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Sphingosine Inhibition of Protein Kinase C Activity and of Phorbol Dibutyrate Binding *in Vitro* and in Human Platelets*

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Sphingosine inhibited protein kinase C activity and phorbol dibutyrate binding. When the mechanism of inhibition of activity and phorbol dibutyrate binding was investigated *in vitro* using Triton X-100 mixed micellar methods, sphingosine inhibition was subject to surface dilution; 50% inhibition occurred when sphingosine was equimolar with *sn*-1,2-dioleoylglycerol ($diC_{18:1}$) or 40% of the phosphatidylserine (PS) present. Sphingosine inhibition was modulated by Ca^{2+} and by the mole percent of $diC_{18:1}$ and PS present. Sphingosine was a competitive inhibitor with respect to $diC_{18:1}$, phorbol dibutyrate, and Ca^{2+}. Increasing levels of PS markedly reduced inhibition by sphingosine. Since protein kinase C activity shows a cooperative dependence on PS, the kinetic analysis of competitive inhibition was only suggestive. Sphingosine inhibited phorbol dibutyrate binding to protein kinase C but did not cause protein kinase C to dissociate from the mixed micelle surface. Sphingosine addition to human platelets blocked thrombin and *sn*-1,2-dioctanoylglycerol-dependent phosphorylation of the 40-kDa (47 kDa) dalton protein. Moreover, sphingosine was subject to surface dilution in platelets. The mechanism of sphingosine inhibition is discussed in relation to a previously proposed model of protein kinase C activation. The possible physiological role of sphingosine as a negative effector of protein kinase C is suggested and a plausible cycle for its generation is presented. The potential physiological significance of sphingosine inhibition of protein kinase C is further established in accompanying papers on HL-60 cells (Merrill, A. H., Jr., Sereni, A. M., Stevens, V. L., Hannun, Y. A., Bell, R. M., Kinkade, J. M., Jr. (1986) *J. Biol. Chem.* 261, 12610–12615) and human neutrophils (Wilson, E., Olcott, M. C., Bell, R. M., Merrill, A. H., Jr., and Lambeth, J. D. (1986) *J. Biol. Chem.* 261, 12616–12623). These results also suggest that sphingosine will be a useful inhibitor for investigating the function of protein kinase C *in vitro* and in living cells.

The central function of the phospholipid, Ca^{2+}, and *sn*-1,2-diacylglycerol-dependent protein kinase (protein kinase C) in transducing intracellularly extracellular signals has recently been recognized (1). These extracellular agents which include neurotransmitters, hormones, and growth factors (1, 2) are bound by specific cell surface receptors and elicit by transmembrane signaling the generation of two second messengers by stimulating the degradation of phosphatidylinositols (1, 3, 4). Inositol 1,4,5-trisphosphate mobilizes intracellular calcium (4), whereas, *sn*-1,2-diacylglycerol (DAG[1]) activates protein kinase C by a mechanism whereby the enzyme undergoes translocation from the cytosol to the plasma membrane (1, 5). The membrane contains phosphatidylserine which is required for activation (6). Protein kinase C has been demonstrated to be the intracellular receptor of the tumor promoting phorbol esters which activate the enzyme by interaction at the *sn*-1,2-diacylglycerol site (7–10). The pleiotropic effects of protein kinase C activation and the recognition that it functions as a transducer of DAG and calcium second messengers make detailed understanding of its mechanism of regulation desirable. Our laboratory has undertaken detailed structure-function analysis of diacylglycerol-protein kinase C interactions and studies on the mechanism of regulation by phosphatidylserine, calcium, *sn*-1,2-diacylglycerols, and lipids in Triton X-100 mixed micelles (11–13).

The discovery or development of inhibitors of protein kinase C has the potential to further the understanding of specific functions of the enzyme in cells and animals. Several inhibitors of protein kinase C have been reported. These include calmodulin antagonists (14–16), H7 (17), adriamycin (15), alkyllysophospholipid (18), a nonsteroidal anti-estrogen, tamoxifen, (19), anti-neoplastic lipoidal amine (20), amiloride (21), verapamil (14), bilirubin (22), and palmitoylcarnitine (12). Herein, we report on the discovery that sphingosine is an inhibitor of protein kinase C.

In this article, we report on the inhibition of protein kinase C by sphingosine and related compounds, the phosphatidylserine, diacylglycerol (phorbol diester), and Ca^{2+} modulation of this inhibition, and the inhibition of phorbol dibutyrate binding. Furthermore, studies in human platelets demonstrate sphingosine inhibition of thrombin and diC_8-dependent activation of protein kinase C and inhibition of phorbol ester binding. Sphingosine inhibition of protein kinase C may have physiological significance in that a regulated metabolic cycle may exist whereby sphingosine functions as a negative effector of protein kinase C. In two accompanying manuscripts (23, 24), the effects of sphingosine on neutrophil function and on HL-60 cell activities are documented.

