RECEPTOR-MEDIATED CALCIUM ENTRY IN RETINAL AMACRINE CELLS

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ABSTRACT

In the vertebrate retina, multiple cell types express g protein-coupled receptors linked to phospholipase C. The signaling pathway engendered by activation of this enzyme can involve Ca\textsuperscript{2+}-permeable transient receptor potential (TRP) channels. To begin to understand the role of these channels in the retina, we undertake an immunocytochemical localization of two TRPC channel subunits, TRPC1 and TRPC4. TRPC1 expression was observed in amacrine cells and their process in the chicken retina. TRPC4 expression was much more widespread with some degree of labeling found in all layers of the retina, and was shown to be expressed in Müller glial cells. Thus, the distributions of these two subunits indicate that different retinal cell types express TRPC channels containing different subunits.

Recently, several sphingolipids have been demonstrated to play key roles in Ca\textsuperscript{2+} mobilization in neurons. Sphingosine-1-phosphate is a sphingolipid metabolite that has been shown to activate a class of g protein-coupled receptors (S1PRs) in other cell types. In the present study, we examine the signaling properties of S1P in retinal amacrine cells. S1P produced a noisy, inward cation current in amacrine cells that occurred through activation of S1P1R and S1P3R. The S1P-induced current was PLC-sensitive and was eliminated with La\textsuperscript{3+} and Gd\textsuperscript{3+}, suggesting activation of TRPCs. S1P also elicited cytosolic Ca\textsuperscript{2+} elevations. The S1P-induced Ca\textsuperscript{2+} increase was mediated by S1P1R and S1P3R and was a result of both release of Ca\textsuperscript{2+} from internal stores and Ca\textsuperscript{2+} influx. Single-cell PCR amplification of TRPC channel subunits 1, 4, and 5 confirmed expression of these subunits in amacrine cells, suggesting that S1P is capable of activating TRPC-mediated Ca\textsuperscript{2+} entry in retinal amacrine cells through a novel lipid signaling pathway.
CHAPTER 1

INTRODUCTION
The vertebrate retina is an accessible part of the brain that has been studied extensively as a model for signal processing. After over 100 years of study, we have a good understanding of phototransduction and many of the patterns of connectivity. Nonetheless, we are unable to predict ganglion cell outputs based on our understanding of retinal circuitry alone. A major gap in our understanding is that relatively little is understood about how retinal function is regulated under different visual conditions. One approach to understanding at least some pieces of this puzzle is to explore the signaling molecules and pathways utilized in the retina.

The retina is a thin layer of specialized sensory tissue located in the back of the eye, and is composed of six major neuronal cell types: rods, cones, horizontal cells, bipolar cells, amacrine cells, and ganglion cells. Of these, amacrine cells are both the most numerous and the most poorly-understood. This disparity stems partially from the larger number (>30) of amacrine cell types (MacNeil and Masland et al 1998, MacNeil et al. 1999) and the complex patterns of their synaptic interactions (Marc and Liu et al. 2000). Amacrine cell bodies sit at the inner border of the inner nuclear layer and in the ganglion cell layer and extend their processes into the inner plexiform layer where they ramify in specific sublaminae (Figure 1). These cells form numerous synapses in the inner plexiform layer with bipolar cells, retinal ganglion cells, as well as with other amacrine cells. Because of the extreme complexity of these synaptic interactions, we utilize a simplified culture system containing morphologically identifiable GABAergic amacrine cells as previously described from Gleason et al (1993), which allows us to observe signaling at the level of even a single synapse. Though cultured from embryonic chick retinæ, these cells are physiologically mature (Huba and Hoffman 1991, Huba et al. 1992), and form functional synapses and autapses after about one week in culture (Gleason et al. 1994, Frerking et al. 1995).
It is well-established that numerous cellular processes, such as enzyme function, mitochondrial function and gene expression are Ca\(^{2+}\)-sensitive. Of particular interest to our lab is the process of synaptic transmission, which is completely dependent on Ca\(^{2+}\). One of the biggest obstacles in our understanding of amacrine cell function is the complex Ca\(^{2+}\) signaling that occurs within these cells. By examining the numerous Ca\(^{2+}\) signaling cascades in amacrine cells, we hope to gain a better understanding of the way that these cells function in the retina.

Previous experiments in our lab have examined Ca\(^{2+}\) increases resulting from activation of g protein-coupled receptors. Sosa et al (2002) observed a biphasic increase in cytosolic Ca\(^{2+}\) upon activation of metabotropic glutamate receptor 5 (mGluR5) in retinal amacrine cells in culture. The first phase was due to Ca\(^{2+}\) release from internal stores; and the more variable second phase was determined to be Ca\(^{2+}\) influx, through a process called store-operated calcium entry. Store-operated calcium entry involves release of Ca\(^{2+}\) from internal stores. This store release, through a poorly understood mechanism, activates store-operated ion channels on the plasma membrane that allow external Ca\(^{2+}\) to enter the cell and replenish depleted stores. Several classes of ion channels were experimentally eliminated as the Ca\(^{2+}\) entry pathway in these cells. The best remaining candidate is a class of non-selective cation channels from the TRP (transient receptor potential) superfamily.

TRP channels are a family of 20 proteins divided into 3 subfamilies based on sequence similarity: TRPV (vanilloid), TRPM (melastatin), and TRPC (canonical). TRPC channels are linked to g protein-coupled receptor activation, and Ca\(^{2+}\) release from internal stores (see Harteneck et al. 2000, Clapham et al. 2001 for review), making them good candidates for mediating the mGluR5-induced Ca\(^{2+}\) influx in amacrine cells. So far, seven TRPC channel subunits have been identified, and 4 subunits combine as homo- and hetero-multimers to form
functional ion channels in native systems. In Chapter 2, I explore the expression of TRPC1 and TRPC4 in cultured cells as well as in the intact chicken retina using polyclonal antibodies raised against these TRPC subunits (Crousillac et al. 2003).

TRPC1 was shown to be expressed by amacrine cells in cell cultures. In retinal tissue sections, TRPC1 expression was confined almost entirely to the inner plexiform layer where it labeled cell processes in three broad stripes. The TRPC1 antibody occasionally labeled cell bodies in the inner nuclear layer, the inner plexiform layer, and the ganglion cell layer, a pattern consistent with TRPC1 expression by amacrine cells (see Figure 1 and legend), which was confirmed by double-labeling with a polyclonal antibody against brain nitric oxide synthase (Fischer and Stell, 1999).

TRPC4 expression was observed primarily in glial cells in retinal cell cultures. In the chicken retina, TRPC4 labeling was widespread with expression observed in all layers of the retina. The most intense labeling was seen in radially-oriented cells in the inner nuclear layer, and in vertical elements just below the ganglion cell layer. Double-labeling experiments using a glial cell marker indicated expression of TRPC4 by Müller glial cells in the chicken retina.

One of the limitations in my efforts to examine TRPC channel expression was the poor reliability and specificity of commercially available TRPC antibodies. Of the TRPC antibodies used, TRPC1 and TRPC4 were the only two for which I could confirm specificity in chicken tissue. Thus, I decided to take physiological and molecular approaches to begin to elucidate the role of these channels and the signaling molecules that can lead to their activation.

In Martin Wilson’s lab at U.C. Davis, Sarah Lindstrom observed an increase in the frequency of transient Ca\textsuperscript{2+} elevations occurring in Ca\textsuperscript{2+}-store-depleted amacrine cell processes in response to various sphingolipid metabolites (unpublished observations). These Ca\textsuperscript{2+}
elevations were demonstrated to be due to Ca\(^{2+}\) influx. Sphingosine-1-phosphate (S1P) was particularly effective in increasing the frequency of these events. These observations indicated that amacrine cells might express receptors for S1P that can mediate Ca\(^{2+}\) entry in these cells. The majority of studies on S1P have examined its role in cell differentiation and growth in numerous cell types. However, relatively little is known about the role of S1P in neuronal signaling, and almost nothing is known about its role in the vertebrate retina. To examine S1P signaling in retinal amacrine cells, I used a combination of electrophysiology, calcium imaging, immunocytochemistry, and PCR amplification techniques. In Chapter 3, I present a novel signaling pathway involving S1P activation of g protein-coupled receptors (S1P1R and S1P3R) and phospholipase C. S1P elicits a noisy, inward cation current in amacrine cells and stimulates Ca\(^{2+}\) elevations involving both store release and Ca\(^{2+}\) influx, possibly via TRPC channels. PCR amplification experiments revealed transcripts from TRPC subunits 1, 3, 4, 5, 6 and 7 in a mixed population of retinal cells, and transcripts from TRPC 1, 4, and 5 in single amacrine cells. Together, these studies present S1P as a potent lipid signaling molecule in the inner retina, and further implicate TRPC channels as mediators of non-voltage-dependent Ca\(^{2+}\) entry in retinal amacrine cells.

REFERENCES


Figure 1.1 The vertebrate retina is a laminar structure containing: photoreceptors (PR), horizontal cells (HC), bipolar cells (BP), amacrine cells (AC), ganglion cells (RGC), and Müller glial cells (MC). Amacrine cell bodies are located at the border of the inner nuclear layer (INL) and the inner plexiform layer (IPL), as well as in the ganglion cell layer (GCL), and extend processes into the IPL. Müller glial cell bodies are located in the INL and extend processes radially throughout the entire length of the retina.


CHAPTER 2

IMMUNOLOCALIZATION OF TRPC SUBUNITS 1 AND 4 IN THE CHICKEN RETINA*

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INTRODUCTION

In the vertebrate retina, both ionotropic and metabotropic receptors mediate the flow and the processing of the visual signal in the retina. Ionotropic receptors can function to depolarize cells, to hyperpolarize cells, or to suppress voltage changes. Through their indirect interactions with ion channels, G protein-coupled, metabotropic receptors can mediate these same changes as well as a host of other modifications in cellular function. Many of the G protein-coupled receptors expressed in the retina are known to activate the IP3 pathway. Among these are the group 1 metabotropic glutamate receptors (mGluRs 1 and 4, Koulen et al., 1997; Cai & Pourcho, 1999; Kreimborg et al., 2001), neurotensin receptors (Borges et al., 1996), muscarinic receptors (Fischer et al., 1998), and P2Y purigenic receptors (Liu & Wakakura, 1998; Neal et al., 2001).

Metabotropic glutamate receptors 1 and 5 are expressed by amacrine cells in the chicken retina and in cultures containing identifiable amacrine cells (Kreimborg et al., 2001). In cultured amacrine cells, activation of mGluR5 produces phospholipase C (PLC)-dependent Ca\(^2+\) elevations that initially involve release of calcium from internal stores. A second and variable phase of these Ca\(^2+\) elevations is dependent upon the influx of Ca\(^2+\) across the plasma membrane (Sosa et al., 2002). Experiments to identify this influx pathway ruled out the involvement of L-type voltage-gated Ca\(^2+\) channels and cAMP-gated channels. Another potential Ca\(^2+\) influx mechanism known to be linked to the activation of the IP3 pathway is the transient receptor potential (TRP) channel (for review see Harteneck et al., 2000; Clapham et al., 2001). Demonstration of TRPC subunit-like immunoreactivity in cultured amacrine cells suggested that TRPC channels might form the entry route for the mGluR5-dependent Ca\(^2+\) elevations (Sosa et al., 2002). The TRP channels form a large (~20 genes in mammals) family of channel subunit proteins. The TRP channel family can be separated into three subfamilies (TRPC, TRPV, and
TRPM) based upon sequence homology. Of these subfamilies, it is the TRPC (TRPC1-7, C for "canonical") subfamily that is linked to activation of G protein-coupled receptors. Although the regulation of this group of TRP channels is poorly understood, there is growing evidence that multiple mechanisms of regulation are related to the activation of the IP₃ pathway, including Ca²⁺ store depletion, diacylglycerol production, activation of phospholipase C, and changes in internal Ca²⁺ (for review, see Clapham et al. 2001). Currently, the vast majority of functional studies of TRPC channels have been performed on cloned and heterologously expressed subunits, and two themes have emerged from these studies. The first is that the functional properties of a subunit can vary depending upon the cellular environment (Lintschinger et al., 2000; McKay et al., 2000). The second is that the functional properties of the channels are highly dependent upon their particular subunit combination (Lintschinger et al., 2000; Strübing et al., 2001).

