Sphingosine Activates Protein Kinase A Type II by a Novel cAMP-independent Mechanism*

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Protein kinase A (PKA) has long been recognized as playing a major role in many regulatory processes in cells through its activation by the ubiquitous second messenger cAMP. We show here a novel mode of activation of PKA type II that is independent of cAMP and is, instead, dependent on sphingosine. PKA type II is specifically activated by sphingosine and its analog, dimethylsphingosine, but not by sphingosine-1-phosphate or other lipids. Like cAMP, sphingosine activates PKA holoenzyme but not the catalytic subunit alone, suggesting that the activation is mediated by the regulatory subunits. However, sphingosine-activated PKA, but not cAMP-activated PKA, is inhibited by phosphatidylinerine, suggesting a distinct mechanism of activation. Furthermore, unlike cAMP, sphingosine does not induce the dissociation of PKA holoenzyme into catalytic and regulatory subunits. Modulation of sphingosine levels in vitro results in alteration in basal membrane-associated PKA activity consistent with a direct effect of membrane sphingosine on PKA type II. Importantly, sphingosine-dependent but not cAMP-dependent activation of PKA specifically phosphorylates Ser58 of the multifunctional adapter protein 14-3-3ζ, promoting the conversion of dimeric 14-3-3 to a monomeric state, thus potentially modulating several biological functions. These results define a new mode of PKA activation that is sphingosine-dependent and mechanistically different from the classical cAMP-dependent activation of PKA. Furthermore, they suggest that stimuli that induce sphingosine accumulation and modulate phospholipid content at the cell membrane have the potential to activate PKA, thereby inducing the phosphorylation of distinct substrates and biological activities.

PKA† was first described 35 years ago as cAMP-dependent kinase (1). Since then, PKA has been demonstrated to play a key role in the signaling of many G protein-coupled receptors through the activation of adenylate cyclase and the consequent production of cAMP (2). Classically, PKA is activated by cAMP-induced disruption of the holoenzyme, releasing the catalytic subunit from the inhibitory activity of regulatory subunits (2). The active catalytic subunit is then available to phosphorylate multiple protein substrates in the cytosol and nucleus. To date, over 100 physiological PKA substrates have been identified that mediate biological functions as diverse as calcium mobilization, gene transcription, and cell survival (2). Thus, PKA is a central player in many cellular processes.

The modulation of protein kinases by second messenger lipids is well established and illustrated by the protein kinase C (PKC) family of enzymes that are activated by the acidic membrane lipid, phosphatidylinerine, and inhibited by the basic lipid, sphingosine (3, 4). Sphingosine is an intermediate in the sphingomyelin pathway that is generated at the plasma membrane upon ceramide breakdown in response to factors such as tumor necrosis factor α and Fas ligand (4). Sphingosine and its naturally occurring analog, dimethylsphingosine (DMS), act as second messengers, inducing apoptosis in many cell types including cancer cells (5). Currently, these sphingolipids are being evaluated as anti-cancer agents, although little is known about the underlying molecular mechanisms involved in sphingosine-induced apoptosis. Sphingosine and DMS have been shown to modulate the activity of several protein kinases, including PKC (3), Src (6), casein kinase II (7), and the epidermal growth factor receptor-associated kinase (8). Additionally, several sphingosine-dependent kinase activities were previously identified in BALB/3T3 fibroblast extracts (9, 10), and one of the activities was shown to phosphorylate members of the 14-3-3 family of adapter proteins (9).

The 14-3-3 family proteins are phosphoserine-binding proteins that interact with and modulate the functions of many key cellular proteins involved in proliferation, cell survival, and cell cycle control (11). The 14-3-3 proteins are dimeric, and each monomer within the dimer is capable of binding a phosphoserine motif in a “client” protein (12). The dimeric structure of 14-3-3 is critical for the regulatory function of these molecules (12). We have recently shown that phosphorylation of 14-3-3ζ by a BALB/3T3 fibroblast extract-derived sphingosine-dependent kinase activity caused monomerization of 14-3-3, thus potentially altering biochemical and biological signals downstream of 14-3-3 (13). We have now found that PKA phosphorylates 14-3-3ζ in response to sphingosine. We show here the characterization of this novel sphingosine-dependent activity of PKA, demonstrating for the first time that not only can PKA type II be activated by sphingosine but that this mode of activation involves a mechanism different from that utilized by cAMP. We show that phosphatidylinerine inhibits sphingosine-induced but not cAMP-induced PKA activation and that sphingosine, unlike cAMP, does not dissociate PKA holoenzyme, indicating that the two modes of PKA activation are differentially regulated. Furthermore, the selective phosphorylation and resulting monomerization of 14-3-3 by sphingosine-activated but not cAMP-activated PKA suggest that this new mechanism may have profound implications for cell signaling and function.

