Sphingosine-1-Phosphate Via Activation of a G-Protein-Coupled Receptor(s) Enhances the Excitability of Rat Sensory Neurons


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INTRODUCTION

Recent work indicates that one of the key mediators released by immune cells, such as mast cells and platelets, is sphingosine-1-phosphate (see recent reviews Olivera and Rivera 2005; Rosen and Goetzl 2005). On activation by certain inflammatory mediators such as tumor necrosis factor, sphingosine-1-phosphate (S1P) is derived from the intracellular metabolism of the membrane lipid, sphingomyelin. In this signaling pathway, the enzyme, sphingomyelinase liberates ceramide, which has been shown to be an important second messenger molecule in a multitude of cellular processes such as apoptosis and cell growth (Ballou et al. 1996; Kolesnick et al. 2000; Mathias et al. 1998; Schütze et al. 1994). Ceramide can be further metabolized by the enzyme ceramidase to sphingosine and sphingosine can then be phosphorylated by sphingosine kinase to form S1P. In addition to ceramide, both sphingosine and S1P can act as intracellular signaling molecules (Hannun and Bell 1989; Olivera and Spiegel 1993; Pyne and Pyne 2000; Spiegel and Milstien 2003). For example, vascular endothelial growth factor increases intracellular S1P, which then mediates the stimulation of Ras with subsequent activation of downstream signaling pathways (Shu et al. 2002). In addition to its role as an intracellular second messenger, S1P can be released or secreted from immune cells by an as yet undefined pathway (Jolly et al. 2004; Prieschl et al. 1999). On its release, S1P can activate different subtypes of a family of G-protein-coupled receptors (GPCRs) known as S1P receptors. This family of GPCRs was described originally as the EDG (endothelial differentiation gene) receptor (Hla and Maciag 1990). Therefore S1P may act as either a first or second messenger to modulate cellular activity (Hla et al. 1999; Spiegel and Milstien 2002, 2003).

It is well established that the inflammation arising from tissue injury is associated with a heightened sensitivity to noxious stimuli, suggesting that there is an important interaction between the activities of immune cells and the sensory neurons activated by noxious stimulation. For example, the pro-inflammatory prostaglandin, prostaglandin E2, which is released by immune cells (Foreman 1987; Higgs et al. 1984; Salmon and Higgs 1987), enhances the excitability or sensitizes nociceptive sensory neurons to both chemical (Baccaglini and Hogan 1983; Handwerker 1976; Nicol and Cui 1994) and electrical (Gold et al. 1996c; Nicol et al. 1997; Weinreich and Wonderlin 1987) stimulation. Because of the novel role of S1P in the inflammatory response and its release from immune cells during injury, it is important to determine whether S1P can augment the excitability of small diameter sensory neurons and thereby contribute to this enhanced sensitivity during inflammation. In this report, we show that direct exposure of isolated sensory neurons to S1P augments AP firing and this results from the modulation of both the TTX-R \( I_{Na} \) and \( I_K \). This action of S1P is mediated by activation of a GPCR(s), suggesting that S1P is a novel lipid mediator that enhances the excitability of capsaicin-sensitive sensory neurons.

METHODS

Isolation and maintenance of rat sensory neurons

Primary cultures of dissociated adult rat dorsal root ganglion (DRG) neurons were prepared as described previously (Lindsay 1988) with slight modification (Jiang et al. 2003). Briefly, male Sprague-
Dawley rats (100–150 g) were killed by placing them in a chamber that was then filled with CO₂. DRGs were removed and collected in a culture dish filled with sterilized Puck’s solution. The ganglia were collected into a conical tube filled with Puck’s solution containing 10 U/ml of papain II and incubated for 10 min at 37°C. The tube was centrifuged for 30 s at low speed, and the pellet was resuspended in Puck’s solution containing collagenase (1 mg/ml, type 1A) and dispase II (2.5 mg/ml). After 10-min incubation at 37°C, the tube was centrifuged for 30 s before the enzyme-containing supernatant was removed. The pellet was resuspended in F-12 medium supplemented with 250 ng/ml 7S-nerve growth factor (Harlan Bioproducts) and mechanically dissociated with fire-polished pipettes until all obvious chunks of tissues were gone. Isolated cells were plated onto plastic coverslips that previously were coated with poly-d-lysine and laminin. The cells were maintained in F-12 medium containing nerve growth factor at 37°C and 3% CO₂ and used within 6–24 h for electrophysiological recordings. All procedures have been approved by the Animal Use and Care Committee of the Indiana University School of Medicine.