EXPERIMENTAL PROCEDURES

Materials

Charles River CD female rats were used for the source of protein kinase C. Ultrogel AcA 44 and Ultrogel AcA 202 were from LKB. [^{32}P]Orthophosphate, Aquasol II, [γ-^{32}P]ATP, and [^{3}H]PDBu (12.5

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[1] The abbreviations used are: DAG, *sn*-1,2-diacylglycerol; PS, phosphatidylserine; $diC_{18:1}$, *sn*-1,2-dioleoylglycerol; diC_8, *sn*-1,2-dioctanoylglycerol; PA, phosphatidic acid; PDBu, phorbol dibutyrate.

Ci/nmol) were from New England Nuclear. Calf thymus histone type III-S, phospholipase C, thrombin, phenylmethylsulfonyl fluoride, stearylamine, bovine serum albumin, phorbol dibutyrate, 4-sphinganine, *threo*sphingosine, and dihydrosphingosine were from Sigma. Leupeptin was from the Peptide Institute (Osaka, Japan). 1,2-Dioleoyl-*sn*-glycerol-3-phosphoserine, 1,2-dioctanoylglycerol, and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine were from Avanti Polar Lipids. *sn*-1,2-Dioleoylglycerol was synthesized from dioleoylphosphatidylcholine as previously described (25). Triton X-100 was from Research Products International Corp. Octylamine was from Aldrich. Swainsonine was a gift from Harry Broquist (Department of Biochemistry, Vanderbilt University), ceramide was a gift from Jim Walsh (Department of Biochemistry, Duke University), *N*-acetylsphingosine was a gift from Barry Ganong (Department of Biochemistry, Duke University), 1,3-dihydroxy-2-amino-3-phenylpropane was a gift from Dennis Liotta (Department of Chemistry, Emory University). Phorbol 12-myristate 13-acetate was from Pharmacia P-L Biochemicals.

Methods

Partial Purification of Protein Kinase C—Protein kinase C was partially purified from rat brain as previously described (13).

Mixed Micellar Assay for Protein Kinase C Activity—Protein kinase C was assayed with Triton X-100 mixed micelles as previously described (13). Sphingosine was dried down with the lipid cofactors.

Mixed Micellar Assay for [^{3}H]PDBu Binding to Protein Kinase C—[^{3}H]PDBu binding was performed as previously described (26).

Preparation of Human Platelets—Human platelets were prepared from freshly drawn blood essentially as described by Siess *et al.* (27). They were then suspended in modified Tyrode's buffer to a concentration of 2.5×10^8 platelets/ml.

[^{3}H]PDBu Binding to Human Platelets—Human platelets, prepared as described above, were suspended at a concentration of 2.5×10^8 platelet/ml. 50 μl of the platelets were then incubated for 5 min with the indicated concentration of sphingosine in Eppendorf microfuge tubes. Sphingosine was prepared in 50% ethanol at a concentration 100-fold the final concentration so that ethanol was kept at 0.5%. [^{3}H]PBDu was added to 10 nM and incubated with the platelets at 37 °C for 10 min. The samples were then filtered on Whatman GF/C filters pre-washed with 5 ml of modified Tyrode's buffer containing 0.1% bovine serum albumin, washed with 10 ml of the same buffer, dried, and counted in 10 ml of Aquasol II in an LKB β counter. Nonspecific binding was determined in the presence of 1 μM unlabeled PDBu and subtracted from the total counts to yield the specific binding.

40-kDa Phosphorylation in Human Platelets—$^{32}P_i$ at 0.2 mCi/ml was added to the platelet suspension and labeling was allowed to proceed for 75 min at 37 °C, after which the platelets were pelleted at $600 \times g$ for 10 min and resuspended in Tyrode's buffer to the same concentration. They were then aliquoted in Eppendorf microfuge tubes and pre-incubated at 37 °C for 5 min with the varying concentrations of sphingosine. Platelets were then stimulated with either 5 μM dioctanoylglycerol (added as an ethanol solution with the final concentration of ethanol 0.5%), 10 μM phorbol 12-myristate 13-acetate, or 1 unit/ml thrombin. The reactions were stopped after 30 s by the addition of an equal volume of 2 × sample buffer, and the samples were then boiled for 3 min. 0.1 ml were then loaded on 10% sodium dodecyl sulfate-polyacrylamide gels and electrophoresis was performed according to the method of Laemmli (28). Gels were subsequently fixed in water/methanol/acetic acid (60:30:10), dried, and autoradiographed.

Phospholipid Quantitation—Phospholipids were extracted from whole platelets by the method of Bligh and Dyer (29). Phosphatidylserine was purified by two-dimensional thin layer chromatography on Silica Gel H plates developed in chloroform/methanol/acetic acid (65:25:10, v/v) in the first dimension and in chloroform/methanol/88% formic acid (65:25:10, v/v) in the second dimension. Phospholipids were quantitated by measuring phosphates according to the method of Ames (30).

The data shown is representative of at least three sets of experiments.

