Regulation of sphingosine-activated protein kinases: selectivity of activation by sphingoid bases and inhibition by non-esterified fatty acids

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Sphingosine has been shown to activate protein kinases in Jurkat T cell cytosol [Pushkareva, Khan, Alessenko, Sahyoun and Hannun (1992) J. Biol. Chem. **267**, 15246–15251]. In this study, two sphingosine-activated protein kinases were distinguished by their substrate specificity, their dose–response to sphingosine and the specificity of their activation by sphingosine and dihydrosphingosine stereoisomers. A p32-sphingosine-activated protein kinase responded to low concentrations of D-*erythro*-sphingosine with an initial activation observed at 2.5 μM and a peak activity at 10–20 μM. This kinase showed a modest specificity for D-*erythro*-sphingosine over other sphingosine stereoisomers, and a preference for sphingosines over dihydrosphingosines. Phosphorylation of a p18 substrate required higher concentrations of sphingosine (20–100 μM) and showed a significant preference for the *erythro* isomers of sphingosine and dihydrosphingosine over the *threo* isomers. The ability of other lipids to modulate sphingosine activation of these kinases was also examined. Oleic acid, but not oleic alcohol or the methyl ester, induced the phosphorylation of a distinct set of substrates (probably through the activation of protein kinase C), and inhibited sphingosine-induced phosphorylation with an IC_{50} of ~ 20 μM. Oleic anhydride failed to induce changes in basal protein phosphorylation but inhibited sphingosine-activated protein kinases, thus distinguishing the effects of fatty acids on protein kinase C from the inhibition of sphingosine-induced phosphorylation. These studies define two distinct sphingosine-activated protein kinases and reveal an important interaction between two classes of putative lipid second messengers.

INTRODUCTION

Sphingolipids, a major class of membrane lipids, play important roles in cell-contact responses, cell growth, differentiation and oncogenesis (Hakomori, 1981; Hannun and Bell, 1989). The discovery that sphingosine is a potent inhibitor of protein kinase C (Hannun et al., 1986) raised the possibility that sphingosine and other sphingolipid breakdown products (Hannun and Bell, 1987) might function as messengers in signal transduction (Hannun and Bell, 1989; Merrill and Stevens, 1989). Recently, ceramide, which is produced from sphingomyelin breakdown, has been implicated as a candidate second messenger in the regulation of cell growth and differentiation (Okazaki et al., 1989, 1990; Kim et al., 1991; Dobrowsky and Hannun, 1992; Hannun, 1992; Merrill, 1992). However, a role for sphingosine itself as a second messenger remains poorly defined.

Sphingosine has been shown to exert multiple biological effects, many, but not all, of which appear to be a consequence of the inhibition of protein kinase C (reviewed in Hannun and Bell, 1989; Merrill and Stevens, 1989; Merrill, 1991).

In a previous study we determined the predominant effects of sphingosine on protein phosphorylation in a cell-free system (Pushkareva et al., 1992). These studies revealed that sphingosine induced protein phosphorylation through the activation of one or more protein kinases. The nature of this kinase(s) and the specificity of action of sphingosine, however, have not been determined.

In this study we evaluate the specificity of sphingosine and of dihydrosphingosine stereoisomers on sphingosine-activated protein kinases. We also report on the inhibition of sphingosine-induced phosphorylation by fatty acids. These studies are beginning to define specific targets for sphingosine action; they also illustrate complex interactions between different putative lipid second messengers.

METHODS

Reagents

D-*erythro*-Sphingosine (from bovine-brain sphingomyelin) and other lipids were from Sigma. Stereoisomers of sphingosine were prepared using the methods of Nimkar et al. (1988). *Erythro* stereoisomers of dihydrosphingosine were prepared using a DL-*erythro* racemic mixture and resolved via their D- and L-salts of *N*-(1-phenylethyl)succinamic acids. D and L-*threo*-Dihydrosphingosines were prepared from the DL-*threo* racemic mixture and separated as D- and L-glutamates using the method described by Stoffel (Stoffel and Bister, 1973). ATP was from Pharmacia and [γ-^{32}P]ATP was from NEN.