Clearly, our understanding of the role and regulation of TRPC channels is in its early stages. Nonetheless, given the functional connection between the IP₃ pathway and the activation of TRPC channels, and the variety of IP₃ pathway-linked G protein-coupled receptors expressed in the retina, it seems likely that, if TRPC channels are expressed, they play an important role in retinal signaling. Unfortunately, almost nothing is known about the expression and function of TRPC channel subunits in the vertebrate retina. To begin to elucidate the role of TRPC channels in retinal function, we have undertaken an immunocytochemical study of the expression pattern of two TRPC channel subunits (TRPC1 and TRPC4) in the adult chicken retina. In our previous work (Sosa et al., 2002), we provided immunocytochemical evidence for the expression of TRPC1 in a subset of cultured chick amacrine cells. Therefore, an additional motivation for this study was to determine whether this expression pattern in culture was relevant to the expression
of these subunits in the intact retina. If the same subunits are expressed by amacrine cells in the intact retina and by amacrine cells in culture, then in the future, we can use the relatively simple culture system to investigate the regulation and the role of these channels in amacrine cell signaling.

MATERIALS AND METHODS

Western Blot Analysis

To obtain the material for Western blot analyses, Sprague-Dawley rats (Laboratory Animal Medicine, LSU) were sacrificed by decapitation and White Leghorn chickens (Poultry Sciences, LSU) by cervical dislocation followed by decapitation. To isolate membrane proteins, brains were homogenized on ice in 320 mM sucrose with Complete Protease Inhibitor (Roche, Indianapolis, IN). Homogenates were centrifuged for 20 min at 4°C and 2500g. Supernatants were centrifuged for 1 h at 4°C and 100,000g. Pellets were resuspended in 320 mM sucrose plus protease inhibitors on ice. For whole-cell lysates, chicken brains and retinas were homogenized in phosphate-buffered saline (PBS) with a protease inhibitor cocktail (Sigma, St. Louis, MO). For both preparations, protein content was quantified using the BioRad (Hercules, CA) protein assay kit.

Membrane preparations were denatured on a 5-20% SDS-PAGE gradient gel. Following electrophoresis, proteins were transferred to nitrocellulose membranes (transfer buffer: 20mM Tris, 192 mM glycine, 15% methanol (v/v), pH 8.3). 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). Anti-TRPC1 and TRPC4 antibodies were purchased from Alomone Labs (Jerusalem, Israel). Anti-bNOS primary antibodies were purchased from Chemicon (Temecula, CA). Membranes were placed in TRPC primary antibodies (1:500) at
room temperature for 1 h and then incubated with a goat anti-rabbit IgG peroxidase conjugate secondary antibody (1:5000) for 1 h at room temperature. The ECL Western Blotting Detection Reagent Kit (Amersham, Piscataway, NJ) was used for visualization of the antibodies. The same procedure was used to examine the binding of bNOS antibody with the exception that the primary antibody was used at a dilution of 1:5000.

Immunohistochemistry

Adult White Leghorn chickens were sacrificed by cervical dislocation followed by decapitation. The eyes were removed and the anterior one-third of the eye was cut away to reveal the eyecup. After removal of the vitreous, 4% paraformaldehyde was added to the eyecup. Ten minutes later, the retinas were dissected from the eyecup and placed in fresh fixative. Retinal tissue remained in fixative at 4°C for 2 hours following dissection. Following fixation, retinas were washed in Dulbecco's phosphate-buffered saline (PBS, Sigma, St. Louis, MO), and then incubated in 30% sucrose in PBS overnight at 4°C. The peripheral 20% of the retinas were discarded and the remaining retinas were embedded in O.C.T. compound (Sakura Finetek, Torrence, CA) by freezing on a slurry of dry ice and isopentane. Embedded tissue was stored at -80°C. Sections (14-17 µm) of retina were cut on an American Optical cryostat. Cell culture methods have been previously reported (Gleason et al. 1992). Cultured cells were fixed in 1% paraformaldehyde for 30 min at 4°C then rinsed in PBS.

Rabbit polyclonal antibodies raised against TRPC1 and 4 subunits were purchased from Alomone Labs (Jerusalem, Israel). The TRPC1 antibodies were raised against a peptide corresponding to amino acids 447-571 from human TRPC1. TRPC4 antibodies were raised against a peptide corresponding to amino acids 943-958 from mouse TRPC4. Polyclonal brain nitric oxide synthase (bNOS) antibodies were purchased from Sigma (St. Louis, MO). The anti-
bNOS antibodies are specific for nitric oxide synthase derived from brain and were generated against a peptide corresponding to amino acids 1409-1429 of bNOS from the rat. Cy3- and FITC-conjugated goat anti-rabbit secondary antibodies were purchased from Chemicon International (Temecula, CA). Vimentin monoclonal and Alexa 488-conjugated goat-anti-mouse antibodies were purchased from Molecular Probes (Eugene, OR).

Retinal sections were incubated for 1 h in 5% normal goat serum in dilution solution (1% bovine serum albumin (BSA); 0.5% saponin in PBS). Appropriate antibody dilutions were determined on retinal sections by examining dilutions ranging from 1:250 to 1:1000 with the optimal dilution being 1:500 for both of the TRP channel antibodies. For experiments with blocking peptides (provided by the manufacturer), undiluted primary antibody was co-incubated with the appropriate antigen peptide at an antibody/peptide ratio of 1:4 and incubated overnight at 4°C. These antibodies were diluted to the same concentrations as the other primary antibodies just prior to use. Sections were incubated in primary antibodies for 1 h at room temperature. Goat anti-rabbit Cy3-conjugated antibodies were diluted in dilution solution to 1:1000. Sections were incubated in secondary antibody for 1 h at room temperature.

The bNOS antibody was tested on sections at dilutions ranging from 1:500-1:3000 with the optimal results at 1:1000. For these experiments, double labeling with the anti-TRPC1 and anti-bNOS antibodies was achieved by applying the TRPC1 antibody and secondary antibody first, followed by the bNOS antibody and secondary antibody. Reversing the order resulted in cross-talk from the secondary antibodies. Goat anti-rabbit Cy3-conjugated secondary antibodies were used for the TRPC1 labeling and goat anti-rabbit FITC-conjugated secondary antibodies (Sigma, 1:250) were used for the bNOS labeling. With this application order, both antibodies labeled some elements uniquely, indicating that the double-labeled elements were not due to
cross-talk from the secondary antibodies. An additional control experiment was performed to confirm that the double-labeling was not artifactual. Sections were incubated in the TRPC1 antibody and then the Cy3-conjugated secondary. This was directly followed by an incubation in the FITC-conjugated secondary (anti-bNOS was omitted). In these sections we observed Cy3 labeling but no FITC labeling. This confirmed that the FITC-conjugated secondary was not binding previously unbound sites on the TRPC1 antibody.

The anti-vimentin monoclonal antibody was tested over a range of dilutions (1:100-1:500) with 1:100 giving the best results. For double-labeling experiments, sections were incubated in the anti-vimentin antibody for 1 h following TRPC4 labeling. Goat anti-mouse Alexa 488-conjugated secondary antibody was diluted to 1:100. Sections were incubated in this secondary antibody for 1 h at room temperature. For all experiments, sections were coverslipped in mounting medium (70% glycerol, 28% PBS, 2% n-propyl gallate, w/v). Labeled sections were observed on a Nikon microphot-FXA upright microscope with epifluorescence optics. For visualization of the double-labeled material, a 515-545 nm band-pass emission filter was used to eliminate the bleed-through from the Cy3 signal into the other channel. Digital images were captured with an Insight RT Slider camera and Spot Advanced software (Diagnostic Instruments, Sterling Heights, MI). Adobe Photoshop was used to assemble the figures. Any adjustments to the appearance of the images were the same for control and experimental images. All results have been confirmed on tissue derived from at least three different animals.

RESULTS

Antibody Specificity

To control for nonspecific binding of goat anti-rabbit secondary on adult chicken retinal tissue, sections were incubated with the secondary antibodies only. Fig. 2.1 shows an example
of tissue labeled with only the Cy3 secondary antibody. The only significant signal is due to the autofluorescence of the photoreceptor outer segments and oil droplets.

Because the antibodies used in this study were raised against mammalian epitopes (TRPC1 from human and TRPC4 from mouse), we first needed to establish that they had the same specificity in chicken brain homogenates as in control rat brain homogenates. The apparent molecular weights for these mammalian proteins are 87 kD (TRPC1, Strübing et al., 2001) and 105 kD (TRPC4, Philipp et al., 2000). These proteins were highly susceptible to degradation as previously reported (Strübing et al., 2001). The best results were obtained when both tissues were freshly prepared. The binding patterns of the antibodies were very similar in the rat and in the chicken preparations (Fig. 2.2). In both the rat and the chicken preparations, the TRPC1 antibody bound at ~200 kD, presumably representing antibody binding to dimers of the TRPC1 subunit protein. For the rat preparation, a much fainter band was also observed near 100 kD. For TRPC4, the antibody bound a single band of ~100 kD in both rat and chicken tissue. To further address the specificity of these antibodies, controls were performed in which each primary antibody was pre-incubated with the appropriate peptide antigen (Figs. 2.3A & 2.8A). For each antibody, pre-incubation with the antigen prevented binding of the primary antibody to the tissue. Together, the similar binding pattern of the antibodies in rat and chicken preparations and the effectiveness of pre-incubation with the peptide antigen in occluding labeling on sections indicate that anti-TRPC1 and TRPC4 recognize TRPC1 and TRPC4 homologues expressed in the chicken.