RESULTS AND DISCUSSION

When the effect of sphingosine on protein kinase C activity was tested using the Triton X-100 mixed micelle assay (13) containing 6 mol % of PS and 2 mol % of $diC_{18:1}$, sphingosine proved to be a potent inhibitor (Fig. 1). Under these conditions, the bulk concentration of PS was 260 μM and $diC_{18:1}$ was 86 μM. Therefore, 50% inhibition (100 μM) occurred on a molar basis equivalent to [$diC_{18:1}$] or 0.4 [PS]. The potency of sphingosine inhibition was markedly affected by the number of Triton X-100 mixed micelles containing 7 mol % of PS and 1 mol % of $diC_{18:1}$ present in the assay (Fig. 2*A*). Thus, the effect of sphingosine was subject to surface dilution. When the data are expressed as mole percent (sphingosine:Triton X-100), sphingosine inhibition at four different levels of Triton X-100 mixed micelles (containing PS and $diC_{18:1}$) was identical (Fig. 2*B*) implying that it is the number of sphingosine molecules present in each mixed micelle that determines the potency and not the absolute concentration. For surface active amphipathic molecules, the expression of inhibitor potencies must be relative to the amount of surface (micelles in this case) as bulk concentrations are misleading. These results also imply that sphingosine interacts with the surface-bound protein kinase C probably by interfering with the function of its regulatory domain. To test this hypothesis, the catalytic domain (protein kinase M) was generated by proteolysis of protein kinase C.[2] The activity of this catalytic

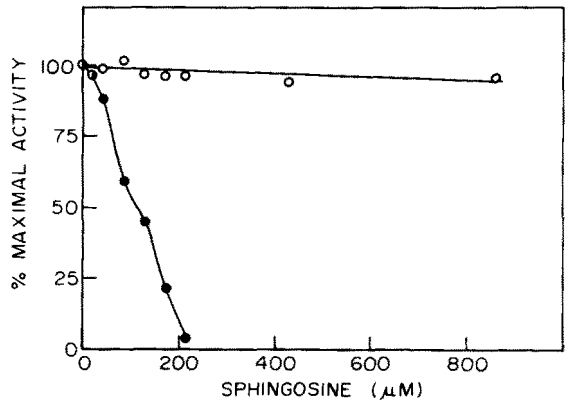

FIG. 1. **Inhibition of protein kinase C activity by sphingosine.** Mixed micelles were formed with 3% (w/v) Triton X-100 containing PS at 6 mol %, $diC_{18:1}$ at 2 mol %, and sphingosine at 10-fold the indicated concentrations. The mixed micelles were then diluted 1:10 into the assay mixture. 1 mol % of sphingosine is equivalent to 43 μM. Effect of sphingosine on protein kinase C (●) and on protein kinase M (○).[2] Identical results were obtained with protein kinase M when sphingosine was added in 0.3% Triton X-100 solution without PS and $diC_{18:1}$.

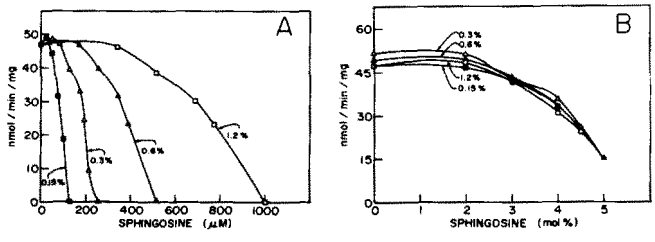

FIG. 2. **Effect of mixed micelle concentration on potency of sphingosine inhibition.** *A*, mixed micelles were formed with Triton X-100 at 12% (w/v, □), 6% (▲), 3% (△), and 1.5% (■) containing 7 mol % of PS, 1 mol % of $diC_{18:1}$, and sphingosine at 10-fold the indicated concentrations. The mixed micelles were then diluted 1:10 into the assay mixture. *B*, mixed micelles were formed with 3% Triton X-100, 7 mol % of PS, 1 mol % of $diC_{18:1}$, and 2–5 mol % of sphingosine. These were then diluted 1:20 (■), 1:10 (△), 1:5 (▲), and 2:5 (□) into the assay mixture. In these experiments, 1 mol % of sphingosine corresponds to 21.5, 43, 86, and 172 μM, respectively.

[2] The catalytic domain was generated by trypsin treatment of purified protein kinase C (31) and purified by Ultrogel AcA 44 molecular sieve chromatography. The fractions showing protein kinase activity independent of Ca^{2+}, PS, and DAG were pooled and used for the above experiments.

domain which is independent of Ca^{2+}, phospholipid, and DAG/phorbol esters (31) was not inhibited by sphingosine (Fig. 1). Thus, sphingosine does not appear to inhibit by interaction with the active site.

To further investigate the mechanism of sphingosine inhibition, the *sn*-1,2-di$C_{18:1}$ dependency of protein kinase C activation was investigated at fixed PS (6 mol %) and Ca^{2+} (50 μM) and at several levels of sphingosine. Importantly, the inhibition by sphingosine was modulated strongly by di$C_{18:1}$ Fig. 3*A*). Double reciprocal plots (Fig. 3*B*) indicated essentially a competitive type of inhibition with respect to di$C_{18:1}$. Similarly, inhibition by sphingosine was overcome by increasing concentration of phorbol dibutyrate (Fig. 4*A*), and double reciprocal plots (Fig. 4*B*) showed competitive inhibition.

