The sphingosine 1-phosphate receptor, S1PR₁, plays a prominent but not exclusive role in enhancing the excitability of sensory neurons.

Running title: S1PR₁ and neuronal excitability

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Abstract

Sphingosine 1-phosphate (S1P) through its interaction with a family of G protein-coupled receptors (S1PR) is proving to have a significant impact on the activation of a variety of cell types, most notably those cells mediating the inflammatory response. Previously, we showed that S1P enhanced the excitability of small diameter sensory neurons and mRNA for S1PR$_{1-4}$ was expressed in sensory neurons. These initial findings did not determine which S1PR subtype(s) mediated the increased excitability. Here, we report that exposure to the selective S1PR$_1$ agonist, SEW2871 produced a significant increase in excitability of some, but not all, sensory neurons. To further examine the role of S1PR$_1$, neurons were treated with siRNA targeted to S1PR$_1$. siRNA reduced S1PR$_1$ protein expression by 75% and blocked the sensitization produced by SEW2871, although some neurons remained responsive to subsequent exposure to S1P. Treatment with scramble siRNA did not alter S1PR$_1$ expression. Recordings from siRNA- and scramble-treated neurons suggested three distinct populations based on their sensitivities to SEW2871 and S1P. Approximately 50% of the neurons exhibited a significant increase in excitability after exposure to SEW2871 and subsequent S1P produced no additional increase; ~25% were not affected by SEW2871 but S1P significantly increased excitability; and ~25% of the neurons were not sensitized by either SEW2871 or S1P. RT-PCR measurements obtained from single neurons revealed that 50% of the small diameter neurons expressed the mRNA for S1PR$_1$. These results indicate that S1PR$_1$ plays a prominent, although not exclusive, role in mediating the enhancement of excitability produced by S1P.

Keywords: sphingosine 1-phosphate, SEW2871, dorsal root ganglia, sensitization, siRNA, action potentials
Introduction

Seminal studies established that the lysophospholipid molecule, sphingosine 1-phosphate (S1P) is an important signaling molecule between cells but also plays an important role as an intracellular second messenger (Hannun and Obeid 2008; Hla 2004; Hla and Maciag 1990; Spiegel and Milstien 2003; Takabe et al. 2008; Van Brocklyn et al. 1998). S1P interacts with a family of five G protein-coupled receptors (S1PR1-5 previously known as Edg receptors) and has a significant impact on the development and activation of different cell types (Anliker and Chun 2004; Meyer zu Heringdorf and Jakobs 2007; Rosen et al. 2009; Sanchez and Hla 2004). Upon activation, a variety of immuno-competent cells (e.g., mast cells and platelets) release S1P where it can function as either an autocrine or paracrine signaling molecule (Goetzl and Rosen 2004; Olivera and Rivera 2005; Rivera et al. 2008; Weigart et al. 2009). In particular, interaction between S1P and the S1P receptor S1PR1 plays a critical role in regulating many aspects of the inflammatory response. Our greatest understanding involves the relationship between circulating levels of S1P, S1PR1, and the trafficking of T lymphocytes in and out of lymph nodes (reviewed Rivera et al. 2008; Rosen and Goetzl, 2005). Reduction of S1P levels or agonist-induced/conditional deletions of S1PR1 results in T lymphocytes failing to leave the node and therefore removing these cells from circulation (Allende et al. 2004; Brinkmann et al. 2002; Chiba et al. 1998; Mandala et al. 2002; Matloubian et al. 2004; Pappu et al. 2007; Schwab et al. 2005). These findings resulted in the idea that removal of S1PR1 (e.g., with the S1PR agonist FTY720) can be a potent immunosuppressant and provides a mechanistic model for the development of selective S1PR1 agonists that produce immunosuppression. Interestingly, because of its immunosuppressive properties, FTY720 has exhibited therapeutic potential in treating multiple sclerosis (Brinkmann et al. 2009; Cohen et al. 2010; Kappos et al. 2010).
Upon activation of immuno-competent cells, the release of multiple mediators can heighten the sensitivity of sensory neurons to a variety of stimuli (reviewed by DeLeo and Yezerski 2001; Miller et al. 2009; Milligan and Watkins 2009; Scholz and Woolf 2007; Thacker et al. 2007; White et al. 2005). However, the impact of these different mediators on neuronal sensitivity is poorly understood. Our previous work showed that S1P enhanced the excitability of small diameter sensory neurons and that sensory neurons expressed the mRNA for S1PR1-4 (Zhang et al. 2006). This work did not explore the idea as to which S1PR subtype(s) mediated increased excitability. In this report we focus on S1PR1 because of the well established S1P/S1PR1 interaction in regulating the inflammatory response, the mechanistic pathways are currently the best understood, and unlike other S1PRs, S1PR1 is activated by the selective agonist SEW2871. Using the combination of activation by S1P or SEW2871, siRNA targeted to S1PR1, and single-cell RT-PCR, we show that S1PR1 plays a prominent role in directly augmenting the excitability of small diameter capsaicin-sensitive sensory neurons, although other S1PRs also are involved.