Cell culture

Jurkat T cells were grown in RPMI-1640 media (Gibco) supplemented with 10% (v/v) fetal bovine serum (Gibco). Cells were maintained at a density of 2×10^5–1.2×10^6 cells/ml.

Preparation of cytosolic fractions

Cytosolic fractions were prepared essentially as described previously (Pushkareva et al., 1992).

Preparation of lipid stocks

Sphingosine solutions in ethanol were diluted in water [final concentration of ethanol 5% or 10% (v/v)], sonicated twice for

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1 min and then used on the same day. Ethanol solutions of appropriate concentrations were used for controls. The same protocol was used to prepare stock solutions of fatty acids.

Protein-kinase assay

The protein-kinase assay was carried out as described previously (Pushkareva et al., 1992) in a total volume of 50 μl containing 5 μl of sphingosine suspension (or ethanol solution), 5 μl of other effectors, 20 μl Mg^{2+}/ATP solution (100 mM Tris/HCl, pH 7.4, 25 mM $MgCl_2$, 62.5 μM ATP and 62.5 μCi/ml [^{32}P]ATP) and 20 μl of diluted cytosol (8–12 μg of protein per sample).

RESULTS

Specificity of effects of sphingosine on cytosolic protein phosphorylation

The ability of sphingosine to induce substrate phosphorylation *in vitro* is specific, since closely related lipids are inactive (Pushkareva et al., 1992) and the amino base, stearylamine, is poorly active (Figure 1). It was therefore important to evaluate the stereospecificity of sphingosine effects. To achieve this, we used the four stereoisomers of sphingosine: D- and L-*erythro*-sphingosines and D- and L-*threo*-sphingosines. These compounds were synthesized chemically and have been well-characterized with respect to optical and chemical purity. We also evaluated the four stereoisomers of dihydrosphingosine (sphinganine). These compounds were prepared by resolving and purifying the enantiomers of racemic mixtures of DL-*erythro*-dihydrosphingosines and DL-*threo*-dihydrosphingosines. The dihydrosphingosines have the same stereostructure as the corresponding sphingosines, but differ by the absence of the 4–5 *trans* double bond.

Initially, the effects of the four stereoisomers of sphingosine on p32 phosphorylation were evaluated. When D-*erythro*-sphingosine was added to cytosolic extracts of Jurkat T cells, it caused a concentration-dependent phosphorylation of a p32 protein, suggesting that a p32-sphingosine-activated protein kinase was being activated (Figure 2a). D-*erythro*-Sphingosine caused an approx. 4-fold increase in p32 phosphorylation, with maximal effects observed at a concentration of 10 μM of sphingosine (Figure 2a). The EC_{50} for D-*erythro*-sphingosine was 8 μM (Table 1). The enantiomer L-*erythro*-sphingosine also activated the p32 kinase to a similar extent but with less potency, such that the maximal effect was reached at a concentration

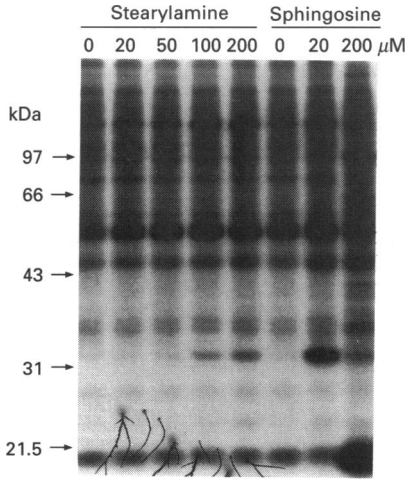

Figure 1 Effects of stearylamine on the phosphorylation of cytosolic proteins from Jurkat T cells

The concentrations of stearylamine and sphingosine indicated were used. The protein-kinase reaction was carried out as described in the Methods section. The position of molecular-mass markers is indicated.

