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Sphingosine-1-Phosphate Elicits Receptor-Dependent Calcium Signaling in Retinal Amacrine Cells

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Crousillac S, Colonna J, McMains E, Dewey JS, Gleason E. Sphingosine-1-phosphate elicits receptor-dependent calcium signaling in retinal amacrine cells. *J Neurophysiol* 102: 3295–3309, 2009. First published September 23, 2009; doi:10.1152/jn.00119.2009. Evidence is emerging indicating that sphingosine-1-phosphate (S1P) participates in signaling in the retina. To determine whether S1P might be involved in signaling in the inner retina specifically, we examine the effects of this sphingolipid on cultured retinal amacrine cells. Whole cell voltage-clamp recordings reveal that S1P activates a cation current that is dependent on signaling through G_i and phospholipase C. These observations are consistent with the involvement of members of the S1P receptor family of G-protein-coupled receptors in the production of the current. Immunocytochemistry and PCR amplification provide evidence for the expression of S1P1R and S1P3R in amacrine cells. The receptor-mediated channel activity is shown to be highly sensitive to blockade by lanthanides consistent with the behavior of transient receptor potential canonical (TRPC) channels. PCR products amplified from amacrine cells reveal that TRPCs 1 and 3–7 channel subunits have the potential to be expressed. Because TRPC channels provide a Ca^{2+} entry pathway, we asked whether S1P caused cytosolic Ca^{2+} elevations in amacrine cells. We show that S1P-dependent Ca^{2+} elevations do occur in these cells and that they might be mediated by S1P1R and S1P3R. The Ca^{2+} elevations are partially due to release from internal stores, but the largest contribution is from influx across the plasma membrane. The effect of inhibition of sphingosine kinase suggests that the production of cytosolic S1P underlies the sustained nature of the Ca^{2+} elevations. Elucidation of the downstream effects of these signals will provide clues to the role of S1P in regulating inner retinal function.

INTRODUCTION

Sphingosine-1-phosphate (S1P) is a sphingosine metabolite that has been linked to numerous cellular functions, including cell signaling, growth, differentiation, and programmed cell death. S1P has also been implicated in neuronal signaling with effects ranging from regulation of neural stem cell proliferation (Harada et al. 2004) and migration (Kimura et al. 2007) to calcium signaling (Giussani et al. 2007; Pollock et al. 2002), increases in excitability (Zhang et al. 2006a,b), and exocytosis (Kajimoto et al. 2007).

S1P is produced through phosphorylation of sphingosine by sphingosine kinase (SphK) (Ghosh et al. 1994; Olivera et al. 1996). There are two forms of SphK (SphK1 and 2); however, most is known about SphK1. SphK1 contains numerous phosphorylation sites and calcium/calmodulin binding sites, suggesting that SphK1 is regulated both by phosphorylation and intracellular Ca^{2+} (Hla et al. 1999). Although the synthesis of S1P is well understood, the mechanism underlying its release is

currently unresolved. It is known that S1P is not released via exocytosis, but there is some evidence that release might occur on an ATP binding cassette transporter (for review, see Kim et al. 2009).

S1P has the potential to act both extracellularly via cell surface receptors and intracellularly as a second messenger (Van Brocklyn et al. 1998). Originally believed to function solely as an intracellular second messenger linked to cell proliferation and survival, it is now well-established that S1P is the endogenous ligand for a family of G-protein-coupled cell surface receptors referred to as S1P receptors (S1PRs). Currently five members of this family (S1P1R–S1P5R) have been identified (see Kluk and Hla 2002 for review). S1P4R, however, has a low affinity for S1P and may have a different endogenous agonist (Candelore et al. 2002).

S1P1R is thought to couple exclusively to the heterotrimeric G protein G_i (Ancellin and Hla 1999; Lee et al. 1998; Windh et al. 1999), consistent with the observation that S1P elicits Ca^{2+} elevations through a pertussis toxin (PTX)-sensitive (and therefore G_i -dependent) pathway in numerous cell types (An et al. 1999; Ancellin and Hla 1999; Kon et al. 1999; Lee O et al. 1999; Okamoto et al. 1998, 1999; Van Brocklyn et al. 1998; van Coppen et al. 1996). Activation of S1P1R results in inhibition of adenylyl cyclase (Zondag et al. 1998), activation of phospholipase C (Okamoto et al. 1998), and mobilization of intracellular Ca^{2+} through IP_3 -sensitive (Formigli et al. 2002; Zhou and Murthy 2004) and IP_3 -insensitive Ca^{2+} stores (Ghosh et al. 1994; Mattie et al. 1994; Meyer zu Heringdorf et al. 1998; Tornquist et al. 1997).

S1P2R and S1P3R can couple to G_i , G_q , and G_{13} (Ancellin and Hla 1999; Ishii et al. 2001; Kon et al. 1999; Okamoto et al. 1999; Sato et al. 1999; Windh et al. 1999; for review, see Sanchez and Hla 2004). Consistent with this promiscuity, activation of these receptors has been linked to both stimulation (Sato et al. 1999) and inhibition of adenylyl cyclase (Okamoto et al. 1999) and stimulation of phospholipase C (An et al. 1999; Ancellin and Hla 1999; Ishii et al. 2001; Okamoto et al. 1999; Sato et al. 1999). There is also evidence for preferential coupling with S1P2R coupling most strongly to G_{13} and S1P3R most strongly activating G_q and subsequent Ca^{2+} mobilization (Ishii et al. 2002). Less is known about S1P5R, but signaling through G_i and G_{12} has been reported (Im et al. 2005; Jaillard et al. 2005; Malek et al. 2001; Novgorodov et al. 2007).

In addition to receptor-mediated pathways, sphingosine derivatives can also act intracellularly. Sphinganine-1-phosphate, which is structurally similar to S1P, activates the entire family of S1P receptors yet does not completely mimic the effects of S1P (Van Brocklyn et al. 1998; Xia et al. 1998), suggesting

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additional sites of action for S1P. Also S1P mobilization of intracellular Ca^{2+} has been observed independent of S1PR expression (Van Brocklyn et al. 1998). Furthermore, effects on proliferation and survival can be achieved through microinjection of S1P as well as through release of intracellular caged S1P; providing further evidence for an intracellular interaction site for S1P (Meyer zu Heringdorf et al. 2003a; Van Brocklyn et al. 1998; Xia et al. 1998).

Very little is known about the signaling capabilities of S1P in the vertebrate retina, but a few of its functions are emerging. S1P can mediate the pathological process of neovascularization that follows retinal injury (Skoura et al. 2007; Xie et al. 2009). There are also recent reports that S1P functions during retinal development. S1P can regulate the proliferation and differentiation of photoreceptors in rat retinal cultures (Miranda et al. 2009) and can also be involved in axonal path-finding by retinal ganglion cells (Strochlic et al. 2008). In amacrine cells, it has been demonstrated that S1P promotes transient Ca^{2+} influx events by a receptor-independent mechanism in store-depleted dendrites (Borges et al. 2008). This indication that S1P can be involved in Ca^{2+} signaling in amacrine cells has led us to ask whether S1P receptor-dependent Ca^{2+} signaling might also occur in these cells.