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‡ The abbreviations used are: PKA, protein kinase A; PKC, protein kinase C; DMS, N,N-dimethylsphingosine; PKI, protein kinase A inhibitor.
**Materials and Methods**

**Reagents**—All sphingolipids were purchased from Biomol, with the exception of sphingosyl-phosphorylcholine (lyso-sphingomyelin) and a set of sphingosine stereoisomers that were obtained from Matreya. All sphingolipids were dissolved in ethanol and stored at −20 °C and either used at ethanol final concentration, 0.1% (v/v) or dried down and resuspended in 5% (v/v) n-octylglucoside (Roche Applied Science) (final concentration, 0.5% (v/v)). Lyso-phosphatidic acid was purchased from Avanti, and all other lipids and lipid metabolites were purchased from Sigma. Phosphatidylycerine and diacetylglycerol were made up in chloroform and used in assays at 3% (v/v) chloroform final concentration. PKI peptide (5–24) was purchased from Biomol, and GF109203X (also known as bisindolylmaleimide I) was developed from Calbiochem. PKA holoenzyme and catalytic subunit were from bovine heart (Sigma). Recombinant wild type and mutant 14-3-3 were purified as described previously (13). Antibody against the catalytic subunit of PKA was from Santa Cruz Biotechnology.

**Cell Culture and Transfection**—COS cells were maintained in RPMI 1640 supplemented with 10% heat-inactivated calf serum and 2 mM L-glutamine. Cells were transfected using Polyfect reagent (Qiagen) following the manufacturer’s protocol and harvested 48 h after transfection. Using a green fluorescent protein reporter construct, the transfection efficiency was found to be 20–30%.

Sphingosine-dependent 14-3-3 Phosphorylation Assays—Sphingosine-dependent phosphorylation of 14-3-3 was essentially carried out as described previously (13) in 20 μl with either cytosolic extract from BALB/3T3 fibroblasts (prepared and described previously) (9), either cytosolic extract with or without sphingolipid (delivered in 0.5% (w/v) ethanol (final concentration, 0.1% (v/v)) or dried down and resuspended in 5% (v/v) n-octylglucoside or 0.25 extract, membrane fraction, or purified PKA (0.7 unit) were added to 20 μl of reaction mixture comprising 50 μM [γ-32P]ATP. Reactions were incubated at 30 °C for 10 min. Reactions were separated by SDS-PAGE, and phosphorylated protein was detected by autoradiography.

Preparation of Membrane Fractions—COS cells were washed with phosphate-buffered saline and then scraped from dishes into phosphate-buffered saline and pelleted by centrifugation. Homogenates were prepared as described previously using 2 × 106 cells/400 μl homogenization buffer (9, 10), and cytosolic and membrane fractions were generated by ultracentrifugation (9, 10). The membrane fractions were resuspended in homogenization buffer supplemented with 0.2% Triton X-100 and 100 mM NaCl to release the membrane-associated PKA enzyme.

**PKA Activity Assays**—Aliquots (10 μl) of eluate fractions, cytosolic extract, membrane fraction, or purified PKA (0.7 unit) were added to 20 μl of reaction mixture comprising 50 μM [γ-32P]ATP in kinase buffer (20 mM Tris-Cl, pH 7.4, 15 mM MgCl2, 25 μM ATP, and 3 mM dithiothreitol), with or without sphingolipid (delivered in 0.5% (v/v) n-octylglucoside with vehicle control) or cAMP and 0.25 μCi of [γ-32P]ATP, followed by incubation at 30 °C for 15 min. After incubation, reactions were separated on 12.5% SDS-PAGE, and phosphorylated protein was detected by autoradiography.