TRPC1 Immunoreactivity in the Adult Chicken Retina

With the TRPC1 antibody, photoreceptors and the outer plexiform (OPL) were completely unlabeled (Fig. 2.3B). Antibody binding was almost entirely confined to the inner
Figure 2.1 The secondary antibody alone does not label the chicken retina. (A,B) Fluorescent and corresponding Nomarski images are shown. (A) This section was incubated with the Cy3-conjugated goat-anti rabbit antibody (1:1000). The fluorescent signal at the outer margin of the retina is due to auto-fluorescence from photoreceptor outer segment fragments and oil droplets. Scale bar is 50 µm. (B), PR is photoreceptor layer, OPL is outer plexiform layer, INL is inner nuclear layer, IPL is inner plexiform layer, and GCL is ganglion cell layer.
Figure 2.2 Antibodies detect proteins of the appropriate molecular weight in the rat brain and the chicken brain. Antibodies raised against TRPC1 (human) and TRPC4 (mouse) were incubated with a blot of proteins from membrane preparations derived from rat and chicken brains. Lanes were loaded with 300µg and 100µg of protein for the anti-TRPC1 and anti-TRPC4 antibodies respectively. For TRPC1, the most prominent band for the rat preparation was at ~200 kD and probably represents antibody binding to dimers of TRPC1 subunits. A second band is present just below the 121.3-kD marker and probably represents monomers of TRPC1. For the chicken membrane preparation, a band is labeled at ~200 kD. Apparently, all of the chicken TRPC1 is in the dimerized form. For TRPC4, the antibody recognized proteins near 100 kD in both rat and chicken membrane preparations.
plexiform layer (IPL). Labeling in the IPL could often be resolved into three broad bands; the first band near the inner border of the inner nuclear layer (INL), the second band roughly in the middle of the IPL, and the third extending down to the ganglion cell layer (GCL). The section shown in Fig. 2.3B represents the most basic of the labeling pattern, with punctuate labeling in three bands in the IPL. In addition to these three bands, we often observed processes coursing horizontally, vertically, or diagonally across the IPL (see Figs. 2.4A & 2.6B). Some of the labeled processes were relatively large in diameter (Fig. 2.4B). Interestingly, the labeling pattern could be punctate within a process indicating that the protein may be localized to varicosities (Fig. 2.4C). Although relatively rare, cell bodies could also be labeled. Anti-TRPC1 immunoreactive cell bodies were found either at the innermost border of the INL (Fig. 2.4A, arrow), in the GCL (Figs. 2.3B & 2.7D) or within the IPL (Figs. 2.4A, arrowhead, & 2.4B). In some cases, the labeled cell bodies in the INL and IPL (Figs. 2.4A & 2.4B) were clearly contiguous with labeled processes. These observations indicate that at least a fraction of the labeled processes. These observations indicate that at least a fraction of the labeled processes in the IPL belong to amacrine cells. The positions of the labeled cell bodies are all consistent their being amacrine cells (Dacey, 1989; Famiglietti, 1992; Teranishi & Negishi 1992; but see Cook & Sharma, 1995) although they could also be ganglion cells (see Discussion).

To learn more about the identity of the TRPC1-positive cell bodies and processes in the IPL, we investigated the possibility that TRPC1 might co-localize with another molecule associated with cellular signaling, nitric oxide synthase (NOS). NOS is the Ca^{2+}-sensitive enzyme responsible for generating nitric oxide from arginine. The brain form of this enzyme (bNOS) has been demonstrated to be expressed in the chicken retina (Fischer & Stell, 1999) and the labeling pattern reported in the IPL is similar to that we observed for the TRPC1 antibody.
For our experiments we used a polyclonal antibody raised against the same fragment of rat bNOS previously described (Fischer & Stell, 1999). Because, to our knowledge, the specificity of this antibody in chicken tissue has not been previously reported, we first characterized its binding pattern using western blot analysis. This analysis showed that this bNOS antibody bound a protein at a molecular weight of about 160-170 kD in both chicken brain and retina homogenates (Fig. 2.5A). This is close to the apparent size of the mammalian protein (~150 kD, Anderson et al., 1995). In the chicken brain homogenate, there is also a second band of a slightly lower molecular weight that may represent a proteolytic cleavage product. In retinal sections, anti-bNOS labeled in a pattern similar to that previously described for the chicken retina (Fig. 2.5 B, Fisher & Stell, 1999). This antibody labeled photoreceptor cell bodies as well as the OPL. In the INL, cells close to the inner margin of the INL were labeled and could often be seen extending their processes at the outermost border of the IPL. Labeled cells were also seen to extend processes deeper into the IPL. Bands of immunoreactivity could also be discerned in the IPL and they occurred at about the same levels as those we described for the TRPC1 labeling pattern. Finally, cells in the GCL were also labeled with the bNOS antibody. In double-labeled sections, the patterns of anti-TRPC1 and anti-bNOS labeling were the same as for singly labeled tissue (compare Fig. 2.3B to 2.6B and Fig. 2.5B to 2.6A, respectively). While the anti-bNOS-labeled cell bodies in Figure 2.6A (arrows) are slightly out of focus in this plane, we chose to show this particular view because the pattern of process labeling in the IPL was so distinctive that it is relatively easy to observe the double-labeled processes (asterisks). Only a subset of the IPL labels overlapped and an area containing bNOS-positive/TRPC1-negative processes in the IPL is indicated by the three arrowheads in Figure 2.6A. TRPC1-positive/ bNOS-negative
Figure 2.3 Anti-TRPC1 labeling is found primarily in the IPL. (A) Section of the retina that was incubated with anti-TRPC1 primary antibodies pre-incubated with the antigen peptide followed by Cy3-conjugated secondary antibody. Binding of the primary antibody was occluded by the pre-incubation. (B) Section of retina labeled with the TRPC1 antibody and the Cy3-conjugated secondary antibody. In the IPL, labeled processes are abundant and they occur in three broad bands. The arrow indicates labeled cell bodies in the GCL. Scale bar is 50 µm.
Figure 2.4 Anti-TRPC1 can also label cell bodies and varicosities. (A) The IPL is shown containing labeled processes. Labeled cell bodies can also be observed. One cell body is at the innermost border of the INL (arrow) and the other is in the outermost region of the IPL (arrowhead). (B) Higher magnification view of a tangential section through the IPL. Two cell bodies are indicated with arrows. The asterisk indicates a relatively thick process traversing the IPL. (C) Labeled processes are shown in the IPL. Arrowheads indicate varicosity-like domains. Scale bars are 25 µm.
Figure 2.5 Brain nitric oxide synthase is expressed in the chicken retina. (A) The bNOS antibody detects protein just below the 176.5-kD marker in homogenates from chicken brain and retina. (B) This same antibody labels the OPL, numerous amacrine cells in the INL, three bands in the IPL and cell bodies in the GCL. Arrows indicate two of the labeled cell bodies. Scale bar is 50 µm.
elements were also observed, indicating that the overlapping signals were not due to cross-talk between the secondary antibodies (see Methods). Although few cell bodies were labeled by the TRPC1 antibodies, many of them were also labeled with the bNOS antibody. To quantify this, image overlays were generated for 25 double-labeled sections. In these 25 sections, five TRPC1-positive cell bodies were found in the INL and of these, two were double-labeled with the bNOS antibody (Fig. 2.7 A & 2.7B). Eighteen TRPC1-positive cell bodies were found in the GCL and all of these cells were also labeled with the bNOS antibody (Fig. 2.7 C & 2.7D). We also examined the labeling characteristics of the relatively thick TRPC1-positive processes and found that they were consistently double-labeled with the bNOS antibody (n=5, Fig. 2.7 E & 2.7F).

TRPC4 Immunoreactivity in the Adult Chicken Retina

Binding of the TRPC4 antibody was widespread in the chicken retina (Fig. 2.8B). Labeling was observed at the level of the photoreceptor cell bodies and in the OPL. Cell bodies lying in middle of the INL were labeled along with their radially oriented processes (Fig. 2.8B & 2.8C). In the IPL, there was relatively weak but distinguishable labeling that could sometimes be resolved into two or three broad stripes of immunoreactivity. Labeling was also observed in the GCL at what appeared to be the basal one-third of cell bodies. Finally, vertically oriented structures were labeled below the GCL and these were sometimes contiguous with the labeled elements in the GCL (Fig. 2.8D).

Co-localization of TRPC4 and Vimentin

Two aspects of the labeling led us to postulate that TRPC4 was expressed by Müller glial cells. First, the labeling extended from the level of the outer limiting membrane to the level of the inner limiting membrane. Second, the position of the cell bodies in the INL and the radial orientation of their processes were consistent with their being Müller cells. To test this
Figure 2.6 BNOS and TRPC1 can be co-localized in the inner plexiform layer of the chicken retina. (A,B) Mirror images are shown of the same section double-labeled with the bNOS and TRPC1 antibodies. Asterisks indicate double-labeled processes. (A) The normal bNOS labeling pattern is seen with labeling of amacrine cell bodies in the INL (arrows), and three bands of labeling in the IPL. Processes in the outermost portion of the IPL (arrowheads) are only labeled with the bNOS antibody. (B) The typical TRPC1 labeling pattern is seen with labeling of cell processes in three bands in the IPL. Scale bar is 50 µm.
Figure 2.7 Co-expression of TRPC1 and bNOS is found in cell bodies and processes. (A) A cell body at the inner border of the INL is labeled with the bNOS antibody (arrows). (B) The same cell is also labeled the TRPC1 antibody (arrows). (C) Anti-bNOS labels cell bodies in the ganglion cell layer. (D) Anti-TRPC1 co-labels a subset of bNOS-positive cell bodies in the ganglion cell layer (arrows). (E) bNOS antibodies, and (F) TRPC1 antibodies can co-label long, relatively thick processes in the IPL. Arrowheads denote the same IPL process expressing both bNOS and TRPC1. Scale bars are 25 μm in A-D and 50 μm in E-F.
possibility, we examined the labeling pattern of a monoclonal antibody raised against vimentin, a marker for Müller glial cells in the chicken retina (Lemmon & Rieser, 1983). Double-labeling with this antibody and anti-TRPC4 demonstrated that many of the TRPC4-positive elements also expressed vimentin. Co-localization of the two proteins was seen for the cells in the INL (Fig 2.9A & 2.9B). Although the cells were double-labeled, on difference in the labeling pattern was that the vimentin antibodies strongly labeled the nuclear regions of the cell bodies while TRPC4 labeling in the nuclear regions was not observed. Co-localization of TRPC4 and vimentin was also observed in the basal structures in the GCL and vertical elements at or below the GCL (Fig. 2.9C & 2.9D). This expression pattern is consistent with the expression of TRPC4 by Müller glial cells.

TRPC4 Immunoreactivity in Culture

A future objective is to test the function of these subunits in retinal cultures derived from the chick retina. Thus, we asked whether the labeling pattern in cultured retinal cells was consistent with what we observe in the intact retina. In chick retinal cultures we can identify amacrine cells, cone photoreceptors (Gleason et al, 1992, 1993), and flat glial cells thought to be derived from Müller glial cells (Lemmon and Rieser, 1983; Moyer et al., 1990). The glial cells in culture have been previously shown to be vimentin-positive (Lemmon & Rieser, 1983; Moyer et al., 1990). The TRPC4 antibody labeled glial cells most intensely and, interestingly, the strongest labeling was often observed at the borders between adjacent glial cells (Fig. 2.10B). Cone photoreceptors were also labeled although at a fairly low intensity (not shown), but amacrine cells were not labeled by this antibody. These results are consistent with the pattern observed in the intact retina and support the possibility that TRPC4 labels only photoreceptors and Müller glial cells in the intact retina.
Figure 2.8 TRPC4 is expressed in the chicken retina. (A) Section of the retina that has been incubated with primary antibodies against TRPC4 that were pre-incubated with the antigen peptide. (B) Anti-TRPC4 antibody labels at the level of photoreceptor cell bodies, the OPL, cell bodies and radially-oriented processes in the INL, the IPL, and vertical elements below the GCL. Scale bar in A is 50 µm for A and B. (C) Higher magnification images show that the radially-oriented processes spanning the INL can arise from cell bodies situated near the middle of this layer (arrowheads). (D) TRPC4-positive processes below the GCL can be contiguous with elements in the basal region of the GCL. Scale bars in C and D are 25 µm.
Figure 2.9 TRPC4 and vimentin co-localize in the chicken retina. TRPC4 (A) and vimentin (B) antibodies are co-localized to cell bodies in the INL with radially oriented processes. Arrows indicate cell bodies that express both TRPC4 and vimentin. TRPC4 (C) and vimentin (D) antibodies also co-localize below the GCL. Arrows indicate vertical elements that demonstrate immunoreactivity for both TRPC4 and vimentin antibodies. Scale bars are 25 µm.
Figure 2.10 The anti-TRPC4 antibody labels glial cells in culture. (A) Glial cells in culture that were incubated in Cy3-conjugated secondary antibody alone. This field contains at least 3 glial cells and demonstrates that no immunoreactivity is observed in the absence of the TRPC4 antibody. (B) TRPC4 labeling is demonstrated by glial cells in culture. TRPC4 did not label amacrine cells in culture, but did label some cone cells (not shown). Scale bars are 25 µm.
DISCUSSION

