Effects of Sphingosine on Peripheral Membrane Interactions: Comparison of Adriamycin, Cytochrome *c*, and Phospholipase A_2[†]

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ABSTRACT: As revealed by resonance energy transfer utilizing pyrene-labeled phosphatidylcholine donor, the mainly electrostatically controlled binding of adriamycin (Adr) and cytochrome *c* (cyt *c*) to mixed egg yolk phosphatidic acid/phosphatidylcholine (eggPA/eggPC, 15:85 molar ratio) liposomes was reversed upon the inclusion of increasing contents of sphingosine. At a [sphingosine]/[eggPA] molar ratio of ≈2:1, the degree of fluorescence quenching by cyt *c* and Adr was approximately the same as when using liposomes lacking eggPA. Similarly, the increase in the surface pressure of sphingosine/eggPA monolayers on an air/water interface due to the membrane penetration of either cyt *c* or Adr was progressively reduced by increasing the content of sphingosine in the monolayers. The above critical [sphingosine]/[acidic phospholipid] stoichiometry yielding dissociation of the positively charged ligands Adr or cyt *c* from membrane acidic phospholipids was shifted from 2:1 to 1:1 upon substituting egg phosphatidylglycerol (eggPG) for eggPA. Accordingly, charge neutralization of the acidic phospholipids by sphingosine could be involved. One eggPA (having maximally two negative charges) appears to require two molecules of sphingosine whereas the maximally singly charged eggPG is neutralized by one sphingosine. For comparison we also studied the effects of sphingosine on the phospholipase A_2 catalyzed hydrolysis of the pyrene-labeled acidic alkyl–acyl phospholipid analog 1-octacosanyl-2-[6-(pyren-1-yl)]hexanoyl-*sn*-glycero-3-phosphatidylmethanol (C_{28}-O-PHPM) and the corresponding phosphatidylcholine (C_{28}-O-PHPC). In the presence of low Ca^{2+} concentrations (≈50 nM) limiting the rate of the enzymatic reaction, sphingosine gradually inhibited the hydrolysis of phosphatidylcholine, and at 1:6 sphingosine/C_{28}-O-PHPC a nearly complete lack of hydrolysis was evident. In contrast, the presence of about equimolar sphingosine in C_{28}-O-PHPM enhanced by approximately 2-fold the hydrolysis of this lipid. This activation was followed by an inhibition until at approximately 1.1:1 [sphingosine]/[C_{28}-O-PHPM] very little activity was detected. However, sphingosine did not prevent the penetration of PLA2 into monolayers of the nonhydrolyzable dialkylphosphatidic acid. Therefore, unlike for Adr and cyt *c*, sphingosine does not prevent the membrane association of PLA2 while the expression of the catalytic activity of this enzyme is inhibited by sphingosine. The latter is likely to result from changes in substrate surface potential due to the presence of sphingosine, a cationic amphiphile.

Sphingosine is a metabolic intermediate of sphingolipids and influences a variety of cellular activities, including modulation of receptor functions, differentiation, growth inhibition, and cytotoxicity (Hannun & Bell 1989; Merrill & Stevens 1989). Interest in the molecular basis of the cellular effects by sphingosine has rapidly increased since the observation of the inhibition by this compound of protein kinase C (PKC)[1] in vitro (Hannun et al., 1986). The structural requirements for sphingosine and other long chain bases for the inhibition of PKC appear to be the presence of a free amino group and an aliphatic side chain (Merrill et al., 1989). PKC-independent modulatory effects by sphingosine on cellular signaling mechanisms have also been described. In concentrations which inhibit PKC, sphingosine is a potent in vitro inhibitor of several calmodulin-dependent enzymes (Jefferson & Schulman, 1988). Activation of caseine kinase II by sphingosine has been reported (McDonald et al., 1991). Sphingosine inhibits the autophosphorylation of the insulin receptor tyrosine kinase in vitro and in situ (Arnold & Newton, 1991) and alters the phosphorylation of the epidermal growth factor receptor (Faucher et al., 1988; Davis et al., 1988). Inhibition of src-kinase-catalyzed Tyr phosphorylation of synthetic acidic model peptide substrates was seen in the presence of sphingosine (Igarashi et al., 1989).

