor subtypes (9, 12). Our earlier studies demonstrated that S1P₃ receptor knock-out mouse embryonic fibroblasts (MEFs) show a complete loss of S1P-mediated phospholipase C activation and a slight decrease in Rho activation, whereas S1P₂ receptor knock-out MEF cells show almost a complete loss in S1P-stimulated Rho activation (9, 12). Others have used S1P receptor knock-out MEF cells to show that Akt phosphorylation is mediated by the S1P₃ receptor (13) and that the S1P₂

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[§] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Figs. S1–S2.

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² The abbreviations used are: S1P, sphingosine 1-phosphate; GTP γ S, guanosine 5'-3-O-(thio)triphosphate; ERK, extracellular signal-regulated kinase; PTX, pertussis toxin; WT, wild type; M β CD, methyl- β -cyclodextrin; MES, 4-morpholineethanesulfonic acid; MAP kinase, mitogen-activated protein kinase; JNK, c-Jun NH₂-terminal kinase; Cav-3, caveolin-3; MEF, mouse embryonic fibroblast.

receptor negatively regulates platelet-derived growth factor-induced motility and proliferation (14). In addition, studies using receptor knock-out mice have demonstrated that the S1P₂ receptor mediates wound healing in hepatic myofibroblasts after liver injury (15), whereas S1P₃ receptors are required to increase intracellular calcium in endothelial cells (16).

We have previously shown that mRNA for the S1P₁, S1P₂, and S1P₃ receptors is present in the heart. In experiments using S1P_{2,3} receptor double knock-out mice we demonstrated that activation of both S1P₂ and S1P₃ receptors, and subsequent Akt activation, contributes to protection from ischemia reperfusion injury *in vivo* (17). Work from the Levkau group (18) also used knock-out mice to demonstrate that the S1P₃ receptor contributes to cardioprotection during ischemia reperfusion. The role played by the S1P₁ receptor cannot be similarly determined as the S1P₁ receptor knock-out mouse shows embryonic lethality and the cardiac specific knock-out is not yet available. Thus whereas S1P signaling has been increasingly linked to regulation of cardiac function, it has been difficult to identify the role of the various S1P receptor subtypes, particularly the S1P₁ receptor.

In this study, one of the first to delineate signaling pathways for endogenous S1P receptors in terminally differentiated cells, we utilized adult mouse cardiomyocytes from S1P₂, S1P₃, and S1P_{2,3} receptor knock-out mice. The data presented here provide unexpected insights into the role of the S1P₁ receptor and contrast signaling of endogenous S1P₁ receptors with that of endogenous S1P₂ and S1P₃ receptors. Additionally, we show that the S1P₁ receptor is compartmentalized and that localization plays an important role in determining the selectivity of this receptor for coupling to and activating G_i-mediated signaling pathways.

MATERIALS AND METHODS

Reagents—Sphingosine 1-phosphate was obtained from Avanti Polar Lipids. SEW2871 and pertussis toxin were obtained from Calbiochem. Methyl- β -cyclodextrin, isoproterenol, and carbachol were purchased from Sigma. Phospho-Akt, phospho-ERK, phospho-JNK, total Akt, total ERK, and total JNK antibodies were purchased from Cell Signaling Technologies and the caveolin-3 and S1P₁ receptor antibodies were purchased from Abcam. The cAMP enzyme immunoassay was purchased from GE Healthcare. Quantitative PCR primers and reagents were purchased from Applied Biosystems.

Animals—Generation and maintenance of S1P₃ receptor knock-out mice (S1P₃^{-/-}), S1P₂ receptor knock-out mice (S1P₂^{-/-}), and S1P_{2,3} receptor double knock-out mice (S1P_{2,3}^{-/-}) was previously reported (9, 12). Animals had free access to water and food. All experiments reported here were performed using 8–16-week-old male mice. Wild-type littermate animals were used as controls for all experiments with S1P₂ or S1P₃ receptor knock-out mice. For experiments with S1P_{2,3} receptor double knock-out mice, the low frequency of obtaining double knock-out mice (1/16) and WT mice (1/16) from the same litter (1/256) necessitated the use of age-matched wild-type mice of the same background as controls. All procedures were performed in accordance with Guide for

the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee.

Quantitative PCR—Total RNA was isolated from WT and S1P receptor knock-out myocytes using the RNeasy kit and converted to cDNA as previously reported (17). Resulting myocyte cDNA was used for quantitative PCR analysis with TaqMan gene-specific primers for S1P₁, S1P₂, and S1P₃ receptors and glyceraldehyde 3-phosphate dehydrogenase using an ABI7500 system. Values for comparison of a single gene across genotypes were determined using cycle threshold (Ct) data fitted to a standard curve. For comparison of multiple transcripts in a single sample, equal amplification efficiency of primers was confirmed and then the 2^{- $\Delta\Delta$ Ct} method was applied to the Ct values (19). Data represent triplicates for each primer set (in single genotype, multiple gene studies) or triplicates for each genotype (in multiple genotype, single gene studies).

cAMP Immunoassay—Cardiomyocytes were treated with isobutylmethylxanthine (500 μ M) for 10 min, followed by 5–15 min with S1P (1 μ M), SEW2871 (1 μ M), or carbachol (30 μ M), and then stimulated with 1 μ M isoproterenol for 10 min. Cells were lysed and assay was performed according to the kit protocol. Results were obtained by fitting data to a standard curve and then normalizing to total protein per sample. For methyl- β -cyclodextrin studies, cells were treated with 1 mM M β CD for 1 h prior to the start of the assay. For cholesterol repletion experiments, cells were treated with 1 mM M β CD for 30 min and then 100 nM cholesterol was added back for 30 min prior to beginning the assay.

Isolation of Adult Mouse Cardiomyocytes—Cardiomyocytes were isolated from the ventricles of 8–16-week-old WT or S1P receptor knock-out mice according to the method adapted from Refs. 20 and 21 by the Alliance for Cell Signaling. Briefly, animals were anesthetized with pentobarbital, and hearts were removed, cannulated, and subjected to retrograde aortic perfusion at 37 °C, at a rate of 3 ml/min. Hearts were perfused for 4 min in Ca²⁺-free buffer, followed by 8–10 min of perfusion with 0.25 mg/ml collagenase (Blendzyme 1, Roche). Hearts were removed from the cannula and the ventricle was dissociated at room temperature by pipetting with increasingly smaller transfer pipettes. Collagenase was inactivated once the tissue was thoroughly digested, by resuspending the tissue in medium containing 10% bovine calf serum. Calcium was gradually added back to a final concentration of 1 mM and cells were plated on laminin-coated dishes in minimal essential medium/Hanks' balanced salt solution containing 5% serum. After 1 h, cells were washed and serum-free medium was added back. Cells remained in serum-free medium overnight (10) and biochemical responses were measured the next day.

