Regulated secretion is a central issue for the specific function of many cells; for instance, mammalian sperm acrosomal exocytosis is essential for egg fertilization. Sphingosine 1-phosphate is a bioactive sphingolipid that regulates crucial physiological processes. Here we report that this lipid triggers acrosomal exocytosis in human sperm by a mechanism involving a Gβγ-coupled receptor. Real-time imaging showed a remarkable increase of cytosolic calcium upon activation with sphingosine 1-phosphate and pharmacological experiments indicate that the process requires extracellular calcium influx through voltage and store-operated calcium channels and efflux from intracellular stores through inositol 1,4,5-trisphosphate-sensitive calcium channels. Sphingosine 1-phosphate-induced exocytosis requires phospholipase C and protein kinase C activation. We investigated possible sources of the lipid. Western blot indicates that sphingosine kinase 1 is present in spermatozoa. Indirect immunofluorescence showed that phorbol ester, a potent protein kinase C activator that can also trigger acrosomal exocytosis, redistributes sphingosine kinase 1 to the acrosomal region. Functional assays showed that phorbol ester-induced exocytosis depends on the activation of sphingosine kinase 1. Furthermore, incorporation of 32P to sphingosine demonstrates that cells treated with the phorbol ester increase their sphingosine kinase activity that yields sphingosine 1-phosphate. We present here the first evidence indicating that human spermatozoa produce sphingosine 1-phosphate when challenged with an exocytic stimulus. These observations point to a new role of sphingosine 1-phosphate in a signaling cascade that facilitates acrosome reaction providing some clues about novel lipid molecules involved in exocytosis.

Sphingosine 1-phosphate (S1P) is a bioactive sphingolipid metabolite that regulates a variety of cellular processes. For years, researchers thought that the biological effects of S1P were due to its actions as an intracellular second messenger. However, surface receptors have recently been identified, and, since then, many downstream effects of S1P receptor activation have been demonstrated (1). S1P binds to five G-protein-coupled receptors of the lysophospholipid receptor family. These receptors were initially called endothelial differentiation gene receptors and have been renamed as sphingosine 1-phosphate receptors (S1PRs). Fundamental differences in signaling through S1PR relate to variations in G-protein coupling (for a review see Ref. 2). Upon binding to its receptors, extracellular S1P generates diverse cellular responses, including angiogenesis, cardiac development, immune activation, cell motility, and neurite extension (3–5). Intracellularly, S1P mobilizes Ca2+, regulates cell growth, and suppresses apoptosis (6). Recently, Anelli et al. (7) demonstrated that cerebellar granule cells and astrocytes release newly synthesized S1P into the extracellular medium. This finding defines a role for this lipid as an autocrine/paracrine mediator. S1P also facilitates glutamate secretion in hippocampal neurons and may be involved in mechanisms underlying regulation of synaptic transmission (8).

Whether acting intracellularly or extracellularly, S1P is generated through phosphorylation of sphingosine by sphingosine kinase (SK). Two types of mammalian SKs (SK1 and SK2) have been characterized so far (9; for a review see Ref. 10), both of which are primarily cytosolic proteins. Production of S1P through increased SK cellular activity has become the subject of much interest because of the involvement of S1P in such diverse and vital cellular processes. Although SK1 and different types of

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1 The abbreviations used are: S1P, sphingosine 1-phosphate; SK, sphingosine kinase; S1PR, sphingosine 1-phosphate receptor; VOCC, voltage-operated Ca2+ channel; PLC, phospholipase C; IP3, inositol 1,4,5-trisphosphate; DAG, diacylglycerol; SOCC, store-operated Ca2+ channel; PKC, protein kinase C; SNARE, soluble NSF attachment protein receptors; HTF, human tubal fluid medium; SKI, sphingosine kinase inhibitor; 2-APB, 2-aminoethoxy-diphenylborate; BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis; PMMA, 4-phorbol 12-myristate 13-acetate; GTPγS, guanosine 5'-O-3-(thio)triphosphate; GDPβS, guanosine 5’-O-2-(thio)dirophosphate; AR, acrosome reaction; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; PSA, P. sativum agglutinin; Tricine, N-12-hydroxy-1,1-bis(hydroxymethyl)ethylglycine; Pg, progesterone; DMS, N,N-dimethylsphingosine.
S1PRs have been detected in mouse sperm (11), the physiological role of S1P and the enzyme that catalyzes its production remains unknown.

A human spermatozoan is a terminally differentiated and extremely specialized cell that has only one function, to fertilize an oocyte and pass on the genetic information contained in its nucleus. In the proximity of the egg, progesterone and zona pellucida glycoproteins (such as ZP3) stimulate mammalian sperm to release the content of the acrosomal granule, a key event in fertilization. The acrosome is a large membrane-limited granule that overlies the nucleus of the mature spermatozoon (12). Acrosomal exocytosis is an all-or-nothing event that involves the opening of multiple fusion pores between the outer acrosomal membrane and the plasma membrane. This Ca\(^{2+}\)-regulated exocytosis is known as the sperm acrosome reaction. Upon activation, the opening of voltage-operated Ca\(^{2+}\) channels (VOCCs) in the plasma membrane, through a still not well defined transduction mechanism, generates a transient increase in cytosolic Ca\(^{2+}\) (13). The current hypothesis is that this Ca\(^{2+}\) increase activates a phospholipase C (PLC), releasing inositol 1,4,5-trisphosphate (IP\(_3\)). This second messenger opens IP\(_3\)-sensitive Ca\(^{2+}\) channels in the membrane of the acrosome. The emptying of the acrosomal store triggers the opening of store-operated Ca\(^{2+}\) channels (SOCs) causing the second sustained Ca\(^{2+}\) increase, which initiates the acrosomal exocytosis (14–16). The molecular events involved in the release of the acrosomal content have been intensively studied in our laboratory and those of others (17).

Although S1P is involved in neurotransmitter secretion, its mechanism of action remains unelucidated. Thus we decided to use spermatozoa as a model to study the role of S1P in exocytosis. The spermatozoan provides an ideal system to address this question, because it is transcriptionally and translationally inactive, does not possess rough endoplasmic reticulum or Golgi apparatus, and undergoes no endocytosis or intracellular transport; allowing us to study the exocytic process in isolation. The purpose of this study is to determine whether S1P is involved in the exocytic process and, if so, to unveil the signaling network modulated by this lipid. We have also attempted to elucidate how the production of this signaling molecule is regulated. Here we demonstrate that S1P, through its autocrine/paracrine action, triggers acrosomal exocytosis. We also present the first evidence suggesting that S1P is exerting its exocytic effect through G\(_\text{C}\)-coupled membrane receptors, increasing the intracellular calcium concentration. Furthermore, we document the presence of SK1 in human sperm cells and its involvement in acrosomal exocytosis. Our results indicate that a SK1/S1P signaling pathway is activated during the acrosome reaction. This discovery has important implications for new molecules that may be involved in exocytosis, and thus may lead to a better understanding of how human spermatozoa undergo the acrosome reaction and fertilize eggs.

