Depression of excitability by sphingosine 1-phosphate in rat ventricular myocytes

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MacDonell, Karen L., David L. Severson, and Wayne R. Giles. Depression of excitability by sphingosine 1-phosphate in rat ventricular myocytes. Am. J. Physiol. 275 (Heart Circ. Physiol. 44): H2291–H2299, 1998.—Sphingosine 1-phosphate (S-1-P) is a bioactive sphingolipid that is released from activated platelets. Extracellular S-1-P augments an inwardly rectifying potassium conductance in cultured atrial preparations, but the electrophysiological effects of this compound in the ventricle are unknown. The electrophysiological effects of S-1-P were examined in single myocytes from rat ventricular muscle. Action potential waveform and underlying ionic currents in the presence and absence of 3 µM S-1-P (1–6 min) were recorded. S-1-P increased the minimum stimulus current needed to elicit an action potential by ~100 pA. Pertussis toxin or preexposure to S-1-P did not alter this effect. The action potential waveform was unchanged by S-1-P. The inward sodium current (I\textsubscript{Na}) was examined in a range of membrane potentials just negative to the potential for firing an action potential. S-1-P reversibly inhibited peak I\textsubscript{Na} by ~50 pA, whereas the inward rectifier potassium current was not significantly changed. The results of this study suggest that S-1-P inhibits rat ventricular excitability by reducing I\textsubscript{Na}.

SPHINGOMYELIN-DERIVED compounds have recently been recognized as components of a novel signal transduction cascade (reviewed in Ref. 19). Cell membrane sphingomyelin is hydrolyzed by sphingomyelinases, resulting in the intracellular release of ceramide, a bioactive lipid second messenger. Ceramide, in addition to having its own bioactivity, is a substrate of ceramidase that catalyzes the formation of sphingosine. Sphingosine is subsequently converted to sphingosine 1-phosphate (S-1-P) through the action of sphingosine kinase. Sphingosine and S-1-P accumulate rapidly in a variety of cell types on stimulation by growth factors and are thought to act as the intracellular mediators of some of the cellular responses to these agents, such as growth and proliferation, changes in morphology, and intracellular calcium mobilization (Refs. 12, 22, see Ref. 19 for review).

Sphingosine kinase is highly active in platelets, resulting in high levels of intracellular S-1-P (36). During platelet activation, intracellular S-1-P is released into the extracellular space (35, 36). Extracellular S-1-P is metabolically stable in plasma, probably existing in a complex with serum albumin; it has been suggested that S-1-P may circulate in the body (35). Recently, it was demonstrated that S-1-P mediates some of its biological effects as an extracellular agonist (4, 24, 36). For example, Bünnemann et al. (4) reported that, in guinea pig atrium, extracellular S-1-P can activate an agonist-sensitive inwardly rectifying potassium conductance [I\textsubscript{K(ACh)}]. This effect, which occurs with an EC\textsubscript{50} of 1.2 nM, is sensitive to inhibition by pertussis toxin (PTx) and can be desensitized by preincubation of cells with S-1-P, suggesting that a G protein-linked plasma membrane receptor for S-1-P is involved (31). Regulation of I\textsubscript{K(ACh)} is the only cardiac electrophysiological effect of S-1-P that has been reported. The potency of this effect is shared by a number of other biological actions of extracellular S-1-P, including regulation of intracellular calcium levels (EC\textsubscript{50} of 2 nM) (31) and neurite retraction (EC\textsubscript{50} of 1.5 nM) (23). However, relatively low-affinity binding sites for S-1-P on platelets and mouse melanoma cells lines also have been identified (e.g., binding sites with dissociation constants of 110 nM and 2.6 µM in human platelets) (36). These findings suggest that both low- and high-affinity sites for extracellular S-1-P exist.

It is likely that extracellular S-1-P, released from activated platelets, accumulates in certain areas of the myocardium, for example, during ischemia. A study of the electrophysiological characteristics of cardiac cells in the presence of S-1-P is therefore relevant, particularly because specific lipids have been demonstrated to play a pro- or anti-arrhythmic role in ischemic myocardial tissue (reviewed in Refs. 6, 16). We hypothesized that S-1-P can modulate electrophysiological activity in the mammalian ventricle. To test this hypothesis, the action potential in single rat ventricular cardiomyocytes was used as an indicator of electrophysiological actions of S-1-P. Voltage-clamp studies were then performed to identify the underlying S-1-P-modulated ionic current(s). The results of this study show that S-1-P reduces ventricular excitability and does so by inhibition of sodium current (I\textsubscript{Na}) at membrane potentials near the threshold for the firing of action potentials.

