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Multiple non-specific effects of sphingosine on adenylate cyclase and cyclic AMP accumulation in S49 lymphoma cells preclude its use as a specific inhibitor of protein kinase C

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Recent studies with phorbol esters have suggested that protein kinase C (PKC) may play a role in the regulation of adenylate cyclase in mammalian cells. Since D-sphingosine has been reported to specifically inhibit PKC in many cell types, we evaluated its effects on stimulation of cyclic AMP accumulation by adrenaline in S49 lymphoma cells. We found sphingosine to have multiple non-specific effects which could not be explained by an inhibition of PKC. These effects included: (i) inhibition by sphingosine (50 μM) of adrenaline-stimulated cyclic AMP accumulation and sphingosine permeation of the cells which rendered them leaky to ATP; (ii) sphingosine (20 μM) augmentation of adrenaline-stimulated cyclic AMP accumulation; (iii) inhibition by sphingosine of adrenaline-stimulated adenylate cyclase in isolated membranes by up to 95%; and (iv) sphingosine (20 μM) inhibition of cellular mechanisms for the elimination of cyclic AMP. These results demonstrate the importance of evaluating the non-specific effects of sphingosine before concluding that its actions are the consequence of a specific inhibition of PKC.

INTRODUCTION

Studies of the effects of phorbol esters on adenylate cyclase suggest that protein kinase C (PKC) may play a role in β-adrenergic receptor desensitization (Kelleher *et al.*, 1984; Sibley *et al.*, 1984*a*,*b*; Johnson *et al.*, 1986) and in the physiological attenuation of G_i-mediated inhibition (G_i is a guanine-nucleotide-binding protein which inhibits adenylate cyclase) (Katada *et al.*, 1985; Jakobs *et al.*, 1985; Johnson *et al.*, 1986). Sphingolipids have been shown to inhibit PKC potently in cell-free systems (Hannun *et al.*, 1986), and since use of these compounds could be an invaluable tool for elucidating the role of PKC in regulating adenylate cyclase, we evaluated their effects on cyclic AMP metabolism in S49 wild-type (WT) cells. We did this because many recent studies purporting to demonstrate the specific blockade of a PKC-mediated event in intact cells by sphingolipids lacked controls directed towards an evaluation of the sphingolipids' effects on cell integrity and on individual components of the second messenger systems (Merrill *et al.*, 1986; Wilson *et al.*, 1986; Hannun *et al.*, 1986, 1987; McIntyre *et al.*, 1987; Faucher *et al.*, 1988; Hannun & Bell, 1989*a*,*b*).

In this paper, data are presented which initially suggested that sphingolipids block cellular responses which may be mediated by PKC. However, close examination of the mechanism of sphingolipid action in S49 cells revealed multiple effects which cannot be explained by inhibition of PKC. These non-specific effects complicate the interpretation of any specific effects the sphingolipids may have on phorbol ester-induced regulation of cyclic AMP metabolism in S49 WT cells. Various reports demonstrating cytotoxic effects of sphingolipids on cells (Merrill & Wang, 1986; Pittet *et al.*, 1987; Winicov & Gershengorn, 1988), and evidence presented in this paper, argue for more thorough evaluation of the effects of sphingolipids on second messenger systems and cell integrity.

MATERIALS AND METHODS

Materials

(−)-Adrenaline (+)-bitartrate, D-sphingosine and fatty-acid-free BSA were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Dimethyl sulphoxide (Me_2SO) was obtained from Fisher Scientific (Pittsburg, PA, U.S.A.). Sphinganine was purchased from Serdary Biochemicals (London, Ontario, Canada). Forskolin was obtained from Calbiochem (San Diego, CA, U.S.A.). [8-^{14}C]ATP and [8-^{14}C]cyclic AMP (40–60 mCi/mmol each) were obtained from ICN (Irvine, CA, U.S.A.).