Co-expression of TRPC1 and bNOS

We have shown that two TRP channel subunits are expressed in the adult chicken retina. TRPC1 is expressed almost exclusively on processes in the IPL, a subset of which belong to amacrine cells. Co-labeling of retinal sections with antibodies against both TRPC1 and bNOS indicates that many processes in the IPL co-express these proteins. Fischer and Stell (1999) have identified many of the bNOS-positive processes in the chicken retina as belonging to amacrine cells. Four morphological types of bNOS-positive amacrine cells were identified and collectively, their processes could extend from the outermost border to about a 70% depth of the IPL. We found that the majority of bNOS-positive/TRPC1-negative processes were located at the outer border of the IPL. This suggests that either these populations of amacrine cells (types 3 and 4 from Fischer & Stell, 1999) do not express TRPC1, or that its expression is confined to those processes projecting deeper into the IPL. Fischer and Stell (1999) identified relatively thick bNOS-positive processes in the IPL as efferent fibers. Interestingly, we also see thick processes labeled with the anti-TRPC1 antibody (Fig. 2.4B), and we find that these processes co-express TRPC1 and bNOS (Fig. 2.7 E & 2.7F) raising the possibility that efferent fibers also express TRPC1. Additionally, we found that some of the relatively rare cell bodies that labeled with anti-TRPC1 were co-labeled with the bNOS antibody. Based on their position, the double-labeled cell bodies in the INL are probably amacrine cells. The double-labeled cells in the GCL are most likely ganglion cells, however, as Fischer and Stell (1999) identified these cells based on their sensitivity to toxic reagents.
The co-expression of TRPC1 and bNOS helps to further define the TRPC1-positive elements in the inner retina, but is there any functional relevance to this co-expression? One possible link is that the bNOS enzyme is Ca\(^{2+}\)-sensitive and in combination with other subunits, TRPC1 forms channels that can support Ca\(^{2+}\) influx (see below). It is not known, however, whether Ca\(^{2+}\) elevations engendered by TRP channel activity would be targeted or substantial enough to activate this enzyme. In Drosophila photoreceptors, TRP channel function has been shown to be regulated by phosphorylation (Warr & Kelly, 1996; Huber et al., 1998; Liu et al., 2000). Thus, another possibility is that TRP channels themselves are a substrate of nitric oxide-mediated modulation.

Expression Pattern of TRPC4

We find a labeling pattern for TRPC4 that indicates expression of this subunit in Müller glial cells and possibly photoreceptors. One question that remains is the identity of the basal elements located at the bottom of the GCL (Figs. 2.8D & 2.9C-D). In the initial labeling, these elements looked like they might be the lower one-third of cell bodies in the GCL but the co-localization of TRPC4 and vimentin makes this interpretation seem unlikely. Another explanation might be that this labeling represents a close association of Müller glial cell processes with neurons in the GCL.

Functional Relevance of TRP Channel Expression Pattern

The physiological properties of TRPC1 have been explored by expression as homomers and as heteromers. As homomers, heterologously expressed TRPC1 subunits produce a current activatable by a diacylglycerol analogue (OAG) but only in Ca\(^{2+}\)-free external solution (Lintschinger et al., 2000). Strübing et al. (2000) also observed that no currents were detectable in the presence of physiological levels of Ca\(^{2+}\) externally. Receptor-activated currents through these homomeric channels have not yet
been observed. A splice variant of human TRPC1 (TRPC1A) has been shown to mediate
currents that are stimulated by store depletion and are also inhibited by physiological levels of
extracellular calcium (Zitt et al., 1996). The paucity of activity observed with TRPC1 homomers
under physiological conditions may indicate that they normally function as part of heteromeric
channels along with other TRPC subunits. Currents through TRPC1 subunits co-expressed with
TRPC3, TRPC4 and TRPC5 have been previously examined. Co-expression of TRPC1 and 3
produced OAG- and Ca$^{2+}$-sensitive currents that were constitutively active but the presence of
the TRPC1 subunit suppressed the receptor-mediated currents produced by TRPC3 alone
(Lintschinger et al., 2000). TRPC4 and 5 have relatively high sequence similarity (Philipp et al.,
1998) so it is not surprising that the properties of TRPC1 and TRPC4 heteromers and the
properties of TRPC1 and TRPC5 heteromers were similar. These heteromers produced receptor-
activated currents that were not sensitive to store depletion (Strübing et al., 2001). In the chicken
retina, although there is a broad overlap of expression “zones” in the IPL, no specific co-
localization of TRPC1 and TRPC4 was discernable at the current level of resolution. It remains
possible that TRPC1 is co-expressed with another subunit whose expression pattern has not yet
been determined.

The activity of channels produced by expression of TRPC4 homomers is dependent upon
the cellular environment. In Chinese hamster ovary cells, expression of TRPC4 produced a
constitutively active current that was not enhanced by receptor activation or store depletion
(McKay et al., 2000). Expression of TRPC4 in HEK-293 cells (Philipp et al., 1996) and oocytes
(Tomita et al., 1998; Kinoshita et al., 2000), however, produced currents sensitive to store
depletion. In neuroendocrine cells (PC12 and adrenal chromaffin cells), TRPC4 channels are
receptor-operated but not store-operated (Obukhov & Nowycky, 2002). However, in a cell line
derived from bovine adrenal cortex, over-expression of TRPC4 subunits produced channels that appeared to be store-operated. The effects of different environments on the function of these channels may also relate to the other, non-TRPC proteins that can associate with TRPC4. Co-immunoprecipitation experiments have demonstrated an association between TRPC4 (and TRPC5 and phospholipase C) and the Na\(^{+}/\)H\(^{+}\) exchanger regulatory factor, a protein that can also associate with the cytoskeleton (Tang et al., 2000). Additionally, binding sites for calmodulin have been identified on TRPC4 (Trost et al., 2001). Thus, even if it is expressed as a homomer, TRPC4’s function may be retinal cell-type specific.

Comparison of the Labeling Pattern for Cells in the Retina and in Culture

One motivation for this study was to establish the relevance of the pattern of expression of TRPC subunits seen in cultured amacrine cells to that found in the intact retina. In culture, TRPC1 labeled a subset (~20%) of amacrine cells (Sosa et al., 2002). For the labeled cells, the protein appears to be expressed both in cell bodies and processes. In the retina, we find distinctively labeled processes in the IPL that can occasionally be traced to a labeled amacrine cell body, indicating that TRPC1 is expressed by amacrine cells in the retina as well as in culture. Cell body labeling in the intact retina is relatively rare and suggests that in culture, a cue that directs the localization of the TRPC1 protein primarily to processes is absent. Alternatively, it might be that the small subset of amacrine cells that express TRPC1 at the cell body are somehow selected for by the timing of dissociation or the conditions in the culture dish.

The most intense labeling for TRPC4 in culture was on glial cells, which is consistent with the labeling of vimentin-positive cells in the intact retina. The TRPC4 antibody also labeled cones in culture and this is consistent with the labeling observed at the level of the photoreceptor cell bodies in the intact retina. The labeling observed in culture was quite faint compared to the
level of labeling observed in the intact retina. This observation raises the possibility that some of the labeling at the level of the photoreceptors might be due to Müller cell processes. Amacrine cells in culture did not label with the anti-TRPC4 antibody (unpublished observations) and this is consistent with the pattern we observe in the intact retina. Nonetheless, TRPC4 labeling was observed in the IPL which could be due, at least in part, to expression of TRPC4 on amacrine cell processes. An immuno-electron microscopy study would resolve the TRPC4-positive elements in the IPL.

The present study confirms that TRPC channel subunits can be expressed in the vertebrate retina. Understanding the function of TRP channels in different retinal cell types will be important in our understanding of cellular signaling capabilities in the retina. Defining the expression pattern of the other TRPC subunits as well as examining the functional properties of these channels in retinal cells will be significant steps in this endeavor.

REFERENCES


CHAPTER 3

SPHINGOSINE-1-PHOSPHATE SIGNALING IN AMACRINE CELLS
INTRODUCTION

Recent studies have indicated a role for sphingolipid metabolites in neuronal signaling. Both ceramide and sphingosine have been shown to activate a Ca^{2+}-dependent inward current in DRG cells, suggesting signaling capabilities for these sphingolipids in neurons (Pollock et al. 2004). Sphingosine-1-phosphate (S1P) is a sphingosine metabolite that has been linked to numerous cellular functions, including cell growth, differentiation, and programmed cell death. S1P is produced through phosphorylation of sphingosine by sphingosine kinase (Ghosh et al. 1994, Olivera et al. 1996). Sphingosine kinase is activated by extracellular stimuli such as phorbol esters and platelet derived growth factor (Olivera et al. 1993). The sphingosine kinase protein itself contains numerous phosphorylation sites and calcium/calmodulin binding sites, suggesting that the enzyme might be regulated by both intracellular Ca^{2+} levels and phosphorylation (Hla et al. 1998).

S1P is a member of a novel class of lipid mediators that have the potential to act both extracellularly via cell surface receptors and intracellularly as second messengers (Van Brocklyn et al. 1998). Originally believed to function solely as a second messenger linked to cell proliferation and survival, it is now well-established that S1P is the endogenous ligand for a family of receptors cloned as an immediate-early gene induced during differentiation of human endothelial cells (Lee et al. 1998). The receptors were originally designated endothelial differentiation gene receptors, but are now commonly referred to as S1P receptors (S1PRs). To date, 5 members of this family (S1P1R-S1P5R) have been identified, and are grouped together according to sequence similarity and their selectivity for S1P over lysophosphatidic acid (see Kluk et al. 2002 for review).
The two most well-characterized S1PRs are S1P1R and S1P3R. 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 (Zhou et al. 2004, Formigli et al. 2002) and IP\(_3\)-insensitive Ca\(^{2+}\) stores (Ghosh et al. 1994, Mattie et al. 1994, Tornquist et al. 1997, Meyer zu Heringdorf et al. 1998). S1P1R is thought to couple exclusively to the heterotrimeric g protein G\(_i\) (Lee et al. 1998, Ancellin et al. 1999, Windh et al. 1999), consistent with the observation that S1P elicits transient Ca\(^{2+}\) elevations through a PTX-sensitive pathway in numerous cell types (van Coppen et al. 1996, Okamoto et al. 1998, van Brocklyn et al. 1998, An et al. 1999, Ancellin et al. 1999, Kon et al. 1999, Lee et al. 1999, Okamoto et al. 1999).