When the concentration of sphingosine was varied at 5, 6, and 7 mol % of PS and fixed di$C_{18:1}$ (2 mol %), the potency was modulated markedly (Fig. 5). This was especially true when PS was not saturating at 5 mol %. Shifting from 6 to 7 mol % of PS, a level at which PS becomes saturating (Fig. 6), revealed that the curves were simply not displaced on a mole for mole basis. When PS was in excess, higher concentrations of sphingosine were required for inhibition (Fig. 5).

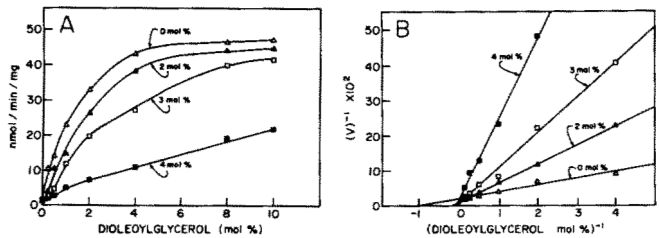

FIG. 3. **Interaction of sphingosine with dioleoylglycerol.** Mixed micelles were formed at 3% Triton X-100, 6 mol % of PS, 0–10 mol % of di$C_{18:1}$, and sphingosine at 0 mol % (△), 2 mol % (▲), 3 mol % (□), and 4 mol % (■). *A*, protein kinase C activity assayed in the presence of 50 μM $CaCl_2$. *B*, double reciprocal plots with di$C_{18:1}$ concentration in mole percent.

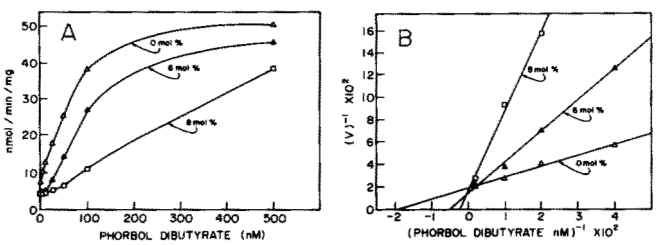

FIG. 4. **Interaction of sphingosine with phorbol dibutyrate.** Mixed micelles were formed with 10 mol % of PS and 0 mol % (△), 6 mol % (▲), and 8 mol % (□) of sphingosine. PDBu was added as an aqueous solution. *A*, protein kinase C activity assayed in the presence of 10 μM $CaCl_2$. *B*, double reciprocal plots of data in *A*.

Next, the effect of 2 and 4 mol % of sphingosine on the PS dependence of protein kinase C activation was investigated (Fig. 6). Interestingly, sphingosine caused a displacement of the PS dependence to higher levels which remained strongly cooperative in the presence or absence of inhibitor. When the PS dependencies were then plotted according to Hill, Hill numbers of 5.4, 6.9, and 8.8 were obtained for the curves generated at 0, 2, and 4 mol % of sphingosine, respectively. Double reciprocal plots were not constructed because of the cooperativity observed with PS and the sphingosine-dependent change in Hill numbers. In that additional PS completely overcame the inhibition by sphingosine, a competitive form of inhibition appears likely.

To further explore the mechanism of sphingosine inhibition, the effect of the level of Ca^{2+} employed was examined at fixed PS and di$C_{18:1}$ and at 0, 3, and 4 mol % of sphingosine. Double reciprocal plots of the calcium dependencies were linear and appeared competitive (Fig. 7).

The effect of sphingosine on phorbol ester binding was examined to further substantiate that sphingosine inhibition occurs by interfering with the regulatory domain of protein kinase C. Sphingosine inhibited phorbol ester binding to Triton X-100 mixed micelles containing 16 and 20 mol % of PS (Fig. 8). The concentration dependence of sphingosine displacement of phorbol dibutyrate, paralleled its ability to inhibit protein kinase C activation by PDBu (Fig. 4*A*). Sphingosine also inhibited phorbol binding when phosphatidic acid was used as the lipid cofactor (PA supports only 50% of the binding measured with PS).

The possibility that sphingosine causes inhibition of activity and phorbol binding by preventing protein kinase C interaction with and binding to mixed micelles was tested (Fig. 9*A*) by molecular sieve chromatography of the appropriate complexes (13). As seen in Fig. 9*B*, protein kinase C remained bound to Triton X-100 mixed micelles containing PS in the presence of sphingosine; sphingosine caused displacement of phorbol dibutyrate without breaking the association of protein kinase C with Ca^{2+} and the Triton X-100/PS mixed micelles.