**EXPERIMENTAL PROCEDURES**

*Reagents*—Human tubal fluid medium (HTF) contains 5.94 g/liter NaCl, 0.35 g/liter KCl, 0.05 g/liter MgSO\(_4\)-7H\(_2\)O, 0.05 g/liter KH\(_2\)PO\(_4\), 0.3 g/liter CaCl\(_2\)-2H\(_2\)O, 2.1 g/liter NaHCO\(_3\), 0.51 g/liter d-glucose, 0.036 g/liter sodium pyruvate, 2.39 g/liter sodium lactate, 0.06 g/liter penicillin, 0.05 g/liter streptomycin, and 0.01 g/liter phenol red. S1P, sphingosine, and dimethylsphingosine were from Avanti Polar Lipids, Inc. (Alabalber, AL). [\(\gamma\)-\(^{32}\)P]ATP was from PerkinElmer Life Sciences. Nifedipine, U73122, U73343, chelerythrine, xestospongin C, 2-[\(\gamma\)-hydroxyanilino]-4-[(\(\mu\)-chlorophenyl)thiazole, HCl, sphingosine kinase inhibitor (SKI), edelfosine, and 2-aminoethoxydiphenylborate (2-APB) were from Calbiochem (Merck Quimica Argentina SAIC, Buenos Aires, Argentina). TLC aluminum sheets silica gel 60 were from Merck KGaA (Darmstadt, Germany). BAPTA, pluronic acid, and Flu-o-3 AM were from Molecular Probes (Carlsbad, CA). Phorbol 12-myristate 13-acetate (PMA), 4α-phorbol 12-myristate 13-acetate (αPMA), 4-methyl-4’-[3,5-bis(trifluoromethyl)-1H-pyrazol-1-yl]-1,2,3-thiadiazole-5-carboxanilide (YM-58483), and 1-[\(\beta\)-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl]-1H-imidazole (SKF-96365), verapamil, pertussis toxin, and progesterone were from Sigma-Aldrich. A23187 was from Alomone Laboratories Ltd. (Jerusalem, Israel). Albumin and fluorescein isothiocyanate-coupled *Pisum sativum* lectin (FITC-PSA) were from ICN (Eurolab SA, Buenos Aires, Argentina). Horseradish peroxidase-coupled anti-rabbit antibody and Cy3-labeled goat anti-rabbit antibody were from Jackson Immunochemicals (Sero-immuno Diagnostics, Inc. Tucker, GA). Recombinant streptolysin O was obtained from Dr. Bhakdi (University of Mainz, Mainz, Germany). Rabbit polyclonal anti-human sphingosine kinase 1 antibody was prepared by the Medical University of South Carolina antibody facility as explained by Johnson et al. (18), and recombinant sphingosine kinase was obtained as described by Stahelin et al. (19). R-Rab3A-GDP\(\beta\)S and R-Rab3A-GTP\(\gamma\)S were generously provided by Cecilia I. Lopez from Instituto de Histología y Embriología, Consejo Nacional de Investigaciones Científicas y Técnicas, School of Medicine, Cuyo National University, Mendoza, Argentina.

**Acrosome Reaction Assays**—Human semen samples were obtained from healthy donors. Highly motile spermatozoa were recovered following a swim-up separation for 1 h in HTF medium supplemented with 5 mg/ml of bovine serum albumin when indicated (capacitating medium) at 37 °C in an atmosphere of 5% CO\(_2\)/95% air. Cell concentration was then adjusted to 5–10 \(\times\) \(10^6\) sperm/ml with HTF, followed by incubation (capacitating conditions) for at least 2 h. In some experiments, sperm were permeabilized as described by Yunes et al. (20) and resuspended in ice-cold sucrose buffer (250 mM sucrose, 20 mM HEPES-K, 0.5 mM EGTA, 2 mM dithiothreitol, pH 7). Cells were treated with inhibitors or stimulants immediately after capacitation as indicated in the legends to the figures. At the end of the experiment, 10–15 \(\mu\)l aliquots from each condition were spotted on slides and fixed/permeabilized in ice-cold methanol and washed four times with distilled water. We evaluated the acrosomal status by staining sperm cells with FITC-PSA according to Mendoza et al. (21). At least 300 cells were scored using a Nikon microscope equipped with epifluorescence optics. Negative (no stimulation) and positive controls (stimulated with calcium in permeabilized cells and with A23187 or progesterone in intact sperm) were included in all experiments. For each experiment, acrosomal exocytosis indexes were calculated by subtracting the number of reacted...
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spermatozoa in the negative control (range 3.6–23.7%) from all values and expressing the resulting values as a percentage of the acrosome reaction (AR) observed in the positive control (range 15–39.1%). The average difference between positive and negative control was 14% (experiments where the difference was <10% were discarded).

**Single Cell [Ca\(^{2+}\)]\(_i\) Measurements—** Motile cells were adjusted to a concentration of 5–10 × 10\(^6\) cells/ml and incubated with 2 μM Fluo-3 AM and 0.02% pluronic acid at 37 °C during 30 min. Cells were washed once with human sperm medium (HSM): 120 mM NaCl, 4 mM KCl, 2 mM CaCl\(_2\), 15 mM NaHCO\(_3\), 1 mM MgCl\(_2\), 10 mM HEPES, 5 mM d-glucose, 1 mM sodium pyruvate, 10 mM L-(+)-lactic acid adjusted to pH 7.4. [Ca\(^{2+}\)]\(_i\) imaging was performed in the same medium. Sperm were immobilized in poly-l-lysine-coated glass, which were mounted on a chamber and placed on the stage of an inverted microscope equipped with a charge-coupled device cool SNAP camera operated with Andor IQ software. Fluo-3 AM was excited with a stroboscopic light-emitting diode-based fluorescence illumination system as previously described (22). Fluorescence measurements were performed by collecting images every 500 ms using a filter cube with the following band widths: excitation 450–490 nm, dichroic mirror 505 nm, and emission 520–560 nm and a Plan Apo 60× objective to estimate the protein loads in each lane. Nonspecific binding sites were blocked with 5% skim milk dissolved in washing buffer. Detection was accomplished with Western Light-Imaging Chemiluminescence Reagent Plus (PerkinElmer Life Sciences). The images of the bands were obtained using a Luminous Image Analyzer LAS-4000 (Fujifilm). When specified, the blots were reprobed with an anti-synaptotagmin VI antibody to estimate the protein loads in each lane. Nonspecific binding sites were blocked with 5% skim milk dissolved in washing buffer and incubated for 2 h with a rabbit polyclonal anti-SK1 IgG (1 μg/ml) in the blocking solution for 2 h at room temperature. Horseradish peroxidase-conjugated goat anti-rabbit-IgG was used as a secondary antibody (1:2000 in 1% fat-free milk in washing buffer, 60 min at room temperature). Excess first and second antibodies were removed by washing the nitrocellulose membranes 5 × 10 min in washing buffer. Detection was accomplished with Western Light-Imaging Chemiluminescence Reagent Plus (PerkinElmer Life Sciences). The images of the bands were obtained using a Luminous Image Analyzer LAS-4000 (Fujifilm). When specified, the blots were reprobed with an anti-synaptotagmin VI antibody to estimate the protein loads in each lane. Nonspecific binding sites were blocked with 5% skim milk dissolved in washing buffer and incubated for 2 h with a rabbit polyclonal anti-synaptotagmin IgG (Abcam, ab24250) diluted 1:500 in 1% fat-free milk in washing buffer. The secondary antibody was used as described for the anti-SK1 Western blot.

