PKC-dependent Activation of Sphingosine Kinase 1 and Translocation to the Plasma Membrane

EXTRACELLULAR RELEASE OF SPHINGOSINE-1-PHOSPHATE INDUCED BY PHORBOL 12-MYRISTATE 13-ACETATE (PMA)*

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Sphingosine-1-phosphate (S1P) is a highly bioactive sphingolipid involved in diverse biological processes leading to changes in cell growth, differentiation, motility, and survival. S1P generation is regulated via sphingosine kinase (SK), and many of its effects are mediated through extracellular action on G-protein-coupled receptors. In this study, we have investigated the mechanisms regulating SK, where this occurs in the cell, and whether this leads to release of S1P extracellularly. The protein kinase C (PKC) activator, phorbol 12-myristate 13-acetate (PMA), induced early activation of SK in HEK 293 cells, and this activation was more specific to the membrane-associated SK. Therefore, we next investigated whether PMA induced translocation of SK to the plasma membrane. PMA induced translocation of both endogenous and green fluorescent protein (GFP)-tagged human SK1 (hSK1) to the plasma membrane. PMA also induced phosphorylation of GFP-hSK1. The PMA-induced translocation was abrogated by preincubation with known PKC inhibitors (bisindoylmaleimide and calphostin-c) as well as by the indirect inhibitor of PKC, C12-ceramide, supporting a role for PKC in mediating translocation of SK to the plasma membrane. SK activity was not necessary for translocation, because a dominant negative G82D mutation also translocated in response to PMA. Importantly, PKC regulation of SK was accompanied by a 4-fold increase in S1P in the media. These results demonstrate a novel mechanism by which PKC regulates SK and increases secretion of S1P, allowing for autocrine/paracrine signaling.

Sphingolipids are ubiquitously found in mammalian cell membranes, where they were originally thought to serve only as structural components. Subsequent studies clearly demonstrated evidence of important roles for sphingolipids and their metabolites as signaling molecules involved in such cellular processes as cell growth, differentiation, senescence, and apoptosis (1-4). One particularly interesting sphingolipid metabolite, sphingosine-1-phosphate (S1P), has emerged as a highly bioactive lipid implicated in both extracellular and intracellular signaling processes (5). S1P has been shown to be secreted into serum from platelets; extracellular S1P, as a first messenger, binds members of the endothelial differentiation gene (EDG) receptor family (now termed S1P1, 2, 3, 4, and 5) on the surface of endothelial cells, triggering such cellular processes as differentiation, migration, and mitogenesis (6). S1P is also suggested to act as a second messenger participating in signaling cascades leading to cytoskeletal changes, motility, release of intracellular calcium stores, and protection from apoptosis (7-10). Whether acting intracellularly or extracellularly, S1P is generated from phosphorylation of sphingosine by the action of SK. Therefore, because of the involvement of S1P in such diverse and vital cellular processes, production of S1P through increased SK cellular activity has become the subject of much interest.

Mammalian cells growing in culture possess intrinsic SK activity, generating low basal levels of S1P. Meanwhile, the agonist-induced increase in SK activity results in significant increase in S1P levels, responsible for cell signaling effects. Reported agonists of SK include platelet-derived growth factor (11), tumor necrosis factor-α (12, 13), nerve growth factor (14, 15), muscarinic acetylcholine agonists (16), serum (11), and phorbol esters (17, 18). However, the mechanisms by which SK is activated and where in the cell S1P is generated remain unknown.

In this study we have addressed PMA-induced activation of SK. PMA stimulated a significant increase in SK activity as assessed by a 4-fold increase in S1P in the media. These results demonstrate for the first time PKC-mediated translocation of SK to the plasma membrane with a concomitant increase in secretion of S1P into the media, indicating that S1P may generate biological responses through an autocrine/paracrine signaling response.

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The abbreviations used are: S1P, sphingosine-1-phosphate; SK, sphingosine kinase; hSK, human SK; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; GFP, green fluorescent protein; EGFP, enhanced GFP; EDG, endothelial differentiation gene; TNF, tumor necrosis factor.
EXPERIMENTAL PROCEDURES

Materials—Human embryonic kidney (HEK) 293 and Hela cells were purchased from American Type Culture Collection. Eagle's minimum essential medium, high glucose Dulbecco's modified Eagle's medium, heat-inactivated fetal bovine serum, phosphate-buffered saline, LipofectAMINE 2000, and pCDNA4-myc/HIS were purchased from Invitrogen. A23187, bisindolylmaleimide, calphostin-c, 4-phorbol-12,13-didecanoate, and phorbol-12-myristate-13-acetate (PMA) were purchased from Calbiochem. S1P, sphingosine, and C6-ceramide were purchased from Matreya, Inc. (Pleasant Gap, PA). [γ-32P]ATP (3000 Ci/mmol) was purchased from PerkinElmer Life Sciences. [3H]EGTA, [3H]sphingosine-bovine serum albumin (0.3%) and [32P]ATP were purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO). Restriction enzymes were purchased from Roche Molecular Biochemicals.

