Inhibition of phosphatidic acid phosphohydrolase activity by sphingosine

Dual action of sphingosine in diacylglycerol signal termination

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Recent evidence indicates that a major fraction of diacylglycerol that is produced in hormonally stimulated cells arises by phosphatidylcholine hydrolysis via the sequential action of phospholipase D and phosphatidic acid phosphohydrolase (PAP). We have previously reported that sphingoid bases stimulate phospholipase D activity in NG108-15 cells. The evidence presented here demonstrates that in sphingosine-treated NG108-15 cells, elevated phosphatidic acid levels are accompanied by a parallel, time- and dose-dependent decrease in diacylglycerol levels. DL-propranolol, a known inhibitor of PAP, exerted similar effects, suggesting that the action of sphingosine may have been due to inhibition of PAP activity. This prediction was confirmed in in vitro experiments in which it was demonstrated that sphingosine is as potent an inhibitor of both cytosolic and membrane-associated PAP activity as propranolol. The hypothesis that sphingoid bases may exert a dual action in diacylglycerol signal termination is proposed.

Sphingoid base; Signal transduction; NG108-15 cell

1. INTRODUCTION

Long-chain amino (sphingoid) bases form the backbone of sphingolipids, important membrane constituents which are particularly concentrated in brain and nerve tissue [1]. Over the past 3 years, sphingoid bases emerged as a new class of putative, physiologic bio-regulatory molecules, primarily because they inhibit the activity of the Ca^{2+}/phospholipid-dependent enzyme protein kinase C (PKC) in vitro and of various cellular events which depend on its activation [2,3]. Intriguingly, 1,2-*sn*-diacylglycerol, the second messenger activator of PKC, can stimulate sphingomyelinase activity in GH, pituitary cells and elevate free sphingosine levels [4]; moremover, the action of sphingomyelinase can reverse PKC activation [5,6]. Hence, it was hypothesized that free sphingosine may funcion as an endogenous, negative modulator of PKC [2-6]. In a previous study we demonstrated that sphingosine stimulates cellular phospholipase D activity and causes a marked increase in phosphatidic acid levels [7]. We now report that sphingosine also inhibits the activity of phosphatidic acid phosphohydrolase (PAP), an enzyme that hydrolyses phosphatidic acid to produce diacylglycerol [8].

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2. EXPERIMENTAL

2.1. *Determination of [^{3}H]phosphatidic acid and [^{3}H]diacylglycerol levels in intact cells*

NG108-15 cells were routinely cultured as previously described [9]. To metabolically label phospholipids, 4×10^4 cells (grown in a 75-cm^2 flask) were preincubated for 24 h with 50 μCi of [^{3}H]oleic acid (or 10 μCi of [^{3}H]arachidonic acid, as in Fig. 2), in Dulbecco's Modified Eagle's Medium that contained 0.1% fatty acid-free bovine serum albumin (DMEM/BSA). The cells were then rinsed once, detached, suspended in DMEM/BSA and allowed to recover for 3 h at 37°C before experiments began. Cells (2×10^5 cells in 1 ml of DMEM/BSA) were incubated in 12×75-mm glass tubes at 37°C with 50 μM sphingosine for the indicated time. Incubations were terminated by adding 1 ml of ice-cold acidified methanol and vortexing. Lipids were extracted from the cells essentially as described [10]. Phosphatidic acid was separated from other lipids by double-development thin layer chromatography (TLC) on 10×20-cm silica gel 60 plates according to Bocckino et al. [11]. Bands corresponding to phosphatidic acid carrier are scraped off and quantitated by liquid scintillation spectrometry. [^{3}H]Diacylglycerol was quantitated by TLC on Silica Gel 60 aluminum sheets using benzene/ethyl acetate (7:3) as the mobile phase.