Next, we tested in human platelets whether sphingosine would inhibit protein kinase C activation by thrombin. This was determined by monitoring the protein kinase C-induced phosphorylation of the 40 (47)-kDa polypeptide (32) (Fig. 10*A*). As shown in Fig. 10*B*, sphingosine caused nearly com-

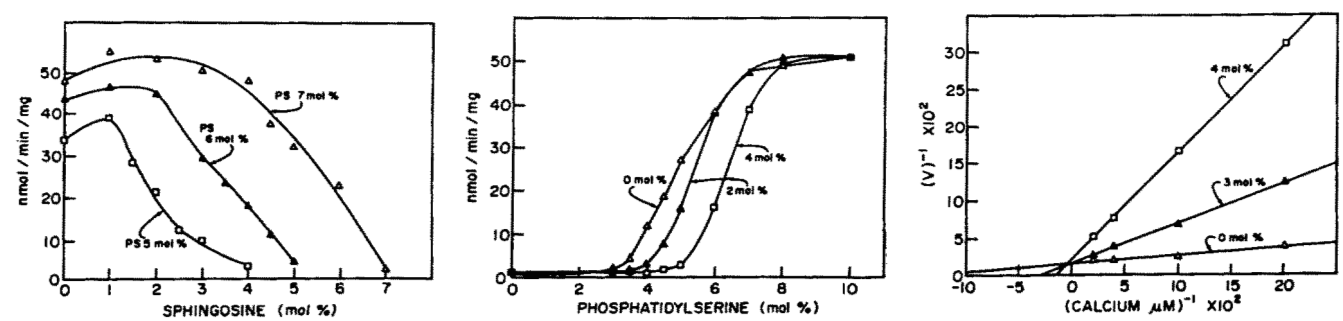

FIG. 5 (*left*). **Potency of sphingosine inhibition as a function of phosphatidylserine.** Mixed micelles contained 2 mol % of di$C_{18:1}$ and 5 mol % (□), 6 mol % (▲), and 7 mol % (△) of PS, and variable mole percent of sphingosine.

FIG. 6 (*center*). **Interaction of sphingosine with phosphatidylserine.** Mixed micelles were formed with di$C_{18:1}$ at 1 mol % and sphingosine at 0 mol % (△), 2 mol % (▲), and 4 mol % (□), and activity was measured in the presence of 50 μM $CaCl_2$.

FIG. 7 (*right*). **Interaction of sphingosine with Ca^{2+}.** Mixed micelles contained 6 mol % of PS, 1 mol % of di$C_{18:1}$, and 0 mol % (△), 3 mol % (▲), and 4 mol % (□) of sphingosine.

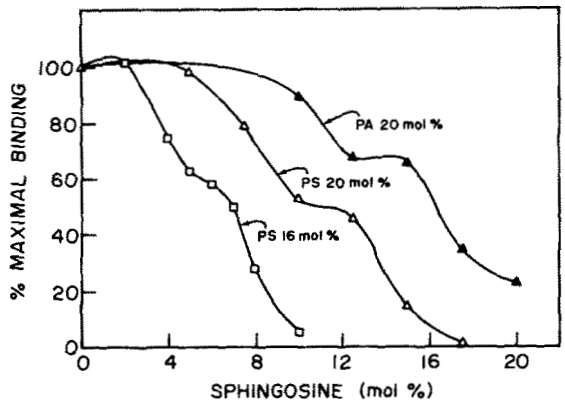

FIG. 8. **Sphingosine inhibition of phorbol dibutyrate binding.** Mixed micelles contained PS at 16 mol % (□) and 20 mol % (△) or PA at 20 mol % (▲) and 0–20 mol % of sphingosine. Binding studies were performed as described under "Methods." The data are plotted as percent of maximal binding under each condition. With 16 mol % of PS there was 82% of the [^{3}H]PDBu binding seen with 20 mol % of PS. At 20 mol % of PA there was only 51% of the binding seen with 20 mol % of PS.

plete inhibition of 40-kDa phosphorylation at a concentration of 25 μM. Partial inhibition was seen with concentrations as low as 10 μM. Sphingosine also inhibited phorbol 12-myristate 13-acetate and diC$_8$-induced 40-kDa phosphorylation (data not shown).

To further analyze the mechanism by which sphingosine inhibits protein kinase C activation, PDBu binding to whole human platelets was studied (Fig. 11). Saturable and displaceable binding of [^{3}H]PDBu to platelets was demonstrated (data not shown). Sphingosine was able to inhibit this binding in concentrations similar to those required to inhibit 40-kDa phosphorylation.[3]

Since sphingosine is an amphiphilic molecule and would be expected to partition into a bilayer or micelle, a direct comparison of its biologic effects to its *in vitro* potency in inhibiting protein kinase C requires that its bulk concentration be expressed as mole percent of sphingosine to phospholipids. To accomplish this, the amount of PS and total phospholipids in platelet membranes were quantitated. Human platelets were found to have 20% of their total phospholipids as PS and the absolute PS concentration under the assay conditions was 200 μM. Therefore, the sphingosine concentrations (expressed as mole percent of sphingosine:phospholipids) required to inhibit platelet 40-kDa phosphorylation were similar to those required for *in vitro* inhibition of protein kinase C: 3–5 mol % of sphingosine required for complete inhibition (or 15–25% of PS). In addition, this inhibition demonstrated the same reversibility observed in the *in vitro* system in that increasing the concentration of diC$_8$ or of platelets (*i.e.* PS) overcomes the inhibition (data not shown).