**Electrophysiology**

Recordings were made using the whole cell patch-clamp technique as previously described (Hamill et al. 1981; Zhang et al. 2002). Briefly, a coverslip with the sensory neurons was placed in a recording chamber where the neurons were bathed in normal Ringer solution containing (in mM) 140 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, 10 HEPES, and 10 glucose; pH was adjusted to 7.4 with NaOH. Patch pipettes were pulled from borosilicate glass tubing and fire-polished. Whole cell voltages or currents were recorded with an Axopatch 200 patch-clamp amplifier (Axon Instruments, Union City, CA). The data were acquired and analyzed using pCLAMP 6.04 or pCLAMP 8.2 (Axon Instruments). The whole cell recording configuration was established in normal Ringer solution. Both capacitance and series resistance compensation (typically 80%) were used. The mean series resistance before compensation was 2.1 ± 0.04 (SE) MΩ (n = 106). Leakage currents were subtracted by using the P/4 protocol for the measurements of TTX-R \( I_{\text{Na}} \). Leak subtraction was not used for the measurement of \( I_{\text{K}} \) so that any effects of these agents on the holding current could be determined. To assess excitability in the current-clamp experiments, neurons were held at their resting potentials (range: −50 to −65 mV) and a depolarizing ramp of current (1 or 1.8 s in duration) was applied. The amplitude of the ramp was adjusted to produce two to four action potentials (APs) under control conditions; the same ramp was then used throughout the recording period for each individual neuron.

To isolate \( I_{\text{K}} \), neurons were superfused with a Ringer solution wherein NaCl was substituted with equimolar \( N \)-methyl-glucamine chloride (NMG-Cl, 140 mM); pH was adjusted to 7.4 with KOH. Recording pipettes typically had resistances of 2–4 MΩ when filled with the following solution (in mM) 140 NaCl, 5 MgCl₂, 4 ATP, 0.3 GTP, 2.5 CaCl₂, 5 EGTA (calcium-free Ca²⁺ concentration of −100 nM, MaxChelator), and 10 HEPES; pH was adjusted to 7.3 with KOH. This pipette solution was used in the current-clamp recordings as well. The TTX-R \( I_{\text{Na}} \) was isolated by superfusing the neurons with (in mM) 30 NaCl, 65 NMG-Cl, 30 tetraethylammonium (TEA), 0.1 CaCl₂, 5 MgCl₂, 10 HEPES, 10 glucose, 10 sucrose, and 500 mM TTX; pH adjusted to 7.4 with TEAOH. These recording pipettes were filled with (in mM) 110 CsF, 25 CsCl, 10 NaCl, 5 MgCl₂, 4 ATP, 0.3 GTP, 1 CaCl₂, 10 EGTA, 10 glucose, and 10 HEPES; pH was adjusted to 7.3 with CsOH. The pipettes used for the recording of TTX-R \( I_{\text{Na}} \) had an average resistance of 1.45 ± 0.12 MΩ (n = 6, range: 1.2–2.0 MΩ) and an average series resistance of 1.52 ± 0.19 MΩ. For the peak values of TTX-R \( I_{\text{Na}} \), shown in Fig. 6, the uncompensated series resistance gave a voltage error of approximately −1 mV. The membrane was held at −60 mV; this value was chosen so that current measurements could be ascertained at a voltage that reflected the normal resting potential in these sensory neurons. Activation of the currents was determined by voltage steps of 300 or 30 ms for \( I_{\text{K}} \) or TTX-R \( I_{\text{Na}} \), respectively, which were applied at 5-s intervals in +10-nM increments to +60 nM. After obtaining the control response, the bath solution was changed to the appropriate Ringer solution, and cells were superfused continuously for the appropriate times. 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 capsaicin sensitivity and that a neuron is a nociceptor 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 capsaicin-sensitive neurons only. All experiments were performed at room temperature (−22°C).

**Data analysis**

Data are presented as the means ± SE. The excitability parameters described in Table 1 were determined, in part, by differentiating the voltage trace (dV/dt) in the current-clamp recordings (sampling frequency of 250 Hz). 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 two data points before the take-off point of the AP. The rheobase was measured as the amount of ramp current at the firing threshold. The resistance at threshold \( R_{\text{th}} \) was calculated as the difference between the firing threshold and the resting membrane potential divided by the rheobase current. The voltage dependence for activation of TTX-R \( I_{\text{Na}} \) and \( I_{\text{K}} \) was fitted with the Boltzmann equation where \( G_{\text{max}} = I/[1 + \exp(V_{\text{m}} - V_{\text{th}})/k] \), where \( G \) is the conductance \( G = I/[V_{\text{m}} - E_{\text{rev}}] \), \( G_{\text{max}} \) is the maximal conductance obtained from the Boltzmann fit under control conditions, \( V_{\text{m}} \) is the voltage for half-maximal activation, \( V_{\text{th}} \) is the membrane potential, and \( k \) is a slope factor. The Boltzmann parameters were determined for each individual neuron and were used to calculate the means ± SE. 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. In a separate series of time control experiments, the peak amplitudes for either \( I_{\text{K}} \) or TTX-R \( I_{\text{Na}} \) did not vary significantly over a 20-min time period. Values of \( P < 0.05 \) were judged to be statistically significant.

