Vol. 198, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 14, 1994 Pages 1-9

REGULATORY EFFECT OF PHORBOL ESTERS ON SPHINGOSINE KINASE IN BALB/C 3T3 FIBROBLASTS (VARIANT A31): DEMONSTRATION OF CELL TYPE-SPECIFIC RESPONSE - A PRELIMINARY NOTE*

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Received November 22, 1993

Summary: Sphingosine-1-phosphate (Sph-1-P) has been implicated as a second messenger in control of cell motility and proliferation (e.g., Sadahira Y, et al., PNAS 89:9686, 1992; Olivera A & Spiegel S, Nature 365:557, 1993). The control mechanism for its synthesis, as catalyzed by sphingosine kinase, is crucial in signal transduction. Synthesis of Sph-1-P in Balb/c 3T3 fibroblasts (A31 variant) is strongly up-regulated by brief treatment of cells with 12-O-tetradecanoylphorbol-13-acetate (TPA). Level of Sph-1-P in PKC-depleted cells is 10-fold higher than in undepleted cells, and a further 5-fold increase occurs after treatment with TPA. In Swiss 3T3 and B16 melanoma cells, Sph-1-P level was unaffected by TPA treatment. Thus, the effect of TPA on Sph-1-P synthesis appears to be cell type-specific.

Sphingosine-1-phosphate (Sph-1-P[1]), the initial catabolite of Sph as catalyzed by Sph kinase (see Discussion), is the subject of much recent interest as a possible second messenger controlling cell growth (1,2) via induction of Ca^{2+} influx (3,4), enhanced phosphatidic acid synthesis (5), or inhibition of cell motility by unknown mechanisms (6). These effects of Sph-1-P are considered to be independent of protein kinase C (PKC), whereas effects of Sph and N,N-dimethyl-Sph are modulated via PKC-dependent pathways (7-9). Sph kinase was partially purified from rat brain and human platelets, and its activity was found to be inhibited by DL-threo-dihydro-Sph (10). Activation of Sph kinase and enhanced formation of Sph-1-P was recently shown to be induced by PDGF and closely associated with PDGF-dependent

*This study was supported by funds from The Biomembrane Institute, in part under a research contract with Otsuka Pharmaceutical Co. S.H. is supported by National Cancer Institute Outstanding Investigator Grant CA42505.

Abbreviations used are: DEAE, diethylaminoethyl; PDGF, platelet-derived growth factor; PKC, protein kinase C; PMSF, phenylmethylsulfonyl fluoride; Sph, sphingosine; TPA, 12-O-tetradecanoylphorbol-13-acetate.

0006-291X/94 $5.00

stimulation of Swiss 3T3 cells (11). However, response of Sph kinase may vary considerably from one cell type to another. We reported previously that Sph-1-P has no effect on proliferation of various types of tumor cells, but does inhibit at very low concentrations (nM range) chemotactic cell motility, migration through Matrigel, and phagokinetic activity of tumor cells (6).

A number of phenotypic changes occurring via PKC-dependent phosphorylation and gene expression are provoked by brief treatment of cells with TPA (12,13). Until recently, PKC was the only known phorbol ester receptor. Very recently, several other proteins have been claimed to be phorbol ester- or diacylglycerol-binding proteins; e.g., DK kinase (14), human n-chimaerin (in neurons) (15), human synaptotagmin (p65) (16), *Raf*-1 kinase (17), *unc-13* of *Caenorhabditis elegans* (related to uncoordinated movement) (18), and *vav* oncoprotein in human hematopoietic cells (19). These proteins all possess zinc finger/ butterfly sequence resembling that in the PKC regulatory region (20,21).

Prolonged treatment of cells with TPA results in PKC depletion and also allows observation of PKC-independent processes. To clarify the regulatory mechanism of Sph kinase, which determines Sph-1-P level, we examined the effects of *in vitro* short-term treatment of cell lysates and *in vivo* long-term treatment of cells with TPA on Sph kinase activity of A31 (Balb/c 3T3 variant), F10 (mouse melanoma variant), and Swiss 3T3 cells. In both cases, strong, PKC-independent, specific enhancement of Sph kinase activity was observed only in A31 cells, indicating that TPA produces a cell type-specific response.

