Sphingosine 1-phosphate protects mouse extensor digitorum longus skeletal muscle during fatigue

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Sphingomyelin derivatives exert important biological actions in different cell types (25–27, 37, 43). The receptor-mediated activation of sphingomyelinase sequentially generates different biologically active compounds, namely, ceramide, sphingosine (SPH), and sphingosine 1-phosphate (S1P). Ceramide activates pathways that regulate stress responses, cell senescence, and apoptosis (21). The enzyme ceramidase generates different biologically active compounds, namely, ceramide, sphingosine (SPH), and sphingosine 1-phosphate (S1P). Ceramide activates pathways that regulate stress responses, cell senescence, and apoptosis (21). The enzyme ceramidase converts ceramide to SPH, which can be phosphorylated to S1P by a specific kinase: sphingosine kinase (SK) (35). SPH regulates the activity of intracellular targets (20), while S1P shows both intracellular and extracellular actions, the latter by stimulating specific cell surface G protein-coupled receptors (25, 27, 37, 43). SPH usually inhibits cell proliferation and promotes apoptosis, while S1P stimulates growth and protects against apoptosis (2, 37, 43). Important to their actions is the considerable serum level of both SPH and S1P (9, 10, 13, 27, 47). Because of the different and sometimes opposite roles of these lipids, great importance is attributed to the expression level and presence of the converting enzymes ceramidase and SK, whose activity may control the “sphingolipid rheostat” wherein the ratio of sphingolipids with opposing actions becomes important in determining cell fate (27, 37).

Recent evidence shows that many of the signaling components of the sphingomyelin cascade are present in skeletal muscle (19, 40). Data from rabbit, rat, and pig show that SPH is endogenous to skeletal muscle (6, 14, 39). In fact, T-tubular membranes are rich in both SPH and the SPH precursor sphingomyelin (15, 39), and T tubules express high levels of sphingomyelinase activity (19, 40). Ceramide is elevated in fast-twitch glycolytic rat skeletal muscles and low in oxidative muscles (14). Importantly, during prolonged exercise, sphingomyelinase activity of skeletal muscle increases, ceramide level decreases, and S1P level increases (15), suggesting that the whole sphingomyelin pathway is activated in exercised muscles. SPH is reported to act on the ryanodine receptor, the sarcoplasmic reticulum (SR) Ca2+ release channel (5, 6, 34, 38, 42), and the T tubule dihydropyridine receptor (DHPR) voltage-dependent Ca2+ channel, i.e., the protein complex responsible for the L-type Ca2+ current in cardiac muscle cells (29, 44). SPH is also known to regulate the activity of protein kinase C and related downstream pathways (20); thus it also may act in skeletal muscle, especially considering that this kinase is localized to the T-tubular membranes (41). The level of S1P in resting skeletal muscle is estimated to be in the low nanomolar range (47), but its possible role in contractility and fatigue has not been evaluated. S1P mobilizes Ca2+ in C2C12 myoblasts, apparently by activating membrane voltage-sensitive Ca2+ channels and intracellular Ca2+ release channels (18, 30). The extracellular action of S1P occurs by activation of specific G protein-coupled cell surface receptors (25, 37, 43), which have been described in skeletal muscle (4). SK is also expressed in skeletal muscle extracellular surfaces (30), suggesting that some of the SPH effects could be explained by...
SPH’s enzymatic conversion to S1P. Consistent with this view is our recent demonstration that SPH released from cardiomyocytes is rapidly converted to S1P (7).

Because both SPH and S1P are present at very high levels in the blood (9, 10, 13, 45), muscle fibers are continuously bathed in elevated levels of the two lipids. Thus we decided to investigate the possible extracellular actions of SPH and S1P in skeletal muscle contractility and fatigue. Our results indicate that exogenous SPH and S1P administration makes the muscle more resistant to fatigue. Moreover, because pretreating muscle with SK inhibitors abolished the protective effect of SPH, it appears that SPH acts through its conversion to S1P and that S1P is responsible for the observed effects of SPH.

MATERIALS AND METHODS

All the experiments were conducted according to the Declaration of Helsinki regarding the treatment of animals during experimentation. The study was approved by the Ethics Committee of the Medical Faculty of the University of Padua. All efforts were made to minimize animal suffering and to use only the number of mice necessary to obtain reliable data.