**S1P receptor expression**

The expression of mRNA for the S1P receptors S1P₁ (EDG-1), S1P₂ (EDG-5), S1P₃ (EDG-3), S1P₄ (EDG-6), and S1P₅ (EDG-8) in lung, the intact DRG, and isolated DRG sensory neurons grown in culture for 24 h in 250 ng/ml NGF were examined by RT-PCR. The isolated lung and intact DRG were homogenized in RNA Stat-60 (Tel-Test, Friendswood, TX) using an autoclaved glass pestle. Total RNA from these tissues and isolated neurons were isolated using RNA Stat-60, treated with DNase I (Invitrogen, Carlsbad, CA), and reverse transcribed using an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). The cDNA was amplified using specific primers for each S1P receptor (Invitrogen) and Platinum PCR SuperMix (Invitrogen). Taq polymerase was activated by incubation at 95°C for 3 min followed by 47 cycles of 40 s at 94°C, 40 s at 47°C and 75 s at 65°C with a final extension phase for 4 min at 65°C. The samples were then held at 4°C until they were run on the gel. As a negative control, PCR products were examined from samples wherein the reverse transcrip-
TABLE 1.  Effects of external S1P (1 μM) on current-clamp parameters

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Resting $V_m$, mV</th>
<th>Latency, ms</th>
<th>Threshold, mV</th>
<th>Rheobase, pA</th>
<th>S1P Rheo</th>
<th>S1P Rheo Cont</th>
<th>$R_{th}$, MΩ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>$-57.6 \pm 0.8$</td>
<td>$1242 \pm 205$</td>
<td>$-28.5 \pm 5.4$</td>
<td>$184 \pm 91$</td>
<td>1.0</td>
<td>245.5</td>
<td>$66.4\pm$</td>
</tr>
<tr>
<td>2 min</td>
<td>$-57.5 \pm 0.8$</td>
<td>$1152 \pm 220$</td>
<td>$-30.9 \pm 6.7$</td>
<td>$173 \pm 93$</td>
<td>0.91</td>
<td>244.4</td>
<td>$73.8\pm$</td>
</tr>
<tr>
<td>6 min</td>
<td>$-57.9 \pm 0.7$</td>
<td>$649 \pm 108$</td>
<td>$-34.2 \pm 2.5$</td>
<td>$64 \pm 15$</td>
<td>0.54</td>
<td>406.3</td>
<td>$56.8\pm$</td>
</tr>
<tr>
<td>10 min</td>
<td>$-58.3 \pm 0.7$</td>
<td>$640 \pm 133$</td>
<td>$-33.3 \pm 2.0$</td>
<td>$63 \pm 18$</td>
<td>0.52</td>
<td>480.1</td>
<td>$94.4\pm$</td>
</tr>
<tr>
<td>20 min</td>
<td>$-57.4 \pm 0.6$</td>
<td>$512 \pm 69^*$</td>
<td>$-34.7 \pm 3.1$</td>
<td>$48 \pm 9$</td>
<td>0.41</td>
<td>527.2</td>
<td>$98.4^*\pm$</td>
</tr>
</tbody>
</table>

Values are means ± SE. *P < 0.05 (repeated-measures ANOVA, Tukey post hoc test), n = 4. S1P, sphingosine-1-phosphate.

Although the increased excitability was not accompanied by either depolarization or a change in the firing threshold, a 20-min exposure to S1P significantly decreased the latency of AP firing and the rheobase by 2.8 ± 0.9- and 3.7 ± 1.5-fold, respectively. Exposure to S1P also significantly increased $R_{th}$. The excitatory effects of S1P appear to be time-dependent since significant changes in these parameters occur 6–20 min after the initial exposure. These results demonstrate that exposure to S1P can enhance the excitability of adult sensory neurons without altering the firing threshold.

Internal perfusion with GDP-β-S blocks the S1P-mediated increase in excitability

Our results indicate that an acute exposure to external S1P enhanced the excitability of sensory neurons. This raises the question as to whether S1P acts as an extracellular mediator that signals via plasma membrane receptors or potentially crosses the neuronal membrane to act internally. Abundant evidence indicates that S1P is the ligand for a number of GPCRs first known as endothelial differentiation gene receptors or EDG receptors (Lee et al. 1998; Okamoto et al. 1998; Takeda et al. 1998;)

**RESULTS**

**S1P enhances the excitability of sensory neurons**

To ascertain whether S1P modulates the sensitivity of small-diameter capsaicin-sensitive sensory neurons, we determined if exposure to this sphingolipid altered the capacity of these neurons to fire APs. Indeed, S1P augmented the excitability of adult sensory neurons. A representative recording (Fig. 1, left) illustrates that a given ramp of depolarizing current evoked three APs (resting $V_m$: $-59$ mV) in the isolated neuron. After the bath application of 1 μM S1P for 20 min, the same ramp of current elicited 14 APs without a significant change in resting $V_m$ (right). Although the firing threshold for this cell was shifted by only a small extent after exposure to S1P (control $-37.4$ mV vs. S1P $-40.4$ mV), the rheobase was reduced from 65 to 23 pA. The results for four neurons are summarized in Fig. 2B, wherein bath-applied S1P significantly increased the number of APs elicited by the ramp in a time-dependent manner (RM ANOVA) from an average control value of 3.0 ± 0.0, to 10.5 ± 1.3 after a 20-min exposure to S1P. The effects of S1P on resting $V_m$, firing latency, threshold, rheobase, and resistance at threshold ($R_{th}$) are summarized in Table 1.
Sensory neurons express mRNA for S1P receptors