**Size-exclusion Chromatography**—Bovine heart PKA holoenzyme (30 000; and they were fractionated immediately prior to PKA fractionation by size-exclusion chromatography using proprietary reagents (Amersham Biosciences). The system was operated at 1 ml/min and a linear gradient of 0–400 mM NaCl in equilibration buffer, and a column with a 20 μM Tris-Cl, pH 7.4, 15 mM MgCl2, 25 μM ATP, and 3 mM dithiothreitol, with or without sphingolipid (delivered in 0.5% (v/v) n-octylglucoside with vehicle control) or cAMP and 0.25 μCi of [γ-32P]ATP, followed by incubation at 30 °C for 15 min and then spotted onto phosphocellulose filters (Whatman P81). Filters were washed 10 times in 0.75% phosphoric acid and finally washed in acetone before liquid scintillation counting.

Anion-exchange Chromatography—Cytosolic extract (5 mg, total protein) was applied to a MonoQ 5/5 column (Amersham Biosciences) equilibrated in 20 mM potassium phosphate, pH 6.8, 2 mM EDTA, and 1 mM β-mercaptoethanol and operated at 1 ml/min and 4 °C. The column was washed extensively in equilibration buffer, and a linear gradient of 0–400 mM NaCl in equilibration buffer was used to elute bound proteins. Fractions (1 ml) were collected from the entire NaCl elution.

Immunoblotting—Column fractions were separated by SDS-PAGE and transferred to nitrocellulose by electroblotting. Immunoblotting using the anti-PKA catalytic antibody (at 1 μg/ml) was carried out as described previously (13) and developed by enhanced chemiluminescence using proprietary reagents (Amersham Biosciences).

**Results**

**PKA Type II in BALB/3T3 Cell Extract Phosphorylates 14-3-3**—A previously described 14-3-3 phosphorylating activity in BALB/3T3 fibroblast extract phosphorylates 14-3-3 on Ser58 in response to sphingosine and its analog, DMS (9), disrupting the dimeric structure of the protein (13). Computer-based evaluation of the Ser58 region in 14-3-3S using a peptide motif-based scanning algorithm (accessible at ScapeSite.mit.edu) (16) suggested PKA and PKC as candidate kinases for phosphorylation of this site (VVGARRSSWRVVSSI, in which S corresponds to Ser58). Therefore, the effects of PKA and PKC inhibitors on BALB/3T3 fibroblast-derived sphingosine-dependent 14-3-3 phosphorylating activity were examined. A caspase-cleaved form of PKCδ isolated from rat liver was previously shown to phosphorylate 14-3-3 in a sphingosine-dependent manner (17). However, the PKC inhibitor, GF109203X, had no effect on 14-3-3 phosphorylation (Fig. 1), indicating that PKCδ is not the sphingosine-dependent phosphorylating activity in BALB/3T3 fibroblast extract. In contrast, PKA, a PKA-specific inhibitory peptide, did inhibit 14-3-3 phosphorylation (Fig. 1). PhosphorImager quantitation of phosphorylation showed that 0.1 μM PKI inhibited sphingosine-dependent 14-3-3 phosphorylation activity by 70%, whereas 0.5, 1, and 10 μM PKI inhibited sphingosine-dependent 14-3-3 phosphorylation activity by >90% (Fig. 1). Additionally, the PKA inhibitor H89 also abolished 14-3-3 phosphorylation (data not shown). Intriguingly, however, the classical PKA activator cAMP was unable to induce 14-3-3 phosphorylation in BALB/3T3 cell extract (Fig. 1).

To ascertain the biochemical relationship between the 14-3-3 phosphorylation activity in BALB/3T3 fibroblast extract and PKA, we performed anion-exchange chromatography of cell extract. Eluate fractions were assayed for PKA activity as determined by phosphorylation of the PKA peptide substrate, kemptide. A single peak of phosphorylation activity was determined to be fractions 31 and 40 when assayed in the presence of 10 μM cAMP (Fig. 2A). The elution profile of the PKA activity identifies the enzyme as PKA type II and is entirely consistent with previous studies with this cell line (18). Strikingly, 100 μM