Tension recording and fatigue protocols. Adult 3-month-old CD1 mice were used for these studies. Extensor digitorum longus (EDL) muscles were removed and placed in a bath containing Ringer physiological saline solution maintained at room temperature and containing 120 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl2, 3.15 mM MgCl2, 1.3 mM NaH2PO4, 25 mM NaHCO3, 11 mM glucose, and 30 μM t-tubocurarine, pH 7.2–7.4. The solution was continuously bubbled with 95% O2-5% CO2 until used. For the experimental test, muscles were physiologically solution bubbled with 95% O2-5% CO2 at 30°C. Muscles were necessary for 50% tension decay (final force (expressed as percentage of the initial force) and the time equilibrated for 15 min at 30°C in the medium, to which was added 120 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl2, 3.15 mM MgCl2, 1.3 mM NaH2PO4, 25 mM NaHCO3, 11 mM glucose, and 30 μM t-tubocurarine, pH 7.2–7.4. The solution was continuously bubbled with 95% O2-5% CO2 until used. For the experimental test, muscles were mounted in a vertical bath filled with 12 ml of the above physiological solution bubbled with 95% O2-5% CO2 at 30°C. Muscles were electrically stimulated with supramaximal pulses (0.5-ms duration, 0.01 s−1) delivered using a Grass S44 electronic stimulator (Grass Instrument, Quincy, MA) through a stimulus isolation unit (Grass SIU5), and isometric force was recorded with a force transducer (Grass FT03). Muscle responses were recorded via an AT-MIO 16 AD card, and data were analyzed using a virtual instrument created with the LabView software program (National Instruments, Austin, TX) (11). Muscles were stretched to the optimal length for twitch tension and equilibrated for 15 min at 30°C in the medium, to which either 5 μM fatty acid-free bovine serum albumin (BSA) was added in control experiments; otherwise, the indicated compounds were added.

Before and after the incubation period, twitch and tetanic (150 Hz, 200 ms) tension levels were measured. Muscles were then subjected to a fatigue protocol consisting of a train of short tetani (150 Hz, 200 ms, 0.2/s) under the conditions described below. Recovery of muscle tetanic tension after administration of the fatigue protocol was followed for 30 min, with a single tetanus (150 Hz, 200 ms) delivered to muscles every 5 min. Recovery was recorded as the percentage of initial tension.

It is worth noting that we analyzed fatigue development only in selected muscles having about the same initial tetanic tension (N/g) to avoid the effects produced by different accumulation and depletion of metabolites during fatigue (17, 50). The maximum tetanic tension, the final force (expressed as percentage of the initial force), and the time necessary for 50% tension decay (t½) were measured.

Treatments. The fatigue experiments were performed according to the following protocols: 1) control condition, Ringer solution containing 5 μM fatty acid-free BSA; 2) 1, 10, and 100 μM SPH (Sigma); 3) 1, 10, and 20 μM S1P (Avanti Polar Lipids); 4) SPH + N,N-dimethylphosphoglycerine (DMS, 20 μM; Avanti Polar Lipids), an inhibitor of SPH kinase (46) that converts SPH to S1P; 5) 10 or 20 μM DMS; 6) 0.25 μM bis-indoxylmaleimide (bis-IM; Sigma), a cell-permeable inhibitor of protein kinase C; 7) SPH + bis-IM, 10 μM SPH, and 0.25 μM bis-IM; 8) S1P + bis-IM, 10 μM S1P, and 0.25 μM bis-IM; 9) SPH/Ca2+-free Ringer solution, 10 μM SPH in a Ca2+-free solution; and 10) S1P/Ca2+-free Ringer solution, 10 μM S1P in a Ca2+-free solution.

SPH, S1P, DMS, and bis-IM were dispersed by performing sonication on a solution of 5 mM fatty acid-free BSA and then storing it at –80°C until used. Before addition to the bath, SPH, DMS, and S1P were briefly sonicated and delivered to the physiological medium so that the final concentration of albumin was 5 μM.