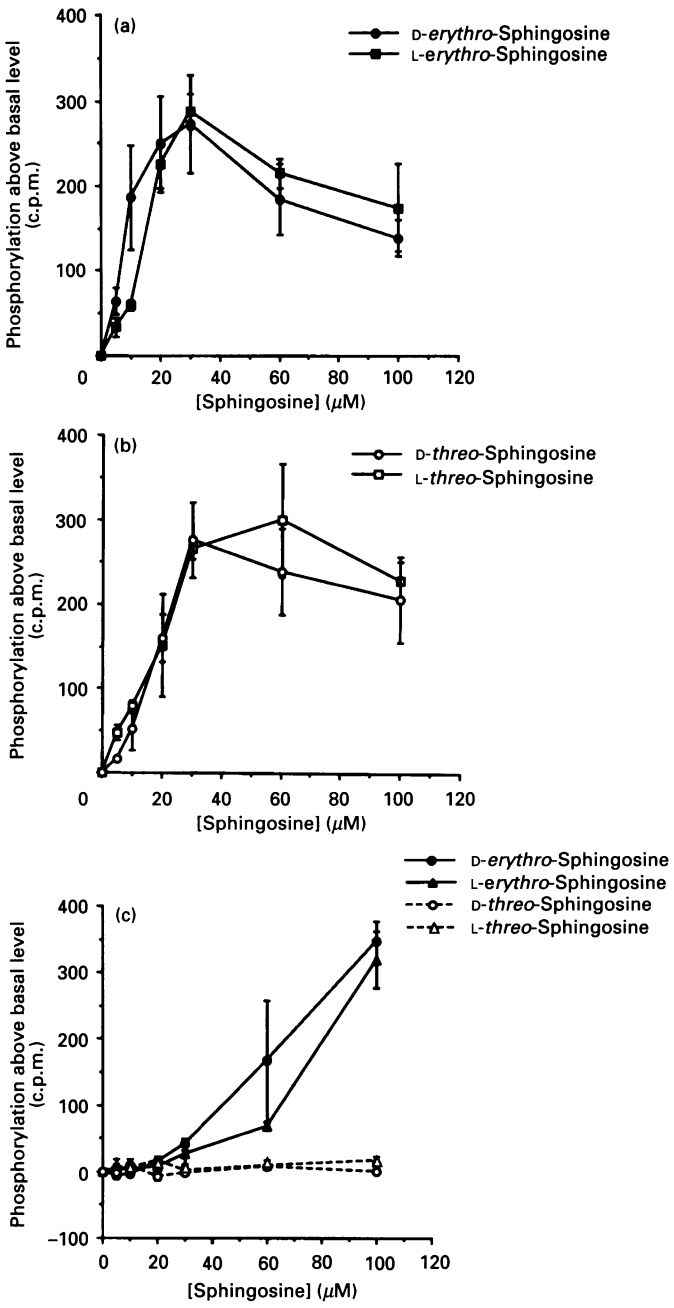

Figure 2 Quantitative analysis of the effects of sphingosine stereoisomers on the phosphorylation of p32 and of p18 cytosolic proteins from Jurkat T cells

(**a**) The effects of *erythro* isomers of sphingosine on p32 phosphorylation; (**b**) the effects of *threo* isomers of sphingosine on p32 phosphorylation; (**c**) the effects of the four stereoisomers of sphingosine on p18 phosphorylation. Data are presented as changes in the phosphorylation above basal levels; the basal phosphorylation ranged 100–150 c.p.m. in different experiments. The data represent the averages of three independent experiments.

Table 1 Effects of stereoisomers of sphingosine on p32 phosphorylation

The $V_{max.}$ is expressed as a fraction of the maximal activation that is achieved with D-*erythro*-sphingosine.

Sphingolipid	Phosphorylation of p32	
	EC_{50} (μM) ± S.D.	$V_{max.}$ (% of maximal)
D-*erythro*-Sphingosine	8 ± 3*	100
L-*erythro*-Sphingosine	15 ± 3	103
D-*threo*-Sphingosine	18 ± 2	99
L-*threo*-Sphingosine	20 ± 4	106
D-*erythro*-Dihydrosphingosine	12 ± 2	56
L-*erythro*-Dihydrosphingosine	7 ± 2	46
D-*threo*-Dihydrosphingosine	9 ± 3	58
L-*threo*-Dihydrosphingosine	8 ± 3	63