Amacrine cells are interneurons that participate in complex synaptic interactions in the inner plexiform layer of the retina. Synaptic input and output is not typically segregated in these cells. Instead pre- and postsynaptic sites are intermingled on amacrine cell dendrites. This arrangement implies that signaling between an amacrine cell and its synaptic partners (bipolar, ganglion, and other amacrine cells) is highly localized. Consistent with this, localized Ca^{2+} signals have been demonstrated in amacrine cell dendrites both in the intact retina (Denk and Detweiler 1999; Euler et al. 2002) and in culture (Azuma et al. 2004; Hurtado et al. 2002; Medler and Gleason 2002; Sen et al. 2007). Furthermore local and reciprocal signaling has been established for an inhibitory feedback synapse between amacrine and bipolar cells (Chavez et al. 2006; Vigh and von Gersdorff 2005). This theme of local signaling might also be extended to S1P. Thus far, the only evidence for production of S1P in the inner retina is in amacrine cells (Borges et al. 2008), but given its effects on photoreceptors (Miranda et al. 2009)

and ganglion cells (Strochlic et al. 2008), it is likely to be produced elsewhere as well. Nonetheless it is well established that many cells that synthesize and release S1P also respond to it (for review, see Maceyka et al. 2008).

To further elucidate the signaling role for S1P in retinal amacrine cells, we have examined the effects of S1P on amacrine cells cultured from the chick retina. Here we use a combination of electrophysiology, immunocytochemistry, Ca^{2+} imaging, and molecular biology methods to explore the signaling properties of S1P in these retinal neurons. We demonstrate that S1P activates a receptor-dependent current as well as cytosolic Ca^{2+} elevations. Evidence is presented suggesting that the channels carrying the S1P-dependent current might be transient receptor potential canonical (TRPC) channels. The signaling pathways that underlie the current and the Ca^{2+} elevations appear to be complex and interacting. Intriguingly, we find that synthesis of internal S1P might also contribute to the response.

METHODS

Cell culture

Dissociation and culture methods have been previously reported (Hoffpauir et al. 2006). Briefly, retinas from 8-day-old White Leghorn chicken embryos (*Gallus gallus domesticus*, Animal Sciences Department, Louisiana State University) were dissociated in 0.1% trypsin and plated at 5.0×10^5 cells/35-mm polyornithine-coated (0.1 mg/ml) tissue culture dish. Cells were also plated onto polyornithine-coated glass coverslips for immunocytochemistry. Retinal cultures were maintained in Neurobasal (Invitrogen, Carlsbad, CA) supplemented with B27 (Invitrogen), 1,000 U penicillin/ml, 100 μg streptomycin/ml, and 1 mM L-glutamine (Sigma, St. Louis MO).

Immunocytochemistry

Cells were fixed in 1% paraformaldehyde for 1 h at 4°C. Rabbit polyclonal antibodies raised against sphingosine kinase 1 were purchased from Abgent (San Diego, CA) but produced no labeling in either chicken retinal tissue or cultured cells. Rabbit polyclonal antibodies raised against human S1P1R and S1P3R were purchased from Cayman Chemical (Ann Arbor, MI). Cultures were incubated for 1 h in 5% normal goat serum in dilution solution [1% bovine serum albumin (BSA); 0.1% saponin in PBS]. Anti-S1P1R and S1P3R antibodies were diluted in dilution solution to 1:500 and 1:250, respectively. Cells were incubated in primary antibodies for 1 h at room temperature. After washing, Cy3-conjugated

TABLE 1. Primer sequences for PCR amplifications

Primer	Sequence	Expected Product Size, bp	Annealing Temperatures
TRPC1 forward	5'-GGTATTTTCTGTGAGAAG-3'	483	Ramped from 37–45° C, 1° C/30 sec.
TRPC1 reverse	5'-CTTCTGATAATTCTCGTC-3'		
TRPC3 forward	5'-TCCTATGACGAAGACGGC-3'	600	48°C
TRPC3 reverse	5'-ACAGTTTGGATGAGCCAC-3'		
TRPC4 forward	5'-GCTGCTCCTAAGTTTAAATG-3'	618	44°C
TRPC4 reverse	5'-CCAGTTTCAATCTTGCAAG-3'		
TRPC5 forward	5'-CGCTTTGGTATGACGGTTT-3'	178	48°C
TRPC5 reverse	5'-CCGGTGTGGCAGATAAACTT-3'		
TRPC6 forward	5'-GATGAAGATGGAACACGG-3'	450	45°C
TRPC6 reverse	5'-GCACCTTGGATGTGCTAC-3'		
TRPC7 forward	5'-GAGCTCCTCCTCAAAAAGG-3'	476	47°C
TRPC7 reverse	5'-CTAGTCTGGCCAATTCATTG-3'		
SphK1 forward	5'-GAACCATACCAACCATCAG-3'	221	54°C
SphK1 reverse	5'-TGCAGTTGGTCAGCAGTTTC-3'		
S1P3R forward	5'-GCCATTCTGGTAGCCATTGT-3'	156	53°C
S1P3R reverse	5'-TGACCAACAGGCAATGAAGA-3'		

TRPC, transient receptor potential canonical; SphK, sphingosine kinase; S1P, sphingosine-1-phosphate.

goat anti-rabbit secondary antibodies (Invitrogen) were diluted to 1:1,000 and applied for 1 h at room temperature. Coverslips were mounted in Vectashield (Vector Labs, Burlingame, CA) and viewed under fluorescent optics on an Olympus IX-70 inverted microscope. Digital images were captured using IPLab (Bonn, Germany) version 4.0. Adobe Photoshop 7.0 was used to assemble the figures.

Western blots

Western blot analyses were conducted following the method described in Crousillac et al. (2003). Briefly, tissues for Western blots were obtained from Sprague-Dawley rats (Laboratory Animal Medicine, LSU) killed by decapitation and White Leghorn chickens (Animal Sciences, LSU) killed by CO₂ exposure followed by decapita-

tion. All methods involving animals were conducted in accord with the National Institutes of Health guidelines and with approval of the Louisiana State University Institutional Animal Care and Use Committee. Whole cell lysates of chicken and rat brains were homogenized in IP buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris pH7.4, 1 mM EDTA, 1 mM EGTA, 0.5% NP40) with a protease inhibitor cocktail (Roche, Indianapolis, IN). Protein content was then quantified using the BioRad (Hercules, CA) protein assay kit. Rat brain homogenate (150 μ g) and 50 μ g chicken brain homogenates were loaded onto a 7.5% SDS-PAGE gel. Following electrophoresis, proteins were transferred to nitrocellulose membranes. The membranes were then incubated overnight at 4°C with blocking buffer (1% bovine serum albumin, 0.1% Tween 20, and 2% nonfat dry milk in Tris-buffered saline). Membranes were incubated

FIG. 1. Sphingosine-1-phosphate (S1P) elicits a cation current. *A, top:* a representative amacrine cell is voltage-clamped in the perforated-patch configuration at -70 mV and a control record is obtained. *Middle:* S1P (1μ M) is applied and a small inward current is elicited. A region of the activated current is shown on an expanded time scale (*inset*). *Bottom:* in a recording from another amacrine cell, 10μ M S1P is applied, and after ~ 10 s, a noisy inward current begins to develop. *B:* a voltage ramp is delivered and an S1P-dependent current (leak-subtracted) is measured that reverses at $+8$ mV, suggesting that the current is carried by a mixture of cations. S1P-dependent currents were also recorded during a series of voltage steps (-70 to $+40$ mV). Currents recorded in a representative cell from 6 of those steps are shown in *C*. Outward currents were not observed unless the voltage was stepped positive to 10 mV ($n = 4$). Currents have been offset vertically for clarity.

in S1PR1 antibody (1:1,000, Cayman Chemical) at room temperature for 1 h and then incubated with a goat anti-rabbit IgG peroxidase conjugate secondary antibody (1:5,000) for 1 h at room temperature. The ECL Western blotting detection reagent kit (Amersham, Piscataway, NJ) was used for visualization of the antibodies.