With these data in hand, we investigated the specificity of protein kinase C inhibition by sphingosine using a number of related molecules. As seen in Fig. 12, octylamine did not inhibit at the concentrations tested whereas stearylamine was nearly as effective as sphingosine. Swainsonine, structurally related to sphingosine (33), was not an inhibitor. *N*-Acetylsphingosine, ceramide, and 1,3-dihydroxy-2-amino-3-phenylpropane analogue were without effect. Fatty acids, and cetyltriethylammonium bromide were also inactive. However, 3-ketosphinganine, *erythro*-, and *threo*-sphinganine were all

[3] Sphingosine concentrations that produce 50% inhibition of phorbol binding and 40-kDa phosphorylation are similar (Figs. 10*B* and 11). However, inhibition of phorbol binding in platelets shows more complex kinetics, the significance of which is unknown at present.

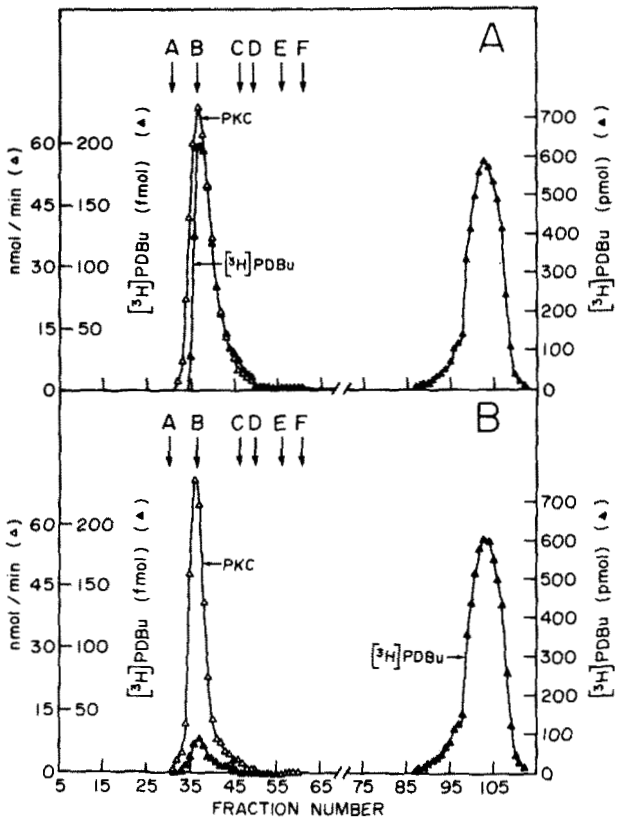

FIG. 9. **Interaction of PDBu and protein kinase C (*PKC*) with mixed micelles containing sphingosine on molecular sieves.** An 80-ml (1.5 × 45-cm) Sephacryl S-200 column was equilibrated with 20 mM Tris-HCl, pH 7.5, 10% sucrose, 50 mM 2-mercaptoethanol, 0.02% Triton X-100, and 200 μM $CaCl_2$. 1 ml of protein kinase C concentrated 10-fold by Amicon (YM 10) ultrafiltration was incubated for 5 min in the presence of 200 μM $CaCl_2$, 100 nM [^{3}H]PDBu, and 1.0% (w/v) Triton X-100 mixed micelles containing 16 mol % of PS with or without 12 mol % of sphingosine. The sample was then chromatographed and the eluate was assayed for protein kinase C activity (△), and for [^{3}H]PDBu (▲, note that the *ordinate* on the left for bound [^{3}H]PDBu is magnified with respect to free [^{3}H]PDBu whose *ordinate* is shown on the right). *A*, mixed micelles contained 16 mol % of PS. A fraction of [^{3}H]PDBu (▲) co-elutes with protein kinase C (△) at an M_r = 200,000. *B*, mixed micelles contained 16 mol % of PS and 12 mol % of sphingosine. Most of the bound [^{3}H]PDBu was displaced. *Arrows* indicate elution position of blue dextran (*A*), amylase (*B*), alcohol dehyrogenase (*C*), Triton X-100/PS mixed micelles (*D*), protein kinase C when it is not bound to mixed micelles (*E*) (and see Ref. 13), and bovine serum albumin (*F*).

inhibitors. Adriamycin, an inhibitor of protein kinase C (15), and sphingosine have similar structural features.

CONCLUDING REMARKS

A number of compounds that inhibit protein kinase C activity have been described, but little data is available on the mechanisms by which such inhibition occurs, leading to the assumption that they "perturb" the lipid bilayer making it unsuitable for protein kinase C activation. The mixed micelle assay for protein kinase C (11, 13) has provided the necessary tool to examine the interaction of amphipathic and hydrophobic molecules with protein kinase C. We have previously shown that protein kinase C interacts with a DAG·4PS·Ca^{2+} complex (12, 13). By competitively interacting with PS, Ca^{2+}, and DAG/phorbol ester, sphingosine prevents the formation of an active lipid-enzyme complex by displacement of the activator (DAG or phorbol ester) from the complex while maintaining the association of the enzyme to the surface (Fig.