Materials and Methods

Isolation and maintenance of adult rat sensory neurons

Sensory neurons were isolated from young adult male Sprague-Dawley rats (100-150 g) using procedures developed by Lindsay (1998) with slight modifications (Chi and Nicol 2007). Briefly, the young rats were killed by placing them in a chamber filled with CO2. The dorsal root ganglia (DRGs) were collected in a culture dish filled with sterilized Puck’s solution. For the experiments examining the acute effects of S1P and SEW2871, the ganglia were transferred to a conical tube with F-12 media containing papain (20 U/ml) and incubated for 15 min at 37°C, followed by incubation with 1 mg/ml collagenase IA and 2.5 mg/ml dispase for 10 min at 37°C.
For the experiments involving siRNA, the ganglia were transferred to a conical tube with F-12 media containing 1 mg/ml collagenase IA and 2.5 mg/ml dispase and incubated for 35 min at 37°C. The suspension was centrifuged for 30 s (1000 x g) after which the enzyme-containing supernatant was removed. The pellet was resuspended in F-12 media supplemented with nerve growth factor (30 ng/ml) and mechanically dissociated with fire-polished pipettes. Isolated cells were plated onto plastic coverslips or the bottom of the culture dish previously coated with poly-d-lysine (100 µg/ml) and laminin (5 µg/ml). Isolated cells were cultured at 37°C and 3% CO₂. The cells were maintained in culture for either 24 hrs (acute electrophysiological recordings) or 7 days (siRNA and electrophysiological studies). All procedures were approved by the Animal Use and Care committee of Indiana University School of Medicine.

Single cell reverse transcription- polymerase chain reaction (RT-PCR)

The presence of gene transcripts for S1PR₁ was detected using techniques described by Chi and Nicol (2007) with modification. Briefly, a small (≤25 µm) or large (>50 µm) diameter sensory neuron was aspirated into a sterilized micropipette (baked at 250°C for 2 hrs) containing diethylpyrocarbonate (DEPC)–treated water. The contents of the micropipette were forced into a 0.2 ml microtube with 5 µl DEPC water and the RNA was reverse transcribed using the iScriptTM cDNA synthesis kit (Bio-Rad, Hercules, CA) for cDNA synthesis in 20 µl reactions according to the manufacturer’s instructions. The cDNA was stored at –20°C before PCR detection. Amplification of S1PR₁ was performed by using the forward primer (278-296 bp): ATGGTGTCCTCCACCAGCATCCC and the reverse primer (725-708 bp): TTAAGAAGAAGAATTGACCTTTCC (accession NM 017301.2, product size 448 bp) using the Platinum PCR Supermix (Invitrogen, Carlsbad, CA). These PCR reactions were run on a PTC-100 programmable thermal controller (MJ Research, Inc., Watertown, MA) for 45 cycles (94°C for 45 s, 46°C for 45 s, 72°C for 2 min). The primers for Kv1.1 are described in Chi and Nicol.
(2007). The PCR products were sequenced using an ABI Prism 3100 genetic analyzer at facilities in the Department of Biochemistry and Molecular Biology, Indiana University School of Medicine.

siRNA treatment

An siRNA sequence was selected according to the software provided by Dharmacon siDESIGN website (http://www.dharmacon.com/designcenter/designcenterpage.aspx). siRNA labeled with the fluorescent tag, DY547, was synthesized by Dharmacon. The sense strand was 5'-GAAGGACCAUGGCAUUAAA-3' and the antisense strand was 5'-AAUGCCAUGGUCCUUCUU DY547-3'. For the scrambled siRNA (SC), the sense sequence was 5'-GCGCGCUUUGUAGGAUUCG-3' and antisense sequence was 5'-CGAAUCCUACAAAGCGCGC DY547-3'; SC was used as a negative control. Isolated sensory neurons were maintained for 2 days in normal media with 30 ng/ml NGF. Normal media was replaced with F-12 media lacking antibiotics and bathed the cells for ~12 hrs. The cells were rinsed once with Optimem media and incubated at 37° C for ~30 min. The metafectene-siRNA complex (100 μl, 200 nM siRNA) was added wherein the neurons were exposed to the siRNA targeted to S1PR1, SC, or metafectene alone for 48 hrs at 37°C. After 2 days, the metafectene -/+ siRNA was washed out and the normal media containing antibiotics and NGF was then added to the neurons and allowed to incubate for 24 hrs before electrophysiology recordings or Western blots were performed.