* The differences between D-*erythro*-sphingosine and each of the other stereoisomers of sphingosine are significant at $P < 0.05$.

of 20 μM, and the EC_{50} was 15 μM (Figure 2a; Table 1). The diastereomers D- and L-*threo*-sphingosine showed a similar activity to each other (Figure 2b) but were less active than D-*erythro*-sphingosine with EC_{50}s of 18 μM and of 20 μM respectively (Table 1). Therefore, D-*erythro*-sphingosine is a very potent activator of p32-kinase, and this activation shows a modest preference for D-*erythro*-sphingosine over L-*erythro*-sphingosine and the *threo* stereoisomers.

Next, the effects of the four isomers of sphingosine on the phosphorylation of p18 (Figure 2) were quantified. D- and L-*erythro*-Sphingosines induced a concentration-dependent effect on the phosphorylation of p18 (Figure 2c), with a 5-fold increase in phosphorylation caused by 100 μM of *erythro*-sphingosines. The kinase that phosphorylated p18 in response to sphingosine exhibited a modest preference for D-*erythro*-sphingosine compared with L-*erythro*-sphingosine (Figure 2c). However, neither D- nor L-*threo*-sphingosine had an effect on p18 phosphorylation (Figure 2c), even at concentrations of up to 400 μM (results not shown). Thus, the p18-kinase is activated only by the *erythro* enantiomers of sphingosine, and the *threo* isomers are inactive.

Next, the specificity of the activation of cytosolic protein phosphorylation was examined for the four stereoisomers of dihydrosphingosine. D-*erythro*-Dihydrosphingosine caused about a 2.5–3-fold increase in p32 phosphorylation, with the maximal effect being observed at a concentration of 20 μM (Figure 3a). The EC_{50} for D-*erythro*-dihydrosphingosine was 12 μM (Table 1). L-*erythro*-Dihydrosphingosine had a very similar effect on p32 phosphorylation to that of D-*erythro*-dihydrosphingosine, but was a little more potent, with the maximal effect observed at a concentration of 10 μM (Figure 3a), with an EC_{50} of 7 μM (Table 1). Thus, the *erythro*-dihydrosphingosines activated approx. 60% of the maximal p32 phosphorylation that was obtained with the *erythro*-sphingosines, but with a similar potency (Table 1). Both D- and L-*threo*-dihydrosphingosines exhibited a similar effect on p32 phosphorylation, with maximal effects observed at a concentration of 20 μM (Figure 3b) and with EC_{50}s of approx. 9 and 8 μM respectively (Table 1).

The effects of the stereoisomers of dihydrosphingosine on p18 phosphorylation were also quantified. D-*erythro*-Dihydrosphingosine caused an approx. 2-fold increase in p18 phosphorylation, with maximal effects observed at concentrations of 60–100 μM (Figure 3c). In contrast with the results with the D- and L-*erythro* enantiomers of sphingosine, L-*erythro*-dihydrosphingosine was a more potent activator

