Sphingosine-1-phosphate elicits receptor-dependent calcium signaling in retinal amacrine cells

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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 upon 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 TRPC channels. PCR products amplified from amacrine cells reveal that TRPCs 1, 3, 4, 5, 6, 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 (Pollock, et al. 2004, Giussani et al. 2007), 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 et al. 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 Gi (Lee et al. 1998, Ancellin and Hla 1999, Windh et al. 1999), consistent with the observation that S1P elicits Ca\textsuperscript{2+} elevations through a pertussus toxin (PTX)-sensitive (and therefore Gi-dependent) pathway in numerous cell types (van Coppen et al. 1996, Okamoto et al. 1998, Van Brocklyn et al. 1998, An et al. 1999, Ancellin and Hla 1999, Kon et al. 1999, Lee et al. 1999, Okamoto et al. 1999). 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\textsuperscript{2+} through IP\textsubscript{3}-sensitive (Zhou et al. 2004, Formigli et al. 2002) and IP\textsubscript{3}-insensitive Ca\textsuperscript{2+} stores (Ghosh et al. 1994, Mattie et al. 1994, Tornquist et al. 1997, Meyer zu Heringdorf et al. 1998).

S1P2R and S1P3R can couple to Gi, Gq, and G13 (Ancellin and Hla 1999, Kon et al. 1999, Okamoto et al. 1999, Sato et al. 1999, Windh et al. 1999, Ishii et al. 2001; 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, Okamoto et al. 1999, Sato et al. 1999, Ishii et al. 2001). There is also evidence for preferential coupling with S1P2R coupling most strongly to G13 and S1P3R most strongly activating Gq and subsequent Ca\textsuperscript{2+} mobilization (Ishii et al. 2002). Less is known about S1P5R, but signaling through Gi and G12 has been reported (Malek et al. 2001, Im et al. 2005, Jaillard et al. 2005, 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 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 (Van Brocklyn et al. 1998, Xia et al. 1998, Meyer zu Heringdorf et al. 2003A).

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 pathfinding 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 cells, ganglion cells 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 (Hurtado et al. 2002, Medler et al. 2002, Azuma et al. 2004, Sen et al. 2007). Furthermore, local and reciprocal signaling has been
established for an inhibitory feedback synapse between amacrine cells and bipolar cells (Vigh and von Gersdorff, 2005, Chavez et al. 2006). 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, 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 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.

**MATERIALS AND 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, LA) were dissociated in 0.1% trypsin and plated at 5.0 X 10\(^5\) cells/ 35mm polyornithine-coated (0.1mg/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), 1000U penicillin/mL, 100µg streptomycin/mL, and 1mM L-glutamine (Sigma, St. Louis MO).

**Immunocytochemistry**

Cells were fixed in 1% paraformaldehyde for 1 hour 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 Sphingosine-1-phosphate receptor 1 (S1P1R) and S1P3R were purchased from Cayman Chemical (Ann Arbor, MI). Cultures were incubated for 1 hour 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 hour at room temperature. After washing, Cy3-conjugated goat anti-rabbit secondary antibodies (Invitrogen) were diluted to 1:1000 and applied for one hour 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) sacrificed by decapitation and White Leghorn chickens (Poultry Sciences, LSU) sacrificed by CO2 exposure followed by decapitation. All methods involving animals were conducted in accord with the NIH 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, 150mM NaCl, 10mM Tris
pH7.4, 1mM EDTA, 1mM 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. 150µg rat brain homogenate and 50µg of 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 in S1PR1 antibody (1:1000, Cayman Chemical) at room temperature
for 1h and then incubated with a goat anti-rabbit IgG peroxidase conjugate secondary antibody
(1:5000) for 1h 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). 10-20 amacrine cells were collected into patch pipets. The pipet 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 BLAST search revealed predicted *Gallus gallus*
gene sequences encoding TRPC protein subunits 1, 3, 4, 5, 6 and 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
Integrated DNA Technologies (Coralville, IA). Messenger RNA isolation was performed using
Dynabeads® mRNA DIRECT Micro Kit (Invitrogen,) following the manufacturer’s isolation
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 (85V for 1.25hr) in 0.5x 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.