Preparation of Caveolar Fractions—Caveolar fractions were isolated from cardiomyocytes using an alkaline, detergent-free procedure (22). Cardiomyocytes from a 10-cm dish were scraped into 1 ml of carbonate buffer (150 mM sodium carbonate, pH 11, 1 mM EDTA), lysed by passing through a 23-gauge needle 10 times, sonicated, mixed with 1 ml of 80% sucrose in MBS (25 mM MES, pH 6.5, 150 mM NaCl, 2 mM EDTA), and loaded into the bottom of a 12-ml ultracentrifuge tube. Next 6 ml of 35% sucrose was layered on top of the lysate, and finally 4 ml of 5% sucrose was layered on top. Tubes were centrifuged at

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4 °C for 3 h at $39,000 \times g$ in a SW41 swinging bucket rotor. Ten fractions, each 1.2 ml in volume, were removed. Equal volumes of each fraction were analyzed by Western blotting. For studies with M β CD, cells were treated with 1 mM M β CD for 1 h prior to stimulation.

Measurement of Cardiomyocyte Contractility—Cardiomyocytes were resuspended in Tyrode's solution and placed on chambers fitted for the IonOptix contractility system. To assess contractile responses, changes in sarcomere length were measured. Cells were paced at 0.5 Hz and allowed to equilibrate to a steady state of contraction. Cells were then treated with S1P (1 μ M), SEW2871 (1 μ M), or carbachol (30 μ M) plus or minus isoproterenol (10 nM). For studies with M β CD, cells were treated with 1 mM M β CD for 1 h prior to addition of agonists.

Immunoprecipitation Experiments—Cardiomyocytes were lysed in RIPA buffer as previously reported (17). Equal amounts of protein were subsequently incubated with 1 μ g of antibody to either Cav-3 or S1P₁ receptor and protein A/G-agarose overnight at 4 °C. Immunocomplexes were washed with RIPA buffer four times, resuspended in loading buffer, boiled, and then analyzed by Western blotting.

RESULTS

Pertussis Toxin Sensitivity of S1P-mediated MAP Kinase and Akt Activation—Adult mouse ventricular myocytes were pretreated with 100 ng/ml PTX overnight and then stimulated with 5 μ M S1P for 5 min. Activation of ERK, JNK, and Akt were assessed by Western blotting with phosphospecific antibodies. S1P induced a 5-fold increase in ERK phosphorylation, a 4-fold increase in JNK phosphorylation, and a 1.6-fold increase in Akt phosphorylation relative to vehicle control (Fig. 1). After PTX treatment, S1P-mediated activation of ERK was reduced by 70%, activation of JNK was reduced by 90%, and activation of Akt was reduced by 77%. These data demonstrate that a significant component of S1P-mediated Akt and MAP kinase activation in cardiomyocytes occurs through a G_i-coupled S1P receptor.

Loss of S1P-mediated MAP Kinase and Akt Activation in S1P Receptor Knock-out Myocytes—Cardiomyocytes from S1P₂, S1P₃, and S1P_{2,3} receptor double knock-out mice were isolated and stimulated with S1P (5 μ M) for 5 min. Activation of ERK, JNK, and Akt was assessed. ERK activation, relative to WT cells, was reduced by 25% in S1P₃ receptor knock-out myocytes, by 60% in S1P₂ receptor knock-out myocytes, and by 88% in S1P_{2,3} receptor double knock-out myocytes. JNK activation was reduced by 25% in S1P₃ receptor knock-out myocytes, by 70% in S1P₂ receptor knock-out myocytes, and by 77% in S1P_{2,3} receptor double knock-out myocytes. Finally, Akt activation was reduced by 35% in S1P₃ receptor knock-out myocytes, by 65% in S1P₂ receptor knock-out myocytes, and fully inhibited in S1P_{2,3} receptor knock-out myocytes (Fig. 2). Thus nearly all S1P-mediated Akt and MAP kinase activation is abolished in S1P_{2,3} receptor double knock-out myocytes, indicating that the S1P₁ receptor, which is still present in the S1P_{2,3} receptor double knock-out myocytes, is not a major mediator of these S1P-promoted responses. This is particularly surprising because the S1P₁ receptor has been shown to couple

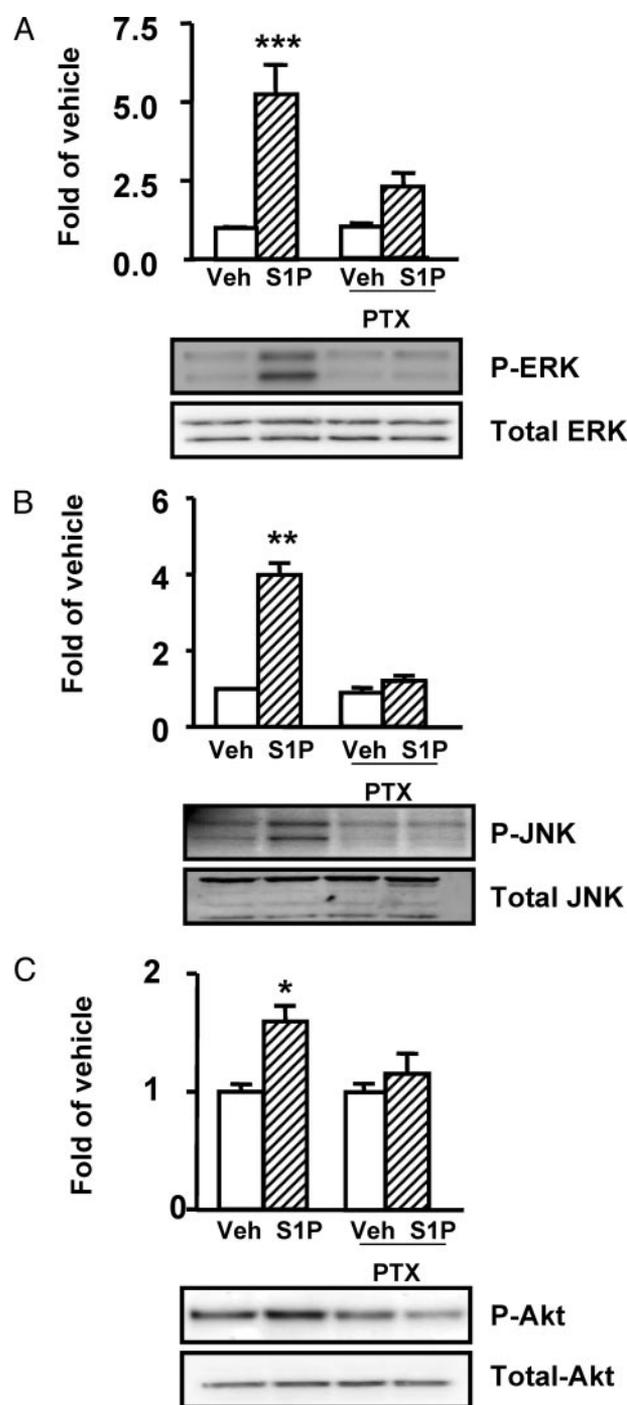


FIGURE 1. Pertussis toxin sensitivity of S1P-mediated activation of ERK, JNK, and Akt in WT adult mouse myocytes. WT myocytes were stimulated with 5 μ M S1P for 5 min and then assayed for phosphorylation of ERK, JNK, and Akt by Western blotting. Representative blots are shown. Phosphorylation was quantitated by densitometry and normalized to vehicle (Veh). Values are mean \pm S.E. ($n > 5$ for each group). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ versus vehicle.

exclusively to G_i, the G-protein that should mediate the pertussis toxin-sensitive Akt and ERK activation shown in Fig. 1.