MATERIALS AND METHODS

Materials. D-erythro-Sph, caproic anhydride, octyl-β-D-glucopyranoside, H7 (1-(5-isoquinolinyl-sulfonyl)-2-methylpiperazine), staurosporine, and benzamidine were purchased from Sigma Chemical Co. (St. Louis, MO). TPA was from Calbiochem (San Diego, CA). DEAE cellulose anion exchange resin (DE52) was from Whatman International (Maidstone, England). Leupeptin, aprotinin, trypsin inhibitor, and Triton X-100 were from Boehringer Mannheim Biochemicals (Indianapolis, IN). Polyclonal anti-PKC isoform antibodies were from Gibco Laboratories (Grand Island, NY). ^{125}I-labeled donkey anti-rabbit antibodies (5-20 μCi/μg), and a commercial PKC assay system, were from Amersham Corp. (Arlington Heights, IL). [γ-^{32}P]ATP (specific activity 3000 mCi/mmol) was from Dupont-New England Nuclear (Boston, MA). Reagents for SDS-PAGE, trans-blot nitrocellulose membrane, and tween-20 were from Bio-Rad Laboratories (Richmond, CA). Silica Gel 60 chromatography plates were from Merck (Darmstadt, Germany). Sph-1-P and [^{3}H]Sph were chemically synthesized as previously described (22,23).

Cell culture. Swiss 3T3 and Balb/c 3T3 clone A31 fibroblasts were obtained from the American Type Culture Collection. Mouse melanoma B16 variant F10 was obtained from Isaiah J. Fidler (Univ. of Texas, M.D. Anderson Cancer Center, Houston, TX). Cells were routinely cultured as previously described (6).

Preparation of cell lysates. Confluent cells in 150 mm dishes were washed three times with cold phosphate-buffered saline and harvested in 250 μl of lysis buffer (20 mM Tris [pH 7.5], 0.5 mM EDTA, 0.5 mM EGTA, 1 mM Na_3VO_4, 10 mM NaF, 1 mM PMSF, 10 mM

β-mercaptoethanol, 1% glycerol, 25 μg/ml leupeptin, 25 μg/ml aprotinin, 25 μg/ml trypsin inhibitor, 10 mM benzamidine, and 0.1% Triton X-100). Harvested cells were homogenized on ice by 10-15 strokes in a pre-cooled Dounce homogenizer. Lysates were incubated on ice for another 30 min with slow agitation, and the homogenate was centrifuged at 12,000xg for 2 min. The supernatant (total cell lysate) was collected and either used directly in Sph kinase assays or loaded onto DEAE cellulose (DE52) anion exchange column pre-equilibrated with column buffer (20 mM Tris [pH 7.5], 0.5 mM EDTA, 0.5 mM EGTA, 10 mM β-mercapto-ethanol, 1 mM PMSF). The column was washed with 10 volumes of column buffer. The Sph kinase and PKC fractions were co-eluted with two volumes of column buffer containing 200 mM NaCl and leupeptin and aprotinin (20 μg/ml). Protein concentrations were determined by the Bradford method (24). Samples were diluted to equal protein concentrations and assayed for either Sph kinase or PKC activity.

Sph kinase assay. Previous attempts at quantitating the activity of Sph kinase were hampered by the unusual solubility properties of Sph-1-P (10,25). In this study, we developed a new, simple, reliable method, and compared it with previously-reported methods, i.e., classical organic solvent extraction and phase separation (25), and chemical modification of Sph-1-P to n-caproyl-Sph-1-P (complete recovery in lower phase) following enzyme activity assay in a mixed micelle system (10).