PCR amplification

Retinal amacrine cells were harvested after 7 days in culture. These cells have been previously characterized and can be identified based on morphological criteria (Gleason et al. 1993). Ten to 20 amacrine cells were collected into patch pipettes. The pipette tip was immediately broken into a lysis buffer containing Tris-HCl, LiCl, EDTA, and LiDS. The lysate was kept frozen at -80°C until it was used for RT-PCR. A basic local alignment search tool (BLAST) search revealed predicted *G. gallus* gene sequences encoding TRPC protein subunits 1 and 3–7 as well as S1P3R and SphK proteins. Gene-specific primers for transcripts encoding these proteins were designed using Primer3 (Rozen and Skaletsky 2000) and can be found in Table 1. Primers were obtained from Inte-

grated DNA Technologies (Coralville, IA). Messenger RNA isolation was performed using Dynabeads mRNA DIRECT micro kit (Invitrogen,) following the manufacturer's isolation protocol. RT-PCR was conducted using SuperScript III one-step RT-PCR with platinum *Taq* (Invitrogen) in a PTC-100 thermal cycler (MJ Research, Waltham, MA). See Table 1 for annealing temperatures. Forty cycles were run for each PCR. Actin was used as a positive control and an RT(–) sample was used as a negative control. Samples were then analyzed using 1.5% agarose gel electrophoresis (85 V for 1.25 h) in $0.5\times$ TBE buffer.

PCR products were purified using a ChargeSwitch PCR clean-up kit (Invitrogen) then combined with Big Dye 3.1, ABI buffer, and respective primers. Cycle sequencing was performed in a PTC-100 thermal cycler. EtOH dye terminator removal was conducted prior to sequencing. Samples were then re-suspended in formamide and sequenced using a 3130XL genetic analyzer (Applied Biosystems, Foster City, CA). For all work reported here, PCR product sequences corresponded to regions of the predicted coding transcripts for the individual proteins as determined by BLAST queries.

FIG. 2. Activation of S1P1R and S1P3R may both contribute to the S1P-dependent current. *A*: an untreated cell that is voltage-clamped at -70 mV is exposed to S1P ($10\ \mu\text{M}$), and a noisy, inward current is observed (bottom trace). *B*: cells were pretreated overnight (18–22 h) with pertussis toxin (PTX, $200\ \text{ng/ml}$). A PTX-treated cell that is voltage-clamped at -70 mV exhibits only a small inward shift in baseline current in the presence of S1P. *C*: comparison of the S1P-dependent charge transfer between treated ($n = 21$) and untreated cells ($n = 17$) revealed a significant reduction in the responses of PTX-treated cells ($P = 0.02$). *D*: SEW2871 ($10\ \mu\text{M}$), an S1P1R-selective agonist, also elicits a noisy inward current. *E*: a cell is voltage clamped at -70 mV and exposed to suramin ($100\ \mu\text{M}$), an inhibitor of S1P3R signaling. Suramin reversibly inhibits the S1P-dependent current (middle trace). *F*: analyses of S1P-dependent charge transfer reveals that suramin significantly suppresses the current ($n = 9$, $P = 0.003$). *C* and *F*, error bars are SE.

Electrophysiology

Culture dishes were mounted on the stage of an Olympus IX-70 inverted microscope and a Ag/Ag-Cl pellet served as a reference electrode in the culture dish. Data were acquired using an Axopatch-1D amplifier, Digidata 1322 data-acquisition board, and pClamp 9.2 software (Molecular Devices, Sunnyvale, CA). Unless otherwise noted, voltage-clamp recordings were made in the perforated-patch configuration. Patch pipettes were pulled from borosilicate glass (1.5 mm OD, 0.86 mm ID) using a Flaming/Brown puller (Sutter Instruments, Novato, CA) and had tip resistances of 3–5 M Ω . For perforated-patch experiments, amphotericin B stock was made at 40 μ g/ml in dimethyl sulfoxide (DMSO). Amphotericin was combined 1:2 with Pluronic F-127 (Invitrogen, 25 mg/ml DMSO) and diluted in internal solution for a final amphotericin concentration of 140 μ g/ml. Gigaohm seals were achieved with a brief hyperpolarizing pulse of -140 mV. Then unless otherwise indicated, the voltage was clamped at -70 mV. Voltage values were not corrected for the (unknown) liquid junction potential resulting from the perforated-patch configuration. Recordings were made at room temperature. All current traces shown were leak-subtracted. For experiments in which charge transfers were compared, data from the same time frame were analyzed for each condition, and the mean charge transfer was calculated for five second windows of time.

Fluorescence measurements

Oregon Green bis-(*o*-aminophenoxy)-*N,N,N',N'*-tetraacetic acid (BAPTA) 488 was prepared as a 2 mM stock solution in DMSO, combined 1:1 with Pluronic F127 (Invitrogen, 25 mg/ml DMSO), then diluted to 2 μ M in Hank's balanced salt solution (Invitrogen). Cells were loaded for 1 h at room temperature in the dark. Cells were then washed with external solution and kept in the dark until the beginning of the experiment, typically ~ 20 min. Culture dishes were mounted

on the stage of an Olympus IX-70 inverted microscope, and images were acquired using IP Lab image capturing software version 4.0. Shutter interval for imaging experiments was 1 s (2 s for Fig. 11B), and exposure time was 150 ms. Fluorescence intensity data were collected from amacrine cell bodies. Background fluorescence was subtracted from all data. For display, raw fluorescence intensity values were normalized to baseline values and are reported as F/F_0 .