**Indirect Immunofluorescence and Confocal Microscopy—** Capacitated sperm cells were adjusted to 7 × 10\(^6\) cells/ml in HTF medium supplemented with 0.5% albumin and kept without any treatment or incubated with 100 μM 2-APB for 15 min (to prevent exocytosis) and then with 200 nM PMA or 200 nM αPMA for 15 min at 37 °C. To determine SK1 localization, sperm cells were spotted on poly-l-lysine-covered slides and fixed in 2% paraformaldehyde in PBS for 10 min at room temperature. After fixation, sperm were incubated in 50 mM glycine-PBS for 30 min at room temperature and permeabilized with 1% Triton X-100 for 10 min. Sperm cells were then blocked for 30 min in 5% bovine serum-PBS. Spermatozoa were labeled with a rabbit anti-SK1 antibody (10 μg/ml in 1% bovine serum-PBS) overnight at 4 °C, followed by a Cy3-labeled anti-rabbit IgG as a secondary antibody (1:600 in 1% bovine serum-PBS) for 1 h at room temperature. Slides were washed with PBS between incubations. When indicated, 10 μg/ml of anti-SK1 antibody was preincubated with the recombinant protein (0.6 μg/ml) against which the anti-SK1 antibody was raised. Finally, cells were fixed for 1 min in cold methanol and stained with FITC-PSA (40 min at room temperature, 50 μg/ml in PBS) and washed with distilled water for 20 min at 4 °C. After this procedure, slides were mounted in 1% propyl-gallate/50% glycerol in PBS. Sperm cells were analyzed by confocal microscopy using an Olympus FluoView™ FV1000 confocal microscope (Olym-
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FIGURE 1. S1P triggers acrosomal exocytosis in intact human spermatozoa. A, after swim-up in HTF (5 mg/ml bovine serum albumin) at 37°C, 5% CO2, sperm were incubated for an additional 2 h under capacitating conditions. Intact and streptolysin O-permeabilized sperm were used. Sperm were treated with increasing concentrations of S1P (0–200 nM) for 15 min at 37°C in 5% CO2. As positive control, permeabilized spermatozoa were stimulated with 10 μM free Ca2+ (black bar) and intact sperm with 10 μM A23187 (gray bar). Sperm were fixed and acrosomal exocytosis was evaluated by FITC-PSA binding with at least 300 cells per condition scored. B, an aliquot of a sperm sample was subjected to swim-up in HTF medium under non-capacitating conditions (37°C 5% CO2, without bovine serum albumin). Another aliquot was processed in capacitating conditions: swim-up in HTF (5 mg/ml bovine serum albumin, 37°C, 5% CO2). Cells recovered from swim-up were incubated for an additional 2 h under non-capacitating or capacitating conditions. Sperm were treated or not (control, Co) with 15 μM progesterone (Pg) or 100 nM S1P for 15 min at 37°C 5% CO2. Acrosomal exocytosis was evaluated as explained under “Experimental Procedures.” The data represent the mean ± S.E. of at least four independent experiments. The means of groups NON CAP and CAP were compared with the corresponding control using Dunnett’s test and classified as non-significant (ns, p > 0.05), or significant (*, p < 0.01 or **, p < 0.001).

Sphingosine Kinase Activity—Sphingosine kinase 1 activity in permeabilized spermatozoa was determined as described in Halt et al. and Anelli et al. (25, 26). Sperm cells (50 × 10⁶) were washed with cold PBS after permeabilization and resuspended in SK1 buffer (20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.5 mM deoxypyridoxine, 15 mM NaF, 1 mM β-mercaptoethanol, 1 mM sodium orthovanadate, 40 mM β-glycerophosphate, 0.4 mM phenylmethylsulfonyl fluoride, 10% glycerol, 0.5% Triton X-100, 10 mM MgCl2, and Complete protease inhibitors). Spermatozoa were incubated in 100 μl of SK1 buffer containing sphingosine (50 μM, delivered in 4 mg/ml fatty acid-free bovine serum albumin) and [γ-32P]ATP (5 μCi) for 30 min at 37°C. As a positive control, HeLa cells (30 μg of proteins) were incubated 15 min at 37°C with 200 nM PMA, washed with cold PBS, and harvested in SK1 buffer. A negative control was performed without sphingosine addition. The reaction was stopped with 10 μl of 1 N HCl and 400 μl of chloroform/methanol/HCl (100:200:1, v/v/v). Subsequently, 120 μl of chloroform and 120 μl of 2 M KCl were added, and samples were centrifuged at 3000 × g for 10 min. 200 μl of the organic phase was transferred to new glass tubes and dried. Samples were resuspended in chloroform/methanol/HCl (100:100:1, v/v/v). Lipids were then resolved on TLC plates using 1-butanol/methanol/acetic acid/water (8:2:1:2, v/v/v) as solvent system and visualized by autoradiography. The same experiment was performed using SK1, a specific SK1 inhibitor, instead of DMS (supplemental Fig. 3B).

Statistical Analysis—Data were evaluated by using one-way analysis of variance. Conditions used for data normalization (0 and 100%) were not included in the analysis. We performed the Dunnett post hoc test for comparison of the means with a control condition. When indicated, a Student’s t test was used. Differences were considered significant at the p < 0.05 level.

RESULTS
Sphingosine 1-Phosphate Triggers Acrosomal Exocytosis in Intact Human Spermatozoa—Diverse lipid molecules trigger the AR using signaling pathways similar to those used by ZP3 (27, 28). To determine if S1P is involved in the AR we evaluated the exocytic response of sperm cells challenged with increasing concentrations of S1P. We found that exogenous S1P elicited acrosomal exocytosis by itself in a dose-dependent manner in intact sperm (Fig. 1A). The percentage of cells that underwent AR increased with the S1P concentration reaching a maximum at 100 nM (ED₅₀ 11 nM). Incubation of non-permeabilized sperm with 100 nM S1P triggers the AR as efficiently as the calcium ionophore A23187 used as positive control for intact sperm (Fig. 1A, A23187). Interestingly, S1P did not elicit AR in permeabilized sperm (Fig. 1A). As expected, permeabilized cells underwent exocytosis when were challenged with Ca²⁺ (Fig. 1A, Ca²⁺). These results suggest that S1P triggers exocytosis by a receptor-mediated mechanism rather than by an intracellular second messenger pathway. Additionally, our results support the idea of a ligand-receptor effect of S1P considering that the literature describes that endothelial differentiation gene receptor-dependent actions of S1P are elicited by submicromolar concentrations of S1P (29, 30), and intracellular effects are mimicked by addition of micromolar concentrations of S1P (31, 32). S1P did not affect sperm viability (control, 93.17% ± 0.87; mean ± S.E., n = 5) or motility (control, 89.26% ± 2.28; 100 nM S1P, 90.56% ± 2.21). In conclusion, S1P triggers acrosomal exocytosis and has no toxic effect on cells.