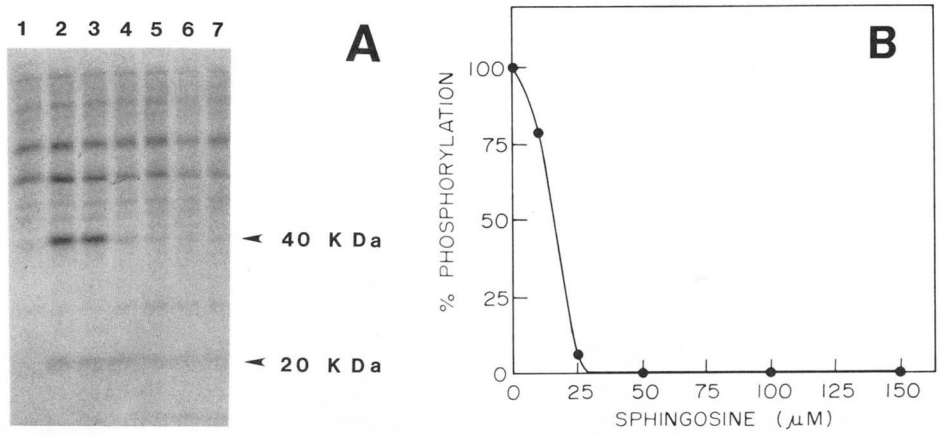

FIG. 10. **Sphingosine inhibition of platelet protein phoshorylation.** *A*, the effect of increasing sphingosine concentration on phosphorylation of the 40-kDa protein induced by thrombin. *Lane 1*, control platelets; *Lane 2*, thrombin (1 unit/ml); *Lane 3*, 10 μM sphingosine; *Lane 4*, 25 μM sphingosine; *Lane 5*, 50 μM; *Lane 6*, 100 μM; *Lane 7*, 200 μM. *B*, the 40-kDa band was cut out and ^{32}P was counted in Aquasol II. The percent of maximal phosphorylation induced by thrombin is plotted as a function of sphingosine concentration.

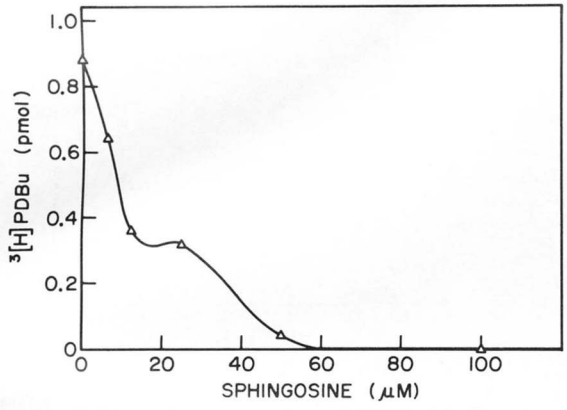

FIG. 11. **Sphingosine inhibition of PDBu binding to platelets.** [^{3}H]PDBu was at 25 nM and platelets at 2.5 × 10^8 platelet/ml.

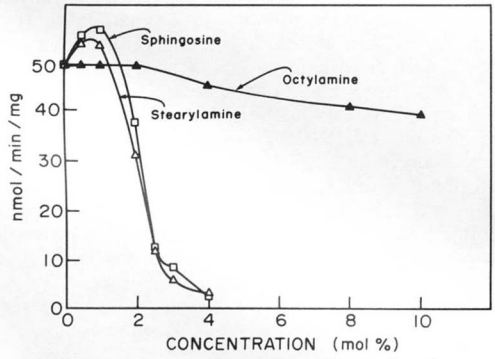

FIG. 12. **Effects of stearylamine and octylamine on protein kinase C.** Mixed micelles contained 5.5 mol % of PS, 1.0 mol % of $diC_{18:1}$ and sphingosine (□), octylamine (▲), or stearylamine (△).

9). The results presented above show that sphingosine is a potent and reversible inhibitor of protein kinase C. This suggests that sphingosine may be a useful inhibitor of protein kinase C in different cell systems. Our results with platelets and the accompanying work in HL-60 cells (23) and neutrophils (24) further attest to the usefulness of sphingosine as a protein kinase C inhibitor.