Several in vivo as well as in vitro studies on the effects of sphingosine connect to acidic phospholipids. For example, activation of L-serine base enzyme (Kanfer & McCartney, 1991), phospholipases D (Kiss & Anderson, 1990; Lavie & Liscovitch, 1990) and C (Hashizume et al., 1992), and inhibition of phosphatidate phosphohydrolase (Lavie et al., 1990; Mullman et al., 1991) by sphingosine lead to increased synthesis of acidic phospholipids. Increased phosphatidic acid levels have been shown to accompany sphingosine-stimulated proliferation of quiescent Swiss 3T3 cells (Zhang et al., 1990b), and a PKC-independent modulatory role for sphingosine on cell growth was suggested (Zhang et al., 1990a). Sphingosine has been shown to enhance phosphatidylserine synthesis by

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[1] Abbreviations: Adr, adriamycin (doxorubicin); C_{28}-O-PHPC, 1-octacosanyl-2-[6-(pyren-1-yl)[hexanoyl-*sn*-glycero-3-phosphatidylcholine; C_{28}-O-PHPM, 1-octacosanyl-2-[6-(pyren-1-yl)]hexanoyl-*sn*-glycero-3-phosphatidylmethanol; cyt *c*, horse heart cytochrome *c*; DHPA, dihexadecylphosphatidic acid; EDTA, ethylenediaminetetraacetic acid; eggPA, -PC, and PG, egg yolk phosphatidic acid, phosphatidylcholine, and phosphatidylglycerol, respectively; π, surface pressure; π_0, original surface pressure; $\delta\pi$, change in surface pressure; PLA2, porcine pancreatic phospholipase A_2; PPDPA, 1-palmitoyl-2-[10-(pyren-1-yl)]decanoyl-*sn*-glycero-3-phosphatidic acid; PPHPC, 1-palmitoyl-2-[6-(pyren-1-yl)]-hexanoyl-*sn*-glycero-3-phosphatidylcholine; PKC, protein kinase C; RFI, relative fluorescence intensity.

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LA-N-2 cells (Singh et al., 1992). Sphingosine prevented the association of histones with phosphatidylserine, and inhibition of PKC by sphingosine was shown to be due to a decrement of the availability of phosphatidylserine in mixed Triton–phosphatidylserine micelles (Bazzi & Nelsestuen, 1987). Likewise, activation of casein kinase II (McDonald et al., 1991) and inhibition of insulin receptor tyrosine kinase (Arnold & Newton, 1991) by sphingosine were reversed by phosphatidylserine. The presence of a charged amino group and an aliphatic side chain have been shown to be necessary for the action of sphingosine in the inhibition of PKC (Merrill et al., 1989) and monocyte tissue factor-initiated coagulation (Conckling et al., 1989). Creation of a positive vesicle surface charge by sphingosine has been suggested to be the mechanism of inhibition of the CTP-phosphocholine cytidylyltransferase activity (Sohal & Cornell, 1990). The above results do suggest that electrostatic interactions with acidic phospholipids could be involved in the effects of sphingosine on cellular membranes. Studies presented in this paper were carried out to examine in vitro this possibility.

Cyt *c* (Nichols, 1974; Mustonen et al., 1987) and Adr (Gianni et al., 1983; Mustonen & Kinnunen 1991) are extensively studied ligands which associate peripherally with membranes. Both cyt *c* and Adr require the presence of acidic phospholipids for efficient membrane binding. Porcine pancreatic PLA2 represents a well-characterized model for membrane-bound enzymes associating with its phospholipid substrates due to a specific interfacial recognition site (Waite, 1987). Several studies have demonstrated acidic phospholipids to be preferred as substrates for this lipolytic enzyme (Volwerk & De Haas, 1982; Thurén et al., 1987a). Importance of membrane surface potential in controlling the expression of the catalytic activity has been proposed (Mustonen & Kinnunen, 1991, 1992). We utilized simple and reasonably well-characterized model membrane systems, liposomes and lipid monolayers on an air/water interface, to investigate the effect of the cationic amphiphile sphingosine on the lipid binding of the above peripherally associating membrane ligands.