MATERIALS AND METHODS

Preparation of isolated cardiomyocytes. Experiments were performed on right ventricular cells from adult rat hearts that were isolated according to a method modified from Bouchard et al. (2). Briefly, male Sprague-Dawley rats (180–250 g) were injected with heparin (600 IU ip), anesthetized with methoxyflurane gas, and killed by cervical dislocation.

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The heart was removed and briefly rinsed in HEPES-buffered Tyrode solution to clear the chambers of blood. The composition of the HEPES-Tyrode solution was as follows (in mM): 140 NaCl, 5.4 KCl, 1 MgCl2, 1 Na2HPO4, 1 CaCl2, 5 HEPES, and 10 D-glucose. All experimental solutions were prepared with Milli-Q grade water. The aorta was cannulated and perfused in a retrograde fashion on a modified Langendorff apparatus with nominally calcium-free HEPES-Tyrode solution for 5 min (15 ml/min, 37°C). The heart was then perfused for 7 min with HEPES-Tyrode solution containing 40 µM CaCl2, 0.02 mg/ml collagenase (500 U/mg; Yakult Honsha, Tokyo, Japan), and 0.004 mg/ml protease (type XIV, 5.4 U/mg; Sigma Chemical, St. Louis, MO). The free wall of the right ventricle was dissected from the heart, placed in 10 ml of Sigma Chemical, St. Louis, MO) and, when filled with internal solution, was superfused in a retrograde fashion on a modified Langendorff apparatus with nominally calcium-free HEPES-Tyrode solution containing 100 µM CaCl2, 0.5 mg/ml collagenase, 0.1 mg/ml protease, and 1% (wt/vol) fatty acid-free BSA (A-6003, Sigma Chemical). After ventricles were finely minced, the tissue pieces were gently agitated in a shaking water bath (35°C). After 30 min, aliquots of isolated cells (300 µl) were removed from the suspension and diluted in storage solution at a ratio of 1:10. The storage solution was HEPES-Tyrode solution with 100 µM CaCl2 and 1% fatty acid-free BSA. Cells were collected at regular intervals for the next 30 min and stored at room temperature for use within the following 10 h in all experiments except for PTx treatments, for which cells were stored for up to 16 h after isolation. Cells used for electrophysiological recording had the microscopic appearance of single cells (i.e., not 2 or more cells), were calcium tolerant and quiescent, and had crisp cross striations without membrane blebs. Cell capacitance, a measure of cell surface area, was 72.9 ± 5.4 pF (n = 22). This indicates that the cells used in this study were single, uncoupled cardiomyocytes, based on a comparison with previously reported mean values (−120 pF) for single adult rat ventricular cardiomyocytes (3, 26).

Electrophysiological methods. Action potentials and whole cell currents were recorded at room temperature (−22°C) using conventional mediated patch recording techniques. Borosilicate electrodes (World Precision Instruments, Sarasota, FL) were pulled on a multiple-stage puller (P87, Sutter Instruments, Novato, CA) and, when filled with internal solution, had a nominal resistance of 2–3 MΩ (action potentials) and 1–2.5 MΩ (current recordings). For action potential, inwardly rectifying potassium current (I_k1), and I_K(ACh) recordings, the internal solution was as follows (in mM): 115 potassium aspartate, 30 KCl, 2.5 Na2ATP, 10 EGTA, and 10 HEPES (pH 7.3, adjusted with KOH; calculated pCa 11). For I_Na recordings, the internal solution contained the following (in mM): 115 cesium aspartate, 30 CsCl, 2.5 Na2ATP, 10 EGTA, and 10 HEPES (pH 7.3, adjusted with CsOH; calculated pCa 11). Liquid junction potentials between the electrode and the external solution were immediately zeroed before seal formation. Because of the low mobility of aspartate in the pipette relative to chloride in the bath, all potential recordings were adjusted for a liquid junction potential of −10 mV. Action potentials were recorded with a unity gain voltage follower Neuroprobe 1600 amplifier (A-M Systems, Everett, WA). The absence of a resistive feedback in the amplifier electronics removed the possibility of artifacts in action potential parameters, such as reduced amplitude and maximum rate of rise of the membrane potential during the phase of rapid membrane depolarization (17). Membrane potential records were low-pass filtered at 20 kHz and sampled at 125 kHz with a 12-bit analog-to-digital converter. Whole cell current recordings were made with a patch-clamp amplifier (EPC-7, List Electronic, Darmstadt, Germany) using the ruptured patch configuration of the patch-clamp technique (8). Currents were low-pass filtered (4-pole Bessel; −3 dB at 3 kHz). I_Na recordings were digitized at 12.5 kHz, and I_k1 and I_K(ACh) records were digitized at 5 kHz. Data were stored and later analyzed on a personal computer. Customized software (Cellsoft, D. Bergman, University of Calgary) controlled data acquisition and stimulation protocols.