Expression of the S1P₁ Receptor mRNA in WT and S1P Receptor Knock-out Myocytes—To determine whether S1P₁ receptor expression was altered in the S1P_{2,3} receptor double knock-out myocytes, levels of S1P₁, S1P₂, and S1P₃ receptor mRNA were assessed by quantitative PCR. Based on mRNA levels, the S1P₁

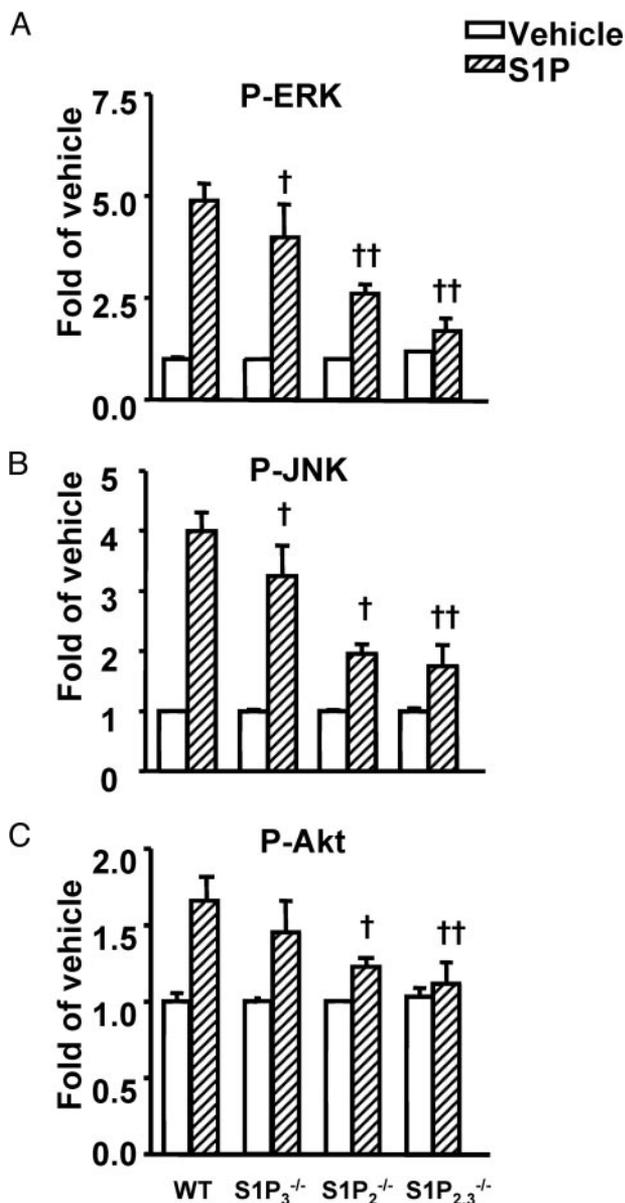


FIGURE 2. S1P-mediated activation of ERK, JNK, and Akt in WT, S1P₃^{-/-}, S1P₂^{-/-}, and S1P_{2,3}^{-/-} adult mouse myocytes. Cardiomyocytes were stimulated with 5 μ M S1P for 5 min and then assayed for phosphorylation of ERK, JNK, and Akt by Western blotting. Phosphorylation was quantitated by densitometry and normalized to vehicle of each genotype. Values are mean \pm S.E. ($n > 5$ for each group). †, $p < 0.05$; ††, $p < 0.01$ versus WT S1P treatment.

receptor is the predominant receptor subtype in WT myocytes, with the S1P₂ and S1P₃ receptor mRNA being expressed at much lower levels (Fig. 3A). S1P₁ receptor mRNA expression is 12-fold higher than that of the S1P₂ receptor, and the S1P₃ receptor mRNA is expressed at over 2-fold the level of the S1P₂ receptor. Notably the levels of S1P₁ receptor mRNA expression did not differ in WT versus S1P₂, S1P₃, or S1P_{2,3} receptor double knock-out myocytes (Fig. 3B). Thus loss of S1P₁ receptor expression is unlikely to account for the markedly attenuated Akt and ERK responses seen in S1P_{2,3} receptor double knock-out myocytes.

The S1P₁ Receptor Mediates Inhibition of cAMP Accumulation through G_i in WT and S1P_{2,3} Receptor Double Knock-

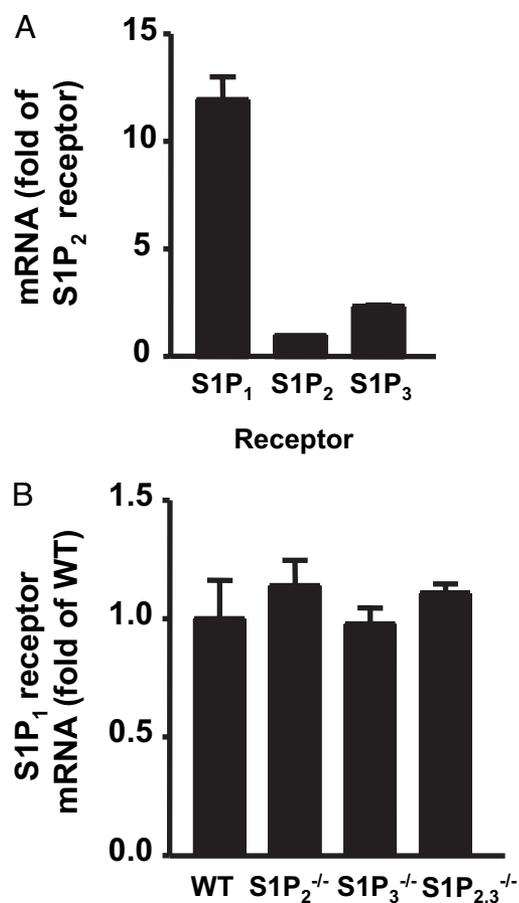


FIGURE 3. Quantitative PCR analysis of S1P receptor mRNA expression in WT and S1P receptor knock-out myocytes. *A*, relative expression of S1P₁, S1P₂, and S1P₃ receptor mRNA in WT mouse myocytes. Results were calculated using the $2^{-\Delta\Delta Ct}$ method. $n > 4$ for each probe set. *B*, expression levels of S1P₁ receptor mRNA in WT, S1P₂^{-/-}, S1P₃^{-/-}, and S1P_{2,3}^{-/-} mouse myocytes. Results were calculated using the standard curve method. $n > 4$ for each genotype.