To establish the effectiveness of the primers used in the RT-PCR studies, the expression of mRNA for S1P receptors was examined in acutely isolated lung from the rat. The S1P1, S1P2, S1P3, and S1P4 receptors are strongly expressed, whereas the mRNA for the S1P5 receptor is weak or inconsistent in the lung (see Table 1, Anliker and Chun 2004). As shown in Fig. 3A, the mRNAs for the S1P receptors, S1P1, S1P2, S1P3, and S1P4, but not S1P5, were detected in the lung. The expected PCR products were of the appropriate sizes wherein S1P1 was 449 bp, S1P2 was 454 bp, S1P3 was 430 bp, and S1P4 was 403 bp. These results are consistent with the observations reported in Anliker and Chun (2004). Using these primers, the mRNAs for the S1P receptors, S1P1, S1P2, S1P3, and S1P4 were detected in samples obtained from both the acutely isolated intact DRG (Fig. 3B) and cells isolated from the DRG and maintained in culture for <24 h (Fig. 3C), which was identical to the protocol used in the electrophysiology studies. The transcript for S1P5 was not detected. In the absence of reverse transcriptase (−) there was no detectable PCR product in any of these tissues indicating that there was no contamination from genomic DNA in these preparations. Therefore because S1P is known to activate GPCRs (see discussion), it is very likely that external S1P augments excitability through activation of one or more of these specific GPCRs.
SIP suppresses $I_K$

It is well established that a reduction in potassium currents enhances the excitability of sensory neurons (Cordoba-Rodriguez et al. 1999; Gold et al. 1996c; Nicol et al. 1997; Weinreich and Wonderlin 1987; Zhang et al. 2002). Therefore we explored the idea that the sensitizing actions of SIP observed in the current-clamp experiments may result from the inhibition of an outward potassium current ($I_K$). Externally applied SIP produced a time-dependent suppression of $I_K$ in adult rat sensory neurons. Figure 4A illustrates a representative recording obtained before (left) and after a 20-min external exposure to 1 μM SIP (middle), wherein SIP reduced the peak $I_K$ by 34% measured at +60 mV. The $I_K$ that was sensitive to SIP was obtained by subtraction and is shown in the right panel. Figure 4B summarizes the effects of SIP on the current-voltage relation obtained from 12 neurons. Under control conditions, the average peak value of $I_K$ was 8.19 ± 1.76 nA as measured at +60 mV whereas after a 20-min exposure to 1 μM SIP the average value was significantly reduced to 5.18 ± 0.98 nA. The extent of inhibition measured after a 10-min exposure (5.46 ± 0.90 nA) was not different from that obtained after 20 min (t-test, $P > 0.05$) suggesting that, for this concentration, the maximal inhibition was achieved after 10 min. The inset shown in B demonstrates that the current (shown as $I_{max}$ where $I_{max}$ is the current obtained for the step to +60 mV) increases with more depolarized voltage steps (−50 to −20 mV) for the control recordings. Conductance values determined from currents before and after the application of SIP were fitted by the Boltzmann relation and were normalized to their respective values for the fits of $G_{max}$ obtained for the control condition. As shown in Fig. 4C, there was a time-dependent decrease in $G$ relative to their control values of $G_{max}$. Boltzmann fits to the measurements obtained from each of the neurons at these different time points showed that there was a small but significant hyperpolarizing shift in the $V_{0.5}$ for the $I_K$s remaining after 6- and 10-min exposures to SIP but not after 20 min (summarized in Table 2). This shift in $V_{0.5}$ might be accounted for by the removal of an SIP-sensitive population of $I_K$, which may then reveal or unmask other populations of potassium channels contributing to excitability. The inset shown in C demonstrates that when normalized to their respective average values determined at −20 mV, the $I_{max}$ and $G_{max}$ relations obtained for the control recordings are very similar over the voltages between −60 and −20 mV. In addition, the inhibition of $I_K$ by SIP was concentration-dependent (data not shown). At 10 nM, SIP had no effect on $I_K$ (a 2 ± 2% reduction, $n = 4$), whereas 100 nM and 1 μM SIP

![FIG. 4.](http://jn.physiology.org/)

![TABLE 2.](http://jn.physiology.org/)

<table>
<thead>
<tr>
<th></th>
<th>$G/G_{max}$</th>
<th>$V_{0.5}$, mV</th>
<th>$k$, mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.03 ± 0.01</td>
<td>−2.9 ± 1.6</td>
<td>15.2 ± 0.7</td>
</tr>
<tr>
<td>2 min</td>
<td>0.91 ± 0.02*</td>
<td>−5.3 ± 1.6</td>
<td>14.7 ± 0.6</td>
</tr>
<tr>
<td>6 min</td>
<td>0.78 ± 0.03*</td>
<td>−7.8 ± 1.9*</td>
<td>14.1 ± 0.5</td>
</tr>
<tr>
<td>10 min</td>
<td>0.71 ± 0.03*</td>
<td>−8.2 ± 2.2*</td>
<td>14.4 ± 0.6</td>
</tr>
<tr>
<td>20 min</td>
<td>0.66 ± 0.04*</td>
<td>−6.1 ± 3.2</td>
<td>15.5 ± 1.1</td>
</tr>
</tbody>
</table>

* $P < 0.05$ (RM ANOVA, Tukey post hoc test), $n = 12$.
significantly decreased the peak $I_K$ by 12 ± 2 and 34 ± 4% ($n = 4$ and 12), respectively, measured at +60 mV after 20-min treatments. In another series of experiments, the question of reversibility was examined. After a 20-min exposure to 1 μM S1P, $I_K$ was suppressed from a control value of 10.43 ± 3.97 to 6.76 ± 2.01 nA ($n = 5$). These neurons then were washed with NMG-Ringer for 10 min after the 20-min treatment with S1P wherein $I_K$ remained unchanged (5.79 ± 2.23 nA), indicating that the S1P-mediated suppression of $I_K$ was not readily reversed within the timeframe of this experiment.