Capacitive transients were recorded as follows. During whole cell current experiments, hyperpolarizing steps of 5 mV from a holding potential of −80 mV were applied to the cell and the resulting transient current was filtered at 10 kHz and digitized at 25 kHz. Cell capacitance was determined from the integral of the current area.

Experimental protocols. Cells were placed in a glass-bottomed chamber on the stage of an Olympus inverted microscope (Olympus Optical, Tokyo, Japan) and were superfused with a HEPES-Tyrode solution containing (in mM) 140 NaCl, 5.4 KCl, 1 MgCl2, 1 Na2HPO4, 1 CaCl2, 10 D-glucose, and 5 HEPES (pH 7.4, adjusted with NaOH). During I_Na recordings, 2 mM 4-aminoipyridine (4-AP) and 3 mM CsCl were also present. For some measurements of I_K1, the concentration of KCl was increased to 10 mM. Experimental protocols began 5 min after the whole cell recording configuration was attained. Action potentials and macroscopic currents were recorded before (control), during, and after (washout) superfusion with 3 µM S-1-P (Biomol, Plymouth Meeting, PA) (±2 ml/min). S-1-P was prepared on the day of the experiment by drying an aliquot of 1 mM S-1-P (dissolved in methanol) under N2 and then S-1-P was resuspended as a complex with 4 mg/ml fatty acid-free BSA. Final concentration of S-1-P in the stock solution was 100 µM. This mixture was incubated at 37°C for 30 min with frequent vortexing, as per the supplier’s recommendation, and was diluted with HEPES-Tyrode solution for superfusion of the cells. Control and washout experiments contained the appropriate concentration of BSA vehicle.

Action potentials were elicited at a rate of 1 Hz with 1.5-ms depolarizing rectangular current pulses, unless otherwise specified. I_Na was elicited by voltage steps from a holding potential of −80 mV to a potential just negative of that which activated a regenerative, uncontrolled I_Na in the range of −62 mV, and then was subjected to only one experimental protocol [action potential, I_Na, I_K1, or I_K(ACh)].

Data were presented as means ± SE, and the number of separate cells is denoted by n values. Statistical significance of effects was determined by paired Student's t-test and one-way repeated measures ANOVA, as appropriate, with the level of significance set at P < 0.05.

RESULTS

Effect of S-1-P on action potentials in isolated rat ventricular myocytes. Characteristics of action potential waveforms in isolated rat ventricular myocytes were examined in the presence and absence of 3 µM S-1-P (Fig. 1). First, the excitability of cells was measured. Excitability was defined as the minimum
strength of current, at a constant frequency and fixed duration, required to elicit an action potential. Cells were stimulated with an incrementally increasing strength of depolarizing current at 1 Hz and 1.5-ms duration. An action potential could be elicited in the myocyte in Fig. 1A with a minimum current of 1,850 pA. After exposure to 3 µM S-1-P for 4 min, the cell failed to fire an action potential when stimulated with the same magnitude of current. After the current was increased by 50 pA, this cell fired an action potential. Similar results from a different cell are shown in Fig 1B. Cumulative data from four cells, describing changes in threshold current by 3 µM S-1-P for 1–6 min, are shown in Fig. 2. S-1-P increased threshold current within 1 min of superfusion by ~75 pA, reaching a maximum of ~100 pA within 3 min (Fig. 2B). This effect persisted for the 6-min treatment period, and it was fully reversible after ~5 min of washout with a solution containing BSA. Conversely, the minimum current required to fire an action potential in separate control cells decreased over the same time period. At time points equivalent to 4, 6, and 11 min after the start of exposure to S-1-P, the minimum stimulus current in control cells decreased by 56.15, 92.77, and 33.73 pA (n = 4).