Solutions and reagents

Reagents were purchased from Sigma unless otherwise indicated. *Normal external solution* consisted of the following (in mM): 5.3 KCl, 136.9 NaCl, 3.0 CaCl₂, 0.4 MgCl₂, 3.0 HEPES, and 5.6 glucose. *Tetraethylammonium (TEA) external solution* consisted of the following (in mM): 5.3 KCl, 116.7 NaCl, 20.0 TEA Cl, 3.0 CaCl₂, 0.4 MgCl₂, 5.6 glucose, and 10.0 HEPES. For electrophysiology, external solutions also contained bicuculline methiodide (3.0 μ M) to block GABAergic synaptic currents and TTX (300 nM) to block voltage-gated Na⁺ channels. *Cesium internal solution* (for ruptured patch) consisted of the following: 100.0 Cs acetate, 10.0 CsCl, 0.1 CaCl₂, 2.0 MgCl₂, 10.0 HEPES, and 1.1 EGTA. *Internal B solution* (for perforated patch) consisted of the following: 135 Cs acetate, 10.0 CsCl, 2.0 MgCl₂, 0.1 CaCl₂, 1.1 EGTA, 10.0 HEPES, and 1.0 NaCl.

S1P (Biomol, Plymouth Meeting, PA) was prepared as a 1 mM stock in methanol then diluted in external solution to a final concentration of either 1 or 10 μ M. The PLC inhibitor U73122 and its less active analog U73343 were used at 10 μ M and purchased from Biomol. The IP₃ receptor antagonist heparin (Calbiochem, San Diego, CA) was included in the patch pipette at a final concentration of 5 mg/ml. The S1PR selective agonist SEW2871 was purchased from Cayman Chemical (Ann Arbor, MI) and prepared as a 10 mM stock in DMSO. PTX (Calbiochem) was used at a final concentration of 200 ng/ml. Cells were incubated for 18 h in PTX diluted in culture

FIG. 3. The S1P-induced current is phospholipase C (PLC)-dependent. *A*: a representative amacrine cell is voltage-clamped at -70 mV. S1P (10 μ M) was applied in the presence of the PLC inhibitor U73122 (10 μ M, middle trace). After 1 min, the U73122 was removed (bottom trace). *B*: the mean charge transfer was significantly reduced by the inhibitor ($n = 6$, $P = 0.02$). *C*: a separate cell is voltage-clamped at -70 mV and then exposed to S1P in the presence of U73343 (10 μ M), a less active analogue of U73122. The analogue did not suppress the S1P current. *D*: the mean charge transfer was unaffected by U73343 ($n = 6$, $P = 0.98$). *B* and *D*, error bars are SE.

medium. Following the incubation period, cells were washed in normal solution and used immediately for electrophysiology experiments. PTX-treated cells used for Ca^{2+} -imaging experiments were washed in normal external solution and loaded with the dye for 1 h prior to recording. Suramin was dissolved directly in the external solution at a concentration of 100 μM . *N,N*-dimethyl-D-erythro-sphingosine (DMS, Biomol) was diluted in ethanol at a concentration of 10 mM then diluted into external solution at a final concentration of 10 μM .

Data analysis

Statistical significance was evaluated using the Student's *t*-test. The data in Figs. 2*E* and 3, *B* and *D*, were evaluated using the paired form of the test. Error bars represent SE.

RESULTS

Individual amacrine cells were voltage-clamped at -70 mV. S1P (1 or 10 μM) elicited a noisy inward current that developed after ~ 10 s of exposure (Fig. 1*A*). Once activated, the current usually persisted after the removal of agonist. Although

the S1P-dependent current could be elicited with 1 μM S1P, the higher concentration was used for most of the experiments because the responses to 10 μM S1P had a faster onset and were more easily distinguishable. To determine the ionic composition of the S1P-dependent current, voltage ramps were delivered during S1P-dependent current activation to reveal its reversal potential (Fig. 1*B*). On average, the current reversed at 7.8 ± 8.5 mV ($n = 11$). The noisy nature of the S1P-dependent current made it difficult to accurately measure its reversal potential and likely contributes to the variability in the values. However, using voltage steps, we found that outward current could only be observed when the voltage was stepped to positive values (Fig. 1*C*, $n = 4$) suggesting that the S1P-dependent current is carried by a mixture of cations.

S1P-dependent current may involve signaling through S1P1R and S1P3R

Because S1P signals through G-protein-coupled receptors and most commonly through G_i , cells were incubated overnight in

FIG. 4. S1P receptors are expressed by amacrine cells. *A*: S1P1R is thought to couple to G_i exclusively, whereas S1P3R can couple to G_i , G_q , or G_{13} . Signaling through these G proteins converges at the level of PLC activation. *B*: Western blot analysis of the S1P1R antibody reveals a single band present for both chicken and rat brain homogenates just below the 50-kDa marker, consistent with the predicted molecular weight for the S1P1R (47 kDa). *C*: secondary-only controls did not show any nonspecific labeling in cultured cells. *D*: the S1P1R antibody labels all amacrine cell bodies (arrows) and their processes in culture. Cone photoreceptors label intensely with this antibody (asterisks) and have been overexposed in this image so that the amacrine cell labeling can be observed. *E*: in a pattern similar to the S1P1R antibody, the S1P3R antibody labels all amacrine cell bodies and their processes in cell culture. All of the cells in this image are amacrine cells. Scale bars are 25 μm . *F*: S1P3R gene-specific primers were used to amplify mRNA from ~ 15 amacrine cells collected from a culture dish. The PCR product produced a single band of the appropriate size (156 bp). The identity of the product as a component of the transcript encoding S1P3R was confirmed by sequence analysis.

PTX (200 ng/ml), an inhibitor of G_i -mediated signaling. In untreated cells, S1P elicited the typical current (Fig. 2A). However, in cells pretreated with PTX, the S1P current was reduced in amplitude (Fig. 2B), suggesting that activation of G_i contributes to the S1P-induced current. Because the S1P-induced current is so noisy, currents were integrated over 5-s windows so that they could be quantified. Comparison of charge transfer revealed a significant reduction in the S1P-dependent current in PTX-treated cells (Fig. 2C). To determine whether the G_i -dependent receptor S1P1R was involved in the response, we used SEW2871, a synthetic agonist selective for the S1P1R receptor (Sanna et al. 2004). SEW2871 (10 μ M) elicited a noisy inward current similar to the S1P-induced current (Fig. 2D).

Because PTX treatment resulted in only partial inhibition of the S1P-mediated current, we asked whether another receptor was being activated by S1P. To explore this, we examined the effects of suramin, a polycyclic anionic compound that can disrupt the interactions between G proteins and G-protein-coupled receptors (Freissmuth et al. 1999). Especially relevant here, however, is that suramin has been shown to inhibit the S1P-dependent activation of S1P3R but does not interfere with signaling via S1P1R or S1P2R (Ancellin and Hla 1999). Suramin can also inhibit other receptors (Sim et al. 2008), but it had no effect on the membrane current when applied alone (not shown). In cells exposed to S1P in the presence of suramin, the noisy inward current was reversibly reduced (Fig. 2E). Charge transfer analysis revealed a significant reduction in the S1P-dependent current in the presence of suramin (Fig. 2F), suggesting that signaling through S1P3R contributes to production of the S1P-dependent current. Note that the small S1P-dependent inward shift in the baseline current persists in the presence of either blocker (Fig. 2, B and E, middle trace). Interestingly, PTX and suramin each inhibited the S1P-dependent current by ~ 75 –80%. This level of inhibition suggests a synergistic relationship between two pathways, such that the total current mediated by S1P and its receptors is greater than the sum of the currents engendered by activation of a single receptor.