Capacitation in the female genital tract is a complex process that prepares the sperm for acrosomal exocytosis and is a hallmark of physiological AR. To unveil the role of capacitation on S1P exocytotic response, sperm were incubated under capacitating (HTF supplemented with 5 mg/ml bovine serum albumin) or non-capacitating (HTF without albumin) conditions and challenged with S1P. As shown in Fig. 1B, only capacitated cells responded to S1P (CAP+SIP and NON CAP+SIP). We also treated sperm cells with 15 μM progesterone to assess their capacitation status (33, 34) (NON CAP+Pg and CAP+Pg). These results suggest that capacitating alterations during the passage of spermatozoa through the female genital tract are crucial for S1P-induced exocytosis.

S1P-induced Exocytosis Requires Extracellular Calcium Influx and Acrosomal Calcium Efflux—We hypothesized that S1P activates exocytosis by a receptor-mediated mechanism
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FIGURE 2. S1P-induced exocytosis requires extracellular calcium influx and acrosomal calcium efflux. A, capacitated spermatozoa were incubated at 37 °C for 15 min without any stimulus (control, Co) or treated with 15 μM progesterone (Pg), 100 nM S1P, or 1 μM cell-permeant prenylated Rab3A activated with GTPγS (R-Rab3A). When indicated, cells were treated before the stimulus with 5 mM BAPTA (BAPTA→Pg, BAPTA→SIP, and BAPTA→R-Rab3A) at 37 °C for 15 min. The data represent the mean ± S.E. of at least four independent experiments. The means of groups with BAPTA were compared with the corresponding group without the chelator using Student’s t-test and classified as significant (**, p < 0.001) or non-significant (ns, p > 0.05). B, capacitated sperm were incubated without any treatment (control, Co) or treated with 10 μM nifedipine, 100 μM verapamil, 0.1 mM NiCl2, 1 μM YM-58483, 50 μM SKF-96365, 100 μM 2-APB, or 1.1 μM xestospongin C (Xc) for 15 min at 37 °C. When specified, acrosomal exocytosis was activated by adding 100 nM S1P, and the incubation continued for an additional 15 min (Nifedipine→SIP, Verapamil→SIP, NiCl2→SIP, YM-58483→SIP, SKF-96365→SIP, 2-APB→SIP, and Xc→SIP). A control with the Ca2+ ionophore A23187 (10 μM) was included. Sperm were then fixed, and acrosomal exocytosis was measured as described under “Experimental Procedures.” The data represent the mean ± S.E. from three to 14 independent experiments and were normalized as described under “Experimental Procedures.” Dunnett’s test was used to compare the means of all groups against the S1P-stimulated condition in the absence of inhibitors. Significant differences at p < 0.001(***) are indicated for each bar.

and wondered if, like known physiological inducers such as progesterone and ZP3, S1P-triggered exocytosis requires extracellular Ca2+ influx. To resolve this issue, we incubated capacitated sperm cells with 5 mM BAPTA to chelate extracellular calcium before the S1P stimulus. BAPTA abolished the stimulatory effect of S1P suggesting that calcium influx is required for S1P-induced AR (Fig. 2A, compare S1P and BAPTA→SIP).

Progesterone-induced AR also depends on calcium influx (35) and Fig. 2A (Pg and BAPTA→Pg). In contrast, Lopez et al. (35) have demonstrated that membrane-permeant Rab3A initiated exocytosis when prenylated and activated with GTP. This small GTPase is involved in a late event of the fusion process and triggers the AR regardless of the extracellular Ca2+ concentration. Thus, we used activated and prenylated Rab3A as a positive control (Fig. 2A, R-Rab3A and BAPTA→R-Rab3A). These results indicate that Ca2+ influx is a prerequisite for S1P-triggered exocytosis.

To elucidate if S1P induces Ca2+ influx and thus the AR through opening VOCCs and SOCCs, we treated the cells with specific calcium channels blockers. Nifedipine is a dihydropyridine Ca2+ antagonist of L-type channel activity (36). Preincubation of sperm cells with 10 μM nifedipine for 15 min before the S1P stimulus completely abrogated S1P-induced exocytosis (Fig. 2B, S1P and Nifedipine→S1P). Verapamil is an arylalkylamine Ca2+ antagonist that acts mainly by inhibiting L-type high voltage-activated Ca2+ currents (37). Pretreatment of human spermatozoa with 100 μM verapamil efficiently blocked S1P-triggered AR (Fig. 2B, Verapamil→SIP, Ni2+, an inhibitor of T-type Ca2+ channel activity (36, 38), also abrogated S1P-induced exocytosis when added to intact sperm cells 15 min before the stimulus (Fig. 2B, S1P and NiCl2→S1P). On the other hand, tri- or divalent metals such as La3+ and Ni2+ have been described as SOCC blockers (38, 39). Thus, to assess if SOCC opening is required for the S1P-induced AR we used potent specific inhibitors of these channels: the pyrazole derivative, YM-58483 (40), and SKF-96365 (41, 42). Both YM-58483 (1 μM) and SKF-96365 (50 μM) strongly inhibited S1P-triggered exocytosis (Fig. 2B, compare S1P, YM-58483→S1P, and SKF-96365→S1P) corroborating that Ca2+ influx through SOCCs is required for the membrane fusion induced by S1P. Together, these results suggest that the signal transduction cascade triggered by S1P leads to the opening of VOCCs and SOCCs.

To determine if S1P-elicited exocytosis requires acrosomal calcium efflux we used specific IP3-sensitive calcium channels inhibitors (2-APB and xestospongin C). Both inhibitors blocked S1P-triggered secretion (Fig. 2B, Xc→S1P and 2-APB→S1P) confirming that S1P leads to calcium release from intracellular stores and, indirectly imply the involvement of a downstream PLC in the signal transduction cascade triggered by S1P.

In summary, these results indicate that S1P-induced exocytosis requires Ca2+ influx from the extracellular medium through VOCCs and SOCCs and Ca2+ release from intracellular stores (likely the acrosome) through IP3-sensitive Ca2+ channels, supporting the notion that S1P governs signaling pathways related to the opening of Ca2+ channels comparable to ZP3 and progesterone.

S1P Increases Intracellular Calcium in Live Sperm—The pharmacological inhibition of the AR shown in Fig. 2B implies that S1P leads to calcium channel activation in sperm cells. To directly assess whether S1P addition increases intracellular Ca2+ in live spermatozoa, sperm cells were loaded with the Ca2+ indicator Fluo-3 AM dye, and Ca2+ changes were measured in single cell experiments. We observed that ~45% of the cells underwent a [Ca2+]i rise when stimulated with S1P during the experiment. Fig. 3A and supplemental Fig. 1 show single cell [Ca2+]i images of human sperm responding to 200 nM S1P. Representative traces of the S1P-induced fluorescence changes are shown in Fig. 3B. Similar results were observed when the experiment was repeated with other sperm batches (Fig. 3C). In summary, these results demonstrate that exogenous S1P increases intracellular calcium in the human sperm head region.