out Myocytes—The question of whether functionally active S1P₁ receptor is expressed in the cardiomyocyte was then addressed. The canonical response regulated through the α subunit of G_i is inhibition of adenylyl cyclase activity. The ability of S1P to inhibit isoproterenol-stimulated cAMP accumulation was assessed using an enzyme immunoassay. Isoproterenol (1 μ M) stimulation for 10 min led to a 6-fold increase in cAMP accumulation. Addition of S1P (1 μ M) or carbachol (30 μ M) 5 min prior to isoproterenol stimulation resulted in 35 and 65% reductions, respectively, in cAMP accumulation. The involvement of G_i in this response was confirmed as pertussis toxin blocked the inhibitory effect of S1P on isoproterenol-stimulated cAMP accumulation (Fig. 4A). The ability of S1P to inhibit cAMP formation was not diminished in S1P_{2,3} receptor double knock-out myocytes (Fig. 4A). Thus the S1P₁ receptor, which is still present in myocytes from the S1P_{2,3} receptor double knock-out mice, is functionally coupled through G_i to the inhibition of adenylyl cyclase activity.

SEW2871 Inhibits cAMP Accumulation but Does Not Activate ERK or Akt in Cardiomyocytes—SEW2871 is an S1P₁ receptor-specific agonist (23, 24). Stimulation of WT cardiomyocytes with 1 μ M SEW2871 resulted in a 30% decrease in iso-

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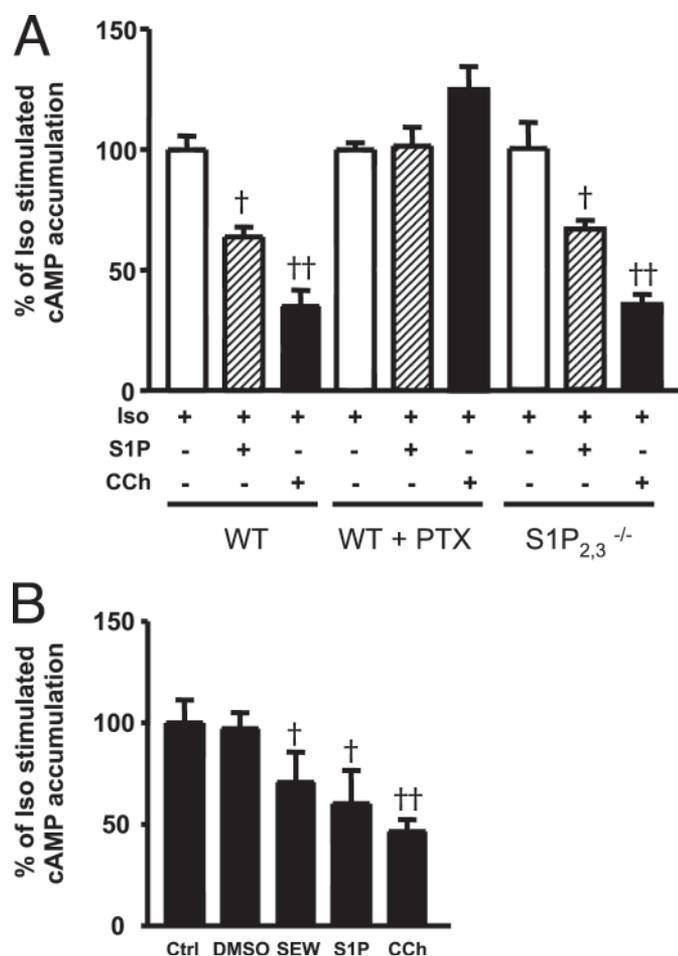


FIGURE 4. S1P- and SEW2871-mediated inhibition (via G_i) of isoproterenol-stimulated cAMP accumulation in WT and S1P_{2,3}^{-/-} myocytes. WT and S1P_{2,3}^{-/-} myocytes were treated with isobutylmethylxanthine for 10 min followed by 10 min of S1P (1 μM), SEW2871 (1 μM), media (control for S1P), or Me₂SO (DMSO) (control for SEW2871, 1:2000) or carbachol (30 μM), and then stimulated with isoproterenol (1 μM) for 10 min. For pertussis toxin, cells were treated overnight with pertussis toxin and the remainder of the assay was performed as above. Cells were lysed and the amount of cAMP was determined by enzyme-linked immunosorbent assay. Amount of cAMP in vehicle-treated sample was subtracted from all other samples and then the amount of cAMP in isoproterenol only-treated samples was normalized to 100%. All other treatments are expressed as a percent of isoproterenol (control). Values are mean ± S.E. (n > 6 for each group). †, p < 0.05; ††, p < 0.01 versus isoproterenol (control). **A**, S1P-mediated inhibition of isoproterenol-stimulated cAMP accumulation is blocked by PTX treatment but still observed in S1P_{2,3}^{-/-} myocytes. **B**, S1P and SEW2871 both significantly decrease isoproterenol-stimulated cAMP accumulation.

proterenol-stimulated cAMP accumulation, an effect comparable with that elicited by addition of S1P (Fig. 4B). This finding provides additional evidence that it is the S1P₁ receptor that mediates adenylyl cyclase inhibition in cardiomyocytes. The effect of SEW2871 on Akt and ERK activation was then examined. Stimulation of WT cardiomyocytes with SEW2871 (5 μM) for 15 min did not elicit ERK or Akt activation (Fig. 5), although this concentration of agonist inhibited cAMP accumulation (Fig. 4B). Additional times of SEW2871 treatment were also examined (5–30 min) and failed to show ERK or Akt activation (data not shown). This contrasts with the ability of S1P to activate both ERK and Akt in these experiments (Fig. 5). These data confirm the observations made using cardiomyocytes from knock-out mice and support the conclusion that the S1P₁