S1P3R has been shown to be coupled to G\(_i\), G\(_q\), and G\(_{13}\) (Ancellin et al. 1999, Kon et al. 1999, Okamoto et al. 1999, Sato et al. 1999, Windh et al. 1999, Ishii et al. 2001). Activation of this receptor has been linked to both stimulation (Sato et al. 1999) and inhibition of adenylyl cyclase (Okamoto et al. 1999), and stimulation of phospolipase C (An et al. 1999, Ancellin et al. 1999, Okamoto et al. 1999, Sato et al. 1999, Ishii et al. 2001). In mouse myoblasts, S1P was shown to stimulate Ca\(^{2+}\) influx mediated by both S1P2R and S1P3R (Meacci et al. 2002). This response was partially inhibited by pertussis toxin, and completely abolished with suramin (a specific inhibitor of G\(_q\)), suggesting that multiple signaling pathways were involved in eliciting the response. A similar result was observed when S1P3R was stably expressed in rat HTC4 hepatoma cells. An et al. (1999) found that S1P was capable of inducing a sustained influx of extracellular Ca\(^{2+}\) that was completely abolished through inhibition of PLC with U73122. This suggested that activation of plasma membrane receptors coupled to PLC was the mechanism for
the Ca\textsuperscript{2+} entry. Partial inhibition of the Ca\textsuperscript{2+} increase was achieved through pre-treatment with pertussis toxin, indicating involvement of separate g protein-sensitive pathways.

In addition to receptor-mediated pathways, there is evidence that S1P can exert some of its effects independently of receptor activation (Van Brocklyn et al. 1998). Sphinganine-1-phosphate, which is structurally similar to S1P, activates the entire family of S1P receptors, yet does not completely mimic the actions of S1P (Van Brocklyn et al. 1998, Xia et al. 1998). Also, mobilization of intracellular Ca\textsuperscript{2+} has been observed independently of S1PR expression (Van Brocklyn et al. 1998). Furthermore, proliferation and survival can be achieved through microinjection of S1P, as well as through release of intracellular caged S1P, further evidence for the existence of an intracellular interaction site for S1P (Van Brocklyn et al. 1998, Xia et al. 1998).

Very little is known about the signaling capabilities of S1P in neurons, and even less is known about its role in the retina. To explore a possible signaling role for S1P in the vertebrate retina, we utilized a simplified culture system containing a class of interneurons called amacrine cells. Amacrine cells form numerous synapses in the inner retina, and are believed to be involved in the processing of the visual signal. In our experiments, we used a combination of electrophysiology, immunocytochemistry, Ca\textsuperscript{2+}-imaging, and molecular biology techniques to explore the signaling properties of S1P in retinal amacrine cells. We find that S1P initiates receptor-dependent signaling in retinal amacrine cells. One consequence of this signaling is the activation of a membrane current that is carried by cations and has properties consistent with the involvement of TRPC channels.
MATERIALS AND METHODS

Cell culture

Dissociation and culture methods have been previously reported (Gleason et al. 1992). Briefly, retinas from 8-day-old White Leghorn chicken embryos (Gallus gallus domesticus, Animal Sciences Department, Louisiana State University, LA, USA) were dissociated in 0.1% trypsin and plated at 5.0 X 10^5 cells/ 35 mm poly ornithine-coated (0.1 mg/mL) tissue culture dish. Cells were also plated onto poly ornithine-coated glass coverslips for immunocytochemistry (see below). Retinal cultures were maintained in Dulbecco's modified Eagle's medium (Gibco, Rockville, MD, USA) with 5% fetal bovine serum (HyClone, Logan, UT, USA), 1000 U penicillin/mL, 100 µg streptomycin/mL, and 1mM L-glutamine (Sigma).

Immunocytochemistry

Adult White Leghorn chickens were sacrificed by inhalation of CO₂ followed by decapitation. The eyes were removed, and the anterior one-third of the eye removed to reveal the eyecup. After removal of vitreous humor, 4% paraformaldehyde fixative was added to the eyecup. Retinae were dissected and placed into fresh fixative. Retinae remained in fixative for one hour at 4°C. Following fixation, retinae were washed in Dulbecco’s phosphate buffered saline (PBS, Sigma, St. Louis, MO), then incubated overnight at 4°C in a 30% sucrose in PBS. The tissue was embedded in O.C.T. compound (Sakura Finetek, Torrence, CA) by freezing on a slurry of isopentane and dry ice. Embedded tissue was stored at -80°C, and 15-20 µm sections were made using a Thermoshandon Cryotome E cryostat. Cultured cells were fixed in 2% paraformaldehyde for one hour, then washed in PBS.

Rabbit polyclonal antibodies raised against Sphingosine-1-phosphate receptor 1 (S1PR1), and calretinin were purchased from Cayman Chemical (Ann Arbor, MI) and Chemicon
International (Temecula, CA) respectively. Retinal sections and cell cultures were incubated for 1 hour in 5% normal goat serum in dilution solution (1% bovine serum albumin (BSA); 0.5% saponin in PBS). Both anti-Sphingosine-1-phosphate receptor 1 and anti-calretinin primary antibodies were diluted to 1:500 in dilution solution. Retinal sections and cultured cells were incubated in primary antibodies for 1 hour at room temperature. After washing (3 X 10 min.), Cy3-conjugated secondary antibody and Alexa 488-conjugated secondary antibody (Molecular Probes, Eugene, OR) were diluted to 1:1000, and were used to visualize the S1PR1 antibody and calretinin antibody, respectively. Retinal sections and cultured cells were incubated in 2° antibody for one hour then washed in PBS. Retinal sections and cultures were mounted in Vectashield (Vector, Burlingame, CA) and viewed under fluorescent optics using an Olympus IX-70 inverted microscope. Digital images were captured using IPLab (Image Capturing software) version 4.0. Adobe Photoshop (version 7.0) was used to assemble the figures.

Electrophysiology

For recordings, cell culture dishes were mounted on the stage of an Olympus IX-70 inverted microscope, and the cells were visualized with Hoffman modulation contrast optics. A Ag/Ag-Cl pellet served as a reference electrode in the culture dish. An Axopatch-1D amplifier (Axon Instruments, Foster, CA) was used in all recordings. Data were acquired using a Digidata 1322 data acquisition board, and pClamp 9.2 software. 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; Sutter Instruments, Novato CA) using a Flaming/Brown puller (Sutter Instruments) 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 (Molecular Probes, Eugene, OR, 25 mg/ml
DMSO) and diluted in internal solution for a final amphotericin concentration of 140µg/ml. All recordings were made at room temperature (22-24°C). Gigaohm seals were achieved by providing a brief hyperpolarizing pulse of -140 mV and unless otherwise indicated, the voltage was clamped at -70 mV. All current traces shown in figures were leak-subtracted. In experiments in which the charge transfers were compared, for a given experiment, equivalent traces were analyzed for each condition, and the mean charge transfer was calculated over 5 second time frames.

Solutions

All reagents were purchased from Sigma (St. Louis, MO) unless otherwise indicated. 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. External solutions also contained bicuculline methiodide (3.0 µM, RBI, Natick, MA) to block GABAergic synaptic currents. Cesium internal solution (for ruptured patch) consisted of the following: Cs Acetate 100.0, CsCl 10.0, CaCl2 0.1, MgCl2 2.0, HEPES 10.0, EGTA 1.1. Internal B solution (for perforated patch) consisted of the following: Cs Acetate 135, CsCl 10.0, MgCl2 2.0, CaCl2 0.1, EGTA 1.1, HEPES 10.0, NaCl 1.0.

Ca²⁺ Measurements

Cells were loaded with 2 µM Oregon Green BAPTA 488 (Molecular Probes) for one hour at room temperature, in the dark. After one hour, the cells were 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 imaging and data information were acquired using IP Lab Image Capturing Software version 4.0. Shutter interval for imaging experiments was 1s, and exposure time was 150 ms unless otherwise
indicated. Background fluorescence measurements were subtracted from all data. Raw
fluorescence intensity values were normalized to baseline values and are reported as F/F₀.

Pharmacological agents

Unless otherwise indicated, Sphingosine-1-phosphate was diluted in external solution to
a final concentration of 10 µM. The PLC inhibitor U73122, and the inactive analog U73343 were
purchased from Biomol (Plymouth Meeting, PA). The IP₃ receptor antagonist heparin was
purchased from Calbiochem (San Diego, CA) and was included in the patch pipette at a final
concentration of 5 mg/ml. The S1P₁-receptor-specific agonist SEW 2871 was purchased from
Cayman Chemical. Pertussis toxin was purchased from Calbiochem and 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
immediately used for electrophysiology experiments. Pertussis toxin-treated cells used for Ca²⁺-
imaging experiments were washed in normal external solution, and loaded with the dye for one
hour prior to recording. Cells were washed in Hank’s balanced salt solution (Sigma) before
imaging experiments were performed.

RESULTS

Individual amacrine cells were voltage-clamped at -70 mV. Control traces were taken in normal
external solution before S1P was applied (Figure 3.1A). S1P elicited a noisy inward current that
developed after 30-40 seconds of exposure (Figure 3.1A). Once activated, the current usually
persisted after the removal of agonist. To determine the ionic composition of the S1P-dependent
current, we delivered a voltage ramp protocol while the S1P-dependent current was activated.
The S1P-dependent current was found to have a mean reversal potential of +8 mV suggesting
that the S1P-dependent current is carried by a mixture of cations.
Figure 3.1 Sphingosine-1-phosphate produces an inward cation current in retinal amacrine cells. **A**, A representative amacrine cell is voltage-clamped in the perforated-patch configuration at -70 mV, and S1P (10µM) is applied. After 30-40 seconds of exposure, a noisy inward current begins to develop. **B**, A voltage ramp produces an S1P-dependent current that reverses at +8 mV, suggesting that the current is carried by a mixture of cations.
S1P Receptors Are Present in Amacrine Cells in Culture and in the Intact Retina.

To determine the mechanism by which the S1P-dependent current is activated, we considered the possibility that S1P might be interacting with g protein-coupled receptors on the plasma membrane. To determine if S1P receptors are expressed by amacrine cells, two receptor-specific polyclonal antibodies were tested on cultured cells, as well as on frozen retinal tissue sections. 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 kD marker in both lanes (Figure 3.2A) consistent with the predicted molecular weight of S1P1R (47 kD). For immunocytochemistry, Cy3-secondary-only control experiments revealed no non-specific immunoreactivity in cultured cells (Figure 3.2B-top panel) or in frozen retinal tissue sections (Figure 3.2B-bottom panel). S1P1R antibodies labeled amacrine cell bodies as well as their processes in cell culture (Figure 3.2C-top panel). To examine the S1P1R expression pattern in the intact retina, S1P1R antibodies were used on frozen retinal tissue sections collected from adult White Leghorn chickens. In retina sections, the S1P1R antibody labeled a subset of cell bodies in the inner nuclear layer near the border of the inner plexiform layer. S1P1R antibodies also labeled several broad stripes at the border of the inner plexiform layer and the inner nuclear layer, and two distinct stripes in the middle of the inner plexiform layer, consistent with its expression by amacrine cells. S1P1R labeling was also present in a group of cells near the ganglion cell layer (Figure 3.2C).

We also examined whether S1P3R is present in amacrine cells. Western blot analysis was performed to confirm specificity of the S1P3R antibody, but no bands were revealed in either chicken brain or rat brain tissues. Thus, any labeling must be considered S1P3R-like. To
Figure 3.2 S1P receptors are expressed by amacrine cells.  

A, Lanes were loaded with 50µg and 150µg of chicken brain and rat brain homogenate respectively. Western blot analysis reveals a single band present in both lanes just below the 50 kD marker, consistent with the predicted molecular weight for the S1P1R (47 kD).  

B, Cy3-secondary-only controls did not show any non-specific labeling in cultured cells (top panel) or in frozen sections of the chicken retina (bottom panel).  

C, The S1P1R antibody labels amacrine cell bodies and their processes in culture (top panel). Immunoreactivity in sections of retina is observed in the inner nuclear layer, the inner plexiform layer, and the ganglion cell layer, consistent with expression of S1P1R by amacrine cells (bottom panel).  