**S1P-sensitive $I_K$ is blocked by TEA**

To characterize the S1P-sensitive $I_K$, the capacity of S1P to inhibit $I_K$ in the presence of tetraethylammonium (TEA) was examined. TEA is a well-established antagonist of some subtypes of delayed rectifier potassium channels (Armstrong 1969; Armstrong and Binstock 1965; Tasaki and Hagiwar 1957). To determine whether the S1P-sensitive $I_K$ may be a TEA-sensitive delayed rectifier type of outward potassium current, the inhibitory effects of S1P were tested in the presence of 10 and 30 mM TEA. Figure 5A shows typical currents recorded from a representative neuron before and after application of 30 mM TEA, and then 1 μM S1P in the presence of 30 mM TEA. Figure 5A (left) illustrates that under control conditions, voltage steps from −80 to +60 mV elicited a family of currents wherein the peak $I_K$ was 6.62 nA at +60 mV. The middle panel of A shows that after a 5-min exposure to 30 mM TEA, $I_K$ was markedly reduced to 0.97 nA (at +60 mV). This sensory neuron then was treated with 1 μM S1P in the presence of 30 mM TEA for 20 min (right), the peak $I_K$ was unchanged by this treatment (1.08 nA) compared with currents obtained in the presence of TEA alone. Figure 5B summarizes the effects of S1P on the current-voltage relations in the presence of 10 mM TEA (left) and 30 mM TEA (right). Currents obtained in the presence of TEA and S1P were normalized to their peak values at +60 mV obtained in the presence of TEA alone. A 20-min treatment with S1P in the presence of 10 mM TEA produced an additional small but significant decrease in $I_K$ obtained at +60 mV (a reduction of 21 ± 1%, $n = 6$, range 20–23%), whereas in 30 mM TEA, S1P had no further effect (3 ± 5%, $n = 4$, range: 0–12%). By itself S1P (1 μM) decreased $I_K$ by 34 ± 4% after a 20-min application as described in the preceding text, indicating that TEA blocked the $I_K$ that was sensitive to modulation by S1P. The results indicate that in the presence of 30 mM TEA, but not 10 mM TEA, S1P was incapable of causing a further inhibition of $I_K$, suggesting that TEA and S1P may act on the same population of potassium channels, and the currents modulated by S1P may be delayed rectifier-type $I_K$.

**S1P augments the TTX-R $I_{Na}$**

One way of classifying sensory neurons is based on their sensitivity to TTX (Caffrey et al. 1992; Campbell 1993; Elliott and Elliott 1993; Ogata and Tatebayashi 1992; Roy and Narahashi 1992). Small-diameter capsaicin-sensitive sensory neurons (presumed to be nociceptive neurons) express the TTX-R $I_{Na}$ at much greater frequency than do the large-diameter capsaicin-insensitive neurons (Arbuckle and Docherty 1995; Pearce and Duchen 1994). Inflammatory mediators augment the peak amplitude TTX-R $I_{Na}$ (England et al. 1996; Gold et al. 1996b; Zhang et al. 2002), suggesting that modulation of this current enhances the excitability of these neurons. Interestingly, inflammatory mediators have little effect on the TTX-sensitive $I_{Na}$ (Gold et al. 1996b; Zhou et al. 2002; but also see Cardenas et al. 1997). The increase in AP firing after S1P treatment raises the question as to whether TTX-R $I_{Na}$ also is modulated by S1P. To address this, TTX-R $I_{Na}$ was isolated by using a Ringer solution containing 500 nM TTX, and the effects of S1P on TTX-R $I_{Na}$ were determined. Figure 6A illustrates the TTX-R $I_{Na}$ recorded under control conditions from a representative neuron (left) and after a 6-min, external application of 1 μM S1P (right). In the presence of S1P, the amplitude of TTX-R $I_{Na}$ was significantly enhanced. The effect of S1P on the current-voltage relationship for TTX-R $I_{Na}$ in six capsaicin-sensitive neurons is summarized in Fig. 6B (left). Under control conditions, the maximum current was obtained
of each respective time in S1P were normalized to the fitted value of conductance-voltage relations and their corresponding Boltzmann conditions and are shown in the normalized to their respective peak currents obtained under control conditions from an adult sensory neuron for steps from −60 to +40 mV. Right: effects of a 6-min exposure to external 1 μM S1P in this same neuron. The lines labeled 0 represent the 0-current values. B: current-voltage relations obtained for 6 neurons under control conditions and after treatment with S1P for different times (left: total current; right: normalized to respective control currents). Treatment times of 2 min produced a significant increase in the peak TTX-R $I_{\text{Na}}$ for the −20 mV voltage step, for both 6- and 10-min exposures, a significant increase in the peak TTX-R $I_{\text{Na}}$ occurred for voltage steps between −40 and −10 mV (RM ANOVA, Tukey post hoc test). The same significant voltages were determined for the $I_{\text{Na}}$ relation. C: conductance-voltage relation; data has been normalized to the fitted value of $G_{\text{max}}$ for each respective control condition. The values of $G/G_{\text{max}}$ for the 2-min exposure to S1P were significantly different from the control values for −20 mV. For the 6-min exposure, values were significantly different for the steps between −40 and −10 mV, and for the 10-min exposure the values were significantly different for the steps between −40 and −20 mV (RM ANOVA, Tukey post hoc test). D shows that a 10-min external exposure to 10 μM Sph has no effect on the current-voltage relation.