S1P Triggers Exocytosis through a Gα1-Coupled Receptor—Sperm cells contain different types of G-proteins, including G11 and G12 (43). Pertussis toxin catalyzes the ADP ribosylation of α subunits belonging to G1 at a cysteine residue near the C termi-
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FIGURE 3. S1P causes a [Ca\(^{2+}\)] \(_i\) increase in human sperm. Capacitated human sperm recovered after swim-up were loaded with Fluo-3 AM (2 \(\mu\)M) in HSM. The fluorescence intensity was visualized before and after S1P addition as described under “Experimental Procedures.” A, representative images of Fluo-3 AM-loaded human sperm before (0, 8, and 16 s) and after (20, 26, 32, 53, and 59 s) the application of 200 nm S1P. The color bar shows fluorescence intensity after background subtraction. B, representative traces of five individual human sperm. The plots indicate the increase in fluorescence intensity in response to 200 nm S1P addition. Fluorescence is expressed as ([Flu3] \(_i\) - Flu3) \(_i\) versus time. C, the increase of fluorescence upon stimulation is shown as the average of all individual human sperm analyzed (\(n = 101\)). Error bars represent the mean \(\pm\) S.E. Note: approximately 45% of the cells responded to S1P addition with an intracellular calcium increase.

Calcium rise is shown clearly in the movies included in supplemental Fig. 1 (200 nm S1P) and supplemental Fig. 2 (pre-incubated for 10 min with 100 ng/ml pertussis toxin before addition of 200 nm S1P). Taken together, these data support the notion that S1P triggers human sperm acrosomal exocytosis through the activation of a G\(_{11}\)-coupled receptor.

S1P-induced Acrosomal Exocytosis Requires PLC, PKC Activities, and Rab3A—The early events in ZP3-induced signal transduction in sperm include the opening of T-type low voltage-activated calcium channels, resulting in a transient calcium influx (50) and the activation of the heterotrimeric G-proteins G\(_{11}\) and G\(_{12}\). These initial responses produce an activation of a PLC (49, 51), which hydrolyzes phosphatidylinositol 4,5-bisphosphate leading to the production of diacylglycerol (DAG) and IP\(_3\). These changes, in addition to others, result in a sustained calcium influx that directly drives exocytosis (52). To test if S1P is activating a signal transduction cascade involving the activity of PLC, we used the PLC thiol-reactive inhibitor U73122 (53). Fifteen \(\mu\)M U73122 completely abolished S1P-induced exocytosis (Fig. 5, U73122\(\rightarrow\)S1P), whereas the inactive analogue U73343 was not able to inhibit S1P-induced AR (Fig. 5, U73343\(\rightarrow\)S1P). Edelfosine, a more specific PLC inhibitor, was also tested. This is a synthetic lysophospholipid analogue that selectively inhibits phosphatidylinositol phospholipase C but does not inhibit phosphatidylycholine-specific phospholipase C or D (54). Edelfosine (30 \(\mu\)M) blocked S1P-induced exocytosis (Fig. 5, Edelfosine\(\rightarrow\)S1P) confirming that a phosphatidylinositol phospholipase C is activated in the S1P-triggered pathway. None of the PLC inhibitors (U73122, U73343, nor edelfosine) showed any effect when added alone (Fig. 5, U73122, U73343, and Edelfosine).

As stated above, activation of PLC generates IP\(_3\) and DAG. This latter molecule is a potent activator of several PKC isoforms. The PKC family of serine/threonine kinases is central to signal transduction pathway in many cell types (55). Phorbol esters, such as PMA, bind to the C1 domain of PKC, mimicking DAG function and thus activating the enzyme (56). The presence and involvement of PKC in the mammalian sperm AR has been previously demonstrated (57, 58). Cheleritrine is a potent and selective inhibitor of PKC, which acts on its catalytic domain and is a competitive inhibitor with respect to the phosphate acceptor. This inhibitor completely abrogated S1P-triggered exocytosis (Fig. 5, Cheleritrine\(\rightarrow\)S1P) indicating that PKC activation is required in the S1P signal transduction pathway.

Ca\(^{2+}\) entry into the cytoplasm causes the activation of Rab3A, a key event that initiates secretion (34, 59). Rab3A effectors are directly or indirectly involved in tethering the acrosome to the plasma membrane. To determine if S1P addition induces AR by using the pathway described for membrane fusion in human sperm, we added the unprenylated permeable Rab3A, His6-R-Rab3A, loaded with GDP\(_\beta\)S (Rab3A-GDP\(_\beta\)S) to sperm cells to compete with the endogenous protein for guanosine nucleotide exchange factors required for its activation. R-Rab3A-GDP\(_\beta\)S inhibited the S1P-elicted AR (Fig. 5, R-Rab3A-GDP\(_\beta\)S\(\rightarrow\)S1P), confirming that S1P is inducing a normal membrane fusion process rather than simply destabilizing membranes or acting through an unknown mechanism.
In addition, this result also suggests that S1P is activating a pathway that finally converges with those activated by proges-
terone and calcium influx (35). In summary, these results indicate
that S1P triggers the AR by activating PLC and PKC and
requires the activity of the small GTPase Rab3A.

Sphingosine 1-Phosphate Triggers Exocytosis

FIGURE 4. S1P triggers exocytosis through a G_1-coupled receptor. A, intact capacitated human sperm were
incubated with or without 100 ng/ml pertussis toxin (Pertussis Tx) for 15 min at 37°C. Acrosomal exocytosis was
then initiated by adding 10 μM A23187 (Pertussis Tx→A23187), 15 μM progesterone (Pertussis Tx→Pg), or 100
nM S1P (Pertussis Tx→S1P) for a further 15 min at 37°C. Several controls were included: background acrosomal
exocytosis in the absence of any stimulation (control, Co); acrosomal exocytosis stimulated by 10 μM A23187
(A23187), 15 μM progesterone (Pg), or 100 nM S1P (S1P). Afterwards, sperm were fixed, and acrosomal exocytosis
was measured as described under “Experimental Procedures.” The data represent the mean ± S.E. from 4
independent experiments. The means of groups with toxin were compared with the corresponding group
without the inhibitor using Student’s t-test and classified as significant (*, p < 0.05; ***, p < 0.001) or non-
significant (ns, p > 0.05). B–F, capacitated human sperm recovered after swim-up were loaded with Fluo-3 AM
and the fluorescence intensity was visualized before and after S1P addition as described under “Experimental Procedures.” B and C, representative single cell spatiotemporal [Ca^{2+}]_i changes after adding 200
nM S1P (blue line) in the absence (B) or presence (C) of 100 ng/ml pertussis toxin (Pertussis Tx→red line). Ionomycin
(20 μM ione, green line) was added at end of the experiment as a positive control. The time frame is indicated
in each panel (seconds). D and E, illustrate the corresponding traces of individual sperm showing the fluores-
ceence change after addition of S1P in absence (D) or presence (E) of pertussis toxin. F, summarizes the S1P
response in the absence (blue bar) or presence of 100 ng/ml pertussis toxin (red bar). Δ(F/F_0)−1 represents the
average of the changes in fluorescence of all individual sperm analyzed (S1P (N = 17, 145 cells) and
Pertussis Tx→S1P (N = 4, 96 cells), ***, p < 0.001)). Error bars represent the mean ± S.E. Note: approximately
45% of the cells responded to S1P addition with an intracellular calcium increase.