Electrophysiological analysis. Skeletal muscle fiber resting membrane potential (RMP) and directly evoked action potential (AP) were studied in control and fatigued fibers in the absence or presence of 10 μM SPH or S1P using intracellular glass microelectrodes filled with 3 M KCl. EDL muscles were incubated for 30 min with SPH or S1P before the experiment was begun at 21 ± 1°C. Direct extracellular stimulation was performed using two-needle Ag/AgCl electrodes with supramaximal electrical stimuli (130–150 V, 0.1-ms duration) as described previously (32). Intracellular recorded signals were sent to a preamplifier (WPI 705; World Precision Instruments) and then to a signal conditioner (CyberAmp; Axon Instruments-Molecular Devices, Union City, CA). The output signal from the conditioner was then fed to a digital oscilloscope (Tektronix 5022N; Tektronix, Richardson, TX). No more than a single AP was generally recorded from the same fiber, because muscle membrane was damaged during each stimulation by the relative movements of the recording electrode with respect to the contracting muscle. The maximal rate of AP rise was determined by calculating the first derivative of the digitized spike.

Determination of SPH levels by reverse-phase HPLC. SPH was determined using HPLC according to a previously described method (9). Briefly, sphingolipids were extracted with butanol and derivatized with o-phthaldehyde (Molecular Probes, Eugene, OR). Samples were separated on a Hi-Pore Reversed Phase Column RP-318 (4.6 mm × 25 cm; Bio-Rad, Hercules, CA) run on a Bio-Rad HPLC system equipped with a PerkinElmer fluorescence detector (excitation, 340 nm; emission, 455 nm). The solvent system was composed of methanol, glacial acetic acid, 1 M tetrabutylammonium dihydrogenphosphate, and nanopure water (82.9:1.5:0.9:14.7, vol/vol) run at 1.0 ml/min. It is worth mentioning that the assay measures SPH content of the whole muscle and that it is not possible to evaluate the contribution of SPH derived from blood, smooth muscle, or endothelial cells. As a consequence, even though the enzymatic apparatus for SPH production has been demonstrated in skeletal muscle T tubules (15, 19, 39), some of the muscle SPH could be generated by non-muscle cells.

Membrane preparation and [3H]ryanodine binding assay. Heavy membrane fractions were purified from mouse hindlimb muscles as previously described (28). Briefly, muscle homogenates were prepared in 10 mM HEPES, pH 7.4, and 20 mM KCl buffer, supplemented with the Complete protease inhibitor cocktail (Roche), and centrifuged at 650 g for 10 min. The myofibrillar sediment was reextracted twice in the same buffer. The three supernatants were then pooled and centrifuged at 120,000 g for 90 min. The total membrane fraction was resuspended in 0.3 M sucrose and 10 mM Tris·HCl, pH 7.4, and stored at –80°C.

SR-enriched membranes (at a protein concentration of 0.1 mg/ml) were incubated with 50 nM [3H]ryanodine in 0.5 ml of 0.1 M KCl, 50 μM CaCl2, and 20 mM HEPES, pH 7.4, for 30 min at 37°C in the presence of various concentrations of SPH (5, 6, 38). The sphingoid base was added to the medium assay as a DMSO solution, and the solute did not exceed 1% of total volume. Control experiments demonstrated that the vehicle had no effect on ryanodine binding. Bound [3H]ryanodine was measured by filtration through Millipore nitrocellulose filters (0.45 μm) and liquid scintillation counting. Specific [3H]ryanodine binding was determined by subtracting the nonspecific binding obtained in the presence of 10 μM unlabeled ryanodine.
induced by repeated brief contractions at 150 Hz in the EDL muscle occurred as a progressive decline of force output during the 6-min stimulation protocol (Fig. 1), reaching 22.8 ± 2.2% of initial tension value, whereas \( t_{1/2} \) was 2.35 ± 0.17 min (Table 1). First, three concentrations of exogenous SPH were tested. In the presence of 1 and 10 \( \mu \)M SPH, a marked slowing of force decline was observed (Fig. 1 and Table 1). In contrast, the reduction of fatigue produced by 100 \( \mu \)M SPH was not significant. Muscle recovery in the presence of SPH was similar to that of control EDL (Fig. 1).

We then evaluated the effects of S1P on fatigue development. Similar to the findings with its metabolic precursor, SPH, S1P at 1 and 10 \( \mu \)M caused a significant reduction of fatigue development (Fig. 2) as indicated by the substantial increase of both final force and \( t_{1/2} \) with respect to controls (Table 1). As also observed with SPH, higher S1P levels (20 \( \mu \)M) slightly but not significantly reduced fatigue development (Table 1). The recovery of the muscles treated with S1P did not differ from that of controls.