for a step to −10 mV and had an average value of $-2.37 \pm 0.47$ nA. After a 6-min exposure to S1P, the peak current significantly increased to $-3.07 \pm 0.28$ nA and was elicited by the step to −20 rather than −10 mV. However, S1P did not influence the rate of current inactivation as the time constants for inactivation measured at −10 mV were not significantly different ($n = 6$, data not shown). These currents were normalized to their respective peak currents obtained under control conditions and are shown in the right panel of Fig. 6B. The conductance-voltage relations and their corresponding Boltzmann fits were determined. The values of $G$ determined for each respective time in S1P were normalized to the fitted value of $G_{\text{max}}$ for the control condition and are shown in Fig. 6C. Significant changes were obtained for $V_{0.5}$ after only a 2-min exposure to S1P, wherein the $V_{0.5}$ for the control condition ($-11.3 \pm 3.3$ mV, $n = 6$) shifted to more hyperpolarized values, moving to $-17.7 \pm 4.2$, $-21.9 \pm 3.3$, and $-24.3 \pm 4.1$ mV after 2-, 6-, and 10-min exposures, respectively. The average value of $k$ remained unchanged ($7.8 \pm 1.4$ for the control vs. $6.3 \pm 0.8, 6.8 \pm 0.9$, and $5.4 \pm 1.1$ mV for 2, 6, and 10 min, respectively). In another series of experiments, the effects of sphingosine (Sph) were examined because Sph is similar in structure to S1P but lacks the capacity to activate the S1P receptors. Unlike S1P, the external application of 10 μM Sph (10 min) had no significant effect on the current-voltage relation for TTX-R $I_{\text{Na}}$ in five sensory neurons (see Fig. 6D). The peak value for TTX-R $I_{\text{Na}}$ under control conditions was $-3.20 \pm 0.62$ nA at −10 mV and remained unchanged at $-3.39 \pm 0.49$ and $-3.11 \pm 0.41$ after 10- and 20-min exposures to 10 μM Sph, respectively. Likewise the values for $V_{0.5}$ were not significantly different after these exposures to Sph wherein the control value was $-5.1 \pm 1.1$ mV compared with $-4.3 \pm 2.9$, $-9.9 \pm 2.4$, and $-3.5 \pm 3.8$ mV after 6-, 10-, and 20-min treatments with Sph, respectively ($P > 0.15$, RM ANOVA). In addition, we previously reported that our recordings of TTX-R $I_{\text{Na}}$, using CsF/CsCl pipettes were stable over time. A 20-min exposure to dihyroceramide, an inactive analogue of ceramide, did not alter the peak TTX-R $I_{\text{Na}}$ over time wherein the average peak current for the control was $-2.01 \pm 0.61$ nA (at −10 mV, $n = 3$) compared with $-2.14 \pm 0.57$ nA (also at −10 mV). Furthermore, in a separate series of time control experiments, the average control TTX-R $I_{\text{Na}}$ was $-2.33 \pm 0.86$ nA (at −10 mV) compared with an average value of $-2.24 \pm 0.72$ nA measured 20 min later. These results demonstrate that over a 20-min period our recordings of TTX-R $I_{\text{Na}}$ with CsF/CsCl pipettes are stable and that external S1P, but not Sph, enhanced the total TTX-R $I_{\text{Na}}$ with a shift in the activation curve to more hyperpolarized potentials.