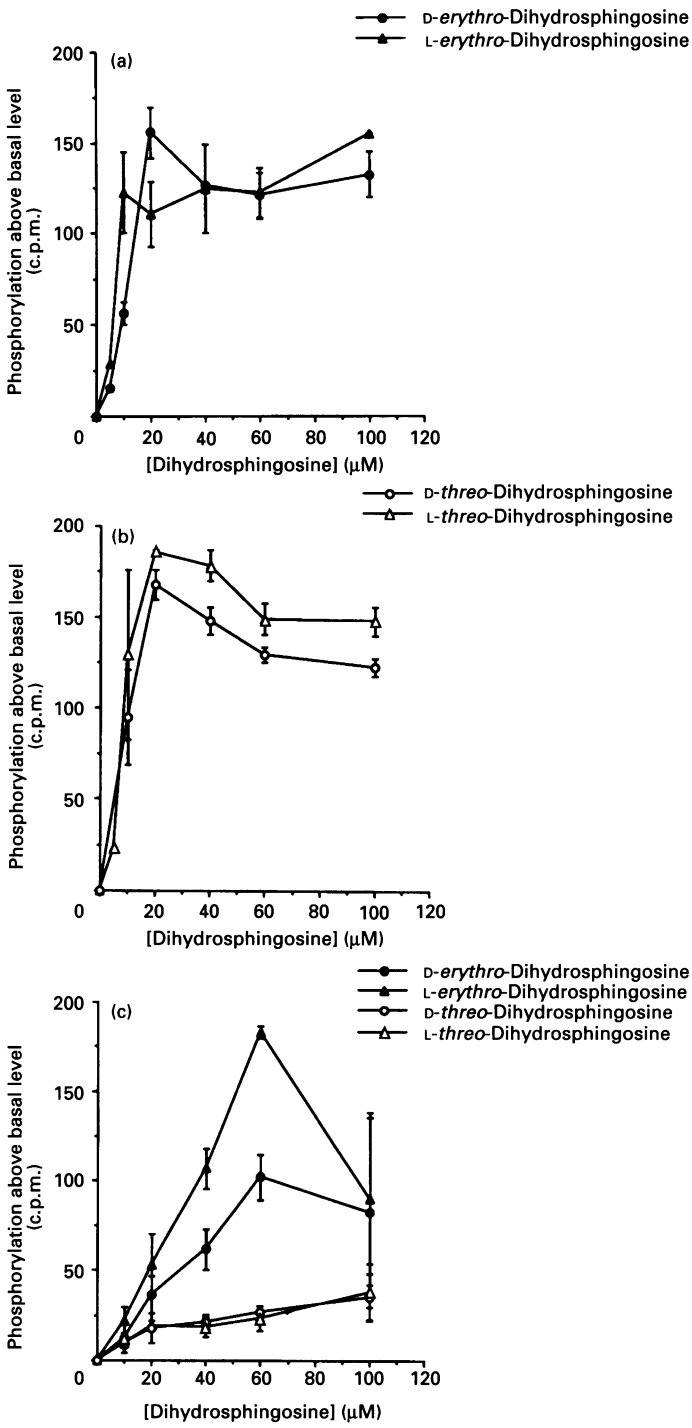

Figure 3 Quantitative analysis of the effects of dihydrosphingosine stereoisomers on phosphorylation of p32 and p18 cytosolic proteins from Jurkat T cells

(**a**) The effects of *erythro* isomers of dihydrosphingosine on p32 phosphorylation; (**b**) the effects of *threo* isomers of dihydrosphingosine on p32 phosphorylation; (**c**) the effects of the four stereoisomers of dihydrosphingosine on p18 phosphorylation. Data are presented as changes in the phosphorylation above basal levels; the basal phosphorylation ranged 100–150 c.p.m. in different experiments. Data represent the averages of three independent experiments.