Cell Culture—HEK 293 cells were cultured in minimum Eagle's medium containing 10% fetal bovine serum and 1% penicillin-streptomycin in a 5% CO2 incubator at 37 °C. Hela cells were cultured under similar conditions with high glucose Dulbecco's modified Eagle's medium. Cells were seeded 24 h before experimentation.

Cloning and Expression of SK—Based on the complete coding sequence for human SK1 in GenBank (accession no. AF238083), gene-specific primers (forward, 5'-CGCCCGCGAGGGAATGACACC-3', and reverse, 5'-GGCTGTCCTCCCCAAAGCATC-3') were used to amplify the entire open reading frame by PCR from human fetal kidney Marathon-ready cDNA (CLONTECH). An additional PCR reaction was required to clone the SK cDNA into the HindIII and EcoRI restriction sites of the mammalian expression vector pEGFP-C3 (CLONTECH) or pcdnas (Invitrogen). The SK expression plasmids were then transfected into HEK 293 cells by transfection with 5'GGATCCCGCGAGGGAATGACACC-3' and reverse, 5'-TTTGAATTCCTCATAAGGGCTTCCCCGCGGCG-3'. Transient expression of SK was accomplished using LipofectAMINE 2000 (Invitrogen) in accordance with the manufacturer's instructions. Following 48 h of expression, transfection efficiencies were determined to be typically between 40–60%.

SK Activity Assay—SK activity was determined as previously described by Olivera et al. (19). Briefly, cell extracts were assayed using 1 mM t-erythro-sphingosine-bovine serum albumin (0.3%) and [γ-32P]ATP (10 μCi, 20 μM) containing 200 mM MgCl2. Lipids were extracted and then resolved by TLC on silica gel G60 with 1-butanol/methanol/acetic acid/water (80:20:10:20, v/v).

In Vivo 32P Labeling of hSK1-GFP—Forty-eight hours after transfection, cell medium was replaced with phosphate-free medium. Following a 2-h incubation, 1 μCi of [32P]Pi, was added to the medium. After 2 h, cells were stimulated with vehicle or 300 nM PMA for 1 h. Cells were then washed once with ice-cold phosphate-buffered saline and lysed in 500 μl of lysis buffer (50 mM Tris, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na2VO4, 10 mM NaF, 10 mM okadaic acid, 10 mM β-glycerol phosphate) containing protease inhibitors. Lysate was centrifuged at 100,000 g for 15 min, and supernatant was immunoprecipitated with a polyclonal anti-GFP antibody (CLONTECH) conjugated to protein A for 4 h at 4 °C. The immunoprecipitate was then resuspended in Laemmli buffer and separated on a 4–15% SDS-polyacrylamide gel, transferred to nitrocellulose, and analyzed by autoradiography.

Preparation of Rabbit Polyclonal Antibody against SK—The antibody was prepared by the Medical University of South Carolina antibody facility. Briefly, a synthetic oligopeptide corresponding to the last 20 amino acids of the C-terminal (CVEPPPSWKPQQMPPPEEPL) of the hSK1 (GenBank™ accession no. AAF73423) was conjugated to keyhole limpet hemocyanin (KLH) and injected into New Zealand White rabbits. Antiserum was affinity-purified over a cyanogen bromide-activated agarose column bound with the same oligopeptide and eluted with 100 mM glycine (pH 2.5).

Western Blot Analysis—Blots were probed with 0.3 μg/ml preimmunized rabbit serum, 0.3 μg/ml immunized rabbit serum, immunized rabbit serum blocked with 2 μg/ml synthetic oligopeptide, or 0.2 μg/ml mouse anti-c-Myc (SC-40, Santa Cruz). Immunoreactive bands were detected with horseradish peroxidase-conjugated anti-rabbit or antimouse IgG (Santa Cruz) and an ECL Plus detection system (Amersham Biosciences).