S1P-induced current is PLC-dependent

The activation of either S1P1R or S1P3R can lead to activation of PLC (Fig. 4A). To determine whether S1P is activating the current through a PLC-dependent pathway, we used the PLC inhibitor U73122 in voltage-clamp experiments. The S1P-dependent current was measured in the presence of U73122 (Fig. 3A), suggesting that the current is PLC dependent. Charge transfer analysis indicated that U73122 caused a significant reduction in the S1P-dependent current (Fig. 3B). In control experiments, the less active analog U73343 was applied along with S1P and had no effect on the S1P-dependent current (Fig. 3, C and D).

S1P receptor expression

Because we had physiological evidence that S1P1R and S1P3R might be involved in the S1P response, the binding of polyclonal antibodies raised to these receptors was examined. Western blot analysis was used to confirm the specificity of the

S1P1R antibody. Antibodies were incubated with a blot of proteins from chicken brain and rat brain homogenates, respectively. A single band was present just below the 50-kDa marker in both lanes (Fig. 4B) consistent with the predicted molecular weight of S1P1R (47 kDa). For immunocytochemistry, secondary-only control experiments revealed no nonspecific binding of the secondary antibodies (Fig. 4C). S1P1R antibodies labeled all amacrine cell bodies as well as their processes in cell culture (Fig. 4D, arrows). Interestingly, another identified cell type in these cultures, cone photoreceptors, showed very strong anti-S1P1R labeling (Fig. 4D, asterisks), suggesting a possible signaling role for S1P in the outer retina.

The antibody raised against S1P3R labeled all amacrine cells in culture (Fig. 4E). Unfortunately we were not able to identify a S1P3R band in Western blots of proteins from either chicken or rat brain. As such, the labeling of amacrine cells is considered S1P3R-like. To support this labeling, we took an alternative approach of RT-PCR amplification of S1P3R mRNA collected from a population of amacrine cells using gene-specific primers (Fig. 4F). A 156-bp PCR product was amplified, which was consistent with the predicted product size. The PCR product was sequenced to confirm its identity as a component of the S1P3R coding transcript. These results are consistent with the S1P3R-like antibody labeling and our physiological results and suggest that amacrine cells express both S1P1R and S1P3R. Nonetheless, complete confirmation of the expression of S1P3R must await a demonstration that the protein is expressed.

S1P-induced current is IP_3 receptor-independent

Activation of phospholipase C results in the hydrolysis of phosphatidylinositol 4,5 bisphosphate (PIP_2) into diacylglycerol (DAG) and inositol trisphosphate (IP_3), which then typically activates IP_3 receptors. To test the involvement of IP_3 receptors specifically, we included the IP_3 receptor antagonist heparin (5 μ g/ml) in the patch pipette and recorded from amacrine cells in the ruptured patch configuration.

FIG. 5. The S1P current is IP_3 receptor-independent. A: the IP_3 receptor blocker heparin (5 μ g/ml) was included in the patch pipette for ruptured-patch voltage-clamp recordings. Before S1P application, the holding current in the heparin-perfused cells was similar to that seen in perforated patch recordings. In S1P, noisy currents develop that are similar in amplitude and appearance to S1P-induced currents recorded in the perforated-patch configuration.

FIG. 6. The S1P-dependent current is lanthanide-sensitive. *A* and *B*, top traces: 2 separate cells are voltage-clamped at -70 mV in the perforated-patch configuration. S1P activates a noisy inward current (middle traces). Most of the S1P-dependent current is eliminated in the presence of either La^{3+} ($10 \mu\text{M}$) or Gd^{3+} ($10 \mu\text{M}$, bottom traces).

ration. The S1P-induced current was not blocked by the inclusion of heparin in the recording pipette ($n = 7$; Fig. 5), even after >20 min of recording time (post-rupture, Fig. 5B), suggesting that the current activation is independent of IP_3 receptor activation.

S1P-induced current is lanthanide-sensitive

In considering the possible mediators of the S1P-dependent current, we were able to rule out activation of GABA_A receptors because the E_{rev} for the S1P-dependent current was too positive ($E_{\text{Cl}^-} = -58$ mV) and because external recording solutions contained bicuculline methiodide to block these receptors. Cyclic-nucleotide-gated channels were also ruled out because 8 Br-cAMP and 8 Br-cGMP do not elicit a current in amacrine cells (Sosa et al. 2002; B. K. Hoffpauir and E. Gleason unpublished observations). We were also able to rule out activation of L-type voltage-gated Ca^{2+} channels based on three considerations. First, the voltage was clamped at -70 mV, well outside the activation range of these L-type voltage-gated Ca^{2+} channels (approximately -40 mV). Second, nifedipine ($10 \mu\text{M}$) was routinely included in external recording solutions to block L-type Ca^{2+} channels. And third, the E_{rev} for the S1P-dependent current was not positive enough to represent Ca^{2+} flux alone. As nonselective cation channels linked to G-protein-coupled receptor activation, TRPC channels were good candidates for the mediators of this current. TRPC channels are sensitive to low micromolar concentrations of the lanthanides La^{3+} and Gd^{3+} . To address the possibility that

TRPC channels mediate the S1P-induced current, we examined the effects of La^{3+} ($10 \mu\text{M}$) and Gd^{3+} ($10 \mu\text{M}$) on the S1P ($10 \mu\text{M}$) current. Both La^{3+} (Fig. 6A) and Gd^{3+} (B) consistently inhibited the S1P current. Note that with lanthanides, as for the G protein inhibitors and PLC inhibitor, a sustained S1P-dependent current persists. Although the origin of this current component is not known, its resistance to all of these agents suggests that it might be due to a direct, nonreceptor-mediated effect of S1P. As for the larger component of the current, however, its regulation, selectivity, and lanthanide sensitivity are all consistent with the possibility that it is mediated by TRPC channels.

TRPC transcripts are present in cultured amacrine cells

To address the possibility that TRPC channels are involved in the S1P-induced current, gene-specific primers were designed for TRPC subunits 1 and 3–7 using predicted sequences in the chicken genome. PCR-amplification of TRPC subunits was performed on small populations (10–20) of amacrine cells collected in a patch pipette. Amplifications produced bands at the appropriate molecular weights (Fig. 7 and Table 1). Individual TRPC subunit PCR product identity was confirmed by sequencing. Because we used mRNA isolated from multiple amacrine cells in our amplifications, we cannot resolve whether an individual amacrine cell can express all of the subunits.

FIG. 7. Transient receptor potential canonical (TRPC) subunit mRNAs are expressed in cultured amacrine cells. *A*: gene-specific primers were designed against TRPC subunits 1 and 3–7. Transcripts were PCR-amplified from 10 to 20 amacrine cells collected in patch pipettes. All of these PCR products were run on the same gel. Expected sizes of the PCR products are in Table 1. Actin was amplified as a positive control.