Preparation of adrenaline and sphingolipid stocks

Adrenaline was dissolved in a solution of 10 mM-ascorbate and 100 mM-thiourea. The final concentrations of ascorbate and thiourea in the adenylate cyclase assays were 0.1 and 1 mM respectively. For stock solutions prepared with Me_2SO, sphingosine or sphinganine was dissolved to final concentrations of 2–20 mM. For the sphinganine stock prepared with fatty-acid-free BSA, 50 mM-sphinganine in 95% ethanol was diluted slowly into a solution of 2.5 mM-BSA prepared in a buffer containing 0.6 mM-$CaCl_2$, 1.5 mM-KH_2PO_4, 2.6 mM-KCl, 0.5 mM-$MgCl_2$, 136 mM-NaCl and 8 mM-Na_2HPO_4, pH 7.4 (Lambeth *et al.*, 1988) to a final concentration of 2 mM. The solution was then incubated in a shaking water bath at 37 °C for 1–2 h prior to use.

Intact cell studies

S49 WT lymphoma cells were cultured at 37 °C in Dulbecco's modified Eagle's medium plus 5% horse serum as previously described (Clark *et al.*, 1982). The ATP pool was prelabelled with [2,8-^{3}H]adenine (40 Ci/mmol, from ICN), and the percentage conversion of [^{3}H]AXP into [^{3}H]cyclic AMP was calculated as described by Barber *et al.* (1980). Briefly, percentage

Abbreviations used: PKC, protein kinase C; WT, wild type; Me_2SO, dimethyl sulphoxide; G_i and G_s, inhibitory and stimulating guanine-nucleotide-binding proteins (G-proteins) respectively.

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conversion of [^{3}H]AXP into [^{3}H]cyclic AMP = {[^{3}H]cyclic AMP/([^{3}H]cyclic AMP + [^{3}H]AXP)} × 100 % where [^{3}H]AXP = [^{3}H]ATP + [^{3}H]ADP + [^{3}H]AMP. Total counts in the AXP fraction were not altered by adrenaline, Me_2SO, ascorbate/thiourea or concentrations of sphingosine ⩽ 30 μM. Sphingosine at 50 μM substantially lowered AXP levels.

Cell lysis and membrane fractionation

Cells were collected by centrifugation and then lysed as previously described (Johnson *et al.*, 1986). The 600 *g* supernatant fraction was layered over a 2-step gradient of 23 and 43 % sucrose in HE buffer (20 mM-Hepes/1 mM-EDTA, pH 8.0). The resulting gradients were then centrifuged at 110000 *g* for 45 min in a Beckman SW27 rotor. Membrane fractions were collected from the sucrose gradients and frozen in 1 ml portions at −80 °C.

Adenylate cyclase assay

Adenylate cyclase was assayed by the method of Salomon *et al.* (1974). The assay solution consisted of 40 mM-Hepes (pH 7.7), 1.4 mM-$MgCl_2$, 1 mM-EDTA, 8 mM-phosphocreatine, 16 units of creatine kinase/ml, 0.2 mM-ATP, 10 μM-GTP, 0.1 mM-1-methyl-3-isobutylxanthine, and approx. 2 μCi of [α-^{32}P]ATP (25 Ci/mmol, from ICN). Each assay was carried out for 10 min at 30 °C.

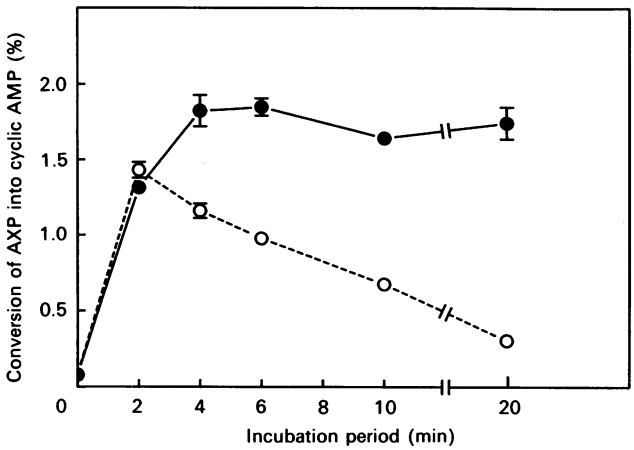

Fig. 2. Effects of 20 μM-sphingosine on adrenaline-stimulated cyclic AMP accumulation in S49 WT cells

S49 WT cells were treated with simultaneous additions of 50 nM-adrenaline and either 20 μM-sphingosine (●) or 0.4 % Me_2SO (○) for the times indicated. [^{3}H]Cyclic AMP accumulation was determined as described in the legend to Fig. 1. Data are plotted as described in the legend to Fig. 1 for a single experiment which is typical of five.