Whereas excitability was reduced by 3 µM S-1-P, the shape of the action potential remained essentially unaffected. This is evident in Fig. 1 and also in Table 1, which describes several parameters of the action potential waveform recorded before, during, and after exposure to 3 µM S-1-P. Two examples of the action potential recordings with S-1-P are shown in Fig. 1 to demonstrate that, on average, the latency to threshold potential was unchanged by S-1-P. Although the action potential duration at 90% of repolarization was apparently prolonged in the absence of S-1-P, the absence of reversibility suggests that this was a time-dependent phenomenon and not an agonist-specific effect (Table 1). As shown in Table 2, a trend toward decreasing values of action potential duration at 90% repolarization with respect to time was also seen in control action potentials.

Effect of S-1-P on $I_{Na}$ and charge contributed by $I_{Na}$. Reduction of excitability by S-1-P, as reflected by an increase in threshold current with no consistent change in the action potential waveform, suggested that the basis for this electrophysiological effect involved those ionic currents that interact to determine threshold. The action potential threshold in single ventricular cells can be described as the membrane potential at which sufficient activation of inward $I_{Na}$ occurs to exceed outward potassium current (33). In the mammalian ventricle, the primary potassium conductance at sub-threshold potentials is the inward rectifier, $I_{K1}$ (20, 28). Accordingly, we investigated the effect of S-1-P on $I_{Na}$ and $I_{K1}$ under voltage-clamp conditions with an emphasis on determining the magnitude of current at membrane potentials at which the cell fires an action potential. For this reason, the magnitude of $I_{Na}$ was measured under conditions similar to those used to measure action potentials (i.e., physiological concentrations of extracellular sodium) as opposed to conditions needed to measure the entire current-voltage relationship of $I_{Na}$ (i.e., reduced extracellular sodium with replacement of the balance with nonpermeant cations with or without sodium channel blockers and reduced...
Fig. 2. Effect of S-1-P on rat ventricular excitability as measured by changes in stimulus current at threshold. Cells were stimulated with increasing strength of current (10- to 50-pA increments, 1 Hz, 1.5 ms) until an action potential was elicited (threshold current). A: threshold currents before (control, time 0), during 3 μM S-1-P exposure (1–6 min), and after washout of S-1-P (−5 min) are expressed as pA at a fixed duration (1.5 ms). Changes in minimum stimulus current were significantly different from control during exposures to S-1-P for 2–6 min (P < 0.05) except at 4 min (P = 0.0573) (1-way ANOVA, Bonferroni ad hoc test). B: absolute differences in threshold current in cells during S-1-P exposure (1–6 min) and washout (−5 min) relative to control conditions are expressed in pA. Values are means ± SE; n = 4 separate cells.

temperatures). In these experiments, I Na was measured in 140 mM external sodium at voltages very close to the threshold for excitability. The holding potential was set to −80 mV, within 5 mV of the resting potential of these cells. When I Na was measured, outward potassium currents that could be activated at these potentials were blocked with 4-AP and internal and external cesium.

Under control conditions, peak I Na was −204 ± 23 pA at −64.0 ± 0.8 mV (n = 6) (Fig. 3A). S-1-P (3 μM) inhibited peak I Na, reaching a maximal reduction of 26% at 4 min (P < 0.05) (Fig. 3, B and C). This effect was reversible within 5 min. The reduction in current, ~50 pA per cell at 4 min of superfusion (Fig. 3C), is comparable to the extra stimulus current necessary to elicit an action potential in the presence of S-1-P (cf. Fig. 2). Washout after S-1-P-mediated inhibition of I Na consistently revealed a larger peak I Na than pretreatment values. This “overshoot” is a reflection of a time-dependent increase in I Na. This was demonstrated by monitoring I Na under control conditions over the time course typical for an S-1-P experiment (recorded current for 11 min starting 5 min after whole cell condition was achieved). I Na showed a small, progressive increase in maximum amplitude over this time period. At the times equivalent to 4, 6, and 11 min after the start of S-1-P exposure, control values of I Na were 109 ± 6.20, 115 ± 9.90, and 135 ± 11.9% of initial values (n = 8). A time-dependent leftward shift in voltage dependence of activation of sodium channels is the likely explanation for such an increase in I Na under control conditions (9). This “run-up” would, of course, lead to an underestimation of the inhibitory effect of S-1-P on I Na. This time-dependent increase in peak I Na amplitude correlates with the observed decrease in minimum current required to fire action potentials in control cells.