Because SK activity has been detected on the extracellular side of cells (48), including skeletal muscle cells (30, 33), we hypothesized that SPH could act indirectly on muscle fatigue development through its phosphorylation to S1P. To verify this hypothesis, the action of the SK inhibitor, DMS (46), was evaluated. The expected beneficial effects of SPH on fatigue parameters shown in Fig. 1 were completely abolished in the presence of DMS (Fig. 2). In contrast, DMS (10 or 20 \( \mu \)M) was without evident effects on muscle fatigue when it was delivered alone (Table 1), suggesting that SPH produced during fatigue is not liberated into the extracellular medium. The recovery of the muscles treated with both SPH and DMS appeared to be slightly delayed (Fig. 2).

Effects of bis-IM on the development of fatigue. To evaluate whether SPH administration could produce, at least in part, an intracellular action through its well-known inhibitory action on protein kinase C activity (20), we tested the effect of another potent inhibitor of the kinase, bis-IM, on the development of EDL muscle fatigue. Bis-IM produced only a moderate but not significant effect on EDL fatigue development (Fig. 3 and Table 1). The simultaneous presence of bis-IM and SPH did

### RESULTS

#### Effects of the different treatments on twitch and tetanic tension levels.

All treatments did not affect twitch properties and tetanic tension in the resting state (data not shown).

#### Effects of S1P and SPH on the development of fatigue.

Because SPH and S1P are normally present at high levels in the blood of experimental animals and humans (9, 10, 13, 45), we hypothesized that they might affect contractility and/or fatigue. Thus we performed fatigue protocols in the presence of exogenously applied SPH and S1P. Normal development of fatigue

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**Table 1. Effects of sphingolipids on development of fatigue in mouse EDL muscle**

<table>
<thead>
<tr>
<th></th>
<th>%Initial tension</th>
<th>( \Delta % )</th>
<th>( t_{1/2} )</th>
<th>( \Delta %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>22.8 ± 2.7</td>
<td></td>
<td>2.35 ± 0.17</td>
<td></td>
</tr>
<tr>
<td>SPH, 1 ( \mu )M</td>
<td>34.7 ± 2.9</td>
<td>+52.2*</td>
<td>3.61 ± 0.22</td>
<td>+53.6*</td>
</tr>
<tr>
<td>SPH, 10 ( \mu )M</td>
<td>31.9 ± 2.7</td>
<td>+39.9*</td>
<td>3.21 ± 0.14</td>
<td>+36.6*</td>
</tr>
<tr>
<td>SPH, 100 ( \mu )M</td>
<td>26.7 ± 2.0</td>
<td>+17.1</td>
<td>2.87 ± 0.20</td>
<td>+22.1</td>
</tr>
<tr>
<td>S1P, 1 ( \mu )M</td>
<td>32.0 ± 3.1</td>
<td>+40.4*</td>
<td>3.38 ± 0.46</td>
<td>+43.8*</td>
</tr>
<tr>
<td>S1P, 10 ( \mu )M</td>
<td>32.5 ± 3.1</td>
<td>+42.5*</td>
<td>3.29 ± 0.39</td>
<td>+40.0*</td>
</tr>
<tr>
<td>S1P, 20 ( \mu )M</td>
<td>29.3 ± 3.4</td>
<td>+28.4</td>
<td>3.01 ± 0.34</td>
<td>+28.1</td>
</tr>
<tr>
<td>SPH + DMS, 10 ( \mu )M + 20 ( \mu )M</td>
<td>23.8 ± 2.4</td>
<td>+4.4</td>
<td>2.46 ± 0.23</td>
<td>+4.7</td>
</tr>
<tr>
<td>DMS, 10 or 20 ( \mu )M</td>
<td>22.2 ± 1.5</td>
<td>-2.6</td>
<td>2.28 ± 0.03</td>
<td>-3.0</td>
</tr>
<tr>
<td>Bis-IM, 0.25 ( \mu )M</td>
<td>27.4 ± 1.4</td>
<td>+20.2</td>
<td>2.58 ± 0.12</td>
<td>+9.8</td>
</tr>
<tr>
<td>SPH + bis-IM, 10 ( \mu )M + 0.25 ( \mu )M</td>
<td>27.4 ± 3.4</td>
<td>+20.2</td>
<td>2.63 ± 0.14</td>
<td>+11.9</td>
</tr>
<tr>
<td>S1P + bis-IM, 10 ( \mu )M + 0.25 ( \mu )M</td>
<td>31.6 ± 3.4</td>
<td>+38.6*</td>
<td>3.04 ± 0.27</td>
<td>+29.4*</td>
</tr>
<tr>
<td>SPH, 10 ( \mu )M/Calc2+ free</td>
<td>23.2 ± 2.2</td>
<td>+1.8</td>
<td>2.44 ± 0.26</td>
<td>+3.8</td>
</tr>
<tr>
<td>S1P, 10 ( \mu )M/Calc2+ free</td>
<td>40.0 ± 4.1</td>
<td>+75.4*</td>
<td>4.27 ± 0.70</td>
<td>+81.7*</td>
</tr>
</tbody>
</table>