RESULTS

Concentration-dependent effects of sphingosine on adrenaline-stimulated cyclic AMP accumulation in S49 WT cells

The experiment in Fig. 1 shows the effects of increasing concentrations of sphingosine on adrenaline-stimulated cyclic AMP accumulation in S49 WT cells. Cells were treated with the indicated concentrations of sphingosine for 5 min, followed by an 8 min pulse with 50 nM-adrenaline. When S49 WT cells are treated for 8 min with 50 nM-adrenaline, a substantial desensitization of cyclic AMP production occurs (Johnson *et al.*, 1986). Sphingosine at concentrations of 20 and 30 μM appeared to partially block this desensitization, resulting in elevations of cyclic AMP accumulation over the non-sphingosine-treated cells of 63 and 138 % respectively. When cells were treated with concentrations of sphingosine ⩾ 50 μM, a dramatic inhibition of adrenaline-stimulated cyclic AMP accumulation was observed, and the intracellular AXP (see the Materials and methods section) levels were lowered by more than 50 %.

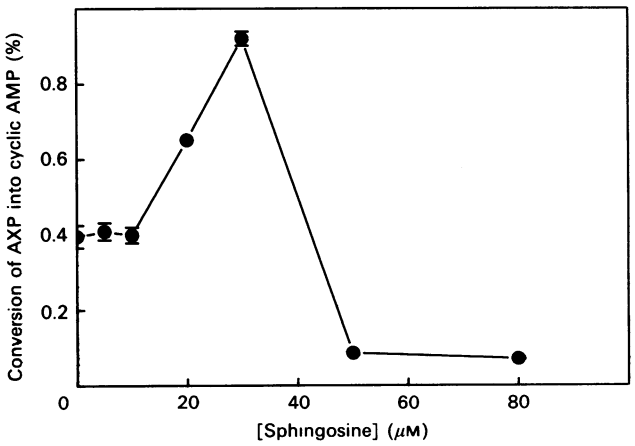

Fig. 1. Concentration-dependent effects of sphingosine on adrenaline-stimulated cyclic AMP accumulation in S49 WT cells

S49 WT cells were pretreated with the indicated concentrations of sphingosine 5 min prior to the addition of 50 nM-adrenaline for 8 min at 37 °C. [^{3}H]Cyclic AMP was isolated and the percenage conversion of [^{3}H]AXP into [^{3}H]cyclic AMP was determined according to the procedures of Barber *et al.* (1980). Data are plotted as the means ± half the ranges for duplicate determinations for a single experiment which is representative of three.

In the experiment shown in Fig. 2, S49 WT cells were treated simultaneously with 50 nM-adrenaline and either 20 μM-sphingosine or 0.4 % Me_2SO. We observed significant elevations of cyclic AMP accumulation in response to stimulation by 50 nM-adrenaline in the cells treated with 20 μM-sphingosine, consistent with the data shown in Fig. 1. The average augmentations observed at 4, 6 and 10 min were 57 ± 24 (n = 4), 57 ± 20 (n = 3) and 76 ± 22 % (n = 5) respectively. No significant effects of 20 μM-sphingosine on basal cyclic AMP levels were detected.

Sphingosine inhibition of cyclic AMP turnover

Although the results of Figs. 1 and 2 suggested that sphingosine was blocking desensitization of the β-adrenergic receptor, it seemed very unlikely that sphingosine was acting on PKC. First, desensitization in these cells in response to 50 nM-adrenaline appears to be primarily mediated by cyclic AMP-dependent protein kinase (Clark *et al.*, 1988). Secondly, when membranes from S49 WT cells, treated as described in Fig. 2, were assayed for adrenaline-stimulated adenylate cyclase activity, we found no significant sphingosine-induced blockade of desensitization (results not shown). Based on these considerations, we decided to investigate other possible mechanisms for the sphingosine-induced elevation of cyclic AMP levels.