2.2. *Assay of phosphatidic acid phosphohydrolase activity in subcellular fractions*

Subcellular fractions were prepared from NG108-15 cells. Cells were suspended at 4°C in 10 mM Tris-HCl, pH 7.4, containing 1 mM dithiothreitol. The cells were sonicated with a probe sonicator (Heat System Ultrasonics), 10 pulses of 5 s cycles at 4°C. The homogenate was centrifuged for 10 min at $18\,000 \times g$ at 4°C. The resulting supernatant was then centrifuged for 80 min at $105\,000 \times g$, to produce a microsomal pellet and a supernatant fraction. The pellet was then resuspended in 10 mM Tris-HCl, pH 7.4, containing 1 mM dithiothreitol and both fractions were stored at −70°C until used. [^{3}H]Phosphatidic acid was prepared from total NG108-15 cells; phospholipids, metabolically labeled with [^{3}H]oleic acid, by incubation with cabbage phospholipase D, essentially according to Eibl and

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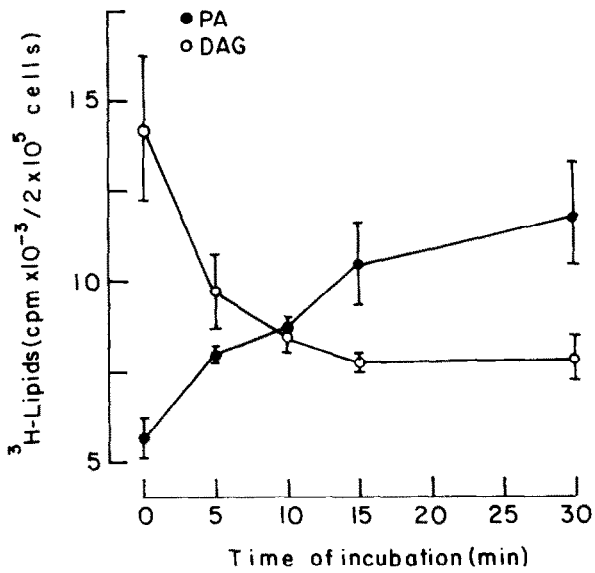

Fig. 1. Reciprocal and concurrent effects of sphingosine on levels of [^{3}H]phosphatidic acid (solid circles) and [^{3}H]diacylglycerol (open circles). [^{3}H]Phosphatidic acid and [^{3}H]diacylglycerol were determined as detailed in section 2. Results are expressed as the mean + SE of two independent experiments, each performed in duplicate.

Kovatchev [12]. [^{3}H]Phosphatidic acid was purified by TLC on Silica Gel 60 plates using the upper phase of a mixture of ethyl acetate /isooctane/acetic acid/water (110:50:20:100) and was 90% pure by 2-dimensional TLC analysis. The specific radioactivity of the purified [^{3}H]phosphatidic acid was 25 Ci/mol. Phosphatidic acid phosphohydrolase activity was assayed by measuring the production of [^{3}H]1,2-diacylglycerol, essentially according to Martin et al. [13]. The incubation mixture, in a final volume of 0.1 ml, contained 100 mM Tris-HCl, pH 7.4, 1 mM dithiothreitol, 2 mg/ml BSA (essentially fatty acid-free), 2 mM $MgCl_2$, 1 mM EGTA, 1 mM EDTA, 0.6 mM phosphatidic acid (Sigma), [^{3}H]phosphatidic acid (5×10^4 cpm), 0.4 mM 1-palmitoyl-2-oleoyl-phosphatidylcholine (Avanti) and 50 μg of cytosol or microsomal protein. To prepare the mixed phospholipid vesicles which served as substrate, appropriate amounts of the lipids were dried under a stream of nitrogen and sonicated in 4 mM EGTA/4 mM EDTA mixture with a probe sonicator for 1 min. The reaction was carried out at 37°C for 30 min and was terminated by the addition of 1 ml methanol/0.1 N HCl (150:1), 1 ml chloroform and 1 ml aqueous EGTA 1 mM. The chloroform phase was collected, dried and [^{3}H]diacylglycerol was separated and counted as described in section 2.1.