**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 (1.5mm OD, 0.86mm ID) using a Flaming/Brown puller (Sutter Instruments, Novato, CA) and had tip resistances of 3-5 MΩ. For perforated patch experiments, amphotericin B stock was made at 40µg/ml in dimethyl sulfoxide (DMSO). Amphotericin was combined 1:2 with Pluronic F-127 (Invitrogen, 25mg/ml DMSO) and diluted in internal solution for a final amphotericin concentration of 140µg/ml. Gigaohm seals were achieved with a brief hyperpolarizing pulse of
-140mV. Then, unless otherwise indicated, the voltage was clamped at -70mV. 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 BAPTA 488 was prepared as a 2mM stock solution in DMSO, combined 1:1 with Pluronic F127 (Invitrogen, 25 mg/ml DMSO), then diluted to 2 µM in Hank’s Balanced Salt Solution (Invitrogen). Cells were loaded for one hour 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 about 20 minutes. 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 1s (2s for Fig. 11B), and exposure time was 150ms. 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/F₀.

**Solutions and Reagents**

Reagents were purchased from Sigma unless otherwise indicated. *Normal external solution* consisted of the following (in mM): KCl 5.3, NaCl 136.9, CaCl₂ 3.0, MgCl₂ 0.4, HEPES 3.0 and glucose 5.6. *Tetraethylammonium (TEA) external solution* consisted of the following (in mM): KCl 5.3, NaCl 116.7, TEA Cl 20.0, CaCl₂ 3.0, MgCl₂ 0.4, glucose 5.6, HEPES 10.0. For electrophysiology, external solutions also contained bicuculline methiodide (3.0 µM) 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: Cs Acetate 100.0, CsCl 10.0, CaCl\(_2\) 0.1, MgCl\(_2\) 2.0, HEPES 10.0, EGTA 1.1. Internal B solution (for perforated patch) consisted of the following: Cs Acetate 135, CsCl 10.0, MgCl\(_2\) 2.0, CaCl\(_2\) 0.1, EGTA 1.1, HEPES 10.0, NaCl 1.0.

Sphingosine-1-phosphate (S1P, Biomol, Plymouth Meeting, PA) was prepared as a 1mM stock in methanol then diluted in external solution to a final concentration of either 1 or 10µM. The PLC inhibitor U73122, and its less active analog U73343 were used at 10µM and purchased from Biomol. The IP\(_3\) receptor antagonist heparin (Calbiochem, San Diego, CA) was included in the patch pipette at a final concentration of 5mg/ml. The S1P1R selective agonist SEW2871 was purchased from Cayman Chemical (Ann Arbor, MI) and prepared as a 10mM stock in DMSO. Pertussis toxin (PTX, Calbiochem) was used at a final concentration of 200ng/mL. Cells were incubated for 18 hours in pertussis toxin diluted in culture medium. Following the incubation period, cells were washed in normal solution and used immediately for electrophysiology experiments. Pertussis toxin-treated cells used for Ca\(^{2+}\)-imaging experiments were washed in normal external solution, and loaded with the dye for one hour prior to recording. Suramin was dissolved directly in the external solution at a concentration of 100µM. N,N-dimethyl-D-erythro-sphingosine (DMS, Biomol) was diluted in ethanol at a concentration of 10mM then diluted into external solution at a final concentration of 10µM.

### Data Analysis

Statistical significance was evaluated using the Student’s t-test. The data in Figures 2E and 3B and D were evaluated using the paired form of the test. Error bars represent SEM.
Individual amacrine cells were voltage-clamped at -70mV. S1P (1 or 10µM) elicited a noisy inward current that developed after about 10 seconds of exposure (Fig. 1A). Once activated, the current usually persisted after the removal of agonist. Although the S1P-dependent current could be elicited with 1µM 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. 1B). On average, the current reversed at 7.8 ± 8.5mV (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. 1C, n=4) suggesting that the S1P-dependent current is carried by a mixture of cations.