MATERIALS AND METHODS

Reagents. The pyrene-labeled phospholipids 1-palmitoyl-2-[10-(pyren-1-yl)]decanoyl-*sn*-glycero-3-phosphatidic acid (PPDPA), 1-palmitoyl-2-[6-(pyren-1-yl)]hexanoyl-*sn*-glycero-3-phosphatidylcholine, (PPHPC), 1-octacosanyl-2-[6-(pyren-1-yl)]hexanoyl-*sn*-glycero-3-phosphatidylcholine (C_{28}-O-PHPC), and 1-octacosanyl-2-[6-(pyren-1-yl)]hexanoyl-*sn*-glycero-3-phosphatidylmethanol (C_{28}-O-PHPM) were purchased from KSV Chemicals Co. (Helsinki, Finland). Dihexadecylphosphatidylcholine (DHPC), dihexadecylhosphatidic acid (DHPA), and egg yolk phosphatidylcholine, -glycerol, and phosphatidic acid were from Sigma. The acidic lipids were calcium-free sodium salts. No impurities were detected in the above lipids upon thin layer chromatography on silicic acid using chloroform/methanol/water/ammonia (90:5.5:5.5:5.5, v/v) as the solvent system and examination of the plates for pyrene fluorescence or after iodine staining. Porcine pancreatic phospholipase A2 (PLA2 EC 3.1.1.4) was from Boehringer Mannheim. Adriamycin and horse heart type VI cytochrome *c* (mainly oxidized form), D-sphingosine (from bovine brain sphingomyelin), and other materials of reagent grade were from Sigma.

Preparation of Liposomes. The indicated lipids were mixed in chloroform, whereafter the solvent was evaporated under nitrogen. After at least 2 h under reduced pressure, the lipids were hydrated with 20 mM Tris-HCl, and 0.1 mM EDTA, pH 7.4. The liposomes contained 1.25 μM PPHPC as a fluorescence marker, and the concentrations of eggPA, eggPG, and sphingosine were varied as indicated. To maintain the total phospholipid concentration constant at 25 μM, the amount of eggPC was adjusted appropriately. To obtain unilamellar liposomes, the dispersions were sonicated for 4 min on an ice–water bath and under an argon atmosphere with a Branson sonifier equipped with a microtip probe and operated at 65-W output.

Quenching of Pyrene Fluorescence. The overlap of pyrene monomer emission with the absorption spectra of cyt *c* (Mustonen et al., 1987, Rytömaa et al., 1992) and Adr (Mustonen & Kinnunen, 1991, 1993) allows to use Perrin–Förster resonance energy transfer to assess the binding of these ligands to pyrene lipid-containing liposomes. In these phospholipid probes, the fluorescent moiety is embedded within the hydrocarbon region of the membrane, and negligible perturbation of the ligand–lipid interaction due to the bulky aromatic moiety is anticipated. Excitation at 344 nm and emission wavelengths of 394 and 480 nm (for monomer and excimer fluorescence, respectively) were selected by the monochromators of our SLM 4800S spectrofluorometer (SLM-Aminco Inc., Urbana, IL). The corresponding slits were 1 and 16 nm. Small aliquots (10 μL) of Adr or cyt *c* were added into the indicated liposome solutions (in thermostated and magnetically stirred cuvettes) with a microprocessor controlled, high-precision electronic pipette (Biohit Inc, Helsinki, Finland) while the consequent changes in fluorescence intensity were recorded. The quantum yields decreased maximally by $\approx$15% as the content of eggPA or sphingosine in liposomes was increased. The reasons for this are uncertain at present yet may relate to altered membrane microviscosity. However, the measured degree of fluorescence quenching correlates with the extent of the membrane association of the fluorescence acceptors. Accordingly, the data are given as percentage of relative fluorescence intensity remaining after the binding of either cyt *c* or Adr to liposomes while normalizing the initial values at 100. All experiments were carried out at 25 °C where the lipids used are in liquid-crystalline state.