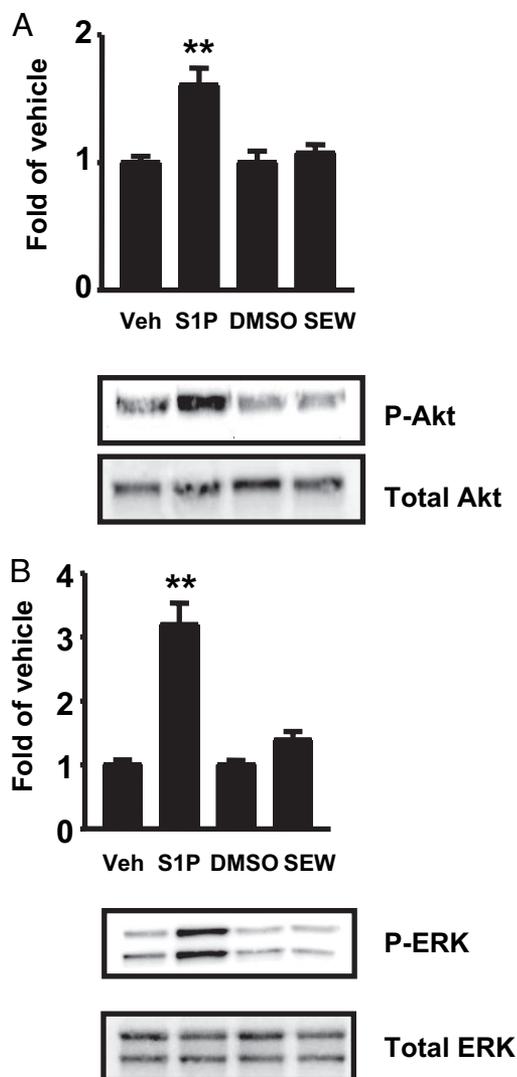


FIGURE 5. S1P- and SEW2871-mediated Akt and ERK activation in WT myocytes. WT myocytes were stimulated with S1P (5 μM), SEW2871 (5 μM), vehicle (Veh) (media control for S1P), or Me₂SO (DMSO) (control for SEW2871, 1:2000) for 15 min and then assayed for phosphorylation of Akt and ERK by Western blotting. Representative blots are shown. Phosphorylation was quantitated by densitometry and normalized to vehicle. Values are mean ± S.E. (n > 4 for each group). **, p < 0.01 versus vehicle.

receptor in adult mouse cardiomyocytes couples to inhibition of adenylyl cyclase but cannot elicit activation of ERK or Akt.

Methyl-β-cyclodextrin Treatment Blocks S1P- and SEW2871-mediated Inhibition of cAMP Accumulation but Not Activation of ERK or Akt—A possible explanation for the ability of the G_i-coupled S1P₁ receptor to regulate adenylyl cyclase, but not ERK or Akt, is that the S1P₁ receptor in the cardiomyocyte is compartmentalized. We hypothesized that S1P₁ receptors were localized to caveolae as this has been shown for S1P₁ receptors heterologously expressed in COS-7 cells (25). Caveolar fractions were isolated from WT myocytes and the presence of the S1P₁ receptor was tested by Western blotting. A band of ~47 kDa, corresponding to the expected size of the S1P₁ receptor, was detected in fractions 4 and 5. This band was not seen when the S1P₁ receptor antibody was preincubated with a blocking peptide for the S1P₁ receptor (data not shown). Caveolin-3 (Cav-3) was also enriched in these same frac-

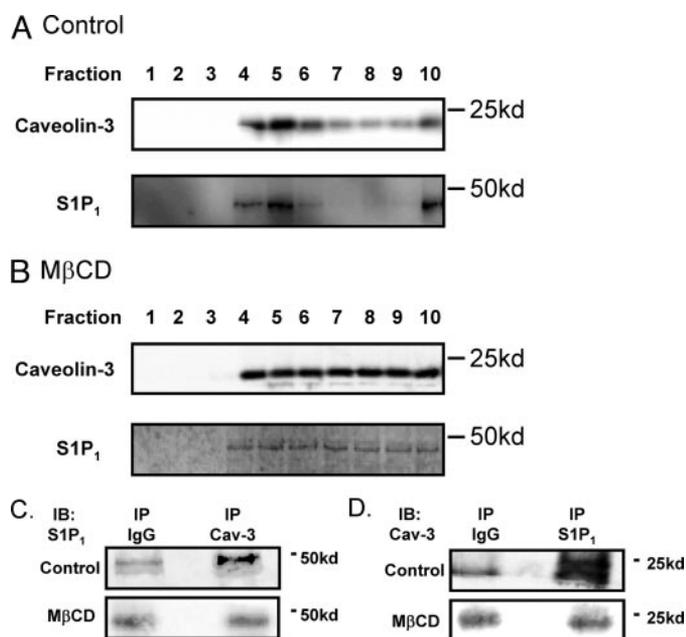


FIGURE 6. Caveolar localization of the S1P₁ receptor. WT cardiomyocytes were lysed in detergent-free bicarbonate buffer and prepared by centrifuging on a discontinuous 40/35/5% sucrose gradient. Equal volumes of each fraction were analyzed by Western blotting. For methyl- β -cyclodextrin experiments, cells were treated with methyl- β -cyclodextrin (1 mM) for 1 h prior to fractionation. Fraction 1 corresponds to the top fraction, whereas fraction 10 corresponds to the bottom fraction. *A*, caveolar fractions of control treated WT cardiomyocytes. *B*, caveolar fractions of M β CD-treated WT cardiomyocytes. *C* and *D*, coimmunoprecipitation (IP) of caveolin-3 and the S1P₁ receptor in WT adult mouse ventricular lysates demonstrating the association between the S1P₁ receptor and caveolin-3 is disrupted by M β CD treatment. *IB*, immunoblot.

tions, confirming the presence of the S1P₁ receptor in caveolae (Fig. 6A).

The experiments above using caveolar fractionation were complemented by immunoprecipitation studies using Cav-3 and S1P₁ receptor antibodies. Immunoprecipitation of the S1P₁ receptor was demonstrated to co-immunoprecipitate Cav-3 and similarly Cav-3 immunoprecipitation was able to pull down the S1P₁ receptor (Fig. 6, *C* and *D*). These data further substantiate an interaction between the S1P₁ receptor and the caveolar protein, caveolin-3. We further demonstrated that this interaction was dependent on intact caveolae as disruption of caveolae by M β CD resulted in a loss of the interaction between the S1P₁ receptor and Cav-3 (Fig. 6, *C* and *D*).

To determine whether caveolar localization contributes to S1P receptor signaling, WT myocytes were treated with M β CD to disrupt caveolae. Treatment of myocytes with 1 mM M β CD for 1 h prior to caveolar fractionation resulted in a redistribution of caveolin-3 and the S1P₁ receptor from their characteristic locations in fractions 4 and 5 into a more even distribution in fractions 4–10 (Fig. 6B). Cells were then examined for the ability of S1P (1 μ M), SEW2871 (1 μ M), or carbachol (30 μ M) to inhibit isoproterenol-stimulated cAMP formation, using the protocol shown in Fig. 4. Isoproterenol elicited a robust increase in cAMP accumulation and carbachol retained its ability to decrease isoproterenol-stimulated cAMP accumulation by 60%. In contrast, S1P and SEW2871 were no longer able to inhibit cAMP accumulation following M β CD treatment (Fig. 7A). The effects of M β CD were demonstrated to be the direct