Additional information concerning the role of I Na in the initiation of an action potential can be obtained by computing the charge contributed by I Na during the time between application of the stimulus current and firing of the action potential, i.e., the latent period. This can be approximated by determining the integral of I Na from voltage-clamp recordings described above and shown in Fig. 3. It is this total current change that charges the membrane capacitance and depolarizes the membrane to the threshold potential. I Na recordings in the presence and absence of S-1-P were integrated

### Table 2. Time-dependent effects on control action potential parameters in rat ventricular cells

<table>
<thead>
<tr>
<th>Action Potential Parameter</th>
<th>0 min</th>
<th>4 min</th>
<th>11 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting membrane potential, mV</td>
<td>−80.74 ± 0.57</td>
<td>−80.82 ± 0.65</td>
<td>−80.90 ± 0.76</td>
</tr>
<tr>
<td>Latency, ms</td>
<td>2.83 ± 0.51</td>
<td>2.56 ± 0.41</td>
<td>2.53 ± 0.41</td>
</tr>
<tr>
<td>dV/dt, mV/s</td>
<td>193.25 ± 13.94</td>
<td>184.20 ± 17.71</td>
<td>182.42 ± 13.46</td>
</tr>
<tr>
<td>Maximum amplitude, mV</td>
<td>37.47 ± 3.21</td>
<td>35.78 ± 3.42</td>
<td>31.15 ± 3.07</td>
</tr>
<tr>
<td>APD 50, ms</td>
<td>35.78 ± 9.67</td>
<td>28.90 ± 6.28</td>
<td>24.71 ± 5.92</td>
</tr>
<tr>
<td>APD 90, ms</td>
<td>69.39 ± 14.73</td>
<td>59.19 ± 10.93</td>
<td>51.24 ± 10.72</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 8 ventricular myocytes. Action potentials recordings were started 5 min after attaining the whole cell configuration (0 min).
during the first 25 ms of the depolarizing voltage step, a time frame that includes the latent period (Fig. 4). Control charge due to \( I_{\text{Na}} \) was \( 3.48 \pm 0.47 \) pC, and this was significantly decreased by 3 µM S-1-P to \( 2.77 \pm 0.48 \) pC after 2 min and to \( 2.67 \pm 0.51 \) pC after 4 min (\( P < 0.05 \), repeated measures one-way ANOVA). The effect was reversible; charge due to \( I_{\text{Na}} \) after 5 min of washout was \( -4.08 \pm 0.84 \) pC. These observations demonstrate an inhibitory effect of 3 µM S-1-P on both peak \( I_{\text{Na}} \) and integrated \( I_{\text{Na}} \) during the period of membrane depolarization to threshold.

Effect of S-1-P on \( I_{K1} \). Excitability is observed in single ventricular cells when the applied stimulus current or \( I_{\text{Na}} \) exceeds outward currents, i.e., the point at which net ionic currents become inward (21). \( I_{K1} \) is the outward potassium conductance that counteracts the applied stimulus and/or \( I_{\text{Na}} \) in the range of membrane voltages near the threshold for firing an action potential (20, 25). With the use of a linear voltage-ramp protocol, the current-voltage relation of background whole cell conductance was obtained in the presence and absence of 3 µM S-1-P (Fig. 5). \( I_{K1} \) was calculated as the current that was sensitive to BaCl\(_2\) (100 µM) (28). A small apparent increase in \( I_{K1} \) in the presence of 3 µM S-1-P was seen at potentials at which \( I_{K1} \) carries outward current, but this effect could not be washed out with BSA-containing solution. To examine outward \( I_{K1} \) current with improved resolution, these experiments were repeated in 10 mM external potassium. Elevated external potassium increases the amplitude of \( I_{K1} \) at negative membrane potentials (25, 28) and thus improves resolution at voltages near the action potential threshold. Under these conditions, maximum outward current averaged \(-170 \) pA at \(-50 \) mV. Again, only an irreversible 10- to 20-pA increase in outward \( I_{K1} \) current was observed in the presence of S-1-P with elevated external potassium (\( n = 3 \), data not shown). However, this small, irreversible increase in \( I_{K1} \) by S-1-P is unlikely to be related to S-1-P-mediated inhibition of excitability, an effect that was readily reversible (cf. Figs. 1 and 2).