**Fig. 1.** PKA inhibitor PKI blocks sphingosine-dependent phosphorylation of 14-3-3S. Sphingosine-dependent 14-3-3 phosphorylation assays were carried out by incubating BALB/3T3 fibroblast cytosolic extract with either cytosolic extract from BALB/3T3 fibroblasts (presumably containing 14-3-3 and PKA) or purified PKA (0.7 unit) and 0.5 μg of 14-3-3S in kinase buffer (20 μM Tris-Cl, pH 7.4, 15 mM MgCl2, 25 μM ATP, and 3 mM dithiothreitol), with or without sphingolipid (delivered in 0.5% (v/v) n-octylglucoside with vehicle control) or cAMP and 0.25 μCi of [γ-32P]ATP, followed by incubation at 30 °C for 15 min. After incubation, reactions were separated on 12.5% SDS-PAGE, and phosphorylated 14-3-3 was detected by autoradiography, and the gel was stained with Coomassie Blue to show equal loading of 14-3-3S substrate even in the absence of phosphorylation. The figure shows a representative result of three experiments.

**Lipid Extraction and Measurement of Sphingosine**—Lipids were extracted from transfected COS cells as described previously (15). Sphingosine levels in the lipid extract were determined by the sphingosine kinase method as detailed elsewhere (15).

**Measurement of cAMP Levels**—Intracellular cAMP levels in transfected COS cells were determined using the cAMP Biotrak enzyme immunoassay system (Amersham Biosciences) following the manufacturer’s instructions.
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DMS also induced phosphorylation of kemptide, with an activity profile identical to that of cAMP-activated PKA but with slightly greater magnitude (Fig. 2A), suggesting that PKA type II fractionated from BALB/3T3 extract may also be activated by sphingolipid. Immunoblotting of all the eluate fractions confirmed the presence of catalytic PKA subunit only in fractions 32–39 (Fig. 2B), consistent with the PKA active fractions (Fig. 2A). 14-3-3ζ phosphorylating activity of all the eluate fractions was also measured in the presence and absence of 100 μM DMS.

The DMS-inducible 14-3-3ζ phosphorylating activity eluted between fractions 31 and 40 and coincided with the peak of PKA activity (Fig. 2C). Furthermore, no 14-3-3ζ phosphorylating activity was detected in the absence of DMS (data not shown), and the DMS-induced 14-3-3ζ phosphorylating activity was inhibited by 10 μM PKI (data not shown), indicating that PKA type II corresponds to the 14-3-3ζ phosphorylating activity in these fractions.

To determine whether PKA type II is capable of phosphorylating 14-3-3ζ in a sphingosine-dependent manner, PKA holoenzyme purified from bovine heart was utilized. By anion-exchange chromatography as described under "Materials and Methods," the presence of PKA catalytic subunit was confirmed by immunoblotting of all the eluate fractions. The activity profile of PKA was similar to that of cAMP-activated PKA, with the exception that PKA type II was activated by sphingosine-dependent phosphorylation.

Materials and Methods. Eluate fractions were analyzed for PKA activity eluted from BALB/3T3 fibroblast extract (Fig. 1). PKA type II holoenzyme phosphorylated 14-3-3ζ only in the presence of DMS and sphingosine, whereas cAMP induced no phosphorylation. This phosphorylation was completely inhibited by the presence of 10 μM PKI, indicating that PKA was indeed responsible for the phosphorylation of 14-3-3ζ (Fig. 3). Additionally, no 14-3-3ζ phosphorylation occurred in the presence of sphingosine-1-phosphate or phosphatidylserine (data not shown), indicating that the phosphorylation of 14-3-3ζ by PKA occurs only in the presence of sphingosine or DMS. Furthermore, PKA did not phosphorylate the S58A 14-3-3ζ mutant (Fig. 3), indicating that Ser58 is the sole target of PKA phosphorylation in 14-3-3ζ.

These results suggest that PKA type II can be activated in a CAMP-independent fashion by sphingosine and DMS to phosphorylate Ser58 of 14-3-3ζ.