The S1P-dependent current may involve signaling through S1P1R and S1P3R

Because S1P signals through G protein-coupled receptors and most commonly through G

Gi, cells were incubated overnight in pertussis toxin (PTX, 200ng/ml), an inhibitor of G

mediated signaling. In untreated cells, S1P elicited the typical current (Fig. 2A). However, in cells pre-treated with PTX, the S1P current was reduced in amplitude (Figure 2B), suggesting that activation of G

 contributes to the S1P-induced current. Because the S1P-induced current is so noisy, currents were integrated over 5 second 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

-dependent receptor S1P1R was involved in the response, we used SEW2871, a synthetic agonist selective for the S1P1R receptor.
(Sanna et al. 2004). SEW2871 (10μM) 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 (Figs. 2B and 2E, 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.

**The 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. The S1P-induced current was inhibited 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. 3C,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 50kD marker in both lanes (Fig. 4B) consistent with the predicted molecular weight of S1P1R (47kD). For immunocytochemistry, secondary-only control experiments revealed no non-specific 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 (Figure 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 156bp 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.

**The S1P-induced current is IP₃ receptor-independent**

Activation of phospholipase C results in the hydrolysis of phosphatidylinositol 4,5 bisphosphate (PIP₂) into diacylglycerol (DAG) and inositol trisphosphate (IP₃) which then typically activates IP₃ receptors. To test the involvement of IP₃ receptors specifically, we included the IP₃ receptor antagonist heparin (5µg/ml) in the patch pipette and recorded from amacrine cells in the ruptured patch configuration. The S1P-induced current was not blocked by the inclusion of heparin in the recording pipette (n=7; Fig. 5), even after > 20 minutes of recording time (post-rupture, Fig. 5B), suggesting that the current activation is independent of IP₃ receptor activation.

**The S1P-induced current is lanthanide-sensitive.**

In considering the possible mediators of the S1P-dependent current, we were able to rule out activation of GABAₐ receptors, because the E_{rev} for the S1P-dependent current was too positive (E_{Cl} = -58mV), 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, Hoffpauir and Gleason unpublished observations). We were also able to rule out activation of L-type voltage-gated Ca²⁺ channels based on three considerations. First, the voltage was clamped at -70mV, well outside the activation range of these L-type voltage-gated Ca²⁺ channels (~40mV). Second, nifedipine (10µm) was routinely included in external recording solutions to block L-type
Ca\textsuperscript{2+} channels. And third, the \( E_{rev} \) for the S1P-dependent current was not positive enough to represent Ca\textsuperscript{2+} flux alone. As non-selective 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\textsuperscript{3+} and Gd\textsuperscript{3+}. To address the possibility that TRPC channels mediate the S1P-induced current, we examined the effects of La\textsuperscript{3+} (10\( \mu \)M) and Gd\textsuperscript{3+} (10\( \mu \)M) on the S1P (10\( \mu \)M) current. Both La\textsuperscript{3+} (Fig. 6A) and Gd\textsuperscript{3+} (Fig. 6B) 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, non-receptor-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, 3, 4, 5, 6, and 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 pipet. 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.

**S1P causes a cytosolic Ca\textsuperscript{2+} increase**
One of the primary functions of TRPC channels is to mediate Ca\(^{2+}\) influx. Thus, if TRPC channels (or other non-selective, Ca\(^{2+}\)-permeable cation channels) are involved, S1P should elicit Ca\(^{2+}\) elevations that are dependent upon 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 below, ethanol) do not elicit a Ca\(^{2+}\) elevation (Fig. 8A). S1P at either 1 or 10μM produced La\(^{3+}\)-sensitive increases in cytosolic Ca\(^{2+}\) (Fig. 8B 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 pre-loaded with Oregon Green BAPTA 488 AM then voltage-clamped in the perforated patch 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.

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

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

Because our fluorescence measurements are non-quantitative, we do not know whether S1P responses in cells pre-treated with PTX are smaller than those in untreated cells. To address whether S1P-dependent \( \text{Ca}^{2+} \) 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 \( \text{Ca}^{2+} \) (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 G\(_i\) by PTX might be incomplete. Alternatively, it is possible that another receptor is involved or that there is a direct (non-receptor-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. 10A,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. 10C, 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 unsuccessful 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, N,N-dimethylsphingosine, (DMS; Yatomi et al. 1996; Edsall et al. 1998) would have any effect on the duration of S1P-dependent Ca\(^{2+}\) elevations. DMS is reported to also inhibit protein kinase C \textit{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 seconds then returned to normal external. After removal of S1P, Ca\(^{2+}\) levels tended to remain elevated for the remainder of the recording period (90sec, Fig. 11B, \textit{solid trace}). We tested for the involvement 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, \textit{dotted trace}). Overall, DMS significantly accelerated the recovery of the Ca\textsuperscript{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\textsuperscript{2+} influx pathway.