D, The S1P3R antibody labels amacrine cell bodies and their processes in cell culture, in a pattern similar to the S1P1R antibody (top panel). S1P3R immunoreactivity is also observed in all levels of the intact retina, consistent with its expression by numerous cell types in the chicken retina including amacrine cells. Scale bars are 25µm in (B), and in top panels (C-D). Scale bars are 50µm in bottom panels (C-D).  

E, S1P1R couples to Gi exclusively, while S1P3R couples to G1, Gq, and G13 in other cell types. Signaling through these g proteins converges at the level of phospholipase C (PLC) activation, which generates inositol trisphosphate (IP3) and elicits cytosolic Ca2+ elevations. PLC activation also produces diacylglycerol (DAG) which activates protein kinase C (PKC).
Adapted from Kluk et al. (2002)
examine the pattern of S1P3R-like expression, polyclonal antibodies raised against S1P3R were used in cell cultures. S1P3R-like immunoreactivity was observed in amacrine cells and their processes in cell culture, in a pattern similar to the S1P1R expression pattern (Figure 3.2D-top panel). In frozen retina sections, S1P3R-like immunoreactivity was present in every layer of the retina, suggesting its expression by numerous cell types in the chicken retina, including amacrine cells (Figure 3.2D-bottom panel).

The S1P-dependent Current Results from Activation of Both S1P1R and S1P3R in Amacrine Cells.

Upon confirming the expression of S1P1R and S1P3R by amacrine cells, we sought to determine if S1P was eliciting a noisy inward current through activation of these receptors. S1P1R is coupled to Gi and is linked to both inhibition of adenylate cyclase and activation of phospholipase C (Figure 3.2E). To examine whether S1P1R contributes to the S1P current, cells were incubated overnight in pertussis toxin (PTX, 200 ng/ml), an inhibitor of Gi-mediated signaling. In untreated cells, S1P application elicited a noisy inward current (Figure 3.3A-bottom trace). However, in cells pre-treated with PTX, the S1P current was reduced (Figure 3.3B-bottom trace), suggesting that activation of S1P1R contributes to the S1P-induced current. Because the S1P-induced current was observed to be extremely noisy, measurements of current amplitude would be meaningless. Thus, to quantify the level of activation of the S1P-induced channel activity, currents were integrated to represent charge over time. Charge transfer analysis revealed a significant reduction in the S1P current in PTX-treated cells compared to untreated cells. (Figure 3.3C). To examine whether activation of S1P1R alone is sufficient to activate the S1P-induced current, we employed SEW 2871, a synthetic agonist specific for the S1P1R
receptor. SEW 2871 (10μM) elicited a noisy inward current similar to the S1P-induced current, further indicating that S1P was activating this receptor at the plasma membrane (Figure 3.3D). Because PTX pre-treatment resulted in only partial inhibition of the S1P-mediated current, we asked whether S1P3R was also being activated by S1P. S1P3R can be coupled to Gq, Gi, or G13, and is linked to activation of phospholipase C (Figure 3.2E). To examine the role of S1P3R in the production of the S1P-dependent current, the effects of suramin, a Gq-specific inhibitor, were examined. In cells exposed to S1P in the presence of suramin, the noisy inward current was reversibly reduced (Figure 3.4A-compare middle trace to bottom trace). Charge transfer analysis revealed a significant reduction in the S1P-dependent current in the presence of suramin and S1P compared with S1P alone (Figure 3.4B), indicating that S1P3R contributes to the production of the S1P-dependent current. Interestingly, PTX and suramin each inhibited the S1P-dependent current by ~75-80%, suggesting a synergistic relationship between the two pathways, meaning the total current mediated by both S1P1R and S1P3R is greater than the sum of the currents mediated by the individual receptors.

The S1P-induced Current is PLC-dependent

The two separate signaling pathways for S1P1R and S1P3R converge at the level of phospholipase C (PLC), with activation of the g proteins Gi and Gq and G13 ultimately leading to activation of PLC (Figure 3.2E). To confirm that the S1P-induced current is receptor-mediated, and to examine whether S1P is activating the current through a PLC-dependent pathway, we used the PLC inhibitor U73122 in voltage-clamp experiments. U73122 caused a substantial reduction in the S1P-induced current (Figure 3.5A-middle trace). Upon removal of U73122, the S1P-dependent current became more active, indicating that the current is PLC-dependent (Figure 3.5A-bottom trace). Charge transfer analysis revealed that U73122 caused a significant reduction
Figure 3.3 Activation of S1P1R contributes to the S1P-dependent current. Cells were pre-treated overnight (18-22 hr.) with pertussis toxin (PTX, 200 ng/ml). A, An untreated cell that is voltage-clamped at -70 mV is exposed to S1P (10µM) and a noisy, inward current is observed (bottom trace). B, A PTX-treated cell that is voltage-clamped at -70 mV is exposed to S1P and only a small inward shift in baseline current is observed. 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=0.019). D, SEW 2871(10µM), an S1P1R-specific agonist, was applied to determine if activation of S1P1R alone is sufficient to produce a current (bottom trace). SEW 2871 produced a noisy inward current, similar to the S1P-induced current.
A. Untreated cell

B. PTX-treated cell

C. Mean Charge Transfer

D. Control
**Figure 3.4** S1P3R also contributes to the S1P-dependent current. **A,** A cell is voltage-clamped at -70mV and exposed to suramin (200μM), a Gq-specific antagonist, and S1P. Suramin caused a reversible (bottom trace) reduction in the S1P current (middle trace). **B,** Analyses of S1P-dependent charge transfer revealed that suramin significantly suppressed the current (n=9, p=0.003).
Figure 3.5 The S1P-induced current is PLC-dependent. **A,** A cell is voltage-clamped at -70 mV. The S1P-induced current was suppressed in the presence of the PLC inhibitor (middle trace). Upon removal of the PLC inhibitor U73122, the S1P current became more active (bottom trace). **B,** The mean charge transfer was significantly reduced by the inhibitor (n=6, p=0.023). **C,** A separate cell is voltage-clamped at -70 mV then exposed to S1P in the presence of U73343 (10 μM), an inactive PLC inhibitor analog. The inactive analog had no effect on the S1P current (compare middle and bottom traces). **D,** The mean charge transfer was unaffected by U73343 (n=6, p=0.983)
in the S1P-dependent current (Figure 3.5B). In control experiments, the inactive analog U73343 was applied along with S1P and had no effect on the S1P current (Figure 3.5C-middle trace). The 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$_3$). To test the involvement of IP$_3$ receptors specifically, we included the IP$_3$ receptor antagonist heparin (5µg/ml) in the patch pipette and performed ruptured patch voltage-clamp recordings on amacrine cells. The S1P-induced current was not blocked by the inclusion of heparin in the recording pipette (n=7; Figure 6-bottom trace), suggesting that the current activation is independent of IP$_3$ receptor activation. The S1P-induced Current is Lanthanide-sensitive.

When determining the possible entry pathways for the S1P-dependent current, we were able to rule out activation of GABA$_A$ receptors, because the $E_{rev}$ for the S1P-dependent current (+8 mV) was too positive to represent Cl$^-$ efflux from the cell ($E_{Cl^-} = -58$ mV), and because external recording solutions contained bicuculline methiodide to block signaling through 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 (Gleason et al. un-published observations, Sosa et al. 2002). We were also able to rule out activation of L-type voltage-gated Ca$^{2+}$ channels for several reasons. Primarily, voltage was clamped at -70 mV in the electrophysiology experiments, outside the activation range of L-type voltage-gated Ca$^{2+}$ channels. Secondly, nifedipine (10 µm) was routinely included in external recording solutions to block activation of these channels. And finally, the $E_{rev}$ for the S1P-dependent current was too negative to represent Ca$^{2+}$ influx alone through a membrane ion channel. The current is likely carried by a mixture of cations. Upon ruling out the involvement of these channels, the most likely candidate is a group of non-
Figure 3.6 The S1P current is IP$_3$ receptor-independent. A, The IP$_3$ receptor blocker heparin (5 mg/ml) was included in the patch pipette for ruptured-patch voltage clamp recordings. Before S1P, 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 (compare to the S1P-induced current in Fig. 3A).
selective cation channels called TRPC channels. TRPC channels have been linked to g protein-coupled receptor activation, activation by tyrosine kinases, and through a poorly understood mechanism, activation by release of Ca^{2+} from internal stores. TRPC channels are sensitive to low 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 µm) and Gd^{3+} (10µm) on the S1P current. Both La^{3+} (Figure 3.7A-bottom panel) and Gd^{3+} (Figure 3.7B-bottom panel), consistently abolished the S1P current. Another hallmark of TRPC-mediated current is a reversal potential close to 0 mV, consistent with the E_{rev} measured for the S1P-dependent current (Figure 3.1B).

S1P Causes a Cytosolic Ca^{2+} Increase that Involves Ca^{2+} Influx

One of the primary functions of TRPC channels is to mediate Ca^{2+} influx to replenish depleted Ca^{2+} stores. Thus, upon application of S1P, we should observe Ca^{2+} elevations dependent, at least in part, on external Ca^{2+}. To determine the effects of S1P on cytosolic Ca^{2+}, we performed Ca^{2+}-imaging experiments on amacrine cells in culture. Cells were loaded with the Ca^{2+}-sensitive dye Oregon Green BAPTA 488. S1P (10 µM) increased cytosolic Ca^{2+}. The sensitivity of these increases to La^{3+} indicates that at least a portion of the Ca^{2+} increases were a result of Ca^{2+} influx (Figure 3.8A). To confirm this, we performed Ca^{2+}-imaging experiments in 0 mM external Ca^{2+}. In 0-Ca^{2+}, S1P elicited small Ca^{2+} increases, which were presumably due to release of Ca^{2+} from internal stores. When external Ca^{2+} was re-introduced, substantial cytosolic Ca^{2+} increases were observed, which were reduced upon subsequent removal of Ca^{2+} (Figure 3.8B), further supporting the notion that S1P is stimulating Ca^{2+} entry across the plasma membrane.
Figure 3.7 The S1P-dependent current is sensitive to lanthanides. A, B (top traces) Two separate cells are voltage-clamped at -70 mV in the perforated patch configuration. Middle traces, S1P activates a noisy inward current. The S1P-dependent current is eliminated in the presence of either La$^{3+}$ (10 µM) or Gd$^{3+}$ (10 µM) (bottom traces).
Figure 3.8 S1P generates Ca$^{2+}$ elevations in amacrine cells. Cells were loaded for one hour with the Ca$^{2+}$-sensitive dye Oregon Green BAPTA 488 (2µM) A, Upon exposure to S1P (10µM), a cytosolic Ca$^{2+}$ increase was observed that was La$^{3+}$-sensitive (10µM). B, Cells were bathed in 0-Ca$^{2+}$ external solution and exposed to S1P in 0-Ca$^{2+}$. A small Ca$^{2+}$ increase was observed, possibly 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.
S1P-induced Ca\textsuperscript{2+} Elevations Either Precede or Co-occur With the S1P-dependent Current

To examine the temporal relationship between the S1P-dependent Ca\textsuperscript{2+} elevations and the S1P-dependent current, we performed simultaneous Ca\textsuperscript{2+}-imaging and voltage-clamp experiments on amacrine cells in culture. Cells were pre-loaded with Oregon Green BAPTA 488 then voltage-clamped in the perforated patch recording configuration. S1P stimulated cytosolic Ca\textsuperscript{2+} increases in some cells that preceded the S1P current (Figure 3.9A). In other cells, S1P caused Ca\textsuperscript{2+} elevations that began at about the same time as the S1P-dependent current (Figure 3.9B). The S1P-induced membrane current never preceded elevations in cytosolic Ca\textsuperscript{2+} (n=11).