The disadvantages of the use of pyrene lipids in resonance energy transfer studies have been discussed previously (Kaihovaara et al., 1991; Mustonen & Kinnunen, 1991, 1993; Rytömaa et al., 1992). Briefly, the kinetics of excited state pyrene relaxation are complex, sensitive to oxygen, and include both monomeric and excimeric decays which are both sensitive to spectral quenchers (e.g., Lemmetyinen et al., 1989; Kinnunen et al., 1993). Approximately 15% higher emission intensities were measured in the absence of oxygen. However, the relative values remained essentially unaltered, and for more facile experimentation the fluorescence data were collected in the presence of atmospheric oxygen. Importantly, one membrane-associated acceptor causes the quenching of several pyrenes thus prohibiting the straightforward use of energy transfer as a spectroscopic ruler (Stryer, 1978; Drake et al., 1991). Accordingly, while the degree of fluorescence quenching does correlate to the number of membrane-associated acceptors, this correlation is not linear. While pyrene-labeled lipids are thus poorly suited for quantitative energy transfer studies, they allow for the sensitive semi-quantitative assessment of the extent of membrane association of cyt *c* and adriamycin. Because of the high affinity of cyt *c* and adriamycin to acidic phospholipids, such experiments

can be performed at low concentrations of both lipids and these ligands thus resulting in an insignificant inner filter effect (maximally <5% of the initial emission). All experiments were repeated two or three times with essentially identical results. When the fluorescence data were collected, ten signals were averaged twice using the built-in function of the instrument. These data points exhibited only minimal scatter. Error bars were typically contained within the symbols used to label the graphs and accordingly, are not shown.

Monolayer Experiments. Penetration of Adr and cyt *c* into eggPA/sphingosine and eggPG/sphingosine monolayers was monitored using a magnetically stirred (250 rpm) circular well (surface area 31 cm^2, volume 50 mL) drilled in Teflon as described earlier (Mustonen & Kinnunen, 1991). Surface pressure was recorded with a platinum Wilhelmy plate connected to an electrobalance. Unless otherwise indicated the aqueous subphase was 20 mM Tris, and 0.1 mM EDTA, pH 7.4. All experiments were performed at an ambient temperature of approximately 23 °C. The lipids were spread on the air/buffer interface from a chloroform solution with a microsyringe. Adr, cyt *c*, and PLA2 were injected through the film into the subphase after the stabilization of the monolayers for approximately 15 min. Collapse pressures for eggPA, eggPG, DHPA, and sphingosine films were 44.5, 47.6, 49.2, and 46.2 mN/m, respectively.