Values are means ± SE. EDL, extensor digitorum longus; \( t_{1/2} \), time necessary for 50% tension decay; SPH, sphingosine; S1P, sphingosine 1-phosphate; DMS, N,N-dimethylsphingosine; bis-IM, bis-indolylmaleimide. At least four experiments were performed for each condition. The final tension output refers to 6-min fatigue protocol. \(*P < 0.05, \dagger P < 0.01\) vs. control values.
not change the effect produced by bis-IM alone, suggesting that SPH action on EDL fatigue is not mediated by protein kinase C. In contrast, bis-IM was not able to alter the positive effect of S1P (Table 1).

Effects of S1P and SPH on the development of fatigue in Ca\(^{2+}\)-free solution. Several workers have demonstrated that S1P not only mobilizes Ca\(^{2+}\) from intracellular stores but also can facilitate entry of Ca\(^{2+}\) from extracellular medium (24, 43). Accordingly, we tested the action of this sphingolipid on EDL muscle fatigue produced in Ca\(^{2+}\)-free Ringer solution to evaluate whether S1P’s actions on fatigability were dependent on extracellular Ca\(^{2+}\). As Fig. 4 shows, S1P maintained its beneficial effect on fatigue development in Ca\(^{2+}\)-free solutions. The final force output and \(t_{1/2}\) values were similar to those obtained in the presence of S1P in normal Ringer solution and significantly higher (\(P < 0.05\)) than those produced by fatigued control muscles (Table 1). Surprisingly, the beneficial effects of SPH were completely abolished by the absence of extracellular Ca\(^{2+}\) (Fig. 4). The recovery was not significantly different from control values.

Effects of S1P and SPH on electrophysiological properties. We next evaluated whether S1P and SPH effects on fatigue development could be explained by the effects of sphingolipid mediators on the skeletal muscle AP. SPH is known to induce depolarization of cardiomyocytes, probably by acting on the DHPR (29, 44). S1P also appears to influence skeletal muscle membrane potential (4), an action that is in part receptor mediated. These data thus suggest another possible site of action of the sphingolipids during EDL muscle fatigue. Accordingly, skeletal muscle fiber RMP and directly evoked APs were studied in control and fatigued fibers in the absence or presence of either S1P or SPH (10 \(\mu\)M). As previously reported by others (for review, see Ref. 17), fatigue caused a significant fall in RMP and a decrease in AP amplitude and maximum rate of rise (Table 2). For muscles at rest, the addition of S1P or SPH did not affect the RMP or the AP amplitude, while the maximum rate of rise of the AP was decreased. Interestingly, the presence of S1P or SPH prevented the fatigue-induced decrease in AP amplitude, while it did not alter the maximum rate of AP rise (Table 2).

Endogenous SPH levels of mouse EDL. Next, we determined the concentration of SPH using HPLC in mouse EDL muscles at rest and after fatigue. The resting level of SPH was \(13.2 \pm 4.2\) nmol/g fresh tissue (\(n = 4\)), while fatigue caused a significant increase of endogenous SPH concentration to \(28.0 \pm 3.2\) nmol/g fresh tissue (\(n = 4\); \(P < 0.03\)), confirming previous observations that this lipid mediator is increased in fatigued rat muscles (14). The skeletal muscle endogenous level of S1P at rest is reported to be at least one order of magnitude lower than that of SPH (47).
membrane preparations isolated from mouse skeletal muscles. Because the binding of \(^{3}H\)ryanodine is dependent on the open state of the Ca\(^{2+}\) release channel (31), these data indicate that SPH acts as an inhibitor of the channel. The SPH concentration able to inhibit 50% of ryanodine binding (IC\(_{50}\)) was \(\sim 18 \mu M\). As also reported by others (42), S1P was without appreciable effects on \(^{3}H\)ryanodine binding (Fig. 5).