S-1-P-mediated decrease in ventricular excitability does not require PTx-sensitive G proteins. Some functional effects of S-1-P, including regulation of an \( I_{K1} \) in
guinea pig atrium, require functional PTx-sensitive G proteins (4). Therefore, we examined whether PTx-sensitive G proteins were involved in the signal transduction mechanism leading to the increase in threshold current mediated by S-1-P. Cells were incubated with 3–5 µg/ml PTx for 4–13 h at room temperature. Threshold current was measured by stimulating the cell at incrementally increasing current strength (1 Hz, 1.5 ms) until an action potential was elicited during exposure to 3 µM S-1-P, and after 5-min washout. $I_{K1}$ is expressed as the difference between current in the presence and absence of 100 µM BaCl$_2$. Data are representative of results from 3 other experiments.

![Fig. 5. Inwardly rectifying potassium current ($I_{K1}$) in the presence of S-1-P. Whole cell $I_{K1}$ in a ventricular cell in 5.4 mM external potassium was recorded using a linear voltage ramp (inset) before (control), after 4-min exposure to 3 µM S-1-P, and after 5-min washout. $I_{K1}$ is expressed as the difference between current in the presence and absence of 100 µM BaCl$_2$. Data are representative of results from 3 other experiments.](Image)

Inactivation of PTx-sensitive proteins was confirmed by examining the muscarinic agonist-stimulated whole cell $I_{K1(ACh)}$ current-voltage relationship in control and PTx-treated cells. Methacholine (3 µM) increased this potassium current in control cells (~180 pA of outward current at ~55 mV, data not shown), which is very similar to ACh-stimulated current in feline ventricular cells (15). No detectable change in whole cell conductance by methacholine (3 µM) was observed in PTx-treated cells, demonstrating that the PTx incubation was effective.

S-1-P effects on threshold current persist after prolonged S-1-P exposure. Specific inhibitors of S-1-P effects are not available, but other approaches have been used to evaluate the specificity of S-1-P-mediated actions. Preexposure of cells to S-1-P has been shown to diminish effects ascribed to an S-1-P-specific receptor, e.g., activation of $I_{K1(ACh)}$ in guinea pig atrial cells (4) and release of calcium from intracellular stores in HEK cells (31). However, preincubation of ventricular cells with 3 µM S-1-P did not diminish the ability of a subsequent addition of S-1-P to increase threshold current. Threshold current was reversibly increased by 91.6 ± 29.4 pA after 4 min of exposure to 3 µM S-1-P in cells that had been preincubated with 3 µM S-1-P for 3.5–6 h ($P < 0.05$, one-way repeated measures ANOVA, $n = 3$). This suggests that homologous desensitization of an S-1-P receptor (or some essential factor in the putative signaling process) did not occur, even though the myocytes were treated at a higher concentration and for up to threefold longer than required to diminish activation of atrial $I_{K1(ACh)}$ (4).

**DISCUSSION**

S-1-P, cellular excitability, and underlying ionic mechanisms. Cardiac excitability, at the single cell level, can be described operationally in terms of the amplitude and duration of a current necessary to initiate an action potential. We observed that 3 µM S-1-P depressed ventricular cell excitability without affecting the action potential waveform or resting membrane potential. This observation suggested that the effects of S-1-P were selective, as opposed to generalized disruption of normal membrane integrity and ion channel function. The stability of the resting membrane potential in the presence of S-1-P argued against important changes in background $I_{K1}$ and led to the hypothesis that S-1-P depressed excitability by decreasing $I_{Na}$. Our observations that S-1-P rapidly and reversibly decreased $I_{Na}$ at membrane potentials very close to the threshold for excitability, in the absence of agonist-specific changes in $I_{K1}$, support this hypothesis. This is the first report, to our knowledge, that describes the effects of a sphingolipid on the electrogenesis of the cardiac action potential in terms of modulation of $I_{Na}$ by S-1-P. The concentration of S-1-P (3 µM) used in this study was comparable to that measured in human serum (0.5 µM) (35), indicating that inhibition of cardiac excitability by S-1-P may be an important (patho)physiological effect.