Cell Fractionation—Forty-eight hours after transfection, cells were treated with 300 nM PMA for the indicated times. Cells were immediately washed twice with ice-cold phosphate-buffered saline and then scraped in 500 μl of lysis buffer (20 mM Tris, pH 7.5, 10 mM EDTA, 2 mM EGTA, 250 mM sucrose, 1 mM phenylmethylsulfonyl fluoride, 10 mM okadaic acid, 1 mM dithiothreitol, 5 mM NaF, 1 mM Na3VO4, 0.5 mM 4-deoxyribofuranose, and 10 μg/ml leupeptin, aprotinin, and soybean trypsin inhibitor). Cell suspensions were sonicated, followed by centrifugation at 1,000 × g for 10 min. Supernatants were separated into membrane and cytosolic fractions by centrifugation at 100,000 × g for 60 min at 4 °C. The membrane fractions were resuspended in 350 μl of lysis buffer containing 0.8% Triton X-100, sonicated, and left on ice for 45 min. Triton-insoluble membrane fractions were collected by centrifugation at 10,000 × g for 10 min at 4 °C and the pellet resuspended in 60 μl of lysis buffer.

Construction of Catalytically Inactive SK—A dominant negative mutation was introduced into GFP-tagged SK in the pEGFP-expression vector using oligonucleotides (forward, 5'-ATCCTCAGGAGACAGTCTGATGCAC-3', and reverse, 5'-GTCACCGGCGGGCCG-3'), generating a substitution of Gly-82 to Asp as previously reported by Pitson et al. (20).

Confocal Microscopy—Approximately 2 × 105 HEK 293 cells were plated onto 35-mm glass-bottom culture dishes (Mattek Corp.) 24 h prior to transfection. After 48 h, cells were viewed under an Olympus IX-70 confocal microscope with a Plan Apo ×60 oil objective (NA 1.4), and images were captured using PerkinElmer ultraview software that was set for 488 nm excitation and 516–560 nm emission for GFP fluorescent signal.

Immunostaining—Hela cells grown on 35-mm glass-bottom culture dishes were washed with phosphate-buffered saline and fixed and permeabilized with 100 μl of 100% ice-cold methanol at −20 °C. Cells were blocked with 2.5% fetal bovine serum for 20 min prior to incubation with 7 μg/ml rabbit anti-hSK1 for 1 h and 10 μg/ml goat anti-rabbit conjugated with rhodamine (Jackson Immunoresearch Laboratories, Inc., West Grove, PA) for 1 h, both diluted in 1.5% fetal bovine serum-phosphate-buffered saline.

In Vivo Labeling of Cells with Sphingosine—HEK 293 cells were seeded at 1 × 106 cells/60 mm dish and transfected with 2 μg of plasmid as described above. After 48 h, the volume of media was adjusted to 2 ml, and cells were treated with 300 nM PMA for the indicated times. Ten minutes before the end of the time point, cells were pulsed with 300 nM [3H]-t-erythro-sphingosine (2.5 μCi). Lipids were extracted by the addition of 2.5 volumes chloroform/butanol/HCl (50/50/1), and the organic layer was dried under nitrogen. Lipids were separated using thin layer chromatography in chloroform/methanol/15 mM calcium chloride (60/35/8). Bands corresponding to S1P standards were scraped and the radioactivity measured using a scintillation counter.

RESULTS

PMA-induced Activation of SK in the Membrane Fraction—We tested the ability of the phorbol ester PMA to activate SK in HEK 293 cells. PMA treatment resulted in a greater than 2-fold increase in whole cell lysate SK activity (Fig. 1A). Upon fractionation of control or PMA-treated cells into cytosolic and membrane fractions, there was a modest increase in SK activity in the cytosolic fraction and a significant increase in SK activity in the membrane fraction (Fig. 1B). This PMA-induced increase in membrane-associated SK activity paralleled the increase in plasma membrane-associated PKC activity following PMA treatment (data not shown).

PMA-induced Translocation of hSK1-GFP to the Plasma Membrane—We wondered if the increase in membrane-associated SK activity was accompanied by SK translocation to the membrane upon PMA stimulation. To test this, we cloned hSK1 from human fetal kidney Marathon-ready cDNA (CLONTECH) and tagged the N terminus with GFP. Transient overexpression of hSK1-GFP significantly increased the whole cell lysate SK activity by more than 100-fold over vector control cells (data not shown), indicating that the GFP-tagged hSK1 was a functional fusion protein. Next, we treated HEK 293 cells expressing hSK1-GFP with PMA and followed the hSK1-GFP protein using confocal microscopy. Upon PMA stimulation, hSK1-GFP was translocated to the plasma membrane. This was detectable as early as 15 min and was complete by 1 h (Fig. 2). To determine whether translocation of GFP-hSK1 seen by confocal microscopy was mirrored by Western blot analysis, we developed a polyclonal antibody against the C-terminal region of hSK1 (Fig. 3A). Immunoblot analysis of overexpressed hSK1-GFP from fractionated HEK 293 cells demonstrated a 2-fold increase of
hSK1-GFP in the membrane fraction in response to PMA treatment, with concomitant slight decrease in the cytosolic fraction and a significant decrease in the Triton-insoluble membrane fraction (Fig. 3, B and C). SK was previously shown to be cytosolic as well as to be associated with the cytoskeleton (21). From our data, it appears that the cytoskeletal pool of SK predominantly translocates to the plasma membrane in response to PMA.