Phospholipase A_2 Assay. Phospholipase A_2 activity was determined by the endpoint assay described in detail elsewhere (Thuren et al., 1985), with minor modifications (Mustonen & Kinnunen, 1992). In brief, ethanol-injected vesicles of C_{28}-O-PHPC and C_{28}-O-PHPM were used as substrates. Data points represent mean values from triplicate assays with the average error varying between 5% and 10%. Reactions were started by the addition of the indicated amounts of PLA2. The enzymatic activity was quantitated by measuring the monomer intensity at 400 nm due to the liberated (pyren-1-yl)hexanoate after a 20-min incubation at 37 °C. The assay was calibrated by measuring the fluorescence intensity due to the addition of known picomolar concentrations of (pyren-1-yl)hexanoate into the reaction mixture in the absence of the enzyme. In 20 mM Tris and 1.0 mM $CaCl_2$ (pH 7.4) at 37 °C, the specific activities were 2.4 nmol/(min·μg) and 25 pmol/(min·μg) for C_{28}-O-PHPM and C_{28}-O-PHPC, respectively (Mustonen & Kinnunen, 1991). Unless otherwise indicated, the PLA2 measurements were carried out in the absence of added $CaCl_2$. The $CaCl_2$ concentration thus present is ≈50 nM and due to residual concentration of this cation in our purified water and buffering materials (Thurén et al., 1986). Pancreatic PLA2 contains Ca^{2+} tightly bound in its active site and required for catalysis. These conditions should mimic the intracellular aqueous phase where Ca^{2+} concentration is generally in the nanomolar range. Accordingly, factors influencing PLA2 activity under conditions where Ca^{2+} concentrations is limiting can be investigated. This approach has been used by us in our previous studies, revealing under proper conditions the activation of PLA2 by polyamines, platelet activating factor, adriamycin, and phorbol myristoyl acetate (Thurén et al., 1986, 1990; Mustonen & Kinnunen, 1991, 1992). Depending on the substrate used this residual Ca^{2+} concentration can support between 25% and 50% of the catalytic activity of PLA2 maximally activated by mmolar Ca^{2+} concentrations (Mustonen & Kinnunen, 1991). Complete loss of activity was seen due to the inclusion of excess (0.1 mM) EDTA. The above procedure results in the presence of 0.9 mM ethanol in the final assay medium. However, our previous studies provided no evidence for an interference by this alcohol concentration with the action of the enzyme (Mustonen & Kinnunen, 1991).

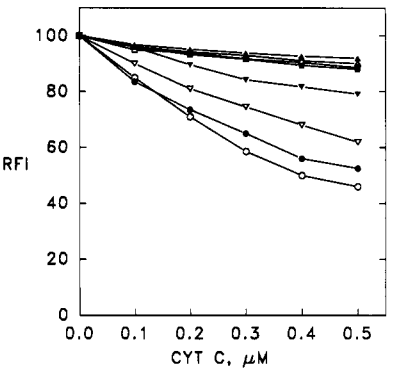

FIGURE 1: Quenching of the pyrene monomer fluorescence (RFI) as a function of cyt *c* concentration. Total lipid concentration was 25 μM with 15 mol % eggPA and 5 mol % PPHPC in eggPC liposomes. The content of sphingosine in the liposomes was 0 (○), 5 (●), 10 (▽), 15 (▼), 20 (□), 25 (■), and 30 mol % (△). For comparison are shown data for eggPC liposomes in the absence of eggPA and sphingosine (▲). The buffer was 20 mM Tris-HCl and 0.1 mM EDTA, pH 7.4. The temperature was 25 °C.

RESULTS

Reversal by Sphingosine of the Electrostatic Association of cyt c and Adr with Phosphatidic Acid-Containing Liposomes. Resonance energy transfer allow one to monitor the binding of cyt *c* to liposomes containing pyrene-labeled phospholipids as fluorescence energy donors (Mustonen et al., 1987). In Figure 1 the quenching of PPHPC-labeled eggPC liposomes containing 15 mol % eggPA is shown as a function of increasing cyt *c* concentration A clear decrease in the membrane association of cyt *c* due to an increasing content of sphingosine (up to 30 mol %) in these vesicles is evident. At sphingosine concentrations exceeding 20 mol %, the binding of cyt *c* was identical to that using phosphatidylcholine vesicles lacking the acidic phospholipid. Lack of cyt *c* association to eggPA-containing membranes in the presence of sufficient amounts of sphingosine was also confirmed by the lack of effect on pyrene quenching due to the addition of 200 mM NaCl which dissociates cyt *c* from negatively charged phospholipids (data not shown).

Similarly to cyt *c*, the spectral overlap of the emission of pyrene-containing lipids and Adr absorption allows monitoring of the membrane association of this drug (Mustonen & Kinnunen, 1991). The reversal of the binding of Adr due to an increase in the content of sphingosine in the liposomes containing 15 mol % eggPA is shown in Figure 2. A saturation in the reversal of the quenching was observed at [sphingosine]/[eggPA] molar ratios above 2:1 and corresponding to the level of quenching by Adr of eggPC liposomes in the absence of eggPA. Adr has been reported to bind to phosphatidylcholine membranes also in the absence of acidic lipids (Burke & Tritton, 1985).