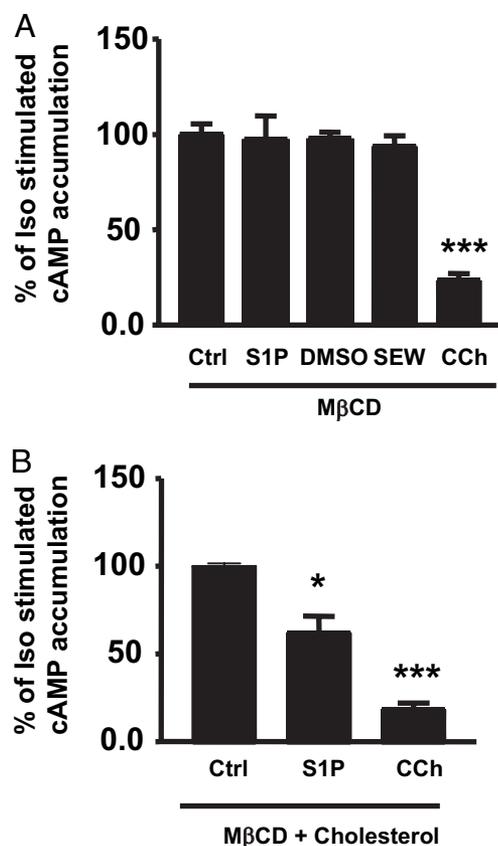


FIGURE 7. Methyl- β -cyclodextrin treatment blocks S1P- and SEW2871-mediated inhibition of isoproterenol-stimulated cAMP accumulation. *A*, WT cardiomyocytes were treated with methyl- β -cyclodextrin (1 mM) for 1 h followed by 500 μ M isobutylmethylxanthine treatment for 10 min, and then S1P (1 μ M), SEW2871 (1 μ M), media (control for S1P), or Me₂SO (DMSO) (control for SEW2871, 1:2000) for 15 min. Finally cells were stimulated with isoproterenol (1 μ M) for 10 min. Cells were lysed and the amount of cAMP was determined by enzyme-linked immunosorbent assay. Amount of cAMP in vehicle-treated sample was subtracted from all other samples and then the amount of cAMP in the isoproterenol only-treated sample was normalized to 100%. All other treatments are expressed as a percent of isoproterenol (Ctrl)-stimulated cAMP accumulation. Values are mean \pm S.E. ($n > 6$ for each group). *B*, following M β CD treatment for 30 min, cholesterol is added back for 30 min and then assay is performed as above. Values are mean \pm S.E. ($n > 6$ for each group). *, $p < 0.05$; ***, $p < 0.001$ versus control.

result of cholesterol depletion and caveolar disruption as the ability of S1P to inhibit isoproterenol-stimulated cAMP accumulation was restored in M β CD-treated cells following cholesterol repletion (Fig. 7B).

S1P and SEW2871 Reduce Isoproterenol-stimulated Positive Inotropy—Isoproterenol treatment leads to increases in cAMP and is known to exert its positive inotropic effect on cardiomyocytes through cAMP-dependent pathways (26, 27). Because we established that S1P and SEW2871 both reduce isoproterenol-stimulated cAMP accumulation, we hypothesized that these agonists would also reduce isoproterenol-stimulated myocyte contractility. As shown in Fig. 8, treatment of myocytes with 10 nM isoproterenol markedly increased contractility as assessed by sarcomere length. Neither S1P nor SEW2871 alone induced any change in basal contractility (Fig. 8A). However, when cells were treated with S1P, SEW2871, or carbachol prior to addition of isoproterenol, the positive inotropic effects of isoproterenol were completely blocked (Fig. 8A). Thus, the ability of S1P and SEW2871 to decrease isoproterenol-stimu-

Localization Confers S1P Receptor Selectivity

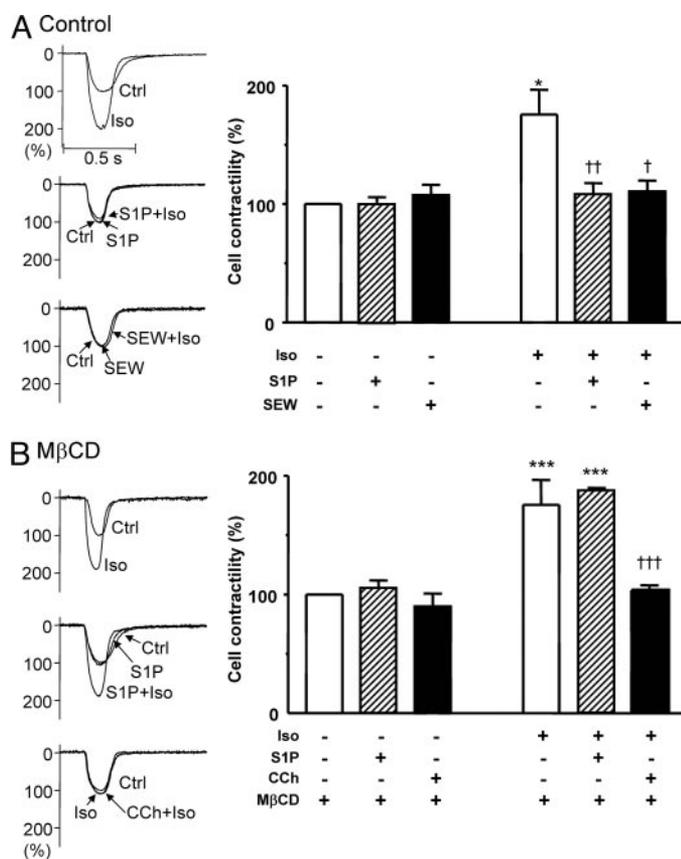


FIGURE 8. S1P- and SEW2871-mediated inhibition of isoproterenol-induced positive inotropy. WT myocytes were paced at 0.5 Hz and S1P (1 μ M), SEW2871 (1 μ M), carbachol (30 μ M), or vehicle were added 10–15 min prior to the addition of isoproterenol (10 nM). Contractility was measured by changes in sarcomere length of control cells (A) or M β CD-treated cells (B). Representative traces reflecting sarcomere length are shown to the left. *, $p < 0.05$; ***, $p < 0.001$ versus control; †, $p < 0.05$; ††, $p < 0.01$; †††, $p < 0.001$ versus isoproterenol (Iso).

lated cAMP accumulation translates into an ability to block isoproterenol-induced positive inotropy.

We demonstrated in Fig. 7A that M β CD treatment blocks S1P- and SEW2871-mediated inhibition of isoproterenol-stimulated cAMP accumulation. This observation suggested that caveolar disruption with M β CD might also impact upon the negative inotropic effects of S1P and SEW2871. To test this, cells were treated with M β CD prior to examining contractile responses to isoproterenol and S1P or carbachol. Remarkably, whereas the effect of carbachol remained intact, there was no longer any inhibition of isoproterenol-stimulated positive inotropy by S1P. Thus by disrupting caveolae, M β CD prevents the S1P₁ receptor from coupling to G_i pathways that reduce cAMP accumulation and myocyte contractility.