One possible interpretation of these results was that sphingosine blocked the removal of cyclic AMP. The term 'cyclic AMP elimination' was previously defined as all potential mechanisms by which the concentration of cyclic AMP in cells can be decreased, with cyclic nucleotide phosphodiesterase and cyclic AMP egress being the only two means. Cyclic AMP egress is the term given to the active process by which cells pump intracellular cyclic AMP out into their extracellular environment. This mechanism accounts for only a small percentage of the total cyclic AMP eliminated by S49 cells. The major mechanism by

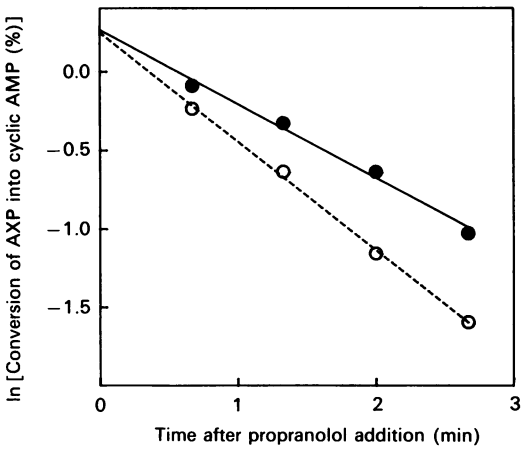

Fig. 3. Effects of sphingosine on the elimination of cyclic AMP from S49 WT cells

S49 WT cells were treated with 10 μM-adrenaline in the presence (●) or absence (○) of 20 μM-sphingosine, followed by the addition of 10 μM-propranolol after 1 min, for the times shown. [^{3}H]Cyclic AMP levels were monitored as described in the legend to Fig. 1. The average decrease (±S.E.M.) in the rate of cyclic AMP elimination by sphingosine in seven experiments was 32±7%. The slope of the resulting lines (k_{dy} values) represent the rate of cyclic AMP eliminaton from these cells. In the absence of sphingosine, $k_{dy} = -0.69$ and in the presence of sphingosine, $k_{dy} = -0.47$. The lines were drawn by linear regression ($r^{-1} \geqslant 0.99$ for each line). The data shown represent a single experiment typical of seven.

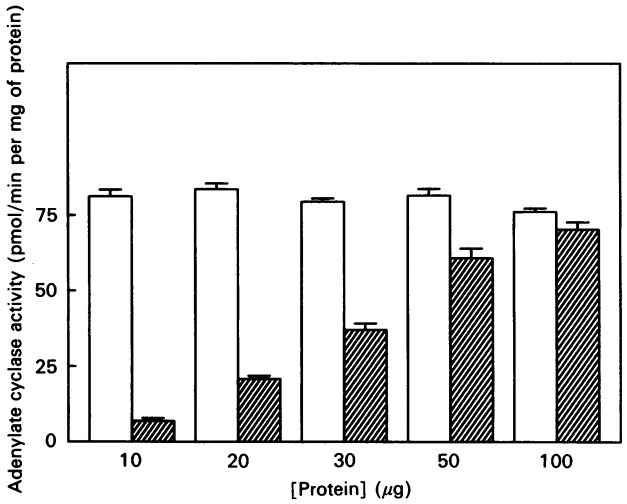

Fig. 4. Addition of sphingosine directly to S49 WT membranes inhibits adrenaline stimulation of adenylate cyclase

Membranes were isolated from S49 WT cells as described in the Materials and methods section. Adenylate cyclase activity stimulated with 50 nM-adrenaline was assayed with (▨) or without (□) 25 μM-sphingosine. Data are plotted as the means±half the ranges for duplicate determinations for a single experiment. Similar results have been obtained in seven experiments, but not all of the concentrations of membrane protein shown were analysed in each experiment. The quantities (μg) of protein used in the seven experiments were: 10, 20, 30, 50, 100; 20, 30, 50, 100; 10, 30, 100 (2 experiments); 30, 100; and 30 (2 experiments) respectively.