Assay mixtures (total volume of 75 μl) contained 25 μl of either total lysates or DEAE-purified fractions with equal amounts of total protein (50 μg) for all cell types examined. Sph substrate (5 μg/5 μl) was delivered either in mixed micelles (10) or as a bulk solution of 10% ethanol in 0.5 M NaCl. Reaction buffer (20 μl) of 50 mM Tris-HCl (pH 7.5), with different effectors such as TPA (24 μg/ml), calcium acetate (12 mM), α-phospholi-dyl-L-serine (8 mole %), H7, or staurosporine. The reaction was started by addition of 25 μl of 150 μM ATP, 45 mM magnesium acetate in 50 mM Tris-HCl (pH 7.5), and 0.5 μCi of [γ-^{32}P]ATP. Reactions were carried out for 10 min at 30°C and stopped by addition of either: (a) 400 μl of methanol/$CHCl_3$ (2:1 v/v) when the procedure of Buehrer & Bell (10) was followed for chemical modification of Sph-1-P; (b) 400 μl of $CHCl_3$/methanol (2:1) as a biphasic system for lipid extraction as in the method of Bligh & Dyer (26); (c) 100 μl of a stop reagent from Amersham's PKC assay system containing an aqueous solution of diluted acidic reaction-quenching reagent. Equivalent volumes from all types of reactions were spotted on silica G-60 plates and chromatographed in $CHCl_3$/pyridine/formic acid (60:30:7) (a) or butanol/acetic acid/water (3:1:1) (b,c). Plates were subjected to autoradiography with X-Omat (Kodak) film using intensifier screens. Exposures were carried out for 12 hr. Phosphorylation was quantitated using an LKB ultrascan imaging scanner. The three methods produced similar qualitative and quantitative data. Therefore, throughout this study, we utilized the new direct assay method (c) for monitoring Sph kinase activity.

PKC activity assay. DEAE samples were diluted to equal protein concentrations (50 μg) and assayed with a commercially available PKC assay system (Amersham) in the presence or absence of TPA or PKC inhibitors (H7, staurosporine). The system utilizes synthetic, PKC-specific, substrate peptides which become phosphorylated with the radiolabel-ed phosphate group from [γ-^{32}P]ATP. At the end of the reaction, the radiolabeled peptide was separated from unincorporated ^{32}P by use of an affinity paper for the peptide. Degree of phosphorylation was determined by liquid scintillation counting.

Western blot analysis for PKC isoforms. DEAE samples were diluted to equal protein concentrations (50 μg) and electrophoretically separated on a 10% SDS-PAGE. Protein migration was compared to pre-stained molecular weight marks (Gibco). Proteins were electrically transferred to nitrocellulose membranes. Western blotting was performed with polyclonal anti-PKC isoform antibodies (Gibco) according to the manufacturer's instructions. We used 2-5 μCi/membrane of donkey anti-rabbit ^{125}I-labeled antibodies (5-20 μCi/μg, Amersham) for detection of reactive bands. The membrane was subjected to autoradiography with X-Omat (Kodak) film overnight.