must consider the possibility that exogenous S1P could be synthe-
sized in the female genital tract, or that sperm cells might produce S1P by themselves when chal-
lenged by a stimulus (autocrine/paracrine mechanism). To resolve
these possibilities, we set out to determine whether SK1 was present
in human spermatozoa, performing a Western blot on an extract of 10 × 10^6 sperm prepared as described under “Experi-
mental Procedures.” Proteins were separated on 8% Tris-Tricine gels and transferred to Immobilon membranes, which were probed
with specific antibodies to detect the presence of SK1. As shown in
Fig. 6A (sperm), the anti-SK1 antibody recognized a single protein
band in sperm lysates with an apparent molecular mass of ~43
dozen with those of the purified recombinant SK1 (data not shown) and rat testis homoge-
nate (Fig. 6A, Testis lane). Johnson et al. (18) demonstrated that PMA
induces PKC-mediated phosphorylation and targeting of SK1 to the
plasma membrane in human embryonic kidney (HEK) 293 cells, which
leads to the release of S1P to the extracellular medium. We
hypothesized that sperm SK1 might utilize a similar mechanism
to synthesize S1P. If this were the case, SK1 would translocate from
the cytosol to membranes upon PMA treatment to produce S1P.
To directly address this hypothesis, we isolated membranes from
50 × 10^6 sperm cells treated (Fig. 6B, 2-APB→PMA) or not (Fig. 6B,
2-APB) with PMA. Sperm were pretreated with 2-APB, to avoid
membrane loss due to the AR. Classic isoforms of the PKC family

Sphingosine Kinase 1 Is Present in Human Spermatozoa—
Under physiological conditions S1P is generated from phos-
phorylation of sphingosine by the action of SK. Therefore, pro-
duction of S1P through increased SK cellular activity became a
focus of our interest. In the particular case of spermatozoa, we
depend on Ca^{2+} for their activity. Thus, we also prepared
membrane extracts from human spermatozoa stimulated with the Ca^{2+} ionophore A23187 (Fig. 6B, 2-APB→A23187).
Western blot analysis revealed that PMA and Ca^{2+} increased endogenous SK1 association with sperm membranes by 4- to
8-fold, demonstrating that, after PMA or Ca^{2+} stimulus, SK1
translocates to the membrane fraction. The blot was repro-
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B) indicate that SK1 is present in human sperm cells and
translocates to a membrane fraction when stimulated with PMA or Ca^{2+}.

SK1 Translocates from the Postacrosomal to the Acrosomal Region in Stimulated Sperm—We reasoned that if SK1 is involved in the signal transduction pathway triggering the AR its localization should be in accordance with its function. To analyze the distribution of SK1 we performed indirect immunofluorescence on fixed cells and double-labeled with FITC-PSA, to distinguish between reacted and intact spermatozoa, and with an anti-SK1 antibody (Fig. 7, FITC-PSA and αSK1, respectively). Under control conditions with or without the addition of 2-APB, most of the cells (69.2% ± 7.2%, mean ± S.E., n = 5) showed a postacrosomal or a diffuse immunolabeling pattern (Fig. 7, C and G, arrowheads). Only 8.5% ± 2.1% of the cells showed acrosomal staining. The fluorescent pattern displayed by the anti-SK1 polyclonal antibody was abolished by preincubation with the recombinant protein against which the anti-SK1 antibody was raised (Fig. 7, red and green). As an antibody-specificity control 10 μM 2-APB alone (Fig. 7, B and I) at 37 °C as explained in the legend to Fig. 6B (E, I, M, Q, and U). The cells were then fixed and double-stained with an anti-SK1 antibody followed by an anti-rabbit Cy3 antibody (Fig. 7, red). FITC-PSA was used to differentiate between reacted and intact sperm (green: B, F, J, N, R, and V). The merged images are shown in D, H, L, T, and X. Some batches were further incubated with 200 nM PMA (2-APB→PMA) or its inactive analogue αPMA (2-APB→αPMA). As an antibody-specificity control 10 μg/ml of the anti-SK1 antibody was preincubated with 0.6 μg/ml of the SK1 protein (2-APB→PMA, αSK1+protein, asterisk). Bar = 6 μm.
Sphingosine 1-Phosphate Triggers Exocytosis

Lenging human spermatozoa with the inactive analogue of PMA (Fig. 7W, 2-APB→αPMA) caused a staining pattern similar to that observed in control cells (Fig. 7, C and G), with more cells displaying the postacrosomal or diffuse (66.7% ± 13.7) than the acrosomal pattern of labeling (15.2% ± 9.75). The last result rules out the possibility of a nonspecific effect of PMA. These findings reaffirm the notion that SK1 translocates to the acrosomal region after stimulus.

SK1 Activity and S1P Production Are Required for the PMA-

triggered Signal Transduction Cascade Leading to Acrosomal

Exocytosis—N,N-Dimethylsphingosine (DMS) acts as a specific competitive inhibitor of SK in diverse cell types and is a useful tool in defining the role of S1P (60). To elucidate whether SK is involved in the signal transduction cascade leading to the AR we tested DMS in functional assays.

Taking into account that PMA triggers exocytosis in intact sperm (61) and that it translocates SK to the membranes (Fig. 6B), we wondered if SK was involved in the signal transduction cascade initiated by PMA. If SK were involved in PMA-induced exocytosis, we would expect an inhibition of PMA-induced AR when DMS was present during the functional assay. Indeed, DMS partially abrogated PMA-stimulated exocytosis (Fig. 8A, compare DMS→PMA with PMA). Its effect was reversed by the addition of S1P (Fig. 8A, DMS→PMA+S1P) confirming the involvement of SK and S1P in the PMA exocytotic pathway.

As expected, A23187 bypassed the inhibitory effect of DMS, because Ca2+ is downstream in the S1P-induced AR pathway (Fig. 8A, compare DMS→A23187 and A23187). This is in accordance with the results shown in Figs. 2A, 2B, and 3 and supplemental Fig. 1). Progesterone-induced AR was not sensitive to DMS inhibition of SK (Fig. 8A, compare DMS→Pg with Pg) suggesting that it might be activating a signal transduction pathway that does not need SK activity. As a control, we treated human spermatozoa with S1P, the product of SK activity after DMS incubation. S1P-triggered AR was not inhibited by the SK inhibitor DMS (Fig. 8A, DMS→PMA+S1P) consistent with the idea that the SK inhibitor blocks exocytosis by preventing S1P production. Additionally, we tried SKI, a potent and selective inhibitor of SK. This compound exhibits non-

ATP-competitive inhibition of human recombinant GST-SK1 with an IC50 value of 0.5 μM, with no inhibition against ERK2, phosphatidylinositol 3-kinase, or PKCα at concentrations up to 60 μM (62). SKI partially abrogated the PMA-induced AR showing an inhibition similar to that produced by DMS (Fig. 8A, compare PMA and SKI→PMA). Furthermore, we confirmed every result obtained in Fig. 8A but using SKI instead of DMS (supplemental Fig. 3A). These observations are consistent with the idea that SK is involved in acrosomal exocytosis.