Because treatment with M β CD clearly affected S1P-mediated inhibition of adenyl cyclase, we examined the effect of this treatment on the ability of S1P to activate Akt and ERK. WT myocytes were treated with 1 mM M β CD for 1 h prior to S1P stimulation and then activation of ERK and Akt was measured by Western blotting. M β CD increased basal ERK activation, consistent with previous findings showing enhanced basal ERK activation in M β CD- or caveolin small interfering RNA-treated cells (28) or in caveolin knock-out myocytes (29). Importantly, however, M β CD treatment did not block the abil-

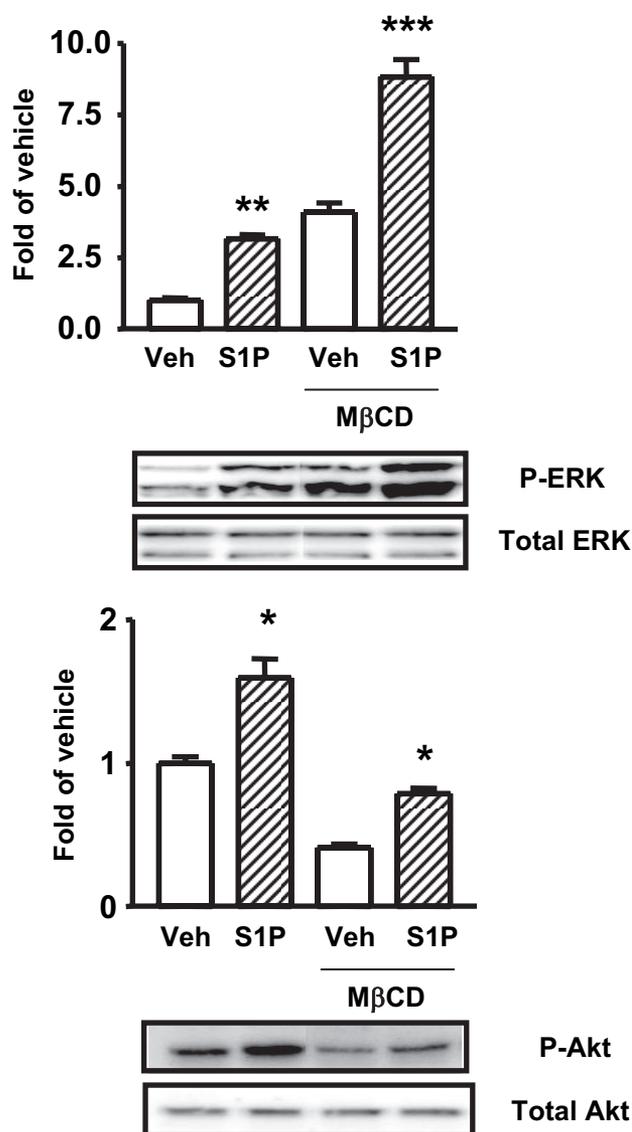


FIGURE 9. Methyl- β -cyclodextrin treatment does not block S1P-mediated activation of ERK or Akt. WT cardiomyocytes were treated with methyl- β -cyclodextrin (1 mM) for 1 h prior to stimulation with S1P (5 μ M) for 5 min and then assayed for phosphorylation of ERK and Akt by Western blotting. Representative blots are shown. Phosphorylation was quantitated by densitometry and normalized to vehicle (Veh). Values are mean \pm S.E. ($n > 4$ for each group). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ versus vehicle.

ity of S1P to further activate ERK. Additionally, M β CD treatment lowered basal Akt activation but the stimulatory effect of S1P on Akt was unchanged (Fig. 9). The lack of effect of M β CD on S1P-mediated Akt and ERK activation contrasts sharply with its disruptive effect on S1P-mediated inhibition of cAMP formation.

DISCUSSION

Sphingosine 1-phosphate signaling pathways have been under intense investigation since the cloning of the first S1P receptor subtypes nearly 10 years ago. Due to a paucity of pharmacological tools, much of the research investigating the role of these receptors has relied upon heterologous expression and biochemical assays to analyze S1P receptor signaling. In the current study we aimed to determine which signaling pathways

occur downstream of endogenous S1P receptor subtypes using a system of differentiated adult mouse ventricular cardiomyocytes isolated from S1P receptor knock-out mice. Remarkably we show that S1P₂ or S1P₃ receptor coupling to G_i elicits ERK and Akt activation but does not contribute significantly to adenylyl cyclase inhibition, whereas coupling of the S1P₁ receptor to G_i inhibits adenylyl cyclase and contractility, but does not regulate Akt and ERK. These findings indicate that the cellular functions of the G_i-coupled S1P₁ receptor are limited, and that S1P receptor selectivity for particular signaling pathways is considerably different in the native cellular context than anticipated from data obtained with heterologous expression systems.

It is generally accepted that the S1P₁ receptor couples exclusively to the G_i family of heterotrimeric G-proteins (4, 30). It has also been documented that MAP kinase and Akt activation by G protein-coupled receptors occurs primarily through a pertussis toxin-sensitive G_i-mediated pathway (31–34). We show in the present study that S1P receptors in cardiomyocytes do in fact activate ERK and Akt through a G_i-mediated pathway as PTX blocks the ability of S1P to elicit these responses. Notably, however, our experiments using an S1P₁ selective agonist, and our findings with cardiomyocytes from S1P receptor knock-out mice reveal that the S1P₁ receptor couples to G_i, but is unable to mediate activation of ERK or Akt in cardiomyocytes.

To understand why the S1P₁ receptor cannot elicit Akt or ERK activation, we examined S1P₁ receptor expression in cardiomyocytes. The S1P₁ receptor is known to be expressed in the heart but it was important to verify that it is in fact expressed on cardiomyocytes as S1P receptors are also present on endothelial, fibroblasts, and other cardiac cell types. Our studies using quantitative PCR show that the S1P₁ receptor is the most abundantly expressed S1P receptor transcript in isolated adult mouse cardiomyocytes. Furthermore, we showed that expression of the S1P₁ receptor is not affected by deletion of the S1P₂, S1P₃, or both S1P₂ and S1P₃ receptors.

To confirm that the S1P₁ receptor is expressed and functional in cardiomyocytes we measured the classical G_i-mediated response, inhibition of adenylyl cyclase activity (35). S1P has been shown to inhibit cAMP accumulation in cells overexpressing the S1P₁ receptor (6, 11), and we previously reported that S1P can inhibit cAMP accumulation in MEF cells from S1P_{2,3} receptor double knock-out mice (9). Our data confirm that the S1P₁ receptor in cardiomyocytes is functionally coupled to adenylyl cyclase inhibition as the S1P₁ receptor selective agonist, SEW2871, is able to inhibit isoproterenol-stimulated cAMP accumulation. Most importantly, we demonstrate that S1P is still able to inhibit isoproterenol-stimulated cAMP accumulation in S1P_{2,3} receptor double knock-out myocytes, thus the S1P₁ receptor in these cells is expressed and functional. We also confirmed that S1P-mediated inhibition of cAMP accumulation in cardiomyocytes is PTX-sensitive. Thus the failure of the S1P₁ receptor to activate Akt or ERK cannot be due to a failure of this receptor to couple to G_i.