**DISCUSSION**

The present work shows that the exogenous administration of S1P and SPH individually caused the significant slowing of fatigue. However, our data show that SPH action depends on its conversion to S1P. Hence, this report is the first to suggest that S1P may exert beneficial effects on the promotion of fatigue. Normally, S1P produces important effects on the AP. In fact, our results show that S1P action depends on its conversion to S1P. Hence, this report is the first to suggest that S1P may exert beneficial effects on the promotion of fatigue. However, our data show that SPH action depends on its conversion to S1P. Hence, this report is the first to suggest that S1P may exert beneficial effects on the promotion of fatigue.

Table 2. Effects of SPH and S1P on electrophysiological properties of mouse EDL muscle

<table>
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<tr>
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<th>Control (n = 138 and 3)</th>
<th>Control + SPH (n = 86 and 3)</th>
<th>Control + S1P (n = 34 and 3)</th>
<th>Fatigued control (n = 50 and 3)</th>
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<td>78.0 ± 0.9</td>
<td>77.0 ± 1.6</td>
<td>76.5 ± 1.3 (^*)</td>
<td>77.7 ± 1.5</td>
<td>75.2 ± 1.6</td>
<td></td>
</tr>
<tr>
<td>Amplitude, mV</td>
<td>96.1 ± 1.1</td>
<td>93.9 ± 1.3</td>
<td>76.2 ± 2.2 (^*)</td>
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<td>MRR, V/s</td>
<td>299.9 ± 7.6</td>
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Values are means ± SE. Numbers in parentheses refer to number of fibers and animals, respectively. Resting membrane potential (RMP), amplitude, and maximum rate of rise (MRR) of action potentials of single fibers in control and after 30 min of incubation in either 10 \(\mu M\) SPH or 10 \(\mu M\) S1P. \(^*\)P < 0.05 vs. resting control values; \(^\dagger\)P < 0.05 vs. fatigued muscle control values.

We recently reported that a substantial amount of SPH produced by cardiomyocytes is released into the extracellular medium and rapidly phosphorylated to S1P (7). In the current study, we discovered that when muscle fatigue was produced in the presence of the SK inhibitor, DMS, the effects produced by SPH were completely abolished. Additional support for a role of SK is the finding that SPH is not effective in retarding fatigue when muscles do not have Ca\(^{2+}\) in the extracellular fluid. It is known that SK activity is dependent on Ca\(^{2+}\) (27, 35). We suggest that a “rheostat” mechanism operates in skeletal muscle cells as it does in other cell types (27, 37) whereby S1P is generated from SPH directly at the extracellular surfaces by a Ca\(^{2+}\)-dependent SK localized in the muscle cell basement membrane as suggested by observations in C\(_{2}C\(_{12}\) myoblasts (30).

An interesting result is that exogenously applied S1P had the same effect in either the presence or absence of external Ca\(^{2+}\), suggesting that S1P does not act by mobilizing extracellular Ca\(^{2+}\). It has consistently been reported that in skeletal muscle, exogenous S1P induces Ca\(^{2+}\) transients independent of L-type channels (4). Thus S1P appears to act on the extracellular side by activating cell surface receptors and downstream signaling pathways, leading to an overall beneficial effect on decreasing the development of fatigue. Normally, S1P produces important intracellular actions by activating specific cell surface G protein-coupled receptors (25, 43), and it is important to note that skeletal muscle possesses these receptors (4). SK is activated by several signals (35, 37, 43), among which is the depletion of endoplasmic reticulum Ca\(^{2+}\) stores (24) and Ca\(^{2+}\) mobilization (48), events that are known to occur during sustained low-frequency fatigue stimulation (1, 3, 17, 50).