Figure 3, panel A, illustrates the increase in the binding of cyt *c* and Adr to eggPC liposomes upon increasing the content of eggPA from 0 to 15 mol %. Exceeding a critical molar fraction of approximately 5 mol % of the acidic phospholipid, the fluorescence intensity was quenched progressively by cyt *c* as a function of increasing eggPA concentration in liposomes. This sigmoidal dependency on the content of the acidic phospholipids results from the requirement for a critical negative surface charge density for the membrane association

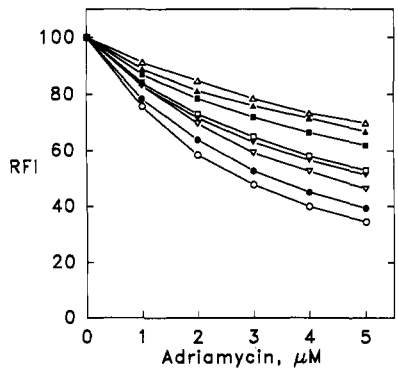

FIGURE 2: Quenching of the pyrene monomer emission as a function of Adr concentration. The content of sphingosine in liposomes containing 5 mol % PPHPC was 0 (○), 5 (●), 10 (▽), 15 (▼), 20 (□), 25 (■), and 30 mol % (△). For comparison for eggPC liposomes are also shown results in the absence of eggPA and sphingosine (▲). Experimental conditions were identical to those described in the legend to Figure 1.

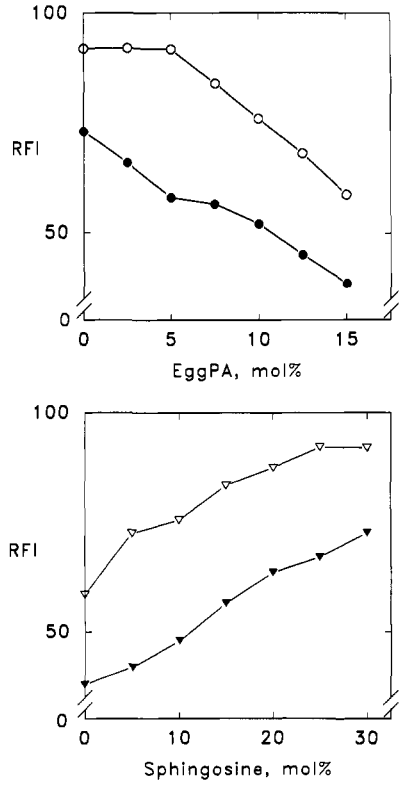

FIGURE 3: Quenching of the pyrene monomer fluorescence by 0.3 μM cyt *c* (○) and by 3 μM Adr (●) as a function as of the content of eggPA in eggPC liposomes (panel A, top). Panel B (bottom) shows the quenching of pyrene fluorescence by 0.3 μM cyt *c* (▽) and 3 μM Adr (▼) as a function of sphingosine content (from 0 to 30 mol %) in eggPC liposomes containing 15 mol % eggPA. Total lipid was 25 μM with 5 mol % PPHPC. Experimental conditions were identical to those described in the legend to Figure 1.

of cyt *c* (Mustonen et al., 1987; Rytömaa et al., 1992). In accordance with our earlier data measured with cardiolipin-containing liposomes (Mustonen & Kinnunen, 1993), there was also a linear decrease in pyrene fluorescence due to membrane binding of Adr as a function of increasing eggPA concentration in eggPC vesicles. Panel B of Figure 3 illustrates the effect of further inclusion of increasing concentrations of shingosine (from 0 to 30 mol %) into eggPC vesicles containing 15 mol % eggPA. Clear reversal of the membrane association of both cyt *c* and Adr is evident. At contents of sphingosine exceeding 30 mol %, there was a plateau in both titration curves corresponding to the quenching of eggPC liposomes in