\textbf{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, non-selective cation current similar to what we have found in amacrine cells. It is well-established that S1P can induce increases in cytosolic Ca\textsuperscript{2+}. However, the source of the S1P-mediated Ca\textsuperscript{2+} increase varies from one cell type to another. In Swiss 3T3 fibroblasts, Mattie et al. (1994) observed a transient increase in intracellular Ca\textsuperscript{2+} in response to S1P. The Ca\textsuperscript{2+} increase was independent of extracellular Ca\textsuperscript{2+} and was abolished when internal Ca\textsuperscript{2+} stores were depleted with thapsigargin. In contrast, Formigli et al. (2002) demonstrated in myoblast cells that S1P elicits a transient Ca\textsuperscript{2+} increase propagating as a wave throughout the cell, and that the Ca\textsuperscript{2+} increase required both intracellular and extracellular Ca\textsuperscript{2+} mobilization. Here, in amacrine cells, S1P elicits a cytosolic Ca\textsuperscript{2+} increase that is dominated by Ca\textsuperscript{2+} influx but also involves release of Ca\textsuperscript{2+} from internal stores. The influx pathway, however, appears to be different for myoblasts and amacrine cells. In myoblast cells, the S1P-mediated Ca\textsuperscript{2+} response was significantly reduced through pre-treatment of cells with the L-type calcium channel blocker nifedipine (Formigli et al. 2002), indicating the involvement of these voltage-dependent Ca\textsuperscript{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 subsequent 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 Figure 10B 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, Tornquist et al. 1997, Spiegel et al. 2002, Im et al. 2005). 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 (Igataki and Hauser 2003, Borges et al. 2008). 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 upon 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 upon 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 mechanisms expressed by amacrine cells.

The evidence presented in this study points to S1P activation of a Ca\textsuperscript{2+}-permeable cation channel at the plasma membrane. One potential function of this sort of channel is to mediate localized receptor-dependent Ca\textsuperscript{2+} entry and allow for neurotransmitter release without the involvement of voltage-gated Ca\textsuperscript{2+} channels. Indeed, Chavez et al. (2006) demonstrated GABAergic feedback in amacrine cells through activation of postsynaptic ionotropic glutamate receptors and Ca\textsuperscript{2+}-induced-Ca\textsuperscript{2+} release (CICR). 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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GRANTS

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DISCLOSURES

There are no disclosures to be made.
REFERENCES


FIGURE LEGENDS

Figure 1. Sphingosine-1-phosphate elicits a cation current. A top, A representative amacrine cell is voltage-clamped in the perforated-patch configuration at -70mV and a control record is obtained. Middle, S1P (1µM) is applied and a small inward current is elicited. A region of the activated current is shown on an expanded timescale (inset). Bottom, In a recording from another amacrine cell 10µM S1P is applied and after ~10 seconds, 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 +8mV, suggesting that the current is carried by a mixture of cations. S1P-dependent currents were also recorded during a series of voltage steps (-70mV to +40mV). 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.

Figure 2. Activation of S1P1R and S1P3R may both contribute to the S1P-dependent current. A, An untreated cell that is voltage-clamped at -70mV is exposed to S1P (10µM) and a noisy, inward current is observed (bottom trace). B, Cells were pre-treated overnight (18-22 hr.) with pertussis toxin (PTX, 200ng/ml). A PTX-treated cell that is voltage-clamped at -70mV 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 (10µM), an S1P1R-selective agonist, also elicits a noisy inward current. E, A cell is voltage clamped at -70mV and exposed to suramin (100µM), 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,F Error bars are SEM.

**Figure 3.** *The S1P-induced current is PLC-dependent.* A, A representative amacrine cell is voltage-clamped at -70mV. S1P (10 μM) was applied in the presence of the PLC inhibitor U73122 (10μM, middle trace). After 1 minute, 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 -70mV and then exposed to S1P in the presence of U73343 (10μM), a less active analogue of U73122. The analog did not suppress the S1P current. D, The mean charge transfer was unaffected by U73343 (n=6, p=0.98). B,D Error bars are SEM.