Our results show that SK and S1P are clearly implicated in the PMA signal transduction cascade driving the exocytosis. To directly prove that PMA activates SK1, we measured the SK1 activity in sperm cells treated or not with 200 nM PMA. S1P synthesis increased 2.5-fold in sperm cells that received the PMA stimulus compared with that treated with DMS even in the presence of PMA (Fig. 8B, DMS and DMS→PMA versus PMA). HeLa cells incubated with sphingosine confirm the Rf of S1P. Also, SKI inhibited S1P synthesis in a similar extent as DMS did (supplemental Fig. 3B, SKI and SKI→PMA versus PMA). These results reinforce the idea that the spermatozoa’s SK1 is directly involved in the production of S1P under the phorbol ester stimulus controlling/regulating the level of this important biomodulator within the spermatozoa.

To establish a connection between the PMA-induced SK activation and the extracellular effects of S1P we performed additional experiments. First, we reasoned that if PMA is inducing acrosomal exocytosis, because it is activating the S1P synthesis, pertussis toxin should be able to inhibit the PMA-induced AR. As shown in Fig. 8C pertussis toxin completely abrogated PMA-triggered AR (Fig. 8C, Pertussis Tx→PMA). This experiment provides additional evidence indicating that the PMA signaling cascade is connected to S1P production and therefore to its autocrine/paracrine effect.

Second, if PMA triggers exocytosis in intact sperm (61) and, (Fig. 8A and SK is involved in the cascade initiated by the phorbol ester, we hypothesized that PMA increases the intracellular calcium, and this rise must be blocked by an SK inhibitor. Effectively, as shown in Fig. 8 (D and E) and supplemental Figs. 4 and 5, PMA induced a calcium increase in live cells that was abolished completely when the cells were preincubated for 10 min with the SK inhibitor SKI. Representative traces of the PMA-induced and SKI inhibition of these responses are shown in Fig. 8, F and G, respectively. DMS also inhibited the calcium increase induced by PMA (Fig. 8H). These results suggest that the PMA-induced AR implies an intracellular calcium increase that relies on S1P synthesized by SK.

DISCUSSION

S1P mediates a wide variety of biological processes, including cell proliferation, survival, motility, angiogenesis, and immune and allergic responses (63). It is now clear that this relatively simple lipid molecule can regulate such a diverse array of processes, because it not only has direct intracellular actions but, importantly, it is also a ligand for a family of five specific seven-transmembrane-spanning G-protein-coupled receptors. These S1P receptors couple to different types of G-proteins, thus enabling them to regulate numerous downstream signaling pathways (64). Although receptors for S1P are abundant in the central nervous system, neuron-specific functions of S1P were unknown until Kajimoto et al. (8) suggested the involvement of this sphingolipid in glutamate secretion in hippocampal neurons. However, S1P molecular mechanism/s of action during exocytosis remains still largely undefined.

Neurons are a well defined system to study secretion; however, in these cells, exocytosis can overlap with other intracellular trafficking processes, such as endocytosis and recycling. In contrast, exocytosis of the acrosome is a synchronized, all-or-nothing process that happens only once in the life of the spermatozoon; hence, it constitutes a particularly attractive system to examine molecular aspects of regulated exocytosis that are not amenable to experimental manipulation in other mammalian models (65). The present study provides new evidence supporting the notion of a novel action for S1P by using human spermatozoa as a model, i.e. an exocytic function whereby S1P
**FIGURE 8.** SKI activity is required for the PMA-triggered signal transduction cascade leading to acrosomal exocytosis. A, human spermatozoa were incubated for 3 h under capacitating conditions. The medium was then supplemented, when indicated, with 5 μM DMS or 1 μM SKI. Acrosomal exocytosis was then initiated by adding 10 μM A23187 (DMS→A23187), 15 μM progesterone (DMS→Pg), 100 nM S1P (DMS→S1P), 200 nM PMA (DMS→PMA and SKI→PMA), 200 nM PMA plus 100 nM S1P (DMS→PMA + S1P), or αPMA (inactive analogue of PMA; DMS→αPMA). The mixture was incubated for 15 min at 37 °C. Several controls were included: background acrosomal exocytosis in the absence of any stimulation (control, Co); acrosomal exocytosis stimulated by 10 μM A23187 (A23187), 15 μM progesterone (Pg), 100 nM S1P (S1P), 200 nM PMA (PMA), or 200 nM αPMA (αPMA). Afterwards, sperm were fixed and acrosomal exocytosis was measured as described under "Experimental Procedures." The data represent the mean ± S.E. of at least four independent experiments. In the case of stimulation with A23187, Pg, or S1P, the means of groups with DMS were compared with the corresponding group without the inhibitor using the Student’s t-test; when PMA or αPMA were used as stimulators, the Dunnett’s t-test was used to compare the means of all groups against the PMA-stimulated condition in the absence of inhibitors (ns, p > 0.05; s, p < 0.001 (**)). B, PMA increases S1P synthesis in human sperm cells. Human spermatozoa (50 × 10⁶) were permeabilized (as described under "Experimental Procedures") and treated for 15 min at 37 °C with 5 μM DMS. Samples were further incubated for 15 min at 37 °C with 50 μM bovine serum albumin-sphingosine, 1 μM of [γ-32P]ATP, and when indicated 200 nM PMA (DMS→PMA and PMA). HeLa cells (positive control, incubated with sphingosine; + SPH, and negative control no SPH added; −SPH) were prepared as described under "Experimental Procedures." Thin-layer chromatograms were developed in 1-butanol/methanol/acetic acid/water (8:2:1:2, v/v) as solvent system and visualized by autoradiography. The figure is representative of three experiments. C, intact capacitated human sperm were incubated with or without 100 ng/ml pertussis toxin (Pertussis Tx) for 15 min at 37 °C. Acrosomal exocytosis was then initiated by adding 200 nM PMA (Pertussis Tx→PMA) and incubating for 15 min at 37 °C. Several controls were included: background acrosomal exocytosis in the absence of any stimulation (control, Co); acrosomal exocytosis stimulated by 10 μM A23187 (A23187) or 200 nM PMA (PMA). Afterwards, sperm were fixed and acrosomal exocytosis was measured as described under "Experimental Procedures." The data represent the mean ± S.E. of five independent experiments. The mean of the group with toxin was compared with the corresponding group without the inhibitor using Student’s t-test and classified as significant (**, p < 0.01). D–H, capacitated human sperm recovered after swim up were loaded with Fluor-3 AM (2 μM) in HSM, and the fluorescence intensity was visualized before and after PMA addition as described under "Experimental Procedures." D and E, representative single cell spatiotemporal [Ca++] changes and their corresponding traces after adding 200 nM PMA (blue line) in the absence (D) or presence (E) of 1 μM SKI (SKI-red line). Ionomycin (20 μM, iono, green line) was added at the end of the experiment as positive control. The time frame is indicated in each panel (seconds). F and G, illustrate the corresponding traces of individual sperm showing the fluorescence change after addition of PMA in the absence (F) or presence (G) of SKI. H, summarizes the PMA response in the absence (blue bar) or presence of 1 μM SKI (red bar) or 5 μM of DMS (yellow bar). Δ(F/F₀) − 1 is the average of the changes in fluorescence of all individual human sperm analyzed (PMA (N = 8, 135 cells), SKI + PMA (N = 5, 67 cells), and DMS + PMA (N = 4, 53 cells)). Error bars represent the mean ± S.E. Dunnett’s test was used to compare the means of all groups against the PMA-stimulated condition in the absence of inhibitors. Significant differences from PMA group for p < 0.001 (**) are indicated for each bar.
triggers acrosomal exocytosis in capacitated human sperm (Fig. 1, A and B). Furthermore, 100 nM S1P was able to produce a maximal effect in intact sperm (Fig. 1A) suggesting that S1P action is mediated by a high affinity receptor (29, 30). Pertussis toxin completely abrogated S1P-induced exocytosis (Fig. 4). Considering that G1 and G2 have been described in sperm cells but not G0, exocytosis inhibition was likely due to S1P binding to a G-coupled receptor.