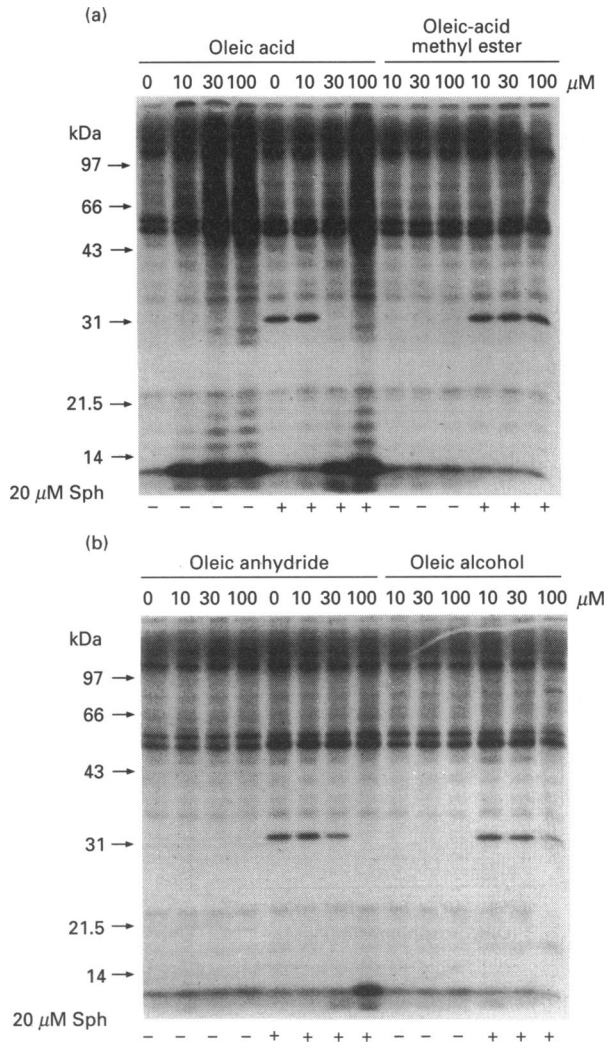

Figure 4 The effects of oleic acid and derivatives on basal phosphorylation and on sphingosine-induced phosphorylation of cytosolic proteins from Jurkat T cells

Cytosolic proteins were incubated with the concentrations of oleic acid or oleic-acid methyl ester (**a**), and oleic anhydride or oleic alcohol (**b**) indicated, in the presence (+) or absence (−) of 20 μM sphingosine (Sph). The position of molecular-mass markers is indicated.

of p18 kinase than D-*erythro*-dihydrosphingosine. L-*erythro*-Dihydrosphingosine caused a 3-fold activation of p18 phosphorylation at a concentration of 60 μM (Figure 3c). Both D- and L-*threo*-dihydrosphingosine were able to activate p18 phosphorylation to some extent, but they were much less potent than the *erythro*-dihydrosphingosines (Figure 3c). Either D- or L-*threo*-dihydrosphingosine at a concentration of 100 μM resulted in an approx. 30% increase in p18 phosphorylation. Thus, the protein kinase that phosphorylates p18 is activated to a much greater extent by *erythro*-sphingosines than by dihydrosphingosines. Also, the D- and L-*threo*-enantiomers of sphingosine and of dihydrosphingosine were largely inactive.

Inhibition of sphingosine-induced protein phosphorylation by fatty acids

While examining the effects of various lipids on sphingosine-induced phosphorylation, we found that fatty acids were potent inhibitors of sphingosine-activated protein kinases. In these experiments, a number of fatty acids were examined: arachidonic acid, oleic acid, elaidic acid (the *trans* isomer of oleic acid), and also some fatty-acid derivatives such as methyl esters, alcohols and anhydrides. In the absence of other lipids, arachidonic acid, elaidic acid (results not shown) and oleic acid (Figure 4a) increased the level of phosphorylation of a number of proteins (16, 18, 19, 28, 30, 36, 38, 44, 70 and 74 kDa) in a dose-dependent manner. This pattern of phosphorylation greatly overlapped that obtained with Ca^{2+}/phosphatidylserine/dioleoylglycerol (results not shown), suggesting that the effects of fatty acids on protein phosphorylation were as a result of protein kinase C activation (McPhail et al., 1984; Murakami et al., 1986; El Touny et al., 1990; Khan et al., 1991).

To determine the effects of oleic acid on sphingosine-dependent phosphorylation, a sphingosine concentration of 20 μM was chosen to obtain the maximal effect on p32 phosphorylation. Oleic acid inhibited the effects of sphingosine on p32 phosphorylation at concentrations of 10–30 μM (Figure 4a). Interestingly, in the presence of 30 μM oleic acid and 20 μM sphingosine, very little phosphorylation over baseline was observed. These results strongly suggest the mutual inhibition of sphingosine-induced phosphorylation by oleic acid and of oleic-acid-dependent phosphorylation (most probably by protein kinase C) by sphingosine.

To try to determine the specificity of the effects of oleic acid, derivatives of oleic acid were tested for their ability to change the basal level of protein phosphorylation as well as the level of sphingosine-induced phosphorylation. Oleic alcohol and the methyl ester of oleic acid did not cause phosphorylation, nor did they inhibit sphingosine-dependent phosphorylation (Figure 4a, 4b). Interestingly, oleic anhydride failed to cause any changes in the basal protein phosphorylation level, but inhibited sphingosine-induced phosphorylation of p32 at a concentration of 30–100 μM (Figure 4b). The inability of oleic anhydride to induce protein phosphorylation is consistent with its lack of effect on purified protein kinase C *in vitro*, thus providing further evidence that phosphorylation in response to oleate is through the activation of protein kinase C. The ability of oleic anhydride to inhibit the effects of sphingosine provides strong evidence that the effects of oleate on the sphingosine-activated protein kinase can be separated from its effects on protein kinase C.