3. RESULTS AND DISCUSSION

Neuroblastoma × glioma hybrid NG108-15 cells were pre-labeled with [^{3}H]oleic acid and the formation of [^{3}H]diacylglycerol and [^{3}H]phosphatidic acid, after treatment with sphingosine, was monitored. As shown in Fig. 1, sphingosine elicits a half-maximal reduction in [^{3}H]DAG levels within 4 min of incubation and a maximal reduction within 15 min. The reduction in [^{3}H]diacylglycerol levels slightly precedes the elevation in [^{3}H]phosphatidic acid levels, which reaches half-maximal levels within 10 min and maximal levels within 30 min. The dependence of diacylglycerol and phosphatidic acid levels on sphingosine concentrations was examined in both [^{3}H]oleic acid-labeled and [^{3}H]arachidonic acid-labeled cells, and compared to the effects of the known PAP inhibitor DL-propranolol [14,15]. Fig. 2 shows that the effect of sphingosine on [^{3}H]diacylglycerol and [^{3}H]phosphatidic acid levels was dose-dependent. During 30 min of incubation, sphingosine induced half a maximal decrease of [*arachidonoyl*-^{3}H]diacylglycerol and [*oleoyl*-^{3}H]diacylglycerol at concentrations of 24 μM and 9 μM, respectively. Concomitantly, sphingosine induced a half-maximal accumulation of [*arachidonoyl*-^{3}H]phosphatidic acid and [*oleoyl*-^{3}H]PA at concentrations of 20 μM and 10 μM, respectively. Thus, with both [^{3}H]fatty acid labels, the concentration of sphingosine required for stimulating phosphatidic acid accumulation is similar to the concentration required for reducing diacylglycerol levels. The sphingosine concentration-response curves resemble those of propranolol (Fig. 2). The 50% effect concentrations of propranolol-induced changes in [^{3}H]diacylglycerol and [^{3}H]phosphatidic acid were 27 μM and 46 μM, respectively, in [^{3}H]arachidonic acid-labeled cells, and 18 μM and 37 μM in [^{3}H]oleic acid-

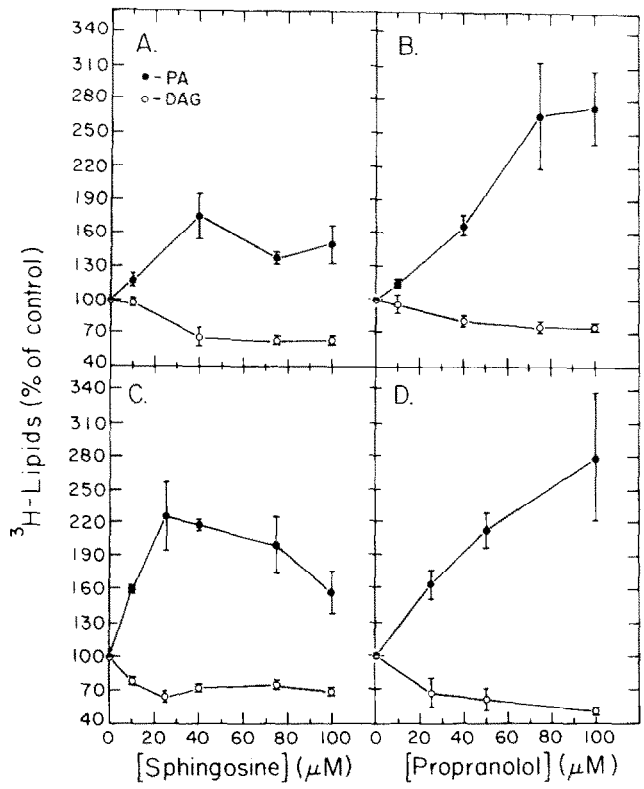

Fig. 2. Dose-dependent effects of sphingosine and propranolol on levels of [^{3}H]phosphatidic acid (solid circles) and [^{3}H]diacylglycerol (open circles). Cells were pre-labeled either with [^{3}H]arachidonic acid (A,B) or with [^{3}H]oleic acid (C,D), and incubated with the indicated concentrations of sphingosine (A,C) or propranolol (B,D) for 30 min. [^{3}H]Phosphatidic acid and [^{3}H]diacylglycerol were determined as detailed in section 2. Results are expressed as the mean ± SE of two (A,B,D) or three (C) independent experiments, each performed in duplicate.