S1P produces calcium-mobilizing effects acting as a second messenger or through G-protein-coupled receptors (66, 67). Here, we revealed that the signal transduction cascade downstream S1P involves a rise in the intracellular calcium concentration in live sperm (Fig. 3) mediated through G-protein-coupled receptor(s) (Fig. 4, A–F). We present evidence indicating that the signaling pathway involves calcium entry by VOCCs and SOCCs (Fig. 2, A and B) and calcium efflux from the acrosomal store through IP3-sensitive channels (Fig. 2B). We also suggest the requirement for PLC, PKC, and Rab 3A activity for S1P-elicited exocytosis (Fig. 5).

Taking into consideration the importance of S1P in the exocytic process, we determined whether this molecule is produced by the spermatozoon. We report here for the first time that SK1 is activated downstream of a PMA exocytotic stimulus. SK1 is not only present in human sperm cells but translocates from cytosol to membranes when the cells are stimulated with calcium or PMA (Fig. 6, A and B). Furthermore, Fig. 7 strongly suggests a redistribution of SK1 to the acrosomal region when challenged with a phorbol ester. The fact that DMS and SKI, both SK inhibitors, abrogated the PMA-mediated exocytosis implies that the SK activity is required for the membrane fusion process triggered by PMA and likely by PLC-mediated DAG production (Fig. 8 and supplemental Fig. 5A).

The increase in S1P concentration after the PMA stimulus measured by the incorporation of 32P to sphingosine corroborates that sperm SK1 is not only present in sperm cells but is activated by PMA (Fig. 8B). Therefore, SK1 activity is likely involved in acrosomal exocytosis, but we cannot rule out a role for SK2 in this process. Furthermore, a PMA-induced calcium increase was completely abolished by SK inhibitors demonstrating that PMA requires S1P for calcium rise and likely for acrosomal exocytosis (Fig. 8, D–H). Phorbol esters are non-hydrolyzable analogues of DAG. This lipid is actively produced by sperm cells upon stimulation with physiological AR inducers, such as progesterone and zona pellucida (49). Our results

![FIGURE 9. Working model for S1P/SK/S1PR interplay during acrosomal exocytosis. The interaction between S1P with a G\(_i\)-coupled receptor (S1PR) activates a VOCC and a heterotrimeric G\(_i\)-protein leading to PLC activation. PLC hydrolyzes phosphatidylinositol 4,5-bisphosphate to generate DAG and IP\(_3\), IP\(_3\) binds to IP\(_3\)-sensitive calcium channels on the acrosomal membrane leading to intra-acrosomal calcium efflux. The emptying of the acrosomal store stimulates the opening of SOCCs at the plasma membrane. The other product of PLC activity, DAG, activates the serine/threonine kinase PKC. PKC may act directly or indirectly through ERK phosphorylating SK1 on residue Ser\(^{225}\) and favoring its interaction with the plasma membrane. Once the enzyme resides on the membrane, it catalyzes sphingosine (SPH) phosphorylation. S1P produced reaches the extracellular medium through an ABCC1 transporter and/or a Spns2 transporter interacting with its receptor and amplifying the signaling pathway. The sustained calcium increase induced by SOCCs opening activates Rab3A leading to acrosomal exocytosis.](image-url)
suggest that DAG and S1P/SK constitute a critical part of the signaling pathway that mediates the AR.

On the basis of our results we built a working model shown in Fig. 9 where the binding of S1P to a G<sub>i</sub>-coupled receptor activates a VOCC and an heterotrimeric G<sub>i</sub>-protein leading to phosphoinositide-specific PLC activation. PLC hydrolyses phosphatidylinositol 4,5-bisphosphate to generate DAG and IP<sub>3</sub>. IP<sub>3</sub> induces calcium efflux from the acrosomal store and consequently stimulates the opening of SOCCs. On the other hand, DAG serves as a second messenger in regulating classic and novel PKC enzymes permitting the phosphorylation of a variety of proteins. In an initial study, Johnson et al. demonstrated that PMA induces PKC-dependent activation of SK1 (18). It was later shown that SK stimulation requires PKC-mediated extracellular signal-regulated kinase (ERK1–2) activation (68–70). ERK phosphorylates SK1 on residue Ser<sup>225</sup> (68), which is required for its translocation to the plasma membrane. Both PKC and ERK1–2 have been identified as regulators of human sperm AR (58, 71) raising the possibility of a connection between the S1P/SK and PKC/ERK1–2 pathways.

The S1P generated by this process has been detected predominantly in the extracellular space in a number of cellular systems (72). S1P is unlikely to flip-flop spontaneously. Accordingly, two ABC transporters have been suggested to have roles in S1P transport: (i) cystic fibrosis transmembrane regulator, which is implicated in S1P entry into the cell (73) and has been described recently in mouse sperm cells (74), and (ii) ABCCL1, which can mediate S1P efflux (75, 76). Melaine et al. (77) performed the molecular cloning of several rat ABC transporters and described the expression of ABCCL1 in rat testis, raising the possibility of the presence of this transporter in sperm cells. Recently, Kawahara et al. (78) identified a new S1P transporter, Spn2, in a mutagenesis screen for regulators of heart development in zebrafish. Spn2 is a multipass transmembrane protein required to export S1P outside the cells. We cannot discount the possibility of the presence of this transporter in sperm cells. Alternatively, SK1 can be secreted to the extracellular medium through an ABC or Spns2 transporter where it interacts with its receptor and closes the activation cycle. However, neither SK1 nor Spns2 is implicated in S1P transport: (i) cystic fibrosis transmembrane regulator, which is required for its translocation to the plasma membrane. Acknowledgments—We thank Marcelo Furlán and Alejandra Medero for excellent technical assistance and Dr. C. Tomes, Dr. S. Patterson, and Dr. Pablo Visconti for critical reading of the manuscript.

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