The S1P-induced Ca\textsuperscript{2+} Increase is G Protein-mediated

To further establish the link between the S1P-dependent Ca\textsuperscript{2+} elevation and the S1P-dependent current, we employed pertussis toxin (PTX) to inhibit G\textsubscript{i}–mediated signaling. In PTX-treated cells, S1P induced increases in cytosolic Ca\textsuperscript{2+} in 50 of 99 cells tested (Figure 3.10A), suggesting that the S1P-induced Ca\textsuperscript{2+} increases were being activated through a different mechanism. However, in a separate experiment, the S1P1R-specific agonist SEW 2871 produced Ca\textsuperscript{2+} elevations, indicating that activation of S1P1R alone was sufficient to elicit a Ca\textsuperscript{2+} increase (Figure 3.10B). Interestingly, in these cells, S1P elicited much larger Ca\textsuperscript{2+} increases than SEW 2871, suggesting that that S1P-induced Ca\textsuperscript{2+} elevations cannot be attributed to activation of S1P1R alone.

To determine whether the S1P-induced Ca\textsuperscript{2+} increases were occurring through a G\textsubscript{q}-mediated pathway, suramin was applied along with S1P in Ca\textsuperscript{2+}-imaging experiments. In the presence of suramin, the S1P-induced Ca\textsuperscript{2+} increases were reduced (Figure 3.10C). After removal of suramin, and 20-30 seconds of exposure to S1P, substantially larger Ca\textsuperscript{2+} increases
Figure 3.9 S1P-dependent Ca$^{2+}$ elevations either precede or co-occur with the S1P-dependent current. 

**A,** An amacrine cell pre-loaded 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. 

**B,** In a different cell, S1P-induces a cytosolic Ca$^{2+}$ increase that occurs simultaneously with the S1P-dependent current.
Figure 3.10 The S1P-induced cytosolic Ca\textsuperscript{2+} increase is mediated by S1P1R and S1P3R. A, Cells were pre-treated overnight (18-22 hr.) in pertussis toxin (200 ng/ml) to inhibit signaling through Gi. A cell is loaded with Oregon Green BAPTA 488 and exposed to S1P. S1P induces a Ca\textsuperscript{2+} increase that is unaffected by pre-treatment with PTX. B, To determine if activation of S1P1R alone is sufficient to elicit a Ca\textsuperscript{2+} increase, SEW 2871 (10\textmu M) is applied. In the presence of SEW 2871, a small cytosolic Ca\textsuperscript{2+} increase is observed. A larger Ca\textsuperscript{2+} increase is observed in the same cell when exposed to S1P (Figure 10B). C, A cell is exposed to suramin (200\textmu M) to inhibit signaling through G\textsubscript{q}. In the presence of suramin and S1P, a small cytosolic Ca\textsuperscript{2+} increase was observed. When suramin was removed, the cell responded with a substantially larger Ca\textsuperscript{2+} increase. C, Cells were pre-treated overnight in PTX and exposed to suramin to inhibit signaling through Gi and G\textsubscript{q}. The majority of cells (n=44) showed no Ca\textsuperscript{2+} increase with pre-treatment with PTX in the presence of suramin and S1P. However, some cells (n=17) exhibited an S1P-induced Ca\textsuperscript{2+} increase in the presence of both inhibitors.
were observed (Figure 3.10C), suggesting that at least a portion of the S1P-induced Ca\textsuperscript{2+} increases were occurring through a G\textsubscript{q}-mediated pathway.

After observing partial inhibition of the S1P-induced Ca\textsuperscript{2+} elevations with exposure to the G\textsubscript{i} and G\textsubscript{q}-specific inhibitors, we investigated whether blocking signaling through both g proteins simultaneously would completely abolish the Ca\textsuperscript{2+} increases. In 44 of 61 PTX-treated cells, in the presence of suramin, S1P had no effect on cytosolic Ca\textsuperscript{2+} levels in the cell (Figure 3.10D). However, a portion of PTX-treated cells (n=17) demonstrated Ca\textsuperscript{2+} increases in response to S1P in the presence of suramin (not shown).

Activation of S1P1R and S1P3R Can Cause Release of Ca\textsuperscript{2+} from Internal Stores

To investigate whether the receptor-mediated Ca\textsuperscript{2+} increase is a result of Ca\textsuperscript{2+} being released from internal stores or external Ca\textsuperscript{2+} influx, cells were incubated overnight in PTX or exposed to suramin to inhibit signaling through G\textsubscript{i} (S1P1R) and G\textsubscript{q} (S1P3R) respectively. In PTX-treated cells S1P resulted in either release of Ca\textsuperscript{2+} from internal stores, followed by external Ca\textsuperscript{2+} influx, or Ca\textsuperscript{2+} influx without detectable store release (n=70; Figure 3.11A,B).

In suramin-treated cells, S1P resulted in release of Ca\textsuperscript{2+} from stores, Ca\textsuperscript{2+} influx, or in most cases a combination of both responses (n=69; Figure 3.11C,D). Together these results indicate that activation of S1P1R leads to elevations in cytosolic Ca\textsuperscript{2+} that can be mediated by internal store release alone, but most often is a combination of store-release and Ca\textsuperscript{2+} influx. Results obtained from suramin-treated cells indicate that the S1P3R-mediated Ca\textsuperscript{2+} can result from both store release and Ca\textsuperscript{2+} influx, and confirm that S1P3R is likely signaling through G\textsubscript{q} rather than G\textsubscript{i} or G\textsubscript{13} in amacrine cells.
Figure 3.11 Activation of S1P1R and S1P3R can cause release of Ca^{2+} from internal stores. Cells were exposed to S1P in 0-Ca^{2+} external, followed by S1P in normal Ca^{2+} external to determine if the S1P-induced Ca^{2+} release is a result of Ca^{2+} released from internal stores, or external Ca^{2+} crossing the plasma membrane. A,B Cells were pre-treated with PTX to inhibit signaling through S1P1R. When exposed to S1P, 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 (n=70). C,D In cells treated with suramin to block signaling through G_{q}, exposure to S1P resulted in store release only, external Ca^{2+} influx only, or most often, a combination of both responses (n=69).
A

PTX-treated
S1P

0 Ca^{2+}
N
0 Ca^{2+}

F/F_o

Time (s)

0 30 60 90 120

1.0
1.2
1.4
1.6

B

PTX-treated

Store release only
Ca^{2+} influx only
Both responses

# of cells

0 10 20 30 40 50 60 70

C

S1P
Sur

0 Ca^{2+}
N
0 Ca^{2+}

F/F_o

Time (s)

0 30 60 90 120 150 180

1.0
1.2
1.4

D

Suramin-treated

Store release only
Ca^{2+} influx only
Both responses

# of cells

0 10 20 30 40 50 60 70
TRPC Transcripts Are Present in Amacrine Cell Cultures

To address the possibility that the S1P-induced current and Ca^{2+} increase are occurring through a TRPC-mediated pathway, 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 a mixed population of retinal cells from scraped culture dishes. Amplification produced a single band in each lane at the appropriate molecular weights (Figure 3.12A), and individual TRPC subunit identity was confirmed through DNA sequencing. To confirm the expression of TRPC by amacrine cells specifically, single-cell PCR was performed on amacrine cells collected in the patch pipette. PCR amplification produced a single band for TRPC 1, 4, and 5 at the appropriate molecular weights (Figure 3.12B), and individual TRPC subunit identity was confirmed with DNA sequencing. TRPC subunits 3, 6, and 7 were not reliably amplified from single amacrine cells. One possible explanation for this is that the expression level of these subunits in single cells is too low to be detected by our methods. Another possibility is that these subunits are not expressed by amacrine cells in our cell cultures, or were not present in those cells collected for analysis.

DISCUSSION

In this study, we provide evidence that S1P activates a receptor-mediated inward cation current in retinal amacrine cells. S1P-dependent currents have also been observed in other cell types. In human umbilical vein endothelial cells (HUVECs), Muraki et al. (2001) demonstrated S1P-activation of a non-selective cation current in a PTX-sensitive, GTP-dependent manner. The S1P-induced current that we characterize in amacrine cells was found to have an $E_{\text{rev}}$ of +8mV, similar to the $E_{\text{rev}}$ for the current observed in human umbilical vein endothelial cells (HUVECs). In HUVECs, the S1P current was followed by a slow-developing sustained increase in cytosolic...
Figure 3.12 TRPC subunits are expressed in amacrine cell cultures. **A**, Gene-specific primers were designed against TRPC subunits 1, 3, 4, 5, 6, and 7. TRPC transcripts were PCR-amplified from a mixed population of retinal cells derived from a scraped culture dishes. Bands were observed at the appropriate molecular weights for each of the TRPC subunits. **B**, TRPC 1, 4, and 5 transcripts were PCR-amplified from single amacrine cells collected in a patch pipet. Bands were present at the appropriate molecular weights. DNA sequencing was performed on all PCR products to confirm their identity.
Ca\textsuperscript{2+}. In amacrine cells, the S1P current occurred simultaneously with the cytosolic Ca\textsuperscript{2+} increase in some cells, and was preceded by the cytosolic Ca\textsuperscript{2+} increase in other cells, perhaps suggesting the presence of a store-operated Ca\textsuperscript{2+} entry pathway in amacrine cells.

It is well-established that S1P can induce an increase in cytosolic Ca\textsuperscript{2+} levels. However, the source of the S1P-mediated Ca\textsuperscript{2+} increase has been shown to vary 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+} pool mobilization. In the current study in amacrine cells, S1P elicited a cytosolic Ca\textsuperscript{2+} increase that involved both release of Ca\textsuperscript{2+} from internal stores, as well as activation of external Ca\textsuperscript{2+} influx (Figure 8,11). 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. However, in the current study, nifedipine was found to have no effect on the S1P current in amacrine cells (data not shown). Furthermore, electrophysiological recordings were performed while cells were voltage-clamped at -70 mV, well outside the activation range of L-type voltage-gated Ca\textsuperscript{2+} channels. Thus, S1P is capable of activating an inward cation current in amacrine cells independent of voltage-gated ion channel activation.

Much debate exists as to whether S1P activates release of Ca\textsuperscript{2+} from internal stores through activation of IP\textsubscript{3}Rs on the endoplasmic reticulum. In Swiss 3T3 fibroblasts, S1P
stimulated an increase in cellular IP3 levels, but the S1P-induced Ca^{2+} increase was unaffected by blocking IP3 receptors with heparin (Mattie et al. 1994), suggesting that S1P stimulates PLC activity, but signals through an IP3R-independent pathway. This S1P-induced Ca^{2+} increase through an IP3R-independent pathway was also observed in other cell types (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 IP3R-independent current (Figure 5,6). At present, the mechanism underlying the IP3R-independence is not known, but it may be that diacylglycerol or protein kinase C is involved, as TRPCs have been shown to be sensitive to both of these signaling molecules.