which S49 wild type cells eliminate cyclic AMP involves destruction of cyclic AMP by phosphodiesterase. To directly investigate the possibility that the augmentation of adrenaline-stimulated cyclic AMP accumulation (Figs. 1 and 2) was the result of a sphingosine-induced blockade of cyclic AMP elimination, the experiment shown in Fig. 3 was conducted. S49 WT cells were stimulated with 10 μM-adrenaline in the presence or absence of 20 μM-sphingosine, and, after 1 min, 10 μM-propranolol was added to block cyclic AMP synthesis. The decay of [^{3}H] cyclic AMP was then observed for an additional time period as indicated. In the experiment shown in Fig. 3, the rate of cyclic AMP elimination (k_{dy}) was decreased from 0.69 min^{-1} to 0.47 min^{-1} following treatment with 20 μM-sphingosine. In seven experiments the range of k_{dy} values for the control and treated cells was 1.15–0.42 min^{-1} and 0.52–0.16 min^{-1} respectively. Individual k_{dy} values differed from day to day, but the difference between control and sphingosine-treated cells was consistently observed. The time course of sphingosine blockade of cyclic AMP elimination (as rapid as 1–4 min following sphingosine treatment) correlates well with the sphingosine-induced augmentation of adrenaline-stimulated cyclic AMP accumulation shown in Fig. 2.

Inhibition of adenylate cyclase by sphingosine

The data in Fig. 1 demonstrated that sphingosine inhibited adrenaline stimulation of cyclic AMP accumulation, and although a part of this inhibition may be attributed to leakage of ATP, the other part of the effect could be inhibition of adenylate cyclase. When we examined the effects of sphingosine on adenylate cyclase activites in sucrose gradient purified WT membranes (Fig. 4), we found that 25 μM-sphingosine inhibited 50 nM-adrenaline-stimulated adenylate cyclase activity. This experiment demonstrates that the inhibitory effects of 25 μM-sphingosine were greatest at low protein concentrations, and that they could be eliminated by increasing the membrane protein in the assay. These results are very reproducible; at high detergent-to-protein ratios (0.15 mg of sphingosine/mg of protein), sphingosine inhibited adenylate cyclase activity by 83±6% ($n = 6$), while lowering the detergent-to-protein ratio to 0.015 mg of sphingosine/mg of protein decreased these inhibitory effects to 22±12% ($n = 7$). The dependency of inhibition on the sphingosine-to-protein ratio is similar to what has been measured

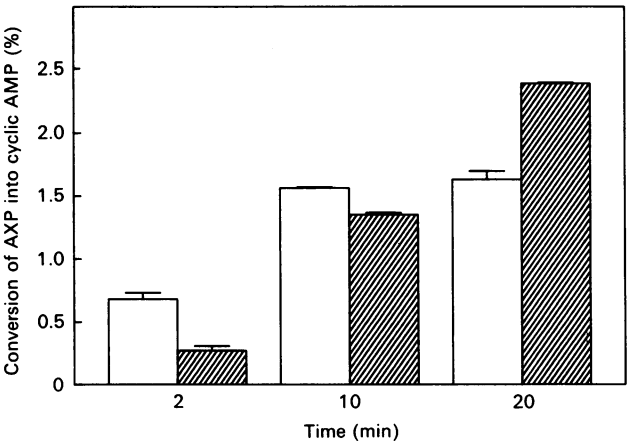

Fig. 5. Effects of 20 μM-sphingosine on forskolin-stimulated cyclic AMP accumulation

S49 WT cells were incubated with 5 μM-forskolin in the presence (▨) or absence (□) of 20 μM-sphingosine (added simultaneously) for the times indicated. Cyclic AMP accumulation was determined as described in the legend to Fig. 1. Data plotted represent the means±half the ranges for duplicate determinations for a single experiment typical of two.

Table 1. Effects of sphinganine prepared in Me_2SO or BSA buffer on forskolin-stimulated cyclic AMP accumulation in S49 WT cells

S49 WT cells were treated with 25 μM-sphinganine (SGA) prepared in Me_2SO or in a BSA-containing buffer 20 s before the addition of 5 μM-forskolin. [^{3}H]Cyclic AMP accumulation was determined as described in the Materials and methods section. Values represent the means ± half the range of duplicate determinations from a single experiment typical of two. Numbers in parentheses represent percentages of values in non-sphinganine-treated controls.