Sphingosine differs from the other known inhibitors of protein kinase C in that it is a natural component of cells comprising a critical component of ceramide, the building block of sphingomyelin and the glycosphingolipids (Fig. 13). Sphingosine and other naturally occurring long-chain (sphingoid) bases are synthesized by serine palmitoyltransferase (34). Sphingosine could also be generated by the action of ceramidases (*N*-acylsphingosine amidohydrolases). These metabolic pathways raise the possibility that the generation of sphingosine intracellularly may serve as a regulated negative effector of protein kinase C activity. Sphingosine levels may be regulated in response to either intra- or extracellular signals. In fact, sphingomyelin was observed to undergo rapid deacylation and *N*-acylation when L-929 fibroblasts were stimulated with specific antibody (35). The deacylation of sphingomyelin leads to the generation of sphingosylphosphorylcholine (lysosphingomyelin). This molecule may lead to the generation of sphingosine through hydrolysis of the phosphorylcholine head group. Therefore, these catabolic pathways for the generation of sphingosine or one of its analogs may play a physiologic role in modulating the activity of protein kinase C. It is unlikely that the biosynthetic pathways of sphingolipids will have such a physiologic function since previous studies have shown the levels of sphingosine and sphinganine to be exceedingly low (36). The potential for a physiologic inhibition of protein kinase C by sphingosine is also substantiated by the finding that sphingosine reversibly inhibits protein kinase C activity in human platelets. Also, the observed cytotoxicity of sphingosine in cells (37) is consistent with sphingosine acting as a potent inhibitor of protein kinase C. The activity of protein kinase C would then be expected to be a function of the concentration of PS (phospholipid), DAG, Ca^{2+}, and the negative effector, sphingosine.[4] Thus, a function of sphingolipids in regulation of transmembrane signaling may emerge.

While such speculation clearly exceeds the available data, the discovery that sphingosine inhibits protein kinase C *in vitro* and in platelets, opens the door to critical tests of the hypothesis. The inhibition of protein kinase C by sphingosine in HL-60 cells (23), and in human neutrophils (24), documented in the accompanying papers (23, 24) represents a beginning in understanding a possible new biologic role for sphingosine in cells. Furthermore, the demonstration that HL-60 cells contain elevated levels of sphingosine bases (23) is consistent with "developmental trapping" occurring by negative regulation of protein kinase C. In this speculative hypothesis, altered metabolism of sphingosine could represent the molecular basis of this form of leukemia.

[4] This suggests an explanation for the different concentration dependencies of protein kinase C activation by the cell permeable diacylglycerol, diC_8, observed in different cell types. In platelets (38), neutrophils (39), and A431 cells (40), micromolar amounts of diC_8 was effective; whereas, in tracheal 2C5 cells (41), pituitary cells (42), and HL-60 cells (10), 10–100 μM amounts were required. Perhaps the higher diC_8 concentrations required reflects the presence of an anti-signal (negative effector) in these cell types, such as sphingosine.

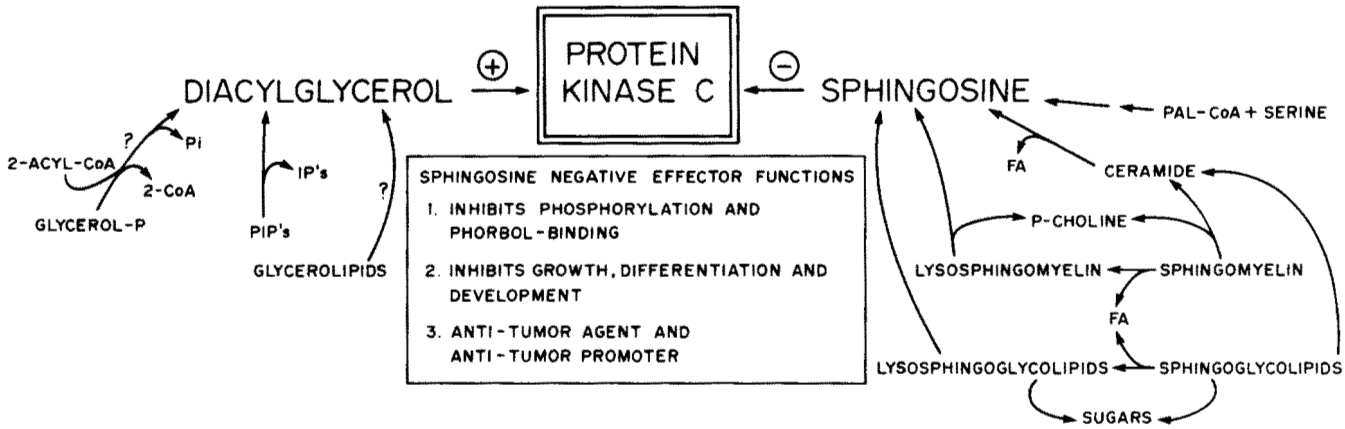

FIG. 13. **Positive and negative lipid effectors of protein kinase C.** An overall view of the metabolism of complex lipids producing diacylglycerol "second messengers" and sphingosine "negative protein kinase C effectors" is shown. *PIP's* stand for the phosphatidylinositol phosphates. We recognize that numerous routes could underlie the *arrows* and that more than one enzyme may lie under a given arrow. The question of whether sphingosine arises directly from palmitoyl-CoA and serine or via dihydroceramide is not illustrated. Sphingomyelin synthesis occurs by the reaction, phosphatidylcholine + ceramide → sphingomyelin + DAG. Evidence that this enzyme resides in the plasma membrane (43) provides another potential route for DAG formation. The figure also points out how the negative effector (and positive effector) functions of sphingosine may be involved in biochemistry, cell biology, and pathology. Consideration of sphingosine providing a functional regulatory link for sphingolipids generally should not be ignored. These are supported fully by the accompanying manuscripts (23, 24).

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