Western blotting

Isolated sensory neurons that were either untreated or exposed to siRNA targeted to S1PR1, SC, or metafectene were sonicated in fresh TNN-SDS buffer (50 mM Tris-HCl, pH 7.5, 150 mM...
NaCl, 0.5% Nonidet P-40, 50 mM NaF, 20 mM EDTA, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 0.1% SDS, and 2 mM phenylmethylsulfonyl fluoride) and incubated at 4°C for 30 min, samples were vortexed every 10 min, followed by centrifugation (5000 x g for 10 min). The supernatant was then stored at -80º C. Protein concentration was determined by the Bradford method (Bio-Rad protein assay, Bio-Rad Life Science Research, Hercules, CA). Equivalent amounts of protein (30-100 µg) were loaded in each well. Prior to loading, samples were kept at room temperature for 30 min and subjected to NuPAGE™ 4-12% Bis-Tris Gel (Invitrogen, Carlsbad, CA) electrophoresis and then transferred to a PVDF membrane (Invitrogen). After serial incubation with specific antibodies, immunoreactive bands on the membrane were developed using an ECL kit (Amersham Biosciences, Piscataway, NJ) and visualized by exposure to ISC Bio Express film. The density of bands was measured using a Kodak 1D 3.6 imaging system (New Haven, CT).

**Electrophysiology**

Recordings were made using the whole-cell patch-clamp technique (Chi and Nicol 2007; Hamill et al. 1981). Briefly, a cover slip with sensory neurons was placed in a recording chamber where neurons were bathed in normal Ringers solution of the following composition (in mM): 140 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, 10 HEPES and 10 glucose, pH at 7.4 with NaOH. Recording pipettes were pulled from disposable borosilicate glass tubing and typically had resistances of 2-5 MΩ when filled with the following solution (in mM): 140 KCl, 5 MgCl₂, 4 ATP, 0.3 GTP, 2.5 CaCl₂, 5 EGTA (calculated free Ca²⁺ concentration of ~100 nM), and 10 HEPES, at pH 7.3 with KOH. Whole-cell currents were recorded with an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA). The whole-cell recording configuration was established in normal Ringers solution. After establishing the whole-cell configuration, both the capacitance
and resistance were compensated by ~80%. To assess excitability in the current-clamp experiment, the neurons were held at their resting potentials (range between -45 to -65 mV) and ramps of depolarizing currents (900 ms in duration) were used to evoke 2-4 action potentials (APs) under control conditions. The same ramp was then used throughout the recording period for each individual neuron. All drugs were applied by external superfusion of the recording chamber using a VC-8 bath perfusion system (Warner Instruments, Hamden CT). The traces were filtered at 5 kHz and sampled at 1 kHz using pClamp 8.0 (Molecular Devices). At the end of each recording, the neuron was exposed to 100 nM capsaicin. This neurotoxin was used to distinguish capsaicin-sensitive sensory neurons as these neurons are believed to transmit nociceptive information (Holzer 1991). However, the correlation between the idea that a neuron is a nociceptor and capsaicin sensitivity is not absolute. Some nociceptive neurons are insensitive to capsaicin and some capsaicin-sensitive neurons are not nociceptors (see Petruska et al. 2000). Therefore this agent was used to define a population of small-diameter sensory neurons that could serve a nociceptive function. The results reported in the following text were obtained from only capsaicin-sensitive neurons. All experiments were performed at room temperature (~23°C).

Data analysis

Data are presented as the means ± s.e.m. The excitability parameters described in Table 1 were determined, in part, by differentiating the voltage trace (dV/dt) in the current-clamp recordings. The voltage and time at which the first AP was fired were taken as the point that exceeded the baseline value of dV/dt by >20-fold. The baseline value of dV/dt was determined by averaging the points between the onset of the ramp and the next 100 ms (135-235 ms). The rheobase was measured as the amount of ramp current at the firing threshold. The resistance at threshold ($R_{th}$) was calculated as the difference between the firing threshold and the resting
membrane potential divided by the rheobase current. Statistical differences between the control
recordings and those obtained under various treatment conditions were determined by using
either a paired t-test, ANOVA, or repeated-measures (RM) ANOVA. When a significant
difference was obtained with an ANOVA, post hoc analyses were performed using a Tukey test.
Values of P<0.05 were judged to be statistically significant.