Purified PKA Type II is a Sphingosine-dependent Kinase—To determine the direct effect of sphingolipid on PKA activity, we tested the ability of DMS to activate purified bovine heart PKA enzyme. The activity of PKA holoenzyme and catalytic subunit was assayed by a standard PKA assay using kemptide as a substrate in the presence or absence of cAMP or DMS. As shown (Fig. 4A), in the absence of exogenous activator, PKA holoenzyme was essentially inactive, whereas the catalytic PKA subunit was active, as expected. The activation of PKA holoenzyme by 10 μM cAMP induced a 9-fold increase in kemptide phosphorylation relative to inactive enzyme. Significantly, a 10-fold increase in kemptide phosphorylation relative to inactive enzyme. Significantly, a 10-fold increase in kemptide phosphorylation was observed on addition of 100 μM DMS to PKA holoenzyme. Furthermore, addition of 10 μM PKI to DMS-activated PKA completely abolished kemptide phosphorylation, confirming that DMS was stimulating PKA (Fig. 4A). This result is consistent with the previous result using BALB/3T3 fibroblast extract, in which DMS induced activation of PKA holoenzyme (Fig. 2A). In contrast, addition of either cAMP or DMS to the catalytic subunit gave no increase in kemptide phosphorylation, indicating that the activity of the PKA catalytic subunit is not affected by DMS.

Sphingosine Activates PKA by Increasing the Activity of the Enzyme—Sphingosine has previously been shown to activate casein kinase II by increasing the activity of the enzyme (Vmax increased) as well as enhancing the affinity of the enzyme for its substrate (Km reduced) (7). To test whether the effect of DMS on PKA holoenzyme is on substrate availability or enzyme activation, PKA activity was determined using different concentrations of kemptide substrate and increasing concentrations of DMS. If DMS affects the substrate rather than the kinase, increasing the DMS concentration should effectively increase the substrate available. However, as shown (Fig. 4B), the maximal activity of PKA assayed using different concentrations of kemptide substrate was achieved with the same concentration of DMS, indicating that DMS acts directly on the enzyme and not on kemptide availability.

The Km and Vmax values of DMS- and cAMP-activated PKA type II were determined using 100 μM DMS and 10 μM cAMP,
Activation of PKA Type II is Specific to Non-acylated Sphingosines—The specificity of PKA holoenzyme activation was examined in a panel of sphingolipids and related molecules. PKA activity was determined by kemptide phosphorylation. PKA holoenzyme was activated to a similar degree in response to 50 μM sphingosine, DMS, trimethyl-sphingosine, dihydro-sphingosine, and phyto-sphingosine, but not by sphingosine-1-phosphate, C2-ceramide, sphingomyelin, spermine, or linoleic acid (Fig. 5). This suggests that activation of PKA type II is restricted to sphingosine molecules with a non-acylated amino group, whereas the more complex N-acylated sphingolipids (ceramide and sphingomyelin) are not capable of activating PKA. Sphingosyl-phosphorylcholine was also tested for its ability to activate PKA holoenzyme. This sphingolipid represents a non-acylated form of sphingomyelin (lysosphingomyelin). Intriguingly, although sphingomyelin failed to activate PKA, sphingosyl-phosphorylcholine did activate PKA (data not shown).

The activation of PKA holoenzyme by sphingosine and DMS was further characterized in a dose-dependent PKA activation study. The EC50 value for PKA activation was 18.8 μM by sphingosine and 25.3 μM by DMS.

The stereospecificity of PKA activation by sphingosine was examined. A panel of sphingosine stereoisomers (D-erythro,-l-erythro,-d-threo-, and l-threo-sphingosine) was tested for the ability to activate PKA holoenzyme. These sphingosines (purchased from Matreya) were relatively insoluble in ethanol and hence were delivered in n-octylglucoside (0.5%, final concentration). At 50 μM, D-erythro-sphingosine from this source was as potent as D-erythro-sphingosine from Biomol. D-Threo-sphingosine induced 50% of the activity induced by D-erythro-sphingosine, whereas l-threo- and l-erythro-sphingosine were unable to induce PKA activity (data not shown).

The Stereospecificity of PKA Activation by Sphingosine—The stereospecificity of PKA activation by sphingosine was examined. A panel of sphingosine stereoisomers (D-erythro,-l-erythro,-d-threo-, and l-threo-sphingosine) was tested for the ability to activate PKA holoenzyme. These sphingosines (purchased from Matreya) were relatively insoluble in ethanol and hence were delivered in n-octylglucoside (0.5%, final concentration). At 50 μM, D-erythro-sphingosine from this source was as potent as D-erythro-sphingosine from Biomol. D-Threo-sphingosine induced 50% of the activity induced by D-erythro-sphingosine, whereas l-threo- and l-erythro-sphingosine were unable to induce PKA activity (data not shown).