Next, with the development of this highly immunospecific anti-hSK1 antibody, we tested the localization of endogenous SK within the cell in response to PMA. Utilizing the Hela cell line that expresses ample amounts of endogenous SK for immunocytochemical detection, significant levels of SK were found to translocate to the plasma membrane in response to PMA, similar to the effects seen with the overexpressed hSK1-GFP fusion protein (Fig. 4). These results demonstrate for the first time that SK translocates to the plasma membrane in response to PMA stimulation.

**PMA-induced Phosphorylation of SK**—PMA is a known activator of PKC, leading to phosphorylation of substrates involved in various signal transduction pathways. To investigate whether SK was a potential substrate of PKC, cells overexpressing GFP-tagged hSK1 were labeled with [32P]orthophosphate in the presence of PMA or vehicle. Immunoprecipitated GFP-hSK1 showed more than a 3-fold increase in 32P incorporation upon treatment with PMA as compared with the vehicle-treated cells (Fig. 5). In vitro PKC activity assays using GFP-hSK1 as a substrate were inconclusive (data not shown).

**Protein Kinase C Inhibitors Block PMA-induced Translocation to the Plasma Membrane**—To address the potential role of PKC in mediating SK translocation, we examined the effects of the direct PKC inhibitors (bisindoylmaleimide and calphostin-c) and the indirect inhibitor C8-ceramide on PMA-induced translocation of SK-GFP to the plasma membrane. Incubation of transiently transfected hSK1-GFP HEK 293 cells with either of two PKC activators, PMA or A23187 ionophore, induced translocation of hSK1-GFP to the plasma membrane. Thirty minutes of preincubation with any one of the above inhibitors prior to 1 h of stimulation with PMA nearly eradicated hSK1-GFP translocation to the plasma membrane (Table I). Therefore, these results demonstrate that PMA-induced SK translocation to the plasma membrane is dependent on activation of PKC.

**SK Activity Is Not Required for PMA-induced Translocation of hSK1 to the Plasma Membrane**—To ascertain whether the activity of the SK enzyme was necessary for translocation, we constructed a catalytically inactive hSK1-GFP plasmid that incorporated a single point mutation (G82D), in accordance with published reports by Pitson et al. (20). Transient expression of this construct in HEK 293 cells did not result in any change in basal activity of SK, thus demonstrating that it lacks activity. Importantly, this mutant abrogated PMA-induced stimulation of SK activity, confirming that it functions as a dominant negative mutant (Fig. 6A). PMA treatment of these cells clearly induced translocation of the catalytically inactive hSK1-GFP to the plasma membrane (Fig. 6B). Thus, SK activity is not required for PMA-induced translocation to the plasma membrane.

**PMA Translocation of SK Is Accompanied by Release of S1P into the Media**—Although it has been shown that stimulation of platelets results in release of preformed S1P, it is not known...
if newly synthesized S1P is secreted. We therefore wondered whether PMA regulation of SK was accompanied by S1P release into the media. To examine such potential regulation, a pulse labeling protocol was established to determine the fate of newly synthesized S1P in response to PMA. HEK 293 cells were transiently transfected with either pEGFP vector (lanes 1 and 2) or pEGFP-hSK1 (lanes 3 and 4). Forty-eight hours after transfection, cells were labeled with 3H]D-sphingosine for 10 min. PMA induced only a modest increase in the incorporation of label into intracellular S1P as compared with vehicle-treated cells (Fig. 7, A and B). In contrast, there was a 4-fold increase in S1P release into the media from PMA-treated cells as compared with untreated cells (Fig. 7, A and B). Furthermore, PKC inhibitors (bisindoylmaleimide and calphostin) prevented the PMA-stimulated intracellular accumulation of S1P and dramatically reduced S1P release into the media (Fig. 7C). These data imply that PMA-induced activation and translocation of SK to the plasma membrane are accompanied by S1P release into the media in a PKC-dependent manner, where it can then access cell surface receptors.