Incubation period (min)	Conversion of AXP into cyclic AMP (%)			
	BSA buffer		Me_2SO	
	−SGA	+SGA	−SGA	+SGA
2	0.29 ± 0.01	0.18 ± 0.01 (62)	1.00 ± 0.01	0.10 ± 0.01 (10)
10	0.86 ± 0.06	1.03 ± 0.00 (119)	2.64 ± 0.01	1.58 ± 0.02 (60)
20	0.94 ± 0.00	2.99 ± 0.01 (319)	2.88 ± 0.03	4.87 ± 0.01 (169)

many times before with a variety of detergents and lipophilic compounds, supporting the possibility that sphingosine acts like a detergent to disrupt adenylate cyclase function.

Biphasic effects of sphingosine on forskolin-stimulated cyclic AMP accumulation

To determine whether the effects of sphingosine were restricted to receptor level stimulation of adenylate cyclase, we investigated the effects of sphingosine on stimulation by forskolin. Activation of adenylate cyclase by forskolin is independent of the β-adrenergic receptor and appears to involve both G_s and the catalytic subunit of adenylate cyclase (Clark *et al.*, 1982). If sphingosine decreases cyclic AMP elimination (Fig. 3) and also inhibits stimulation of adenylate cyclase (Fig. 4), we would expect it to inhibit forskolin stimulation of cyclic AMP production initially, and then, as cyclic AMP levels rise, to block cyclic AMP elimination which would gradually result in the augmentation of forskolin-stimulated cyclic AMP levels. Fig. 5 illustrates an experiment in which S49 WT cells were exposed to simultaneous addition of 5 μM-forskolin and either 20 μM-sphingosine or 0.4% Me_2SO. Sphingosine inhibited forskolin-stimulated cyclic AMP accumulation at 2 min (52 ± 10%, $n = 2$), was less inhibitory at 10–12 min (11 ± 1%, $n = 2$) and caused a substantial enhancement of cyclic AMP accumulation at 20 min (53 ± 5%, $n = 2$). In two additional experiments we found similar responses of cells to forskolin stimulation in the presence of sphinganine (Table 1).

Effect of BSA on the biphasic effects of sphingosine

It has been suggested previously that BSA can prevent the non-specific actions of sphingosine (Lambeth *et al.*, 1988), so we investigated the effects of fatty-acid-free BSA on the sphingolipid-induced inhibition and enhancement of forskolin-stimulated cyclic AMP production (Table 1). In this experiment S49 WT cells were treated with 25 μM-sphinganine prepared with either BSA (see the Materials and methods section) or Me_2SO; the cells were then stimulated with forskolin, and cyclic AMP production was monitored. As controls, cells were treated with either BSA or Me_2SO. BSA alone caused a substantial inhibition of 5 μM-forskolin-stimulated cyclic AMP accumulation compared with cells treated with 0.4% Me_2SO. Despite this considerable inhibitory effect of 30 μM-BSA, the qualitative effects of sphinganine were similar whether the cells were prepared in Me_2SO or BSA. The inhibitory actions of the sphingolipids on forskolin stimulation in S49 cells are consistent with detergent-like effects on the plasma membrane.

DISCUSSION

Sphingolipids have been reported to specifically inhibit PKC in cell-free systems (Hannun *et al.*, 1986) and intact cells (Hannun *et al.*, 1986, 1987; Merrill *et al.*, 1986; Wilson *et al.*, 1986; McIntyre *et al.*, 1987; Faucher *et al.*, 1988). In these studies, however, the non-specific effects of these drugs on second messenger systems were not thoroughly evaluated. Pittet *et al.* (1987) discovered that sphinganine permeabilized the plasma membranes of human neutrophils, rendering them permeable to Ca^{2+}, adenine and intracellular enzymes, and altering the membrane potential. Lambeth *et al.* (1988) argued that the cytotoxic effects reported by Pittet and colleagues occurred because sphinganine solutions were prepared in Me_2SO, and that when sphinganine was prepared in a buffer containing fatty-acid-free BSA, the cytotoxic effects of sphinganine were minimized. However, several important controls were missing from the experiments of Lambeth *et al.* (1988), the most notable of which was measurement of the effects of BSA alone on neutrophil functions. Further, they did not report the consequences of administering BSA with Me_2SO.