We also have investigated whether SPH may exert an intracellular action by modulating the activity of protein kinase C, a well-established target for SPH (20). However, SPH does not seem to produce the positive effects on fatigue through inhibition of protein kinase C, because the moderate, nonsignificant action produced by the cell-permeable inhibitor of the kinase, bis-IM, was not augmented by the addition of S1P. The data presented herein do not exclude the possibility that exogenously added SPH may affect the closure of the ryanodine receptor, a very potent effect of SPH that is shown in Fig. 5. Finally, bis-IM does not influence the effects of S1P on fatigue, suggesting that the signaling pathway activated by S1P does not include protein kinase C.

A possible target of S1P and SPH action could be found in the effects of the lipids on the AP. In fact, our results show that S1P and SPH were able to counteract the fatigue-dependent decrease in AP amplitude. The prolonged duration of AP produced by the two lipids likely results in an increase in the

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</table>

Values are means ± SE. Numbers in parentheses refer to number of fibers and animals, respectively. Resting membrane potential (RMP), amplitude, and maximum rate of rise (MRR) of action potentials of single fibers in control and after 30 min of incubation in either 10 \(\mu M\) SPH or 10 \(\mu M\) S1P. \(^*\)P < 0.05 vs. resting control values; \(^\dagger\)P < 0.05 vs. fatigued muscle control values.
useful voltage-dependent SR Ca\(^{2+}\) release, resulting in a beneficial effect of the lipids in the attempt to counteract the reduction of tension output. Moreover, because it has been shown that AP propagation into the T tubules is impaired during fatigue, causing poor activation of central myofibers (16), the action of S1P and SPH in causing prolongation of the active state could have a beneficial result by favoring the recruitment of all contractile elements. It has consistently been demonstrated that SPH affects the electrophysiological properties of voltage-gated Na\(^{+}\) channels in isolated cardiac myocytes (44) by decreasing the maximum current carried (peak current) and increasing the opening state. This should result in a reduction of the AP maximum rate of rise and an increase in AP amplitude. Moreover, S1P has been demonstrated to favor the receptor-mediated activation of Cl\(^{-}\) currents, resulting in the depolarization of keratinocytes (49), and also to cause depolarization of oligodendrocytes (22), while it did not have an effect on the L-type Ca\(^{2+}\) currents in an insulinoma cell line (23).

The present work also shows that SPH is an endogenous component of mouse skeletal muscle, with concentrations similar to those reported in rabbit, rat, and pig skeletal muscle (5, 6, 12, 34, 38, 42). Moreover, during sustained contractile activity, total endogenous levels of SPH increased in whole muscle homogenates. Similar results were observed previously in rat skeletal muscle (14). The major source of SPH in cells is sphingomyelin, which is sequentilly hydrolyzed by sphingomyelinase and ceramidase to form ceramide and SPH, respectively. The rise in SPH levels (and subsequent S1P production by the kinase) during fatigue may be due to the activation of the lysosomal acid sphingomyelinase as a result of the decline in intracellular pH that accompanies fatigue (17, 51). An additional increase in the level of SPH could be the consequence of the stimulation of a neutral sphingomyelinase and cell surface-specific receptors by circulating TNF-\(\alpha\), IFN-\(\gamma\), and IL-1\(\beta\), known activators of the sphingomyelinase pathway that have been shown to increase during sustained exercise (8, 26; for review, see Ref. 36). We also report that the endogenous SPH elevation may affect fatigue development by direct actions on the SR Ca\(^{2+}\) release channel. It is known that SPH is an endogenous modulator of the ryanodine receptor channel (5, 6, 12, 34, 38, 42), altering ryanodine binding to the receptor (5, 38, 42) and its gating properties at the single-channel level (34). We hypothesized in the present study that the intracellular actions of SPH on coupling also may be mitigated by the rheostat mechanism hypothesized herein, whereby the SPH is converted to S1P, which is known to have no influence on the ryanodine receptor (42).

In conclusion, present results indicate that SPH exerts a protective effect on the development of fatigue through mechanisms mostly accounted for by SPH’s conversion into S1P. The protective effects of these important signaling lipids is most likely attributable to their actions on the skeletal muscle AP; however, the abundant presence of S1P receptors on the skeletal muscle sarcolemma (4) suggests that G protein-coupled receptors may mediate S1P actions. Our laboratory is currently investigating this possibility.

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REFERENCES

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