Chemicals
S1P was obtained from Avanti Polar Lipids, Inc., (Alabaster, AL) and dissolved according to
instructions provided by the supplier (www.avantilipids.com/SyntheticSphingosine-1-
phosphate.asp). SEW2871 was purchased from Cayman Chemical Co. (Ann Arbor, Michigan).
The S1PR1 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA); the actin antibody
was from Thermo Scientific (Waltham, MA). Metafectene was purchased from Biontex-USA
(San Diego, CA). Tissue culture supplies were purchased from Fisher (Pittsburgh, PA). All
other chemicals were obtained from Sigma Chemical Corp. (St. Louis, MO, USA). Capsaicin
and SEW2871 were dissolved in 1-methyl-2-pyrrolidinone (HPLC grade). These stock solutions
were then diluted with Ringers solution to yield the appropriate concentration. We
demonstrated previously that the vehicle 1-methyl-2-pyrrolidinone has no effect on AP firing or
the activation of either TTX-R \( I_{Na} \) or \( I_{K} \) (Zhang et al. 2002).

Results
S1P enhances the excitability of small diameter sensory neurons
Exposure of small diameter (≤25 μm) sensory neurons to externally applied S1P produced a
time-dependent increase in the number of APs evoked by a ramp of depolarizing current. A
representative recording is shown in Fig. 1A where under control conditions (left panel) this
neuron had a resting membrane potential of -49 mV and the ramp elicited 3 APs. After a 5 min
exposure to 1 μM S1P (right panel), the membrane depolarized to -42 mV and now the same ramp evoked 12 APs. Similar results were obtained in this same neuron when a series of current steps was used (see Fig. 1B). Under control conditions, a 50 pA step elicited a small depolarization without generating an AP whereas a step to 300 pA was required to elicit 1 AP. However, after S1P, the 50 and 300 pA steps evoked 1 and 3 APs, respectively. The ability of S1P to increase the number of APs evoked by the ramp is summarized in Fig. 1C. Both 5 and 10 min exposures to S1P significantly increased the number of evoked APs by about 4-fold. Thus, these results indicate that S1P quickly augments the excitability of sensory neurons and is consistent with our previous observations (Zhang et al. 2006).

SEW2871 enhances the excitability of some, but not all, sensory neurons

We previously reported that both the isolated intact DRG and isolated sensory neurons maintained in culture expressed the mRNA for four of the five S1P receptor subtypes (S1PR1-4, but not S1P5, Zhang et al. 2006). These observations raise the question as to which receptor subtype(s) mediates the enhanced excitability produced by S1P. To address this question, sensory neurons were treated with SEW2871, a selective agonist for S1PR1. SEW2871 has no activity at S1PR2-5 (EC50 S1PR1 ~13 nM, Jo et al. 2005; Sanna et al. 2004). As demonstrated in Fig. 2, external exposure to 100 nM SEW2871 produced a rapid increase in the number of APs evoked by the ramp. The increased excitability produced by SEW2871 was associated with a significant time-dependent depolarization of the resting membrane potential (~8 mV) and a reduction in the rheobase (~50%), however, the firing threshold was unaffected (summarized in Table 1). These effects were similar to that observed for S1P. Despite the reduction in rheobase, SEW2871 did not significantly alter RTh (P=0.22, Table 1) although there was a trend to increased values over time. Normalization of RTh to their respective control values indicate a trend towards increased RTh, for example after a 10 min exposure the value was 1.85 ± 0.51.
compared to the controls. The lack of significance may result from the variance in these measurements. Interestingly, of the nine neurons treated with SEW2871 only five exhibited increased excitability; the other four remained unaffected by the S1PR$_1$ agonist. These results suggest that S1P activation of S1PR$_1$ is sufficient to produce sensitization and that about half of the neurons are functionally sensitive to activation of S1PR$_1$. However, this finding does not eliminate the possibility that other S1P receptors may contribute to the increased excitability.

siRNA targeted to S1PR$_1$ blocks the sensitization produced by SEW2871, but not S1P

To further examine the role of S1PR$_1$ in augmenting neuronal excitability, siRNA targeted to S1PR$_1$ was designed and then applied to the sensory neurons maintained in culture (see Methods). Two representative Western blots are illustrated in Fig. 3. Panel A shows that 200 nM siRNA targeted to S1PR$_1$ produced a dramatic reduction in the expression of S1PR$_1$ compared to the untreated control neurons. Neither treatment with metafectene nor the scramble siRNA had any effect on the levels of S1PR$_1$. Panel B shows another tissue harvest and siRNA treatment wherein S1PR$_1$ was greatly reduced even when the protein content for that particular lane was doubled as indicated by the larger actin band. The extent of knockdown for S1PR$_1$ from five different tissue harvests (one tissue harvest corresponds to one siRNA treatment) is summarized in panel C. The level of S1PR$_1$ expression was reduced significantly compared to the untreated control levels (decreased by 75 ± 7%, n=5, P<0.05, ANOVA) whereas metafectene or scramble siRNA had no effect on S1PR$_1$ expression (reductions of 6 ± 9% and 16 ± 6%, respectively).