S1P receptors have been suggested to transactivate, or cooperate in signaling with growth factor receptors such as those for platelet-derived growth factor (14) or epidermal growth factor

(36). The absence of S1P-mediated ERK or Akt activation in the S1P_{2,3} receptor double knock-out myocytes could in theory reside in the loss of such interactions. There is, however, no reason to suspect that the ability of the S1P₁ receptor to engage these receptors would be altered by deletion of either S1P₂ or S1P₃ receptors. Another possibility is that the S1P₁ receptor cannot act alone to activate ERK or Akt but requires concomitant signaling through other S1P receptor subtypes. However, when WT myocytes, which do not have altered S1P₂ or S1P₃ receptor expression, are stimulated with the S1P₁ agonist, SEW2871, no activation of ERK or Akt is seen. The data from SEW2871-treated cardiomyocytes therefore indicates that the absence of S1P₂ or S1P₃ receptors is not the reason that S1P₁ receptor activation fails to couple to these responses. In summary, both genetic and pharmacologic data indicate that despite being functional, and coupling to G_i, the S1P₁ receptor in cardiomyocytes is unable to activate Akt or MAP kinases.

Membrane organization has been proposed as a mechanism of regulating cell signaling by localizing receptors, G-proteins, and effectors (37). Caveolae, or caveolin containing invaginations of the plasma membrane have been well studied as membrane domains heavily enriched in signaling components (38, 39). In particular, caveolae are known to be enriched in adenylyl cyclase (40–42) and more recently the S1P₁ receptor has also been found in caveolae of COS-7 cells overexpressing this receptor (25). Data presented here demonstrate that endogenous S1P₁ receptors in cardiomyocytes are also localized to caveolae. The pharmacological agent M β CD has been shown to disrupt caveolae by depleting cellular cholesterol (43) and has been previously used to disrupt caveolae in rat cardiomyocytes (44, 45). We show here that M β CD treatment not only disrupts caveolin-3 localization but also that of the S1P₁ receptor, which is no longer concentrated at the 5/35% sucrose gradient interface where caveolae are typically found (22, 46). A minor proportion of the total amount of caveolin-3 and S1P₁ receptor are still seen in fractions 4–5, possibly reflecting incomplete disruption or contamination across fractions. Immunoprecipitation studies further confirmed that the S1P₁ receptor interacts with caveolin-3 and that this interaction can be disrupted by M β CD treatment. Thus we have shown that endogenous S1P₁ receptors are localized to caveolae and that disruption of caveolae disturbs the localization of the S1P₁ receptor.

Because the S1P₁ receptor inhibits cAMP accumulation and caveolae are known to be enriched in adenylyl cyclase (40–42), we asked whether the coupling between this receptor and effector requires caveolar compartmentation. In cells treated with M β CD, S1P was still able to activate ERK and Akt. This finding argues against nonspecific effects of M β CD treatment on S1P receptor function, and also implies that S1P-mediated activation of ERK and Akt does not require caveolar integrity. In contrast, M β CD treatment completely abolished the inhibitory effects of S1P and SEW2871 on isoproterenol-stimulated cAMP accumulation and on isoproterenol-induced positive inotropy. Importantly, the effects of M β CD were reversible as cholesterol repletion following M β CD treatment restored the ability of S1P to inhibit isoproterenol-stimulated cAMP accumulation.

Our data using cells from S1P receptor knock-out mice indicate that ERK and Akt are activated almost exclusively through

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S1P₂ and S1P₃ receptors (Fig. 2). This is consistent with our published work suggesting that coupling of S1P₂ and S1P₃ receptors to Akt activation mediates the protective effect of S1P on ischemic injury *in vivo* (17). The lack of effect of M β CD on these responses suggests that these receptors are not confined to the caveolar compartment, although this cannot be formally proven because there are not adequate antibodies available to detect these endogenous receptors in the mouse. The findings discussed above indicate that the S1P₁ receptor is, in contrast, localized within the caveolar compartment where it signals to adenylyl cyclase. An intriguing question is why activation of S1P₁ receptors and G_i in caveolae does not lead to activation of ERK or Akt, and why activation of S1P₂ and S1P₃ receptors and G_i outside of caveolae does not contribute to inhibition of adenylyl cyclase. One possible explanation is that different G_i subunits mediate these responses. Inhibition of the major isoforms of adenylyl cyclase in the heart (ACV/VI) is known to be mediated by the α subunit of G_i (47), whereas activation of MAP kinases and Akt is thought to occur through β/γ subunits (32, 48–51). One might speculate that β/γ subunits released when G_i is activated through the caveolar S1P₁ receptor are unable to access the signaling molecules (*e.g.* phosphatidylinositol 3-kinase and Ras) that ultimately activate Akt and ERK. Conversely α subunits activated through G_i coupling to S1P₂ and S1P₃ receptors may be unable to reach ACV/VI confined within caveolae. Studies using antibodies selective for α subunits of the different isoforms of G_i were carried out to test the possibility that certain G_i isoforms are enriched in or excluded from caveolae. The specificity and sensitivity of the antibodies was not, however, adequate to allow us to reach conclusions on this interesting possibility.

The physiological role played by the cardiomyocyte S1P₁ receptor in regulating cardiac function *in vivo* remains to be determined, as does the question of whether its physiological effects are distinct from those of the cardiomyocyte S1P₂ and S1P₃ receptors. We show that the endogenous S1P₁ receptor reduces isoproterenol-stimulated positive inotropy suggesting that this receptor may be involved in regulating acute adrenergic signaling in the heart. Our previous studies using three lines of S1P receptor knock-out mice showed that activation of endogenous S1P₂ and S1P₃ receptors provides protection against ischemic injury in the heart (17). Work by others has also shown that the S1P₃ receptor is involved in protection from ischemic injury (18). In other recent studies using S1P₃ receptor knock-out mice we have found that this receptor regulates phospholipase C and is involved in development of cardiac hypertrophy after transverse aortic constriction.³ Thus S1P₂ and S1P₃ receptors in the heart appear to predominantly couple to ERK, phospholipase C, and Akt pathways, well established mediators of cardiac growth and protection. S1P₁ receptors on cardiomyocytes appear, in contrast, to be more involved in regulation of contractility and likely in other calcium or ionic responses downstream of cAMP-mediated pathways (52).

In summary, the current studies, focused on endogenous S1P receptor subtypes in differentiated adult cells, provide new

insights into the specificity of these receptors in regulating signaling pathways and reveal that S1P₁ receptor signaling is more specialized than was once thought. Understanding which S1P receptors and signaling pathways are involved in *in vivo* cardiovascular responses will require consideration of the effects of S1P not only on cardiomyocytes but also on other cell types including cardiac fibroblasts and endothelial cells, as well as definition of the source of S1P. The findings presented here nonetheless, provide new and unanticipated information that should aid in ultimately determining whether pharmacological modulation of these receptors is appropriate and which receptors might be targeted to obtain a beneficial response.

Note Added in Proof—A paper that appeared while this manuscript was under review reports experiments using an S1P1 receptor agonist antibody to demonstrate that the S1P1 receptor can activate Akt and thus protect adult mouse myocytes from hypoxia (53).

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