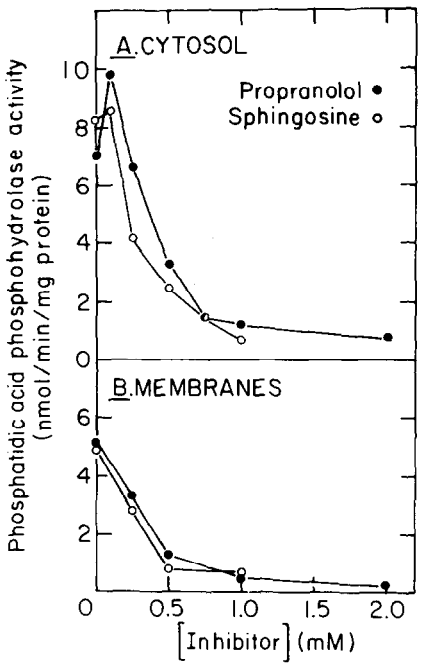

Fig. 3. Effects of sphingosine (open circles) and propranolol (solid circles) on phosphatidic acid phosphohydrolase activity in vitro. (A) Cytosolic PAP. (B) Membrane-associated PAP. Results are expressed as the mean of duplicate determinations from a representative experiment, repeated twice.

labeled cells. The decrease in [^{3}H]phosphatidic acid accumulation evident at high concentrations of sphingosine, but not of propranolol, is most likely due to the known cytotoxic effect of this compound [16]. Apart from that, there was a close correlation between the efficacy of these two compounds.

The similarity between the effects elicited by sphingosine and those of propranolol suggested that sphingosine too may act as an inhibitor of PAP activity. To evaluate this possibility we examined the direct effect of sphingosine on PAP activities present in cytosol and microsomes prepared from NG108-15 cells (Fig. 3). Enzyme activity was measured in vitro by the production of [^{3}H]diacylglycerol from mixed liposomes containing [^{3}H]phosphatidic acid and phosphatidylcholine. The properties of NG108-15 cytosolic and microsomal PAP activities resemble those of the equivalent liver activities (O. Piterman and M. Liscovitch, in preparation). Sphingosine inhibited both the cytosolic and the membrane-associated PAP activities, exhibiting IC_{50} values of 240 and 250 μM, respectively (Fig. 3). Propranolol inhibited PAP activity with IC_{50} values that were somewhat higher than those of sphingosine (380 μM and 330 μM, for the cytosolic and the membrane-associated enzyme, respectively). It was therefore concluded that sphingosine is capable of inhibiting PAP activity in NG108-15 cells. The mechanism of PAP inhibition by sphingosine is presently unknown. The mechanism of PAP inhibition by propranolol is believed to involve its direct interaction with the negatively charged phosphatidic acid [14,15]. Propranolol can be categorized as a cationic amphiphilic drug [17], a class of compounds with which sphingosine shares important structural features, i.e. the positively charged amino group and a hydrophobic domain. Sphingosine may therefore be categorized as a natural cationic amphiphilic molecule, and its mechanism of PAP inhibition may be similar to that of other cationic amphiphilic drugs.

Accumulating evidence indicates that a major fraction of diacylglycerol that forms in signal-activated cells is generated from phosphatidylcholine, by the sequential action of phospholipase D and PAP (Fig. 4) [18,19]. Therefore, the present results suggest that, by virtue of its inhibitory effect on PAP activity, sphingosine may decrease the availability of diacylglycerol and thus may negatively modulate the activity of PKC. Moreover, a recent study has demonstrated that sphin-