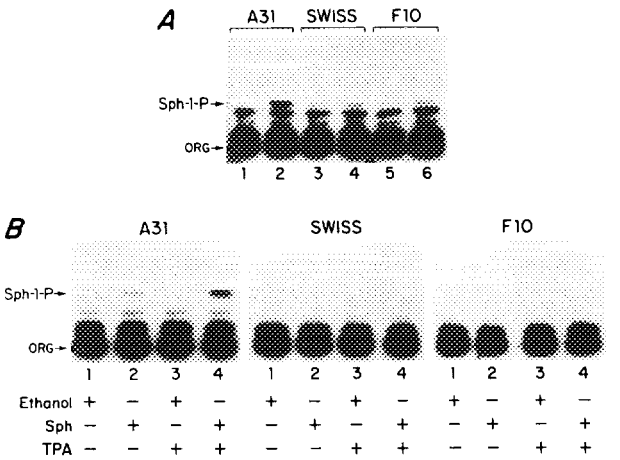

Figure 1. Sph kinase activity in total lysates and fractionated lysates of A31, Swiss 3T3, and F10 cells. **Panel A.** Autoradiogram from a representative experiment demonstrating Sph-1-P conversion. Lanes 1, 3, 5: controls with 1% ethanol added to cell lysates. Lanes 2, 4, 6: 100 μM Sph added to lysates. **Panel B.** Autoradiograms of TLC separations demonstrating the rate of Sph-1-P conversion in DEAE-fractionated lysates. Lanes 1 and 3: controls. Lane 2, 100 μM Sph. Lane 4, 100 μM Sph + 100 nM TPA. Sph kinase assay and TLC separation were performed as described in M&M.

RESULTS

Demonstration of Sph kinase activity (conversion of Sph to Sph-1-P) in total lysates of A31, F10, and Swiss 3T3 cells. Only A31 lysate showed a strong band corresponding to Sph-1-P. This activity was 25-fold higher than that in F10 or Swiss 3T3 cells (Fig. 1A; Table I). Similar results were obtained for DEAE-fractionated lysates; i.e., only the fraction separated by DEAE column chromatography of lysate from A31 cells (but not F10 or Swiss 3T3 cells) showed a strong band corresonding to Sph-1-P (Fig. 1B).

Up-regulation of Sph kinase activity by brief treatment of cells with TPA. The band corresponding to Sph-1-P in DEAE-fractionated lysate of A31 cells was faintly present, but was greatly enhanced (15-fold) when lysate was treated with 100 nM TPA. In contrast, Swiss 3T3 cells showed only a 1.8-fold increase and F10 cells showed no change in Sph-1-P band on TPA treatment (Fig. 1B; Table I).

Sph kinase activity in PKC-depleted A31 cells. It is well established that prolonged treatment of cells with TPA depletes PKC. We wished to learn whether TPA-induced specific enhancement of Sph kinase activity is independent of PKC activity. Levels of seven members of the PKC family in A31, Swiss 3T3, and F10 cells were determined by Western blot analysis. PKCα and PKCζ were present in all three cell lines, but the other five PKCs (β, γ, ξ, δ, η) were not detected in any of the cell lines. It was reported previously that prolonged exposure of quiescent cultures of these three cell lines to TPA induces down-

TABLE I. PKC and Sph kinase activity in Swiss 3T3, A31, and F10 cells

Cell line	Treatment		PKC activity (pmol/ min/ μg)	Sph kinase activity (fold increase)[b]
	Cells[a]	Lysates[a]		
Swiss 3T3	—	—	0.2	1
	—	TPA	6.8	1.8
	TPA	—	0.2	1
	TPA	TPA	0.2	1
A31	—	—	0.2	25 (1)
	—	TPA	7.6	375 (15)
	TPA	—	0.2	250 (10)
	TPA	TPA	0.4	2000 (80)
	—	TPA + H7	1.4	375 (15)
	—	TPA + staurosporine	0.3	375 (15)
F10	—	—	0.2	1
	—	TPA	2.3	1
	TPA	—	0.2	1
	TPA	TPA	0.2	1

Total lysates and confluent and quiescent cultures of cells were prepared as described in M&M. PKC and Sph kinase activity assays were performed as described in M&M. Assays were linear with respect to time and protein concentrations used. Values represent means of triplicate determinations, which varied by less than 15%. Similar results were obtained in at least five additional experiments. Concentrations of H7 and staurosporine used were 20 nM and 5 nM, respectively.

[a] Cells were exposed to 400 nM of TPA for 72 hr. Ten nM TPA was added to DEAE-fractionated cell lysates in the presence of 8 mol % phosphatidylserine and 1 mM Ca^{2+}.