Aside from activation of receptors at the plasma membrane, some evidence indicates that S1P can mediate its effects intracellularly. Meyer zu Heringdorf et al (2003) demonstrated mobilization of internal Ca^{2+} from thapsigargin-sensitive stores upon photolysis of caged S1P while signaling through S1PRs was inhibited. This suggests the existence of an intracellular interaction site for S1P. Ghosh et al (1994) demonstrated the presence of sphingosine kinase in the E.R. membrane of smooth muscle cells, and observed direct Ca^{2+} mobilization in purified E.R. membrane vesicles. These experiments provide evidence for a novel intracellular interaction site for S1P. One possible interaction site is SCaMPER (sphingolipid calcium release-mediating protein of endoplasmic reticulum), a protein identified for another sphingolipid derivative, sphingosyl-phosphocholine (SPC). It was demonstrated that SPC produced Ca^{2+} increases in Xenopus oocytes stably transfected with SCaMPER (Mao et al. 1996). These Ca^{2+} increases occurred independently of activation of either Ryanodine receptors or IP3Rs, indicating that SCaMPER is a novel intracellular interaction site for sphingolipids (Mao et al. 1996).
In the present study, S1P was shown to activate an inward cation current, as well as an increase in cytosolic Ca\textsuperscript{2+} levels in amacrine cells, and both responses appeared to be mediated by S1P1R and S1P3R. Interestingly, in a small fraction of cells, S1P elicited Ca\textsuperscript{2+} responses even when signaling through both G\textsubscript{i} and G\textsubscript{q} was inhibited with PTX and suramin. There are at least three possible explanations for this result. The first is that S1P3R is coupled to the heterotrimeric g protein G\textsubscript{13} in amacrine cells, making it resistant to both PTX and suramin. The second is the existence in amacrine cells of S1PRs other than S1P1R and S1P3R that are coupled to either G\textsubscript{12} or G\textsubscript{13} proteins. The most likely candidates are S1P2R, and S1P5R, both of which have been found in neurons. S1P2R has been shown to couple to G\textsubscript{i}, G\textsubscript{q}, and G\textsubscript{13} (An et al. 1999, Ancellin et al. 1999, Windh et al. 1999), and S1P5R has been shown to couple to G\textsubscript{i} and G\textsubscript{12} ((Im et al. 2000, Malek et al. 2001). The final possibility is that S1P is acting directly on an intracellular interaction site as observed by Ghosh et al. (1994), Mao et al. (1996), and Meyer zu Heringdorf et al. (2003). Further experimentation will be needed to explore these possibilities.

The evidence presented in this study points to S1P activation of a cation channel at the plasma membrane (Figure 3.13). TRPC channels allow cations, including Ca\textsuperscript{2+} to enter cells at negative membrane potentials and are linked to g protein-coupled receptor activation and release of Ca\textsuperscript{2+} from internal stores (see Clapham et al. (2001) for review), making them good candidates for activation by S1P. Though the function of TRPC channels is poorly understood, one idea is that they are expressed in cells as a means of replenishing depleted Ca\textsuperscript{2+} stores. Another possibility is that these channels mediate localized receptor-mediated 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 glutamate receptors and Ca\textsuperscript{2+}-induced-Ca\textsuperscript{2+} release (CICR). While in
these experiments, Ca$^{2+}$ influx occurred through ionotropic glutamate receptors, it might be possible that signaling through metabotropic receptors such as the S1PRs can trigger a similar response. It will be interesting to see whether S1P, through the complex lipid signaling pathway proposed in this study, is capable of mediating such a process in the vertebrate retina.

REFERENCES


Figure 3.13 Sphingosine-1-phosphate (S1P) activates g protein-coupled receptors (S1P1R and S1P3R) on amacrine cells. Signaling through G\textsubscript{i} and G\textsubscript{q} proteins activates PLC and stimulates both release of Ca\textsuperscript{2+} from internal stores and Ca\textsuperscript{2+} influx through TRPC channels. TRPC activation was IP3R-independent and may be mediated by DAG or PKC.


CHAPTER 4

CONCLUSIONS
In Chapter 2, I demonstrate the expression of two TRPC subunits in the chicken retina. Immunocytochemistry experiments revealed unique expression patterns for TRPC1 and TRPC4 in retinal tissue sections. TRPC1 was expressed in amacrine cells, while TRPC4 was found in Müller glial cells. TRPC1 and TRPC4 are structurally similar and have both been implicated as regulators of store-operated Ca\(^{2+}\) entry in other cell types. The expression of TRPC subunits in functionally-diverse multiple cell types in the chicken retina suggests an important role for TRPC channels in the regulation of Ca\(^{2+}\) levels in retinal cells. TRPC channels may function as the primary source of voltage-independent Ca\(^{2+}\) entry in some cells, or even provide a means for localized receptor-mediated Ca\(^{2+}\) entry at retinal synapses.

In Chapter 3, I examine the signaling properties of sphingosine-1-phosphate (S1P) in retinal amacrine cells. S1P was found to activate S1P1R and S1P3R through the g proteins Gi and Gq respectively. Activation of these receptors resulted in the production of an inward cation current and a cytosolic Ca\(^{2+}\) elevation that involved both store release and Ca\(^{2+}\) influx. Of the possible Ca\(^{2+}\) influx mechanisms, TRPC seems the most likely, as TRPC activity has been linked to both g protein-coupled receptor activation and release of Ca\(^{2+}\) from stores. Furthermore, the reversal potential of the S1P-mediated current indicates that the current is carried by a mixture of cations, and the current was consistently abolished with La\(^{3+}\) and Gd\(^{3+}\). Two of the hallmarks of TRPC channels are a non-selective permeability to cations, and a sharp sensitivity to lanthanides. In addition to binding S1P1R and S1P3R, it is also possible that S1P may be directly activating a membrane ion channel at the plasma membrane. Association of TRPC channels with lipid rafts in amacrine cell membranes supports a possible interaction between S1P and TRPC channels (Martin Wilson, unpublished observations).
One thing that is unclear in the S1P signaling pathway is the exact mechanism through which S1P is able to stimulate release of Ca\(^{2+}\) from internal stores. Store-release is generally thought to involve activation of IP\(_3\) receptors, or ryanodine receptors, or both. However, S1P has been shown in numerous cell types to be capable of activating store release through an IP\(_3\) receptor-independent mechanism (Ghosh et al. 1994, Tornquist et al. 1997, Spiegel et al. 2002, Im et al. 2005). Voltage-clamp experiments revealed that S1P activation of membrane ion currents is PLC-dependent, but occurs independently of IP\(_3\) receptor activation (Fig. 6). However, the mechanism for the S1P-induced release of Ca\(^{2+}\) from stores is still poorly understood. Our lab plans to examine the roles of PLC and IP\(_3\)Rs in generating this Ca\(^{2+}\) increase in Ca\(^{2+}\)-imaging experiments. It might also be helpful to determine if release of stored Ca\(^{2+}\) is occurring through ryanodine receptors. This will allow us to better understand the signal transduction pathways resulting from activation of S1P receptors.

One possible method for improving our understanding of S1P as a signaling molecule in the inner retina involves in-vivo recordings from chicken retinal slices. Thus far, our understanding of S1P signaling in amacrine cells stems from experiments performed on isolated amacrine cells in culture. However, in the retina these cells form numerous synaptic contacts with bipolar cells, ganglion cells, as well as with other amacrine cells. It will be interesting to observe how input from other retinal cell types will influence the signaling properties of S1P in amacrine cells.

Another question of interest involves the endogenous production of S1P in retinal amacrine cells. Expression of sphingosine kinase has been reported in the mammalian retina, but its expression in the chicken retina is still unconfirmed. Application of N,N-dimethylsphingosine (DMS), a sphingosine kinase inhibitor, resulted in a decrease in the frequency spontaneous Ca\(^{2+}\)
elevations in cell processes, suggesting that this enzyme is functioning in amacrine cells (unpublished observations). Localization of sphingosine kinase in retinal amacrine cells has been unsuccessful thus far. A polyclonal antibody for sphingosine kinase revealed no immunoreactivity in chicken retinal sections, and antibody-specificity through western blot analysis could not be confirmed. To overcome this, I have designed PCR primers for this enzyme, using predicted sphingosine kinase sequences in the chicken genome. We plan to amplify sphingosine kinase transcripts from cells collected from scraped culture dishes and from chicken retinal tissue. It will also be interesting to determine if this enzyme is inducible in an intact system, and if so, what types of molecules can be used to activate this enzyme. This will allow us to better understand the functional role of an S1P signaling pathway in the retina.

Perhaps the biggest question to us at this point involves the possible consequences of activation of this pathway in amacrine cells. While relatively little is known about the signaling capabilities of S1P in the vertebrate retina, it is interesting to speculate that this sphingolipid, through TRPC activation, might be playing a substantial role in Ca\(^{2+}\) regulation in amacrine cells. Though the function of TRPC channels is poorly understood, one idea is that they are expressed in cells as a means of replenishing depleted Ca\(^{2+}\) stores. Another interesting possibility is that TRPC channels may be capable of mediating synaptic transmission in amacrine cells. It is thought that TRPC channels provide localized Ca\(^{2+}\) increases for spatially-defined signal transduction pathways (Clapham 2002). Thus, these channels might be capable of mediating localized receptor-mediated Ca\(^{2+}\) entry that allows for neurotransmitter release without the involvement of voltage-gated Ca\(^{2+}\) channels.

Synaptic transmission is known to be initiated through depolarization of the presynaptic terminal. This depolarization opens voltage-gated Ca\(^{2+}\) channels at the terminal, which allow
external Ca\(^{2+}\) to enter and trigger neurotransmitter release. Recently, however, two interesting studies demonstrated that synaptic transmission in amacrine cells can also occur as a result of localized Ca\(^{2+}\) increases occurring independently of voltage-gated Ca\(^{2+}\) channels. Chavez et al. (2006) demonstrated that GABAergic feedback in A17 amacrine cells can occur through activation of postsynaptic glutamate receptors. Release of glutamate from rod bipolar cells activates ionotopic glutamate receptors on A17 amacrine cells, and Ca\(^{2+}\) entering through these glutamate receptors localized to the pre-synaptic terminal was shown to stimulate Ca\(^{2+}\)-induced-Ca\(^{2+}\) release (CICR) from internal stores. This localized increase in cytosolic Ca\(^{2+}\) is sufficient to trigger neurotransmitter release in these cells. (Chavez et al. 2006). In addition to this study, Warrier et al.(2005) demonstrated that a least a portion of the cytosolic Ca\(^{2+}\) increase responsible for neurotransmitter release in amacrine cells is due to release of stored Ca\(^{2+}\) through IP\(_3\) receptors on the endoplasmic reticulum. Interestingly, it was also shown that application of an agonist for a metabotropic glutamate receptor known to stimulate release of stored Ca\(^{2+}\) resulted in enhanced neurotransmitter release (Warrier et al. 2005).

Both of these studies provide evidence that localized Ca\(^{2+}\) increases are capable of triggering neurotransmitter release in amacrine cells. It will be interesting to determine whether S1P, through the complex lipid signaling pathway proposed in this study, is capable of mediating such a process in these cells. Future experiments will reveal the impact of this signaling molecule in the inner retina, and provide a better understanding of the mechanism through which amacrine cells shape and refine the visual signal.

REFERENCES


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Title: Immunolocalization of TRPC channel subunits 1 and 4 in the chicken retina
Title: dissertation by Scott Crousillac
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Scott Michael Crousillac was born to Hugh and Norma Crousillac in Baton Rouge, Louisiana, in 1978. He received his primary and secondary education in Greenwell Springs, Louisiana, and graduated from Central High School in May 1996. He enrolled as an undergraduate at Louisiana Scholars’ College in Natchitoches, Louisiana, in August 1996, and completed the requirements for a Bachelor of Arts degree in May 2000. He began work toward his doctorate at Louisiana State University in August 2001 in Dr. Evanna Gleason’s laboratory. He successfully defended his dissertation in October 2007 and will be awarded the degree of Doctor of Philosophy in December 2007.