Having established that treatment with siRNA targeted to S1PR$_1$ effectively reduced expression of this receptor, further studies determined the specific role of S1PR$_1$ in the enhancement of
excitability. Because the siRNA targeted to S1PR₁ was labelled with the fluorescent tag DY547, only neurons that took up siRNA were used for electrophysiology experiments. siRNA-treated neurons were exposed sequentially to 100 nM SEW2871 for 10 min and then 1 µM S1P for 10 min. The results obtained from eleven sensory neurons are summarized in Fig. 4. After treatment with siRNA targeted to S1PR₁, none of the eleven neurons exhibited increased AP firing during the 10 min exposure to the specific S1PR₁ agonist, SEW2871. Additionally, after siRNA treatment, SEW2871 failed to depolarize the resting membrane potential and reduce the rheobase when compared to the effects of SEW2871 on untreated control or the scramble siRNA condition (see Table 1). The SEW2871-induced trend towards increased Rₚ over time that was exhibited for both untreated and scramble siRNA-treated sensory neurons was not observed after siRNA treatment wherein the values remained quite similar to those obtained under control conditions (Table 1). These results indicate that the S1PR₁ siRNA effectively blocked the response to SEW2871 or that none of these neurons expressed S1PR₁ (however, see single-cell results described below). Additionally, nine of these eleven neurons were then exposed to S1P wherein six were not sensitized but three neurons exhibited a significant two-three fold increase in the number of evoked APs after S1P. Assuming that all nine neurons expressed S1PR₁ which was functionally blocked by siRNA, then the six that were not sensitized by S1P did not express other S1P receptors whereas the three that were sensitized expressed additional S1P receptors. As a negative control, the effect of labeled scramble siRNA on the modulation of excitability by SEW2871 and S1P was examined. The results from 16 sensory neurons are summarized in Fig. 5. It appeared that there were three distinct populations of sensory neurons based on their sensitivities to these agents. Panel A shows that seven of the sixteen neurons exhibited a significant increase in AP firing after exposure to 100 nM SEW2871 and that exposure to S1P produced no additional increase in AP firing (P=0.87 compared to the 10 min SEW2871 results). Panel B demonstrates that four of the sixteen
neurons were not affected by SEW2871 but that S1P significantly increased AP firing after 6 and 8 min. Lastly, panel C indicates that five of the sixteen neurons were not sensitized by either SEW2871 or S1P. Taken together, these results would suggest that approximately 50% of these neurons express S1PR₁, 25% do not express S1PR₁ but do express other functional S1P receptors, and that ~25% do not express S1P receptors.

Some, but not all, small diameter sensory neurons express the mRNA for S1PR₁

The sensitivities to SEW2871 and S1P would suggest that some, but not all, sensory neurons express S1PR₁. To determine the frequency of S1PR₁ expression, individual isolated small diameter sensory neurons were aspirated into a large diameter pipette (~25 µm) from which the contents were deposited into an RT-PCR tube. The mRNA from individual cells was then probed for S1PR₁. Two representative gels are presented in Fig. 6. Panel A demonstrates that the mRNA for S1PR₁ was detected in two of four small diameter (<25 µm) sensory neurons (although there is rather faint band for C3). S1PR₁ was also detected in a large diameter neuron (>50 µm diameter). For three individual neurons (two small C5 and C6 and one large diameter L2) no amplicons were detected in the absence of reverse transcriptase (no RT). In addition, for two samples lacking template (labeled B, blank) no amplicons were detected. In another preparation, the cDNA products from individual neurons were split into two samples wherein one (5 µl sample) was probed for S1PR₁ and the other (2 µl sample) was probed for the potassium channel Kv1.1 as a positive control. As shown in panel B, two of the four neurons expressed the mRNA for S1PR₁ whereas all four neurons expressed the mRNA for Kv1.1. Both amplicons were detected in mRNA isolated from intact DRGs whereas no products were detected in no-template reactions (labeled B). A number of these single-cell RT-PCR reactions were run wherein from 10 separate preparations, 46 small diameter neurons were processed for
S1PR₁; of those 46 neurons, 23 exhibited the mRNA for S1PR₁. Consistent with the electrophysiological results described above, the single-cell RT-PCR studies indicate that approximately 50% of the small diameter sensory neurons express S1PR₁. These results indicate that individual sensory neurons can express multiple S1PRs that may have differing functional effects on the capacity of the cell to fire APs.