We have chosen to study the effects of S-1-P on $I_{Na}$ under conditions that complemented the action potential studies. Thus a holding potential of ~80 mV, very similar to the resting membrane potential of the cells, was used, and the voltage-clamp steps depolarized the cell to potentials just below the levels required for activation of a regenerative $I_{Na}$. No attempt was made to measure $I_{Na}$ at more positive potentials, since maneuvers for controlling membrane potential during $I_{Na}$ activation (e.g., extracellular sodium and replacement with nonpermeant cations, sodium channels blockers, and low temperatures) could mask relatively small changes (e.g., 10–15 pA) in $I_{Na}$. Moreover, in our experience (3), escape from voltage control occurs in almost all cases. Whole cell recordings of $I_{Na}$ demonstrate time-dependent hyperpolarizing shifts in both steady-state inactivation characteristics and the conduc-
tance-voltage relationship (9). With time, the former causes an apparent decrease in peak $I_{Na}$ and the latter increases peak $I_{K}$, when $I_{Na}$ is activated by small positive voltage steps from a holding potential of ~80 mV. In our experiments, the net effect of these two dynamic processes was a time-dependent increase in $I_{Na}$ (Fig. 3). Thus the inhibitory effect of S-1-P on $I_{Na}$ develops even in the presence of a time-dependent increase or run-up of $I_{Na}$. This phenomenon results in an underestimation of the actual inhibitory effect of S-1-P on $I_{Na}$. Even so, the approximately 20% decrease in $I_{Na}$ by 3 µM S-1-P agrees closely with the absolute increase in stimulus current, thereby supporting the premise that S-1-P mediates a decrease in cellular excitability mainly by an inhibition of $I_{Na}$. The finding that the relative decrease in the $I_{Na}$ was somewhat larger than the relative increase in the minimum stimulus current is in accordance with experimental findings and mathematical modeling predictions describing the rather complex, nonlinear relationship between sodium channel conductance and threshold in ventricular cells (13).

Inhibition of $I_{Na}$ by other sphingolipids has been observed. Yasui and Palade (34) demonstrated that $I_{Na}$, measured under conditions of reduced extracellular sodium, was markedly inhibited by 50 µM sphingosine in rat ventricular myocytes. The contribution of S-1-P to this effect, through conversion of sphingosine to S-1-P, remains to be established, since it is not known if cardiomyocytes have sphingosine kinase activity. However, not all compounds with a sphingosyl backbone have the capacity to reduce $I_{Na}$, because 50 µM sphingosylphosphorylcholine was without an inhibitory effect (34). It is also interesting to note a similarity between modulation of $I_{Na}$ by S-1-P and cytoskeleton-active compounds. S-1-P affects remodeling of cytoskeletal elements in some cell types (32), and cytochalasin D, which inhibits polymerization of actin, also inhibits whole cell $I_{Na}$ in rat and rabbit ventricular myocytes (30). It remains to be determined whether the inhibitory effects of S-1-P on excitability and $I_{Na}$ in our study are related to changes in myocardial cytoskeletal proteins.

Possible modulatory actions of S-1-P on the outward current at membrane potentials near threshold remained an important consideration in our attempt to elucidate the ionic mechanism(s) of decreased excitability. A precedent for this effect has been established in guinea pig atrial cells, in which S-1-P potently augments a muscarinic $I_{K(ACh)}$ (4, 31). However, in rat ventricular myocytes, we failed to obtain any convincing evidence for a specific effect of S-1-P on barium-sensitive whole cell K⁺ conductance. Outward current did increase by a small amount (Fig. 5), but this effect was irreversible, unlike S-1-P-mediated inhibition of excitability. Experimental conditions that increase $I_{K1}$, namely, increasing extracellular potassium to 10 mM, improved the resolution of our recordings of $I_{K1}$, but S-1-P application still failed to consistently increase this outward current. We conclude from these results that the observed increase in outward $I_{K1}$ at near-threshold voltages was not a specific effect of S-1-P. The absence of an agonist-specific effect on $I_{K1}$ is in agreement with the stability of the resting membrane potential during exposure to S-1-P (Table 1), since in the ventricle the resting potential is primarily determined by the $I_{K1}$ (20, 25).