S1P causes a cytosolic Ca^{2+} increase

One of the primary functions of TRPC channels is to mediate Ca^{2+} influx. Thus if TRPC channels (or other nonselective, Ca^{2+} -permeable cation channels) are involved, S1P should elicit Ca^{2+} elevations that are dependent on external Ca^{2+} . To determine the effects of S1P on cytosolic Ca^{2+} , Ca^{2+} -imaging experiments were done on Oregon Green BAPTA 488-loaded amacrine cells. Control data show that the vehicles for S1P (methanol) and DMS (see following text, ethanol) do not elicit a Ca^{2+} elevation (Fig. 8A). S1P at either 1 or 10 μM produced La^{3+} -sensitive increases in cytosolic Ca^{2+} (B and C). In the absence of La^{3+} , the time course of the Ca^{2+} elevations was more variable and could outlast the

exposure to S1P, sometimes for minutes (Fig. 8C, inset). To examine the source of the Ca^{2+} increase, Ca^{2+} imaging experiments were repeated in the absence of external Ca^{2+} . In 0- Ca^{2+} , S1P elicited small Ca^{2+} increases that were presumably due to release of Ca^{2+} from internal stores (Fig. 8D). When external Ca^{2+} was re-introduced, substantial cytosolic Ca^{2+} increases were observed, further supporting the idea that S1P stimulates Ca^{2+} entry across the plasma membrane.

To examine the temporal relationship between the S1P-dependent Ca^{2+} elevations and the S1P-dependent current, simultaneous Ca^{2+} -imaging and voltage-clamp experiments were done. Amacrine cells were preloaded with Oregon Green BAPTA 488 AM then voltage-clamped in the perforated-patch

FIG. 8. S1P generates La^{3+} -sensitive Ca^{2+} elevations in amacrine cells. Cells were loaded for 1 h with the Ca^{2+} -sensitive dye Oregon Green BAPTA 488. **A:** a representative example of a fluorescence recording testing the effects of the vehicle for S1P (1% Met-OH) and *N,N*-dimethyl-*D*-erythro-sphingosine (DMS, 1% Et-OH), a reagent used in the experiments depicted in Fig. 11. No Ca^{2+} elevations were seen in response to these compounds. **B:** a representative recording shows that 1 μM S1P can elicit La^{3+} -sensitive Ca^{2+} elevations. **C:** data from a different cell showing a La^{3+} -sensitive cytosolic Ca^{2+} increase to 10 μM S1P. **Inset:** data from a different cell showing that in the absence of La^{3+} , the Ca^{2+} elevations can persist well after the removal of S1P (scale bars are 1.1 F/F_0 and 30 s). **D:** in 0- Ca^{2+} , a small S1P-dependent Ca^{2+} increase was observed, likely representing release of Ca^{2+} from internal stores. Re-introduction of external Ca^{2+} produced a dramatic increase in the S1P-dependent Ca^{2+} elevation. **E:** an amacrine cell preloaded with Oregon Green Bapta 488 is voltage-clamped at -70 mV in the perforated-patch configuration. S1P causes a cytosolic Ca^{2+} increase that precedes the inward S1P-dependent current. **F:** in a different cell, S1P induces a cytosolic Ca^{2+} increase that occurs simultaneously with the S1P-dependent current.

recording configuration. In some cells (5 of 13), S1P produced cytosolic Ca^{2+} increases that preceded development of the S1P-dependent current (Fig. 8E). In other cells, (8 of 13), S1P-dependent Ca^{2+} elevations and the S1P-dependent current had a similar onset (Fig. 8F). The S1P-induced membrane current was never observed to precede elevations in cytosolic Ca^{2+} , suggesting that release of internal Ca^{2+} is a prerequisite for the activation of the current.

S1P-induced Ca^{2+} increase is receptor-mediated

To further explore the link between the S1P-dependent Ca^{2+} elevation and the S1P-dependent current, we used PTX to inhibit G_i -mediated signaling. In PTX-treated cells, S1P induced increases in cytosolic Ca^{2+} in 50 of 99 cells tested (Fig. 9A), suggesting that the S1P-dependent Ca^{2+} increases could be activated through a G_i -independent mechanism. However, in a separate experiment, the S1P1R-selective agonist SEW2871 consistently produced Ca^{2+} elevations, indicating that activation of S1P1R alone was sufficient to elicit a Ca^{2+} increase (Fig. 9B). If SEW2871 is activating S1P1R alone and S1P1R is the primary receptor linked to G_i , then we would expect that PTX pretreatment would inhibit SEW-dependent Ca^{2+} elevations. In 65% of PTX-treated cells examined ($n = 46$), SEW2871 did not elicit Ca^{2+} elevations, but Ca^{2+} elevations

were produced by subsequent exposure to S1P (Fig. 9C). The remaining cells (16/46) had very small SEW responses in comparison to the responses engendered by S1P. These small SEW responses might be due to incomplete inhibition of G_i by PTX. These results suggest that in control cells, SEW2871 is eliciting Ca^{2+} elevations by activating S1P1R and G_i .

To test the possible involvement of S1P3R in the S1P-dependent Ca^{2+} elevations, we examined the effects of suramin. Suramin alone had no effect on cytosolic Ca^{2+} . S1P in the presence of suramin, typically produced small elevations in cytosolic Ca^{2+} (Fig. 9D, asterisk) relative to those elicited in the same cell after suramin had been removed (D), suggesting that a substantial fraction of the S1P-induced Ca^{2+} increases occurs through activation of S1P3R.

Because our fluorescence measurements are nonquantitative, we do not know whether S1P responses in cells pretreated with PTX are smaller than those in untreated cells. To address whether S1P-dependent Ca^{2+} elevations typically occurred by signaling via both S1P1R and S1P3R, we examined the effects of blocking signaling through both receptors. In 72% ($n = 61$) of PTX- and suramin-treated cells, S1P had no effect on cytosolic Ca^{2+} (Fig. 9E). The balance of the PTX-treated cells had small but detectable Ca^{2+} increases in response to S1P in the presence of suramin, again suggesting that the inhibition of

FIG. 9. The S1P-induced cytosolic Ca^{2+} increase may be mediated by both S1P1R and S1P3R. A: cells were pretreated overnight (18–22 h) in pertussis toxin (200 ng/ml) to inhibit receptor signaling through G_i . A cell is loaded with Oregon Green BAPTA 488 and then exposed to S1P. S1P still induces a Ca^{2+} increase despite the pretreatment with PTX. B: to determine if activation of S1P1R alone is sufficient to elicit a Ca^{2+} increase, SEW2871 (10 μM) is applied. In the presence of SEW2871, a small cytosolic Ca^{2+} increase is observed. C: in a representative PTX-treated cell, SEW2871 produces little or no Ca^{2+} elevation. S1P, however, elicited a La^{3+} -sensitive response. D: a cell is exposed to suramin (100 μM) to inhibit signaling through S1P3R. In suramin and S1P, a small cytosolic Ca^{2+} increase was observed (asterisk). When suramin was removed, the cell responded with a substantially larger Ca^{2+} increase. E: cells were pretreated overnight in PTX and exposed to suramin to inhibit signaling through G_i and S1P3R. The majority of PTX-treated cells (27/44) showed no S1P-dependent Ca^{2+} increase in the presence of suramin.