Discussion

We demonstrate that S1P via activation of S1PR₁ has the capacity to enhance the excitability of small diameter sensory neurons. This idea is supported by our findings that the S1PR₁ selective agonist, SEW2871, increased the excitability in a manner that was very similar to S1P and treatment with siRNA targeted to S1PR₁ completely blocked the increase in AP firing produced by SEW2871. However, other observations indicate that S1PR₁ is not the only S1PR capable of modulating neuronal excitability. This notion is consistent with the results obtained in untreated neurons where SEW2871 produced increased AP firing in approximately 50% of the recorded neurons as well as in neurons treated siRNA targeted to S1PR₁ where S1P increased firing in only about 25% of the neurons. Furthermore, experiments involving scramble siRNA as a negative control demonstrated that SEW2871, but not S1P, sensitized about 50% of the neurons whereas S1P but not SEW2871 sensitized about 25% of the neurons. These studies also showed that about 25% of the recorded neurons were insensitive to both SEW2871 and S1P. Previously we detected the mRNA for S1PR₁-4 in both the intact DRG and isolated sensory neurons maintained in culture (Zhang et al. 2006). In that study, internal perfusion with GDP-β-S blocked the sensitization produced by external S1P, demonstrating that these effects were mediated by activation of a G protein-coupled receptor(s). The detection of mRNA expression for S1PRs other than S1PR₁ suggests that S1PRs can have a significant impact on
excitability. This will be an area for future investigation to establish the role of other S1PRs in regulating excitability. However, in contrast to S1PR₁, establishing the specific role of other S1PRs will be more elusive as S1PR₂-4 currently lack any selective agonists and receptor antagonists have overlapping specificities (e.g., see VPC44116 below) and in some cases act as partial agonists (Lynch and Macdonald 2008). Using the approach taken in these current studies, siRNA targeted to individual S1PRs and their use in specific combinations may elucidate the role of each S1PR in regulating neuronal excitability.

There are relatively few investigations that have examined the neurophysiological actions of S1P and the role of S1PRs in either the peripheral or central nervous systems (see recent review by Okada et al. 2009). Using S1P-induced $^{35}$S-GTPγS labelling as a measure of receptor localization, different brain regions have quite variable levels of expression (Sim-Selley et al. 2009; Waeber and Chiu, 1999). For example, labelling in the cerebellum is high whereas that in the thalamus is relatively low. Detecting S1PRs in the central nervous system raises interesting questions as to what is the origin of S1P and what role these sphingolipids may serve in neuronal function. An early study demonstrated that depolarization by high potassium caused the release of S1P from rat cerebellar granule cells and that the transcripts for S1PR₁-3 were detected in these neurons (Anelli et al. 2005). Similarly, in isolated rat hippocampal neurons, exposure to high potassium or S1P caused the release of glutamate as measured by either fluorescence detection or enhanced secretion as detected by FM4-64 loading of synaptic vesicles (Kajimoto et al. 2007). These authors also reported that both S1P and high potassium produced a rapid FRET response after transfection with tagged S1PR₁ and β-arrestin. Interestingly, the high potassium effect was suppressed by pretreatment with dimethylsphingosine, which is an inhibitor of sphingosine kinase, whereas the S1P response
was not altered. Such observations may reflect the capacity of S1P to serve as an intracellular second messenger or as an extracellular primary messenger. This dual character has been referred to as the inside-outside nature of S1P signalling (Hla et al. 1999; Takabe et al. 2008).

In these hippocampal cells, siRNA targeted to S1PR$_1$ or S1PR$_3$ partially suppressed the effects of S1P on secretion, however, both siRNAs together produced complete inhibition. These results suggest that depolarization, via an undefined mechanism, results in the production of S1P via activation of sphingosine kinase, and somehow causes the release of glutamate. Subsequently, this S1P could be released from the neuron wherein autocrine and/or paracrine activation of S1PR$_1$/S1PR$_3$ can also release glutamate from hippocampal neurons. In a recent contrasting study, exposure to S1P or SEW2871 decreased the frequency (by ~27%) as well as the amplitude (by ~13%) of spontaneous glutamate-mediated EPSCs recorded from isolated and cultured cortical pyramidal neurons (Sim-Selley et al. 2009). The inhibitory actions of S1P were partially reversed by pretreatment with VPC44116, which is an S1PR$_1$/S1PR$_3$ antagonist as well as a partial agonist at S1PR$_4$/S1PR$_5$, suggesting that both receptors may be involved. It is curious that in hippocampal neurons S1P promotes glutamate release whereas in cortical pyramidal neurons S1P appears to suppress glutamate release. The physiological significance of these different observations are yet to be fully understood and may reflect the varied nature of S1P signalling in the brain.

Our findings in isolated small diameter sensory neurons suggest that elevated levels of S1P associated with peripheral inflammatory conditions can result in the increased sensitivity of these neurons through the modulation of G protein-coupled signaling cascades. The idea that peripheral S1P can be pro-inflammatory is supported by the observation that injection of S1P into the paw of a rat produced a dose-dependent edema that was associated with a significant
infiltration of eosinophils (Roviezzo et al. 2004). Synovial tissue isolated from patients with
rheumatoid arthritis exhibited increased expression S1PR\(_1\) compared to those with
osteoarthritis. In addition, treatment with S1P significantly increased the proliferation of
synoviocytes isolated from patients with rheumatoid arthritis as well as human MH7A cells,
which is a cell line established from intraarticular tissues of the knee joint from rheumatoid
arthritis patients (Kitano et al. 2006).