Elaidic acid (the *trans* isomer of oleic acid) and its derivatives showed similar effects on sphingosine-dependent protein phosphorylation to oleic acid and oleic-acid derivatives (results not shown), although elaidic acid was less potent than oleic acid.

The effect of oleic acid on p18 phosphorylation induced by 100 μM of sphingosine was also examined. The complete inhibition of p18 phosphorylation required concentrations of oleic acid of 30–100 μM (results not shown).

Thus, fatty acids can both activate fatty-acid-dependent protein kinases (most probably protein kinase C) and inhibit sphingosine-induced protein phosphorylation. Fatty-acid anhydrides can mimic the effects of fatty acids on sphingosine-dependent phosphorylation, but do not have effects on the basal level of protein phosphorylation. Both alcohols and methyl esters of fatty acids are inactive.

DISCUSSION

The results from this study confirm the existence of two distinct sphingosine-dependent phosphorylation pathways in the cytosol of Jurkat T cells. These can be distinguished by their phosphorylation of different substrates in response to sphingosine with significantly different potencies and specificities in their

requirements for sphingosine activation. These phosphorylation events can be tentatively assigned to two distinct sphingosine-activated protein kinases: a p32-sphingosine-activated protein kinase and a p18-sphingosine-activated protein kinase. The phosphorylation of p32 shows a preference for activation by sphingosines over that by dihydrosphingosines, with a slight preference for D-*erythro*-sphingosine over other stereoisomers.

The phosphorylation of p18 showed features that were distinct from those of p32 phosphorylation. Only the *erythro* isomers of sphingosine and of dihydrosphingosine were significantly active in inducing p18 phosphorylation; the *threo* isomers were largely inactive. Among the sphingosine stereoisomers, D-*erythro*-sphingosine was more potent than the others, and the sphingosines showed significantly better activity than did the dihydrosphingosines. These features distinguish the phosphorylation of p32 from that of p18, and suggest the existence of two distinct sphingosine-activated protein kinases (to be tentatively designated as p18-sphingosine-activated protein kinase and p32-sphingosine-activated protein kinase) that can be distinguished by (1) their substrates, (2) the potency of their sphingosine activation, and (3) the specificity of their sphingosine activation.

Although multiple *in vitro* targets for sphingosine have been identified, the specificity of the activation caused by sphingosine has been examined in detail only in the case of protein kinase C (Merrill et al., 1989). These studies showed that protein kinase C is inhibited to a similar extent by all of the stereoisomers of sphingosine as well as by both dihydrosphingosine and stearylamine (Merrill et al., 1989). Thus, the stereoselectivity of the activation of the p32- and p18-kinases by sphingosine has not been observed previously.

These results raise the important question of the nature of the sphingosine-activated protein kinases. These kinases appear to be distinct from protein kinase C, cyclic-AMP-dependent protein kinase, calcium/calmodulin-dependent protein kinases and tyrosine kinases (Pushkareva et al., 1992). A major impediment to defining the nature of the sphingosine-activated protein kinases is the lack of defined *in vitro* substrates for these enzymes that would allow their purification and characterization.

These results have interesting implications for the possible physiological interactions between fatty acids and sphingoid bases in the regulation of protein kinases. It has previously been shown that the activation of protein kinase C isoenzymes by fatty acids is inhibited by sphingosine, whereas we show here that the activation of sphingosine-dependent protein kinases is inhibited by fatty acids. Thus, these two classes of lipid breakdown products appear to have opposing effects on protein phosphorylation by exerting antagonistic effects on distinct protein kinases.

In conclusion, these studies demonstrate that sphingosine could exert potent and specific effects on protein phosphorylation *in vitro* through the activation of distinct protein kinases. Further studies are required to determine the nature of these kinases.

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