[b] First value in each row represents Sph kinase activity (in arbitrary units) relative to that of untreated Swiss 3T3 cells, which is defined as 1. Value in parentheses for A31 experiments represents fold increase of Sph kinase activity relative to that of untreated A31 cells, which is defined as 1.

regulation of PKC and eventually total depletion of enzyme activity (12). We found that after prolonged TPA exposure only PKCα was depleted, and PKCζ level remained unchanged, in each of the cell lines (Fig. 2). Sph-1-P level in PKC-depleted A31 cells was 10-fold higher than in undepleted A31 cells (Table I), whereas Sph-1-P levels in PKC-depleted vs. undepleted Swiss 3T3 or F10 cells were roughly the same, in agreement with the findings of Spiegel et al. (2). Sph-1-P level in PKCα-depleted A31 cells showed a further 8-fold increase on addition of TPA as determined using DEAE lysates; i.e., potentiation of Sph kinase in A31 extract by TPA addition was observable. In contrast, Sph-1-P levels in TPA-treated, PKCα-deficient Swiss 3T3 or F10 cells were not significantly affected by addition of TPA (Fig. 3; Table I).

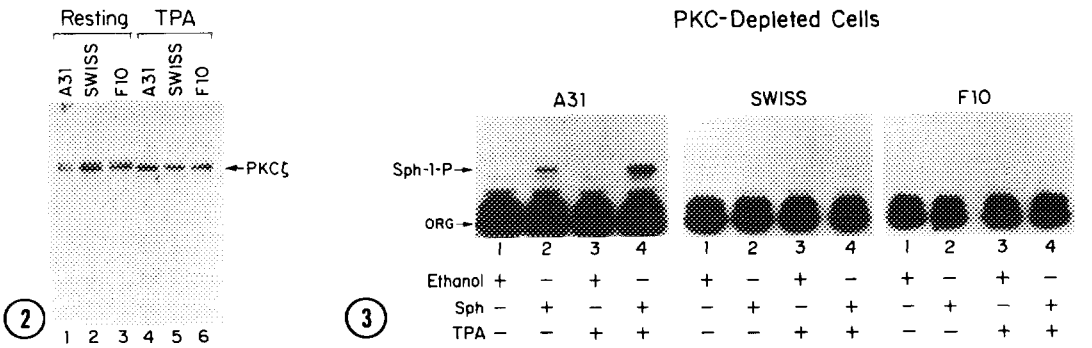

Figure 2. Western blots of PKCζ. Lanes 1-3, resting cells. Lanes 4-6, prolonged exposure to 400 nM TPA. Lanes 1 and 4, A31 cells. Lanes 2 and 5, Swiss 3T3 cells. Lanes 3 and 6, F10 cells. Under the same conditions, PKCα was totally down-regulated and not detected (data not shown).

Figure 3. Sph kinase activity in PKC-depleted DEAE-fractionated cell lysates. Cells were exposed to 400 nM TPA for 72 hr. Sph-1-P conversion was determined as described in the Fig. 1 legend. The autoradiogram is representative of four independent experiments. Lanes 1 and 3, controls. Lane 2, 100 μM Sph. Lane 4, 100 μM Sph + 10 nM TPA.

Effects of PKC inhibitors (staurosporine and H7) on Sph kinase activity. To further assess the enhancing effect of TPA on Sph kinase as observed specifically in A31 cells, we tested the effects of PKC inhibitors. PKC activity of DEAE lysates of A31 cells was strongly inhibited by 5 nM staurosporine or 20 nM H7. In contrast, Sph kinase activity of A31 cell lysates was unaffected by similar or higher concentrations of either PKC inhibitor (Fig. 4; Table I).

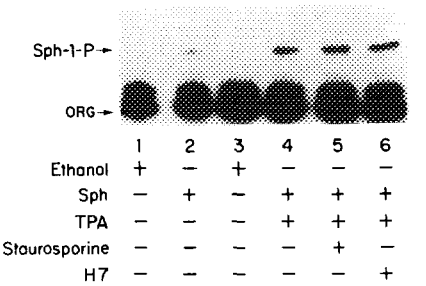

Figure 4. Effects of staurosporine and H7 on Sph kinase activity in DEAE-fractionated lysates of A31 cells. Cell lysate preparation, Sph-1-P conversion, and TLC separation were performed as described in the Fig. 1 legend. The autoradiogram is representative of three independent experiments. Lanes 1 and 3, controls. Lane 2, 100 μM Sph. Lane 4, 100 μM Sph + 10 nM TPA. Lane 5, 100 μM Sph + 10 nM TPA + 5 nM staurosporine. Lane 6, 100 μM Sph + 10 nM TPA + 20 nM H7. Similar results for staurosporine and H7 were observed when TPA was omitted.