In contrast to the peripheral actions of S1P, two recent studies have examined the central
actions of S1P on nociceptive behavioral responses wherein S1P appears to diminish the
sensitivity of neurons. In contrast to peripheral inflammatory conditions that are associated with
increased levels of S1P, in either zymosan- or formalin-induced animal models of inflammatory
pain the levels of cerebrospinal S1P were reduced (Coste et al. 2008). Intrathecal injection of
S1P transiently reduced the secondary phase of nociceptive behaviors (i.e., number of flinches)
produced by injection of formalin into the rat’s paw. It is difficult to know which S1PR mediated
these antinociceptive effects as their RT-PCR measurements from adult rat spinal cord
indicated all five S1PRs were expressed. However, these authors showed that S1P reduced
the forskolin-stimulated increase in cyclic AMP levels and this reduction was prevented by
pertussis toxin; these results suggest that the actions of S1P were mediated by the inhibitory G
protein, Gi. In another investigation, similar observations were obtained wherein 20 min after
intracerebroventricular injection of S1P, the latency of the tail-flick in response to a thermal
stimulus was significantly increased, indicating that S1P was antinociceptive (Sim-Selley et al.
2009). Interestingly, injection of SEW2871 was only about ~50% as effective as S1P and these
effects were not reversed by pretreatment with VPC44116. Both results suggested that S1PRs
other than S1PR\(_1\) were involved in this response. It is difficult to assess the specificity of the
response to S1P since intracerebroventricular injection also elicited catalepsy, hypothermia, and locomotor inhibition.

In conclusion, our current results suggest that enhancement of sensory neuron excitability associated with peripheral inflammation can result through the activation of S1PR\(_1\) although other S1PRs likely have an important role in this sensitization. S1P may prove to be a significant inflammatory signalling mediator that communicates the status of the external environment between immuno-competent cells and sensory neurons.
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Disclosures:

The authors have nothing to disclose.
References


Figure legends

Figure 1. S1P enhances the excitability of small diameter sensory neurons. Panel A demonstrates representative voltage traces recorded under control conditions (left) and after a 5 min exposure to 1 µM S1P (right). Under control conditions, this small diameter sensory neuron had a resting membrane potential of -49 mV and 3 APs were evoked by the ramp of depolarizing current (450 pA, bottom trace). After S1P, the resting potential was depolarized to -42 mV and now the ramp evoked 12 APs. The dashed line indicates the 0 mV level. Panel B shows representative voltage recordings obtained from the same neuron as in A for steps of depolarizing current. The left panels illustrate the response to a 50 pA (top) and a 300 pA (bottom) step of current. The right panels show the response to the same current steps after a 5 min exposure to 1 µM S1P. Panel C summarizes the time-dependent increase in excitability produced by S1P in five sensory neurons for APs elicited by the ramps of depolarizing currents. The asterisks indicate a significant difference compared to the control condition with P<0.05, RM ANOVA.

Figure 2. The S1PR1 selective agonist, SEW2871, increases the number of evoked APs in some, but not all, sensory neurons. In five of nine neurons, 100 nM SEW2871 produced a significant increase in the number of APs evoked by the ramp of current, whereas in four of the nine SEW2871 had no effect. We define sensitivity to SEW2871 as a >2-fold increase in the number of evoked APs after exposure. The asterisks indicate a significant difference compared to the control condition with P<0.05, RM ANOVA.
Figure 3. siRNA targeted to S1PR$_1$ greatly reduces the protein expression of this receptor. Panel A illustrates a representative Western blot where the expression of actin and S1PR$_1$ were measured for four different conditions. The untreated control is indicated by the lane labeled Cont, exposure to only the transfecting detergent metafectene is labeled Met, treatment with 200 nM siRNA targeted to S1PR$_1$ is labeled siRNA, and treatment with 200 nM scramble siRNA is labeled SC. The antibody for actin was used at a dilution of 1:1000, the antibody to S1PR$_1$ was used at a dilution of 1:200. Panel B shows another representative Western blot where the amount of protein added to the lane labeled siRNA was doubled for detection purposes. Panel C summarizes the levels of expression of S1PR$_1$ for the four treatment conditions obtained from five different tissue harvests: untreated control condition (Cont), exposure to only metafectene (Met), treatment with 200 nM scramble siRNA (SC), and treatment with 200 nM siRNA targeted to S1PR$_1$ (siRNA). The densities for the protein bands for S1PR$_1$ under these different treatment conditions have been normalized to their respective levels for actin densities and then all values have been normalized to their respective untreated controls. The asterisk indicates a significant difference compared to the control condition with $P<0.05$, ANOVA.