Attempts to identify the mechanism of action of S-1-P on excitability. In multicellular cardiac preparations, excitability is dependent on a number of parameters, including gap junction conductance (27), a process that is known to be regulated by a variety of lipids (5, 18). The effect of S-1-P on gap junction conductance is unknown, although all available data report that lipids, including a structural analog of S-1-P, lysophosphatidic acid (10), decrease gap junction conductance. On the basis of the measured capacitance, the majority of the preparations used in this study were single cells, but, nonetheless, we cannot rule out the possibility that some may have been pairs of cells. The possibility of gap junction regulation by S-1-P cannot, however, account for its effects on excitability because the electrophysiological effects of reduced gap junction conductance in isolated cardiomyocyte pairs is the opposite of the inhibitory effects of S-1-P on excitability. Thus, during stimulation of action potentials in pairs of isolated cardiomyocytes, a decrease in gap junction conductance would reduce the loss of stimulating charge from the cell, and less current would be required to depolarize the myocytes to threshold. Consequently, an increase in gap junction conductance would have to be invoked to account for the observed effects on excitability, for which there is no experimental evidence.

Although a number of important features of the mechanism involved in decreased excitability and inhibition of $I_{Na}$ by S-1-P remain to be identified, it is evident that PTX-sensitive G proteins are not essential elements. This possibility was examined because PTX-sensitive G proteins are essential components in the mediation of some of the effects of S-1-P, such as inhibition of cAMP production (7, 11, 31), activation of atrial $I_{K(ACh)}$ (4), and activation of the sodium/proton exchange (29). Our results, showing that inhibition of isolated ventricular cell excitability by S-1-P is independent of G/G₉ activation, are consistent with other actions of S-1-P such as phospholipase C activation (11) and phosphatidic acid accumulation (7). Moreover, other signaling mechanisms involving soluble intracellular second messengers or regulation of intracellular calcium are unlikely to be important in the S-1-P-induced changes in excitability observed in this study, since the whole cell recording mode dialyzes small soluble intracellular molecules and 10 mM intracellular EGTA is an effective buffer of divalent cations under steady-state conditions.

Some effects of S-1-P applied extracellularly can be diminished by preincubation with S-1-P or structurally related lipids (4, 31), leading to the suggestion that expression of an S-1-P receptor may be controlled by desensitization/downregulation processes. The lack of such an effect on S-1-P-mediated inhibition of excitability in this study does not necessarily exclude a role for
an S-1-P receptor, but it does indicate that the mechanism for depressed excitability is different from that involved in other S-1-P-mediated electrophysiological events such as \( I_{\text{K(ACh)}} \) activation in guinea pig atrium (4).

A direct interaction of S-1-P with the sodium channel may occur, although this was not specifically tested. Direct lipid-sodium channel interaction has been postulated as the mechanism involved in cardiac sodium channel inhibition by extracellularly applied lipids such as poly- and monounsaturated fatty acids (1, 14). It is interesting to recall that a negatively charged head group apparently is necessary for channel inhibition by unsaturated fatty acids (1). The S-1-P molecule, with a long-chain unsaturated hydrocarbon tail and a polar phosphate group at the end of the sphingosine backbone, has a structure similar to those lipids that modulate sodium channel function.

Formation of blood clots during coronary occlusion and subsequent ischemic events could lead to accumulation of extracellular S-1-P. Concentrations of S-1-P in human serum have been estimated to be 0.5 µM (35), although levels may be higher in ischemic areas of the myocardium, depending on the contribution of non-human serum have been estimated to be 0.5 µM (35), although levels may be higher in ischemic areas of the myocardium, depending on the contribution of non-

In summary, 3 µM S-1-P depressed the excitability of isolated rat ventricular myocytes by reversibly increasing the current necessary to elicit action potentials. The measured decrease in peak whole cell \( I_{\text{Na}} \) by 3 µM S-1-P is comparable in size to the observed increase in stimulus current needed to fire an action potential, and both of these effects were fully reversible. We conclude that S-1-P reduced excitability by inhibiting cardiac sodium channel function at voltages near threshold for activation. PTx-sensitive G proteins are not involved in this process, perhaps suggesting that S-1-P interacts directly with the cardiac sodium channel.

We sincerely thank Dr. Robert B. Clark of the University of Calgary for generously contributing superb technical support to this study. We also thank Dr. Gabor Tigyi at the University of Tennessee for helpful discussions concerning sphingolipid-mediated ion channel regulation.

This research was supported by operating grants from the Medical Research Council of Canada to W. R. Giles and D. L. Severson. W. R. Giles holds a Medical Scientist Award from the Alberta Heritage Foundation for Medical Research. K. L. MacDonell is a recipient of an Alberta Heritage Foundation for Medical Research Fellowship.

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Received 2 February 1998; accepted in final form 29 July 1998.

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