DISCUSSION

Cell growth, cycle, and motility are believed to be controlled by a complex network of transmembrane signaling factors. The role of glycerophospholipid metabolites as second messengers has been well studied (27,28), but less is known about second messengers derived from sphingolipids. However, the roles of gangliosides and their primary degradation products, and of Sph and its derivatives, in control of transmembrane signaling have received increasing attention in recent years (29,30).

Sph-1-P has long been known as the initial product of Sph degradation by Sph kinase (31), which is subsequently degraded by pyridoxal phosphate-dependent lyase (located in endoplasmic reticulum) into phosphatidylethanolamine and palmitaldehyde (32). Recently, Sph-1-P has been implicated as a second messenger for promotion of PDGF- or serum-dependent cell growth (11), a PKC-independent cell motility inhibitor (6), and an enhancer of Ca^{2+} mobilization and phosphatidic acid synthesis. Therefore, understanding the mechanisms of transmembrane signaling requires clear knowledge of how endogenous levels of Sph-1-P are regulated.

As an initial step in this direction, we have examined the effects of brief and prolonged TPA treatment on Sph kinase activity of A31, 3T3, and F10 cells, using cytosolic fractions which do not contain the Sph-1-P degrading enzyme, lyase. TPA is known to be an activator of not only PKC, but also a number of factors (including transcription factors) which alter cell physiology (see Introduction). Long-term treatment of cells with TPA is known to deplete PKC (12). In the present study, we found that Sph kinase activity was strongly promoted by TPA treatment in A31 cells, but not in Swiss 3T3 or F10 cells. This effect was even more pronounced in PKC-depleted A31 cells. Two conclusions emerge from the present findings: (i) Sph kinase regulation occurs not through PKC but rather through direct effect of phorbol ester on Sph kinase or yet-unidentified protein kinases (short-term *in vitro* response), or through enhanced transcription of Sph kinase (long-term *in vivo* response). (ii) Cellular response to TPA, and mechanism for regulation of Sph kinase, differ markedly from one cell type to another.

Spiegel et al. have demonstrated that (i) Sph-1-P enhances phosphatidic acid levels, and promotes insulin-dependent and Con A-dependent cell proliferation in Swiss 3T3 cells (1,2); and (ii) PDGF- or serum-dependent cell growth stimulation is closely associated with up-regulation of Sph kinase and increased Sph-1-P level in 3T3 cells (11). In contrast, we did not observe induction of cell proliferation by Sph-1-P in mouse melanoma B16, F1, or F10, Balb/c 3T3, human fibrosarcoma HT-1080, or cow pulmonary artery endothelial (CPAE) cells. Cell motility was inhibited strongly by Sph-1-P in F1 and F10, to a lesser extent in Balb/c 3T3, and not at all in HT-1080 or CPAE cells. It is conceivable that Sph-1-P

promotes cell proliferation through one pathway, and inhibits cell motility and phagokinetic activity through a different pathway. Although both these hypothesized pathways are unknown, they might involve enhancement of Ca^{2+} mobilization, phosphatidic acid synthesis, or activation of some unknown factor.

This study on effect of TPA on Sph kinase included only three cell lines. More systematic studies will undoubtedly reveal other cell lines having the same property as A31. These data will be useful for further characterization of Sph-1-P as a regulator of cell physiology, and for better understanding of how Sph kinase is regulated.

Acknowledgment. The authors thank Stephen Anderson, Ph.D., for scientific editing and preparation of the manuscript.

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