The Sphingosine-dependent Activation of PKA Type II Is Specifically Inhibited by Phosphatidylserine and Phosphoryl-ethanolamine—Sphingosine inhibits the kinase activity of PKC (3, 4), an enzyme classically activated by the acidic lipid phosphatidylserine. The inhibitory effect of sphingosine on PKC is thought to be related to the charge neutralization of basic sphingosine by acidic phosphatidylserine (3). Therefore, the effect of phosphatidylserine on the sphingosine activation of PKA was studied using PKA type II holoenzyme and kemptide as substrate. As shown (Fig. 6A), 50 μM phosphatidylserine had no effect on PKA type II activity in the absence of activator and had little effect on cAMP activation of PKA, but it completely inhibited DMS-activated PKA. In contrast, 50 μM diacylglycerol had little effect on either inactive or DMS- or cAMP-activated PKA (Fig. 6A). This indicates that the effect of phosphatidylserine on DMS-activated PKA is specific and may, as with the effect on PKC, be related to charge neutralization.
activity was assayed by cAMP-mediated activation of PKA type II. The effect of sphingosine on DMS- and cAMP-activated PKA holoenzyme was determined. A, PKA holoenzyme activity was assayed by kemptide phosphorylation in 0.1% (v/v) ethanol alone (white bars) or in the presence of 10 μM cAMP (black bars) or 100 μM DMS (gray bars) with either vehicle alone (3% chloroform) (Control), 50 μM sphingosylphosphorylcholine (SPS), or 50 μM diacylglycerol (DAG). B, PKA holoenzyme activity was assayed by kemptide phosphorylation in the presence of 10 μM cAMP (black bars) or 100 μM DMS (gray bars) with either vehicle alone (0.1% ethanol) (Control), 10 μM phosphoryl-ethanolamine (PE), or 50 μM lyso-phosphatidic acid (LPA). The values represent means ± S.E. of triplicate determinations, and both experiments were repeated twice.

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Sphingosine Activates PKA by a Mechanism Distinct from cAMP Activation—cAMP induces activation of PKA by binding to the dimeric regulatory subunits, causing them to dissociate from the tetrameric PKA holoenzyme complex and thereby releasing the inhibition on catalytic subunits (2). To determine whether a similar mechanism was involved in sphingosine-induced activation of PKA, the ability of DMS to dissociate the holoenzyme into catalytic and regulatory subunits was examined by size-exclusion chromatography. Purified bovine heart PKA holoenzyme (M, 174,000) and PKA catalytic subunit (M, 38,000) eluted from the column at fraction 11 and fraction 22, respectively. As expected, pre-incubation of holoenzyme with 30 μM cAMP completely dissociated the PKA holoenzyme, with the peak of PKA activity shifting to fraction 22 (Fig. 7A), and the catalytic subunit detected by immunoblotting appearing only in fractions 20–23 (Fig. 7B). In contrast, pre-incubation of holoenzyme with 50 μM DMS and elution in the presence of 50 μM DMS resolved a peak of PKA activity around fraction 11 (Fig. 7A), and the catalytic subunit was detected only in fractions 9–13 (Fig. 7C), consistent with the position of intact holoenzyme. These results indicate that, unlike cAMP, DMS is unable to dissociate the PKA holoenzyme complex.

Modulation of Sphingosine Levels in Vivo Alters Basal PKA Activity—To determine whether the in vitro activation of PKA by sphingosine occurs in vivo, we used direct and molecular approaches to manipulate the level of sphingosine in COS cells and examined the corresponding effect on basal membrane-associated PKA activity, i.e. PKA activity in the absence of exogenously added cAMP.