Figure 4. siRNA targeted to S1PR$_1$ blocks the sensitization to SEW2871 although S1P can still sensitize some neurons. After neurons were treated with 200 nM siRNA targeted to S1PR$_1$ (fluorescently labeled) using our standard protocol (described in the Methods) they were used for recordings. The eleven small diameter sensory neurons that were exposed to 100 nM SEW2871 failed to exhibit an increase in AP firing by the ramp over a 10 min period. Of the eleven neurons, nine were then exposed to 1 µM S1P for another 10 min. Six of the nine neurons remained unaffected after exposure to S1P whereas three of the nine exhibited
increased firing after S1P. The asterisks indicate a significant difference compared to the 10 min SEW2871 condition with P<0.05, ANOVA.

Figure 5. In scramble siRNA treated sensory neurons, SEW2871 and S1P have varying capacities to augment neuronal excitability. Sensory neurons were treated with 200 nM scramble siRNA that was fluorescently labeled using our standard protocol. Recordings were obtained from a total of 16 small diameter neurons during exposure to 100 nM SEW2871 for 10 min and then to 1 µM S1P for an additional 10 min. Panel A summarizes the results obtained from seven of the sixteen neurons wherein SEW2871 significantly increased AP firing and that S1P produced no additional effect. Panel B represents the results obtained from four of the sixteen neurons wherein SEW2871 was ineffective, but S1P did produce a significant increase in AP firing. Panel C shows that five of the sixteen neurons were not affected by either SEW2871 or S1P. The asterisks indicate a significant difference compared to the control condition with P<0.05, ANOVA.

Figure 6. Single-cell RT-PCR analysis demonstrates that some, but not all, small diameter sensory neurons express the mRNA for S1PR1. Panel A illustrates a representative gel for the detection of S1PR1 in five individual sensory neurons. The lanes labelled C1-C6 (cell1-cell6) are the mRNAs from individual small diameter neurons whereas those labelled L1-2 (large cell1 and cell2) are mRNAs obtained from individual large diameter neurons. Lanes C1-L1 represent the detection of S1PR1 amplicons with a product size of 448 bp. Lanes C5-L2 underwent PCR in the absence of reverse transcriptase (No RT). Lanes labeled B (blank) represent reactions performed in the absence of any template. Panel B demonstrates, in a different tissue harvest than shown in A, the detection of S1PR1 and the potassium channel, Kv1.1 from the same four
individual sensory neurons. For small diameter sensory neurons C2-C5 Kv1.1 amplicons were detected whereas S1PR₁ was detected in only two of the four neurons. The lanes labelled DRG were amplicons obtained from mRNA isolated from 10 ganglia.
Table 1 Effects of SEW2871 on excitability properties

<table>
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<tr>
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<th>Untreated neurons</th>
<th>S1PR₁ siRNA treated</th>
<th>Scramble</th>
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<tr>
<td><strong>Resting Membrane Potential (mV)</strong></td>
<td></td>
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<tr>
<td>Control</td>
<td>-56.5 ± 1.7</td>
<td>-61.2 ± 1.3</td>
<td>-59.9 ± 1.4</td>
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<tr>
<td>2 min</td>
<td>-52.1 ± 2.2</td>
<td>-62.6 ± 1.8</td>
<td>-58.8 ± 1.5</td>
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<tr>
<td>4 min</td>
<td>-49.3 ± 4.2*</td>
<td>-63.0 ± 1.8</td>
<td>-56.9 ± 1.8*</td>
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<tr>
<td>6 min</td>
<td>-47.8 ± 4.4*</td>
<td>-63.2 ± 1.9</td>
<td>-55.1 ± 2.2*</td>
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<tr>
<td>10 min</td>
<td>-48.8 ± 4.5*</td>
<td>-62.5 ± 2.0</td>
<td>-51.8 ± 2.6*</td>
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<tr>
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<tr>
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<tr>
<td>10 min</td>
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<td>Resistance at threshold (MΩ)</td>
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<td>379 ± 62</td>
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<td>1.45 ± 0.22</td>
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<td>10 min</td>
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<td>379 ± 62</td>
<td>1.48 ± 0.26</td>
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* Indicates significant difference from control.
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<th>Scramble n=7</th>
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<td>10 min</td>
<td>1.85 ± 0.51</td>
<td>0.99 ± 0.04</td>
<td>1.65 ± 0.38</td>
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*P<0.05, RM ANOVA
A. Control  

S1P 5 min  

B. Control  

S1P 5 min  

C. 

Number of APs

<p>| | | | | |</p>
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<tr>
<th></th>
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<td>S1P (min)</td>
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