In this study we have demonstrated that concentrations of sphingosine and sphinganine in the range 10 to 50 μM (which have been purported to have specific effects on PKC) cause both augmentation and inhibition of hormone and forskolin stimulation of cyclic AMP accumulation. Also, 50 μM-sphingosine was found to permeabilize the S49 cells, resulting in the loss of about 50% of the AXP pool (see the Materials and methods section). Since this would substantially deplete the substrate for adenylate cyclase, it probably contributed to the almost complete blockade of cyclic AMP accumulation by sphingosine. However, we also found that 25 μM-sphingosine inhibited stimulation of adenylate cyclase activity by adrenaline, suggesting that the blockade of cyclic AMP accumulation was in part caused by the direct inhibition of adenylate cyclase. The dependence of the extent of the inhibition of adenylate cyclase on the sphingosine-to-protein ratio was suggestive that the effect of sphingosine was detergent-like. In summary, the inhibition by sphingosine of adrenaline stimulation of cyclic AMP accumulation was most consistent with a general sphingosine-induced perturbation of the plasma membrane which causes both an inhibition of adenylate cyclase activities and, at higher concentrations, permeabilization of the cells.

In contrast with the inhibitory effects of sphingosine, we also found that 20–30 μM-sphingosine caused a time-dependent augmentation of cyclic AMP accumulation in response to stimulation with 50 nM-adrenaline. This effect could be caused by a blockade of the cyclic AMP-dependent protein-kinase-mediated

desensitization, which is the major mechanism involved in the desensitization of adenylate cyclate at these concentrations of hormone (Clark *et al.*, 1988), by a block in cyclic AMP elimination, or by both of these mechanisms. Since we found that sphingosine caused a significant block of cyclic AMP elimination, and yet did not significantly affect the desensitization of adenylate cyclase measured in cell-free assays, we conclude that the augmentation is caused primarily by the blockade of cyclic AMP elimination.

The biphasic effect of 20 μM-sphingosine observed on the stimulation of cyclic AMP accumulation by forskolin is consistent with the conclusions derived from the effects of sphingosine on stimulation by hormones. We would expect the detergent-like effect of sphingosine (the inhibition of forskolin-stimulated cyclic AMP production) to predominate at early times, since the sphingosine-induced inhibition of cyclic AMP elimination would not be quantitatively important until cellular cyclic AMP accumulates to higher levels. At that point the rapid rise in cyclic AMP production due to a sphingosine-induced inhibition of cyclic AMP elimination (mostly by inhibition of phosphodiesterase) would obscure the inhibitory effect of sphingosine on cyclic AMP production. The early inhibition by sphingosine of forskolin stimulation (2 min), which was not observed when S49 WT cells were stimulated with adrenaline, may, in part, reflect the fact that activation of adenylate cyclase by forskolin demonstrates a lag not seen in activation by hormones (Clark *et al.*, 1982). The rapid activation of adenylate cyclase by adrenaline, combined with the sphingosine-induced blockade of cyclic AMP elimination (Fig. 3), might obscure the inhibitory detergent-like effects of sphingosine when intact cells were stimulated with adrenaline.

To summarize, we have found that sphingosine and sphinganine have multiple effects on cyclic AMP accumulation in S49 WT lymphoma cells which cannot be explained by an inhibition of PKC. Inhibition of cyclic AMP accumulation by sphingosine seems most consistent with a generalized detergent-like disruption of the plasma membrane, resulting in inhibition of adenylate cyclase at high concentrations of the sphingolipid. At relatively high concentrations, sphingosine permeabilization of the cells probably contributes as well. Augmentation of cyclic AMP accumulation may result from inhibition of phosphodiesterase activity, cyclic AMP egress from cells, or both. Demonstrating the specific blockade of a physiological PKC-mediated event by sphingolipids in intact S49 WT lymphoma cells, in light of their non-specific effects on cyclic AMP metabolism and cell integrity, could prove to be difficult, if not impossible. Since signal transduction systems are well conserved, it is possible that the non-specific effects of sphingosine that we have observed in S49 lymphoma cells occur in other cell lines and with other second messenger systems. At present, more detailed evaluations of the non-specific effects of the sphingolipids are needed before justifying their use as specific inhibitors of PKC in intact cells.

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