Initially, DMS treatment was used as a means of inducing sphingosine accumulation in cells. The in vivo use of DMS has a dual effect in that, in addition to contributing directly to sphingosine accumulation as an exogenous sphingosine source, DMS also inhibits the sphingosine-converting enzyme, sphingosine kinase (20), thereby enhancing endogenous sphingosine levels. COS cells were treated either with or without 10 μM DMS for 30 min, and the cells were then lysed, and membrane fractions were prepared and solubilized in homogenization buffer containing 0.2% Triton X-100 and 100 mM NaCl to release the membrane-associated PKA enzyme (19). The total kinase activity associated with the membrane was then determined by kemptide assay in the presence of 10 μM cAMP. The kemptide phosphorylating activity was also determined in the absence of cAMP as a measure of basal kinase activity, and this is expressed relative to total kinase activity as shown in Fig. 8. It can be seen that DMS treatment of cells significantly increased the basal kinase level relative to the total kinase activity compared with untreated cells (Fig. 8A). Moreover, determination of basal kemptide phosphorylating activity in the presence of the PKA-specific inhibitor PKI shows that >50% of the basal activity is inhibited, indicating that PKA is responsible for the majority of this basal activity. Thus, exogenous addition of DMS appears to increase basal membrane-associated PKA activity in COS cells, consistent with the accumulation of sphingosine and predicted activation of PKA.

In a second approach, we have used overexpression of sphingosine kinase to manipulate endogenous sphingosine levels. It
We have shown that the activation of PKA type II is specific to non-acylated sphingosines and that acylated sphingolipids and sphingosine-1-phosphate are unable to induce PKA activation (Fig. 5). The activation of PKA holoenzyme by sphingosylphosphorylcholine is intriguing and may account for the previously observed effect of this lipid on protein phosphorylation in Jurkat T cell extracts (22). However, very little is known about the formation of sphingosylphosphorylcholine in the cell (23), making the physiological significance of this finding unclear. Additionally, phosphatidylinositol and diacylglycerol had no effect on PKA holoenzyme activity (Fig. 6A). This is by no means a comprehensive survey of potential lipid activators, but it does indicate that the activation of PKA type II is specific to sphingosines, a group whose members are gaining recognition as lipid second messengers (4).

Our results clearly demonstrate that PKA type II phosphorylates Ser\(^{30}\) of 14-3-3 and that this phosphorylation is specific to sphingosine-activated PKA (Fig. 3). This finding raises the possibility that sphingosine-activated PKA exhibits substrate selectivity, with 14-3-3 representing a prototypic substrate for sphingosine-activated PKA. Unlike cAMP, sphingosine did not induce dissociation of the PKA holoenzyme complex (Fig. 7). It is intriguing to speculate that sphingosine may activate the intact PKA holoenzyme complex, although additional studies are required to confirm this. The exact molecular basis for activation of PKA by sphingosine is unclear, but clues may be drawn from the inhibitory action of the acidic lipid phosphatidylinositol. In the case of PKC, sphingosine is thought to inhibit the enzyme by causing neutralization of acidic phosphatidylinositol (3), suggesting that the basic charge on sphingosine is important for its effect. From our results, the basic nature of sphingosine also appears to be important for PKA activation (Fig. 5), suggesting that the charge on the molecule plays a role in PKA activation, explaining the inhibitory effect of phosphatidylinositol and phosphoryl-ethanolamine on sphingosine-induced PKA activity (Fig. 6). Our results also indicate that sphingosine-induced activation of PKA type II is mediated by the regulatory subunit of the enzyme, which is an acidic protein. It is therefore possible that direct binding of sphingosine to the type II regulatory subunit mediates sphingosine-induced PKA activation. It is currently unclear whether PKA type I can be similarly activated by sphingosine.