G_i by PTX might be incomplete. Alternatively, it is possible that another receptor is involved or that there is a direct (nonreceptor-mediated) effect of S1P as observed by Van Brocklyn et al. (1998).

Activation of either S1P1R or S1P3R can elicit release of Ca^{2+} from internal stores

To further investigate the source of the receptor-mediated Ca^{2+} increases, cells were either incubated overnight in PTX or exposed to suramin to inhibit signaling through G_i (S1P1R) and S1P3R, respectively, and to isolate signaling through the two receptors. Ca^{2+} -free external solution was used to isolate the source of the Ca^{2+} increase. In PTX-treated cells, S1P resulted in either release of Ca^{2+} from internal stores, followed by external Ca^{2+} influx, or Ca^{2+} influx without detectable store release. Store release alone was never observed in PTX-treated cells ($n = 70$; Fig. 10, A and B). In suramin-treated cells, S1P resulted in release of Ca^{2+} from stores, Ca^{2+} influx, or in most cases a combination of both mechanisms ($n = 69$; Fig. 10, C and D). These results suggest that S1P1R-mediated Ca^{2+} elevations (suramin-treated) can result from either Ca^{2+} influx or store release or both. They also suggest that Ca^{2+} influx dominates S1P3R-dependent Ca^{2+} elevations (PTX pre-treated) and that the responses via this receptor are not generated by store release alone.

Synthesis of S1P in amacrine cells

These studies provide multiple pieces of evidence for receptor-mediated S1P signaling in retinal amacrine cells. There is physiological evidence suggesting that cultured amacrine cells express the synthetic enzyme for S1P, sphingosine kinase (SphK) (Borges et al. 2008). We attempted to use polyclonal antibodies raised against SphK1 (see METHODS) but were un-

successful with this approach. To further explore the expression of SphK in amacrine cells, we designed gene-specific primers for SphK 1. A PCR product of the appropriate size was amplified from mRNA isolated from a collection of 10–20 amacrine cells. Sequencing confirmed that the PCR product was from the mRNA encoding SphK 1, consistent with the findings of Borges et al. (2008) and suggesting that it is possible for the enzyme to be expressed in amacrine cells (Fig. 11A).

Because SphK is Ca^{2+} -calmodulin-sensitive (Alemany et al. 2000; Sutherland et al. 2006), it is possible that the receptor-mediated, S1P-dependent Ca^{2+} elevations lead to further production of internal S1P. To address the possibility that cytosolic S1P was being generated, we asked whether a competitive inhibitor of SphK, DMS (Edsall et al. 1998; Yatomi et al. 1996) would have any effect on the duration of S1P-dependent Ca^{2+} elevations. DMS is reported to also inhibit protein kinase C *in vitro* (Igarashi and Hakomori 1989), but this effect was not observed in intact cells from three different cell lines (Edsall et al. 1998). Additionally, results reported for human neutrophils (Igataki and Hauser 2003) and cultured chick amacrine cells (Borges et al. 2008) indicate that the target of DMS is SphK rather than the influx channels themselves. It is important to note here that in the absence of inhibition by lanthanides, both the S1P-dependent current and the S1P-dependent Ca^{2+} elevations often persist after removal of the agonist and that Ca^{2+} influx (as opposed to release from stores) is the primary source of the Ca^{2+} elevation. Here we test the hypothesis that the persistent nature of the response is due to generation of cytosolic S1P subsequent to receptor activation. Control cells ($n = 19$) were exposed to S1P for 30 s then returned to normal external. After removal of S1P, Ca^{2+} levels tended to remain elevated for the remainder of the recording period (90 s, Fig. 11B, solid trace). We tested for the involve-

FIG. 10. Activation of either receptor can elicit release of Ca^{2+} from internal stores. A: an amacrine cell has been pretreated with PTX. In this cell, no S1P-dependent Ca^{2+} elevation is observed in the absence of external Ca^{2+} . Re-introduction of extracellular Ca^{2+} results in a cytosolic Ca^{2+} elevation. B: cells treated with PTX responded with either release of Ca^{2+} from internal stores followed by external Ca^{2+} influx, or Ca^{2+} influx without internal store release (as in A, $n = 70$). C: data from an amacrine cell exposed to suramin. In 0 external Ca^{2+} , a small S1P-dependent calcium elevation is observed. Re-introduction of extracellular Ca^{2+} increased the magnitude of the S1P-dependent Ca^{2+} elevation. D: cells in suramin responded to S1P with either store release only, Ca^{2+} influx only, or most often, a combination of both responses ($n = 69$).

FIG. 11. Inhibition of SphK limits the duration of the S1P-dependent Ca^{2+} elevations. **A:** SphK1 gene-specific primers were used to amplify mRNA from ~15 amacrine cells collected from a culture dish. The PCR product produced a single band of the appropriate size (221 bp). The identity of the product as SphK1 was confirmed by sequence analysis. **B:** control cells were exposed to S1P for 30 s followed by 90 s in normal external. Normalized data from a representative amacrine cell show that the cytosolic Ca^{2+} level does not recover on this time frame (solid trace). However, when DMS (10 μM) was applied (time indicated by the bar, data from a different cell, dotted trace), a decline in cytosolic Ca^{2+} was typically observed. **C:** averaged data are shown from 19 control and 19 DMS-treated cells. To calculate the percent change, raw fluorescence values were obtained for both groups at $t = 120$ s (the onset of DMS application) and $t = 150$ s (when DMS was removed). Error bars are SE.

ment of internal S1P in a separate set of cells ($n = 19$) by applying DMS after washout of S1P. With DMS, cytosolic Ca^{2+} levels typically declined more rapidly than in control (Fig. 11B, dotted trace). Overall, DMS significantly accelerated the recovery of the Ca^{2+} elevations (Fig. 11C, $P = 0.0002$). This suggests that extracellular S1P binding to receptors might lead to activation of SphK, synthesis of S1P, and a relatively sustained activation of a Ca^{2+} influx pathway.

DISCUSSION

In this study, we demonstrate that S1P activates a receptor-mediated cation current in retinal amacrine cells. S1P-dependent currents have been observed in other cell types, including human umbilical vein endothelial cells (Muraki et al. 2001). This current is a PTX-sensitive, nonselective cation current similar to what we have found in amacrine cells. It is well-established that S1P can induce increases in cytosolic Ca^{2+} . However, the source of the S1P-mediated Ca^{2+} increase varies from one cell type to another. In Swiss 3T3 fibroblasts, Mattie et al. (1994) observed a transient increase in intracellular Ca^{2+} in response to S1P. The Ca^{2+} increase was independent of extracellular Ca^{2+} and was abolished when internal Ca^{2+} stores were depleted with thapsigargin. In contrast, Formigli et al. (2002) demonstrated in myoblast cells that S1P elicits a transient Ca^{2+} increase propagating as a wave throughout the cell and that the Ca^{2+} increase required both intra- and extracellular Ca^{2+} mobilization. Here in amacrine cells, S1P elicits a cytosolic Ca^{2+} increase that is dominated by Ca^{2+} influx but also involves release of Ca^{2+} from internal stores. The influx pathway, however, appears to be different for myoblasts and amacrine cells. In myoblast cells, the S1P-mediated Ca^{2+} response was significantly reduced through pretreatment of cells with the L-type calcium channel blocker nifedipine (Formigli et al. 2002), indicating the involvement of these voltage-dependent Ca^{2+} channels. In the current study, however, we rule out the involvement of these channels on several criteria. Thus S1P is capable of activating an inward cation current in amacrine cells independent of voltage-gated ion channel activation.