**DISCUSSION**

In this study, we have demonstrated that PMA stimulates a significant increase in membrane-associated SK activity, and this increase is accompanied by a distinct translocation of SK to the plasma membrane. In addition we have shown that PMA induces phosphorylation of SK. PKC Inhibitors block SK translocation, indicating that this event is mediated by PKC. Interestingly, SK catalytic activity is not required for its translocation, as shown by the ability of a dominant negative SK-GFP to translocate to the plasma membrane. Furthermore, SK activation and translocation by PMA stimulation resulted in a 4-fold increase in membrane-associated SK activity, and this increase is accompanied by a distinct translocation of SK to the plasma membrane.

**TABLE I**

| Treatment                  | Translocation |
|----------------------------|---------------|
| 300 nM PMA                 | 64.3 ± 4.1    |
| 10 μM A23187 Ionophore     | 42.7 ± 6.3    |
| 100 nM 4α-phorbol ester    | 1.4 ± 0.5     |
| 3 μM bisindoylmaleimide + 300 nM PMA | 2.8 ± 1.2 |
| 1 μM calphostin + 300 nM PMA | 5.6 ± 2.7 |
| 40 μM C6-ceramide + 300 nM PMA | 2.7 ± 1.5 |

**FIG. 4.** PMA-induced translocation of endogenous SK to the plasma membrane. Hela cells were treated in the absence or presence of 300 nM PMA for 1 h. Immunocytochemical detection of endogenous SK was performed according to the method described under “Experimental Procedures” using anti-hSK1 antibody. Confocal images are representative of >50% of the cells observed in 3–5 independent experiments.

**FIG. 5.** PMA-mediated phosphorylation of hSK1-GFP. HEK 293 cells were transiently transfected with either pEGFP vector (lanes 1 and 2) or pEGFP-hSK1 (lanes 3 and 4). Forty-eight hours after transfection, cells were labeled with 32P]P and stimulated with either vehicle (lanes 1 and 3) or 300 nM PMA (lanes 2 and 4). Cell lysates were immunoprecipitated with polyclonal rabbit anti-GFP antibody, separated by SDS-PAGE, transferred to nitrocellulose, and analyzed by autoradiography. Immunoblotting the membrane with an anti-rabbit horseradish peroxidase-conjugated antibody confirmed equal loading based on heavy and light chain immunoglobulins.
the cell. In addition, SK is also thought to be a regulated protein in which incubation of cells with any of a number of known SK activators results in a transient increase in S1P over the basal levels, thereby initiating a cell signaling cascade leading to diverse biological processes including calcium mobilization, cell growth, differentiation, survival, motility, and cytoskeleton organization (6–10). Although it is quite clear in the literature and from this study that agonists are able to induce SK activity within the cell, it is not clear how this activation occurs, where subcellularly S1P is being generated, or how S1P initiates cell signaling cascades that trigger such diverse biological responses.

Here we have demonstrated for the first time that PMA-induced activation of SK1 leads to PKC-mediated translocation of SK to the plasma membrane and SK1 phosphorylation and is accompanied by secretion of S1P into extracellular media. Other work supports a role for agonist-induced translocation of SK to the membrane. In a study using platelet-derived growth factor, Rosenfeldt et al. (21) demonstrated an accumulation of SK at membrane ruffles, suggesting that localized S1P production aids in directional migration through binding to EDG-1 receptors. Other agonists activating SK could also be mediated by PKC-induced translocation of SK. In fact, while this work was in progress, Melendez and Khaw (22) showed that IgE cross-linking of FcεRI in mast cells was accompanied by SK translocation to the plasma membrane. The current results suggest that PKC activation may provide a direct mechanism for translocation of SK to the plasma membrane in response to many agents that activate phospholipase C/PKC. Our data demonstrating that SK is phosphorylated in response to PMA raise the possibility that SK is a direct substrate of PKC. At this point we cannot unequivocally rule on this possibility, because further in vitro studies on SK phosphorylation by PKC are required. Whether phosphorylation of SK is required for its translocation also is yet to be determined. These studies are now under way in our laboratory.

Another mechanism by which SK could be found in the proximity of the cell membrane was suggested in a recent
Activation and Translocation of SK Leads to SIP Release

In summary, we present a novel mechanism by which PMA activates SK and induces translocation to the plasma membrane in a PKC-dependent manner. This translocation is accompanied by secretion of SIP into the media, suggesting autocrine/paracrine signaling.

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