**Discussion**

The current results demonstrated that treatment with external S1P augments the capacity of capsaicin-sensitive sensory neurons to fire APs evoked by a depolarizing current. The increased firing was associated with a decrease in both the firing latency and the rheobase as well as an increase in $R_P$, with no significant change in the firing threshold. Our findings with S1P are analogous to a previous report that the inflammatory prostaglandin $E_2$, which alters the sensitivity of nociceptive sensory neurons, increased the number of APs and decreased both the firing latency and threshold of small-diameter sensory neurons (Gold et al. 1996a). Gold et al. reported a decrease in firing threshold with prostaglandin $E_2$; however, they defined threshold as a 20-fold change in $d$ firing latency with prostaglandin $E_2$, whereas we defined threshold as a 20-fold change in $d$ firing latency with prostaglandin $E_2$.
reported for modulation of TTX-R \( I_{\text{Na}} \) by other inflammatory agents such as prostaglandin \( E_2 \) (England et al. 1996; Gold et al. 1996b) or endothelin-1 (Zhou et al. 2002). These mediators act through GPCRs and it has been demonstrated that \( F^- \) can combine with \( Al^{3+} \) to form a complex capable of activating \( G \) proteins (see references in Saab et al. 2003). It is possible that the S1P-induced modulation results from \( Al^{3+} \) in the glass pipette combining with pipette \( F^- \) rather than a direct binding of S1P to its receptor. This does not seem likely because inclusion of a chelator of \( Al^{3+} \), deferoxamine, in pipettes containing CsF had no effect on the peak amplitude of TTX-R \( I_{\text{Na}} \) (Saab et al. 2003). In addition, reports have indicated that recordings of TTX-R \( I_{\text{Na}} \) with CsF pipettes are not stable over time. However, a 20-min application of external Sph (Fig. 6D) had no significant effect on either the peak amplitude or the value of \( V_{0.5} \) for TTX-R \( I_{\text{Na}} \). The hyperpolarizing shift induced by S1P is similar to that reported by Zhou et al. (2002) for the effects of endothelin-1 on TTX-R \( I_{\text{Na}} \), the recordings of which were also performed with CsF pipettes. The shift in \( V_{0.5} \) was blocked by an antagonist of the endothelin-A receptor, which would be inconsistent with the idea that this shift resulted from nonspecific actions of CsF. The \( V_{0.5} \) for the \( I_K \) remaining after S1P exposure also was shifted in a hyperpolarizing direction by \( \sim 5 \) mV even though the currents were reduced by S1P and the number of evoked APs was increased. We previously reported similar observations for the effects of ceramide on the activation properties of these currents (Zhang et al. 2002). Clearly, there is a complex relationship between TTX-R \( I_{\text{Na}} \) and \( I_K(s) \) that regulates the excitability of these neurons and that multiple signaling pathways can modulate their activities (Nicol 2005). Thus understanding the integration of these currents to control the state of neuronal excitability remains a question for future studies.

S1P can enhance the firing capacity of sensory neurons through its actions as an external agonist at an S1P receptor(s). This notion is based on two observations. First, RT-PCR experiments indicate the expression of mRNAs for S1P receptors in the lung, which is a tissue wherein the expression of these receptors is well established (see Anliker and Chun 2004). Samples obtained from the intact DRG and isolated sensory neurons exhibited an analogous expression profile for the S1P_{1-4} receptors although S1P_{5} was not detected in any of these tissues. Because of the similarity between the intact DRG and isolated neurons and the findings that application of S1P augments the excitability of sensory neurons, these results suggest that sensory neurons express the mRNA for these particular S1P receptors. However, such findings do not exclude that possibility that S1P receptors also are expressed in these nonneuronal cells associated with the DRG. Second, internal perfusion with GDP-\( \beta \)-S blocks the increase in the number of evoked APs that occurs with external application of S1P. In contrast to our observations in sensory neurons, the existing literature indicates that in other cell types the actions of S1P are largely to reduce excitability. Perhaps the best characterized is the S1P-mediated activation of an inwardly rectifying \( I_K \) in atrial myocytes that is gated by ACh (Bunemann et al. 1995; Guo et al. 1999; Liliom et al. 2001). These actions of S1P are blocked by pretreatment with pertussis toxin, indicating the involvement of \( G_{\beta\gamma} \), coupling to activation of this current. In cardiac ventricular myocytes, S1P is linked to a decrease in excitability via suppression of \( I_{\text{Na}} \) in a pertussis toxin-independent manner (MacDonell et al. 1998). In brain microglia and fibroblasts, S1P increases intracellular \( Ca^{2+} \) levels, resulting in the activation of a charybdotoxin- andiberiotoxin-sensitive \( Ca^{2+} \)-dependent \( I_K \) (Repp et al. 2001; Schilling et al. 2002). In an analogous fashion, the precursor of Sph, Sph, reduced both \( I_{\text{Na}} \) and \( I_C \) when applied externally to rat ventricular myocytes (McDonough et al. 1994; Yasui and Palade 1996). Similar results have been reported for Sph-induced inhibition of \( I_{\text{Ca}} \) in GH4C1 cells (Titievsky et al. 1998). Whether the actions of Sph on these cells was secondary to phosphorylation by Sph kinase to produce S1P was not determined. Externally applied Sph also blocks the calcium release-activated current, \( I_{\text{CRAC}} \), in the mast cell line RBL-2H3 (Mathes et al. 1998). Interestingly, Mathes et al. demonstrated that ceramide, Sph (but not internally applied Sph), and the Sph kinase inhibitor, DMS, all blocked \( I_{\text{CRAC}} \); however, neither sphingomyelinase (the enzyme that liberates ceramide) nor S1P altered \( I_{\text{CRAC}} \). Mathes et al. speculated that it is the incorporation of Sph and compounds containing the Sph backbone (i.e., ceramide) into the plasma membrane that results in the block of \( I_{\text{CRAC}} \). In contrast to the studies demonstrating that external Sph was capable of modulating membrane currents in other cells, our observations in sensory neurons show that external Sph is ineffective at altering either the generation of APs (Zhang and Nicol, unpublished observation) or the amplitudes of TTX-R \( I_{\text{Na}} \). It is possible that different cell types have distinct capacities that are permissive for the entry of sphingomyelins into the cell and may thus account for the varying effectiveness of external Sph.