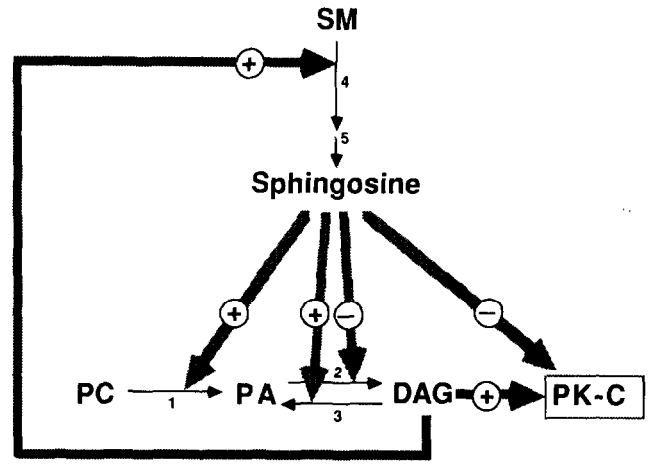

Fig. 4. A schematic and semi-hypothetic diagram of signal-induced generation of diacylglycerol and phosphatidic acid and their interconversion, and negative feedback regulation by sphingosine of diacylglycerol-dependent signalling. Solid arrows indicate metabolic reactions while thick, shaded arrows represent either stimulatory (plus) or inhibitory (minus) regulatory effects. DAG is depicted as being produced from PC by the sequential action of phospholipase D (reaction 1) and PAP (reaction 2). Recent studies indicate that this indirect pathway is quantitatively the most important source of DAG generated in stimulated cells [18,19]. DAG activates PKC, simultaneously and independently stimulating the activity of a neutral sphingomyelinase [6] (reaction 4). This leads (after the action of ceramidase, reaction 5) to production of free sphingosine. Sphingosine has a direct inhibitory effect on PKC activity [21]. Concurrently, sphingosine blocks the generation of DAG by inhibiting PAP activity (present report) and enhances DAG removal by stimulating an 80 kDa diacylglycerol kinase isozyme [20] (reaction 3). This results in decreased DAG levels, inactivation of PKC (accompanied by its translocation back to the cytosol [5]) and hence, signal termination. Thus, sphingosine exert a dual - direct and indirect - inhibitory action on DAG and PKC signalling. The reciprocal regulation of PAP and diacylglycerol kinase by sphingosine, as well as the activation of phospholipase D [7,22] leads to an increase in PA levels. For the sake of simplicity, the possible role of PA as an intracellular messenger in its own right (cf. [18 and 19]) is not depicted in this scheme. See text for a more detailed discussion. *Abbreviations*: DAG, diacylglycerol; PA, phosphatidic acid; PC, phosphatidylcholine; SM, sphingomyelin; PKC, protein kinase C.

gosine markedly activates an 80-kDa diacylglycerol kinase isozyme [20]. This suggests that sphingosine may inhibit the generation of diacylglycerol by PAP while concomitantly stimulating diacylglycerol metabolism. The two enzymes that catalyze the inter-conversion of diacylglycerol and phosphatidic acid - PAP and diacylglycerol kinase - may thus be coordinately and reciprocally modulated by sphingosine. Hence, in addition to its direct, inhibitory effect on PKC, which involves interference with the binding of PKC to the ternary complex formed at the plasma membrane by Ca^{2+}, phosphatidylserine and the signal-generated DAG [21], sphingosine may also terminate PKC activity by decreasing the amount of diacylglycerol available for its activation.

Our hypothesis that sphingosine inhibits diacylglycerol generation complements and completes a model put forth by Kolesnick [5,6] for the termination of PKC activation by endogenous sphingosine (Fig. 4). According to this model, diacylglycerol activates a membrane sphingomyelinase, giving rise (following the action of a ceramidase) to free sphingosine. We here propose that the endogenous sphingosine thus generated may now decrease diacylglycerol production (by inhibiting the activity of PAP) and stimulate its metabolism (by activation of diacylglycerol kinase), thus completing a self-contained negative feedback loop. Whether the inhibitory effect of sphingosine on PAP activity reflects a physiological control pathway and the postulated dual role of sphingoid bases in diacylglycerol signal termination remain to be established by future research.

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