Our data suggest that the S1P-dependent current and Ca^{2+} influx are initiated by activation of both S1P1R and S1P3R in amacrine cells. For S1P1R, this suggestion is supported in part by antibody labeling. Although there can be specificity problems with antibodies against G-protein-coupled receptors (Michel et al. 2009), the ability of SEW2871 to activate both the current and Ca^{2+} influx at a concentration demonstrated to be ineffective on the other S1P receptors (Sanna et al. 2004) tempers this concern.

The evidence for the expression of S1P3R in amacrine cells is somewhat more tenuous. We have amplified a PCR product from amacrine cell mRNA that corresponds to a coding region of the S1P3R gene, and we observe S1P3R-like immunoreactivity but we are not able to confirm the expression of the protein. Several of our physiological results, however, suggest that this receptor is expressed in amacrine cells. First, we find that suramin is an effective inhibitor of S1P-dependent current and Ca^{2+} influx. The effects of suramin must be interpreted with caution because it can have wide ranging effects on G proteins and G-protein-coupled receptors (Freissmuth et al. 1999). Nonetheless in the context of S1P receptors, suramin

has been shown to be effective in inhibiting signaling through S1P3R but ineffective in inhibiting signaling via S1P1R or S1P2R into the millimolar range (Ancellin and Hla 1999). Another indication that S1P is signaling through an additional receptor in amacrine cells comes from data in Fig. 10, B and D. The distinctive Ca^{2+} response patterns with respect to store release versus influx produced in the presence of each inhibitor (PTX or suramin) provide additional physiological evidence for signaling through both S1P1R and S1P3R in amacrine cells.

Intriguingly, there is evidence for an obligate collaboration between S1P1R and S1P3R in some signaling pathways. S1P1R and S1P3R are co-expressed and are both required for endothelial cell proliferation (Kimura et al. 2000), migration (Kimura et al. 2000; Paik et al. 2001), and morphogenesis (Lee et al. 1999). Especially relevant to our results, S1P1R has been shown to actually suppress Ca^{2+} signaling unless it is co-expressed with S1P3R specifically (Meyer zu Heringdorf et al. 2003b). Interestingly, heterodimers of S1P1R and S1P3R have been detected in expressions studies (Van Brocklyn et al. 2002), so it may be that dimerization is a requirement for interactions between these receptors.

Much debate exists as to whether S1P couples to release of Ca^{2+} from internal stores through activation of IP_3 Rs on the endoplasmic reticulum. In Swiss 3T3 fibroblasts, S1P stimulated an increase in cellular IP_3 levels, but the S1P-induced Ca^{2+} increase was unaffected by blocking IP_3 receptors with heparin (Mattie et al. 1994). The IP_3 R-independent but S1P-dependent Ca^{2+} increase has been observed in other cell types as well (Ghosh et al. 1994; Im et al. 2005; Spiegel and Milstein 2002; Tornquist et al. 1997). In the present study, we observed S1P activation of a PLC-dependent but IP_3 R-independent current.

In addition to activation of receptors at the plasma membrane, S1P can also signal internally. There is good evidence that S1P can act as a Ca^{2+} influx factor (Borges et al. 2008; Igataki and Hauser 2003). A potentially relevant intracellular interaction has been shown between S1P and TRPC5 (Xu et al. 2006). It was demonstrated that intracellular S1P acts directly as an internal ligand for the natively expressed TRPC1-5 heteromultimers in the plasma membrane of smooth muscle cells. Furthermore, this interaction was shown to lead to channel activity and Ca^{2+} influx. These observations fit well with the Ca^{2+} /calmodulin-dependence of SphK (Hla et al. 1999), and the idea that receptor-dependent Ca^{2+} elevations could activate SphK, and produce S1P that triggers gating of a Ca^{2+} influx channel. An important link was established when it was demonstrated that Ca^{2+} influx was eliminated in mast cells from SphK2 knockout mice (Olivera et al. 2007). Interestingly, it is emerging that it may not be the activity of SphK that is Ca^{2+} /calmodulin sensitive but the localization. In expression systems, a Ca^{2+} -dependent translocation of SphK1 to the plasma membrane leads to generation of S1P (Alemany et al. 2000, 2001; Sutherland et al. 2006). Although we have not yet confirmed the involvement of TRPC channels specifically, the effects of inhibiting SphK on the receptor-mediated Ca^{2+} elevations provides further evidence that internally generated S1P can gate a Ca^{2+} influx channel in amacrine cells.

Is S1P activating store-operated Ca^{2+} entry (SOCE) in amacrine cells? SOCE is defined as the influx of Ca^{2+} that is dependent on depletion of Ca^{2+} from internal stores (for

review, see Smyth et al. 2006). A corollary of this definition is that the activation of receptors is not involved. The expression of SOCE is variable among neurons (for review, see Putney 2003), but there is strong evidence that SOCE exists in amacrine cell dendrites (Borges et al. 2008). Our current recordings are from the whole cell and our fluorescent measurements have been made from cell bodies but there are some consistencies with SOCE in the results reported here. First, we show that the S1P-dependent Ca^{2+} elevation typically involves release of Ca^{2+} from stores and that the S1P-dependent current does not precede the elevation of cytosolic Ca^{2+} . We also show that the S1P-dependent current is not dependent on the activity of the IP_3 receptor, and we demonstrate that both the S1P-dependent current and the S1P-dependent Ca^{2+} elevations are sensitive to μM concentrations of lanthanides. Nonetheless, our results fail a primary test of SOCE: independence from receptor activation. The inhibitory effects of PTX, the requirement for PLC activity, and the ability for SEW2871 to partially substitute for S1P all point to the involvement of a receptor (or receptors) in the mediation of the effects of S1P reported here. Perhaps the answer lies somewhere in the middle in that receptor-mediated S1P signaling might somehow co-opt the SOCE mechanism expressed by amacrine cells.

The evidence presented in this study points to S1P activation of a Ca^{2+} -permeable cation channel at the plasma membrane. One potential function of this sort of channel is to mediate localized receptor-dependent Ca^{2+} entry and allow for neurotransmitter release without the involvement of voltage-gated Ca^{2+} channels. Indeed Chavez et al. (2006) demonstrated GABAergic feedback in amacrine cells through activation of postsynaptic ionotropic glutamate receptors and Ca^{2+} -induced- Ca^{2+} release. It has also been demonstrated that release from internal stores can elicit GABA release in cultured amacrine cells (Warrier et al. 2005). It will be important to determine whether S1P, through the complex lipid signaling pathway proposed in this study, is capable of mediating synaptic signaling in the inner retina.

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