Based on our RT-PCR results, sensory neurons likely express S1P_{1} (EDG 1), S1P_{2} (EDG 5), S1P_{3} (EDG 3), and S1P_{4} (EDG 6) receptors. These GPCRs are linked via \( G_{\alpha\omega}, G_{\beta\gamma} \), and \( G_{12/13} \) to the activation of various downstream signaling cascades such as phospholipase C, the release of intracellular \( Ca^{2+} \), activation of the Ras-MAP kinase pathway, or inhibition of adenyl cyclase (Ancellin and Hla 1999; Okamoto et al. 1998; Spiegel and Milstien 2002; 2003; Wu et al. 1995). Although our current work does not elucidate the signaling pathways mediated by S1P, it is possible to potentially eliminate unlikely candidates. Many studies demonstrated that the increased sensitivity of sensory neurons to different types of stimuli results, in part, from an increase in cyclic AMP and activation of PKA (Cui and Nicol 1995; Ferreira and Nakamura 1979; Hingten et al. 1995; Taiwo et al. 1989). Therefore the idea that augmentation of excitability is mediated by a decrease in adenyl cyclase activity via S1P receptors seems unlikely. An S1P receptor-induced increase in the levels of intracellular \( Ca^{2+} \) might lead to activation of PKC, and PKC has been shown to play a role in the sensitization of sensory neurons (Aley et al. 2000; Barber and Vasko 1996; Meller et al. 1996; Premkumar and Ahern 2000; Schepelmann et al. 1993). However, our preliminary results (data not shown) indicate that external S1P does not increase intracellular \( Ca^{2+} \) even though intracellular \( Ca^{2+} \) was elevated by KCl and capsaicin. The enhanced excitability might result from activation of the MAP kinase pathway. Indeed, there are numerous reports demonstrating a role for MAP kinase (both the ERK_{1/2} and p38 subtypes) in the enhanced sensitivity of sensory neurons to noxious stimulation after inflammation or nerve injury (Aley et al. 2001; Dai et al. 2002; Ji et al. 1999, 2002; Jin et al. 2003; Karim et al. 2001; Svensson et al. 2003). In addition, MAP

kinase has been shown to modulate the activity of a number of membrane currents (Adams et al. 2000; Aimond et al. 2000; Crepel et al. 1998; Fitzgerald 2000; McLaughlin et al. 2001; Wilk-Blaszcak et al. 1998; Yang et al. 2001; Yuan et al. 2002). Taken together, such observations suggest that S1P binding to an S1P receptor could result in the downstream activation of MAP kinase, and this pathway may be important in modulating the activity of ion channels in nociceptive sensory neurons.

The ability of internally perfused S1P to augment excitability in the presence of GDP-β-S raises the question as to what cellular mechanisms are activated by this sphingolipid to enhance neuronal firing. Presently, the role of S1P as an intracellular signaling molecule in neurons is poorly understood (see review by Colombaioni and Garcia-Gil 2004). However, this role of S1P is better understood in studies examining immuno-competent cells and transfected cell lines. There are many reports demonstrating that intracellular S1P leads to the release of intracellular Ca\(^{2+}\). In permeabilized DDT1 MF-2 cells (a smooth muscle cell line) as well as a preparation of rough endoplasmic reticulum isolated from these cells, S1P produced a transient increase in intracellular Ca\(^{2+}\) levels that was independent of inositol-trisphosphohate (IP\(_3\)) (Ghosh et al. 1990, 1994). Similarly, activation of high-affinity receptors for IgG, IgE, or CsA in immuno-competent cells, such as mast cells, produced an IP\(_3\)-independent release of Ca\(^{2+}\) from internal stores that was associated with an increase in intracellular S1P (Choi et al. 1996; Melendez and Khaw 2002; Melendez and Ibrahim 2004; Melendez et al. 1998). As described in the preceding text, cells have GPCRs activated by S1P; however, Van Brocklyn et al. (1998) showed that many of the stimulating effects of S1P were independent of the activation of S1P receptors. For example, S1P equally increased intracellular Ca\(^{2+}\) in both empty-vector and S1PR1 transfected HEK and National Institutes of Health 3T3 cells and that microinjected S1P stimulated the synthesis of DNA in Swiss 3T3 cells, an effect that mimicked the reported actions of PDGF. Thus these observations indicate that S1P may prove to be an important signaling agent in the release of Ca\(^{2+}\) from internal stores distinct from those pathways liberating IP\(_3\). A role for intracellular S1P in the modulation of intracellular Ca\(^{2+}\) levels in neurons is unknown. In addition, recent studies have shown that TNFα or IL-1β elevated intracellular levels of S1P (Pettus et al. 2003, 2005). The increase in this sphingolipid resulted in the induction of cyclooxygenase-2 and the consequent production of prostaglandin E\(_2\); however, the mechanism whereby S1P modulates the expression of cyclooxygenase-2 is unknown.

In conclusion, our current observations when taken together with our previous findings (Zhang et al. 2002) suggest that activation of sphingolipid metabolism in sensory neurons is an important signaling pathway in modulating the excitability. S1P that is released by other cells, such as platelets or mast cells, may activate S1P receptors on sensory neurons to modulate the activity of ion channels that directly control excitability. Thus the sphingomyelin signaling cascade likely plays an important role as a primary first messenger and perhaps as a second messenger in controlling the sensitivity of sensory neurons after exposure to inflammatory or neuropathic conditions.

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