For PKA type II to be activated by sphingosine in vivo, it must be localized to sites of sphingosine generation. Sphingosine is generated primarily at the plasma membrane from the breakdown of ceramide, which, in turn, is derived from sphingomyelin in the outer leaflet of the plasma membrane. This sphingomyelin pathway is activated by many physiological activators that cause membrane rearrangement and lead to sphingosine accumulation in the membrane (24). Intriguingly, PKA type II is also predominantly found associated with membranes, through association with protein kinase A-anchoring proteins (25), placing it in close proximity to the site of sphingosine accumulation. Consistent with the notion that sphingosine activates membrane-associated PKA type II, we have...
shown that in vivo modulation of sphingosine levels alters the pattern of basal membrane-associated PKA activity (Fig. 8). Indeed, depletion of endogenous sphingosine levels by overexpression of sphingosine kinase was sufficient to reduce basal PKA activity in transiently transfected COS cells, indicating that PKA is sensitive to fluctuations in endogenous sphingosine levels (Fig. 8B). This reduction in basal PKA activity may also be attributable in part to the breakdown product of sphingosine-1-phosphate, phosphoryl-ethanolamine, which we have shown inhibits sphingosine-activated but not cAMP-activated PKA activity (Fig. 6B). Additionally, physiological activators of the sphingomyelin pathway are known to generate levels of sphingosine sufficient to activate PKA. For instance, tumor necrosis factor α treatment induces sphingosine levels equivalent to 10–20 μM in human neutrophils (26). Moreover, the sphingosine-dependent activity of membrane-associated PKA type II would be tightly regulated because, under normal conditions, phosphatidylinerine is found predominantly in the inner leaflet of the plasma membrane. Therefore, the sphingosine-dependent activity of PKA type II would be minimal unless phosphatidylinerine is removed from the inner leaflet through membrane rearrangement, the same conditions that lead to sphingosine accumulation. The concentration of sphingosine required for PKA activation (EC50 values of 18 and 25 μM for sphingosine and DMS, respectively) is comparable with that required for in vitro inhibition of PKC (IC50 values of 50 and 10–15 μM for sphingosine and DMS, respectively) (4). In the case of PKC, the inhibitory effect of sphingosine and DMS has been established in vivo (4), indicating that the levels of sphingosine in vivo are sufficient for PKA activation. Moreover, it appears that the regulation of PKA and PKC by sphingosine and phosphatidylinerine can be directly opposed in vivo: PKA is activated by sphingosine, and this activation is inhibited by phosphatidylinerine, whereas PKC is activated by phosphatidylinerine and inhibited by sphingosine. This suggests that the levels of phosphatidylinerine and sphingosine in the membrane can coordinate regulation of PKA and PKC activity. This requires further investigation and may reveal a new level of control over PKA and PKC signaling.

The identification of PKA as a sphingosine-dependent kinase is at variance with a previous report that indicated that sphingosine had no effect on PKA activity (3). However, the absence of effect reported may reflect the substrate used but not detailed because we found cAMP-activated but not DMS-activated PKA was able to phosphorylate casein (data not shown). Complex sphingolipid-containing gangliosides from the brain have previously been shown to stimulate PKA activity in a cAMP-independent manner (27) and, furthermore, in concordance with our results, did not dissociate the holoenzyme complex. Our results also appear to differ with respect to the phosphorylation of 14-3-3 by PKA. Previous studies failed to detect phosphorylation of 14-3-3 by PKA (9, 28). However, these studies were carried out in the absence of sphingolipid. This is an important factor because we only observe 14-3-3 phosphorylation by PKA in the presence of sphingolipid, but not cAMP (Figs. 1 and 3). The absence of 14-3-3 phosphorylation following cAMP activation of PKA is intriguing and suggests that the release of the PKA catalytic subunit is insufficient to allow 14-3-3 phosphorylation. This is supported by studies with purified catalytic subunit in which no 14-3-3 phosphorylation is detected (data not shown). Additional studies are in progress to examine the molecular basis of 14-3-3 phosphorylation by PKA in the presence of sphingosine. Our findings show that PKA type II phosphorylates Ser56 of 14-3-3, thereby inducing monomerization of the 14-3-3 protein (13). It is intriguing that this activity, as with the phosphorylation of 14-3-3 by caspase-cleaved PKCζ (17), is sphingosine-dependent. Sphingosine, DMS, and physiological activators of the sphingomyelin pathway induce apoptosis in many cell types (4). It is therefore possible that the sphingosine-dependent activity of PKA contributes to this apoptotic process. In particular, the PKA-mediated sphingosine-induced phosphorylation of 14-3-3 on Ser56 disrupts the function of dimeric 14-3-3, which, as discussed previously (13), may lead to an imbalance in the stress- and mitogen-activated protein kinase pathways and contribute to sphingosine-induced cell death. Additionally, removal of sphingosine by sphingosine kinase, an enzyme associated with enhanced proliferation and survival of cells (15), may act to alleviate sphingosine-induced PKA activation and thereby reduce apoptosis of cells. Thus, the sphingosine-dependent activation of PKA type II could potentially affect multiple biological processes, at least some of which are sphingosine-induced signaling events that are involved in apoptosis.

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