**Figure 4.** *S1P receptors are expressed by amacrine cells.* A, S1P1R is thought to couple to G_i exclusively, while S1P3R can couple to G_i, G_q, or G_13. Signaling through these G proteins converges at the level of phospholipase C (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 50kD marker, consistent with the predicted molecular weight for the S1P1R (47kD). C, Secondary-only controls did not show any non-specific 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 over-exposed 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 25μm. F, S1P3R gene-specific primers were use to amplify mRNA from ~15 amacrine cells collected from a culture dish. The
PCR product produced a single band of the appropriate size (156bp). The identity of the product as a component of the transcript encoding S1P3R was confirmed by sequence analysis.

**Figure 5.** The S1P current is IP3 receptor-independent. **A,** The IP3 receptor blocker heparin (5µg/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.

**Figure 6.** The S1P-dependent current is lanthanide sensitive. **A, B** (top traces) Two 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 La3+ (10µM) or Gd3+ (10µM, bottom traces).

**Figure 7.** TRPC subunit mRNAs are expressed in cultured amacrine cells. **A,** Gene-specific primers were designed against TRPC subunits 1, 3, 4, 5, 6, and 7. Transcripts were PCR-amplified from 10-20 amacrine cells collected in patch pipets. 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.

**Figure 8.** S1P generates La3+-sensitive Ca2+ elevations in amacrine cells. Cells were loaded for one hour with the Ca2+- 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 DMS (1% Et-OH), a reagent used in the experiments depicted in Figure 11. No Ca^{2+} elevations were seen in response to these compounds.

**B**, A representative recording shows that 1µM 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 10µM 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/F₀ and 30s). **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 pre-loaded with Oregon Green Bapta 488 is voltage-clamped at -70mV 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.

**Figure 9.** The S1P-induced cytosolic Ca^{2+} increase may be mediated by both S1P1R and S1P3R. **A**, Cells were pre-treated overnight (18-22hr) in pertussis toxin (200ng/ml) to inhibit receptor signaling through Gᵢ. A cell is loaded with Oregon Green BAPTA 488 and then exposed to S1P. S1P still induces a Ca^{2+} increase despite the pre-treatment with PTX. **B**, To determine if activation of S1P1R alone is sufficient to elicit a Ca^{2+} increase, SEW2871 (10µM) 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 (100µM) 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 \( \text{Ca}^{2+} \) increase. E, Cells were pre-treated overnight in PTX and exposed to suramin to inhibit signaling through \( \text{Gi} \) and S1P3R. The majority of PTX-treated cells (27/44) showed no S1P-dependent \( \text{Ca}^{2+} \) increase in the presence of suramin.

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

**Figure 11.** Inhibition of SphK limits the duration of the S1P-dependent \( \text{Ca}^{2+} \) 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 (221bp). The identity of the product as SphK1 was confirmed by sequence analysis. B, Control cells were exposed to S1P for 30 seconds followed by 90 seconds in normal external. Normalized data from a representative amacrine cell show that the cytosolic \( \text{Ca}^{2+} \) level does not recover on this time frame (solid trace). However when DMS (10\( \mu \text{M} \)) 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 sec (the onset of DMS application) and t=150 sec (when DMS was removed). Error bars are SEM.
Table 1. Primer Sequences for PCR amplifications.

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<tr>
<td>S1P3R-reverse</td>
<td>5' TGACCAACAGGCAATGAAGA-3'</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
A

Control

Control

1 µM S1P

1 µM S1P

10 µM S1P

10 µM S1P

B

Current (pA) vs Voltage (mV)

C

30 mV

20 mV

10 mV

0 mV

-10 mV

-20 mV
A

S1P1R

S1P3R

Plasma Membrane

G_i

G_q

G_j

G_{13}

PLC^*

B

S1P1R

Chicken Brain

Rat Brain

50kDa

35kDa

C

2 only

D

S1P1R

E

S1P3R

F

RT-

S1P3R

200 bp
A

Control / Heparin

0 pA

-25 pA

1s

B

S1P / Heparin

0 pA

-50 pA