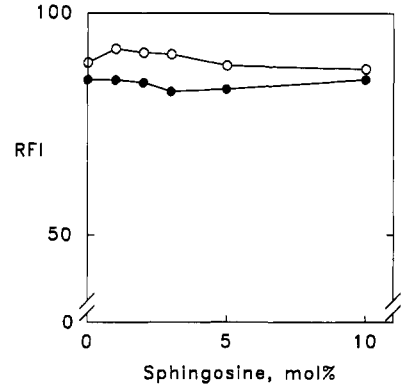

FIGURE 4: Quenching of pyrene fluorescence by 0.25 μM cyt *c* (○) and by 2.5 μM Adr (●) as a function of the indicated sphingosine content in eggPC liposomes. Total lipid was 25 μM with 5 mol % PPHPC. Experimental conditions were identical to those described in the legend to Figure 1.

the absence of eggPA and sphingosine by cyt *c* and Adr (data not shown).

Maximally ionized PA bears two negative charges in its phosphate. Accordingly, it was of interest to repeat the above experiments with eggPG, which upon complete deprotonation has one net negative charge. In brief, the effect of increasing concentrations of sphingosine (from 0 to 20 mol %) in eggPC/eggPG liposomes (85:15 molar ratio) was studied. Compared with the data in Figure 3 measured with eggPA, approximately half the amount of sphingosine (i.e. 15 mol %, corresponding to a 1:1 [sphingosine]/[eggPG] molar ratio) was needed to reverse the binding of cyt *c* and Adr to the liposomes.

Figure 4 depicts control experiments revealing that in the absence of acidic phospholipids the presence of up to 10 mol % sphingosine had no effect on the binding of cyt *c* or Adr to eggPC liposomes.

Effects of Sphingosine on PLA2-Catalyzed Hydrolysis of C_{28}-O-PHPM and C_{28}-O-PHPC. In order to study the effects of sphingosine on PLA2 reaction, vesicles of the alkyl–acyl pyrenephospholipids C_{28}-O-PHPM and C_{28}-O-PHPC containing the indicated concentrations of sphingosine were used as substrates. In Figure 5, panel A, is shown the inhibition by increasing sphingosine concentration of the hydrolysis of 31 μM C_{28}-O-PHPC by porcine pancreatic PLA2. Concentrations of sphingosine exceeding 5 μM (>14 mol %) inhibited the hydrolysis completely. In contrast to the zwitterionic substrate, increasing the sphingosine concentration up to approximately equimolar with C_{28}-O-PHPM caused a 2-fold activation of the hydrolysis of the acidic substrate (panel B). However, above 25 μM sphingosine (corresponding to ≈0.8:1 [sphingosine]/[C_{28}-O-PHPM] molar ratio), a progressive inhibition of hydrolysis was evident. Half-maximal inhibition was obtained at [sphingosine]/[C_{28}-O-PHPM] at ≈1:1 and almost total inhibition at 35 μM sphingosine, corresponding to ≈1.1:1 [sphingosine]/[C_{28}-O-PHPM] molar ratio.

Due to the high affinity of Ca^{2+} to acidic phospholipids and the absolute requirement of this cation for the catalytic action of PLA2 (Verheij et al., 1981), the effect of 4 mM $CaCl_2$ on the hydrolysis of C_{28}-O-PHPM was studied (Figure 5, panel B). In the presence of 4 mM $CaCl_2$, approximately 4-fold activation of the hydrolysis was seen, and low concentrations of sphingosine (<15 μM) had only an insignificant effect. Increasing the content of sphingosine above 35 mol % progressively inhibited the hydrolytic reaction, and at 56 mol % sphingosine (corresponding to 1.3:1 [sphingosine]/[C_{28}-O-PHPM] molar ratio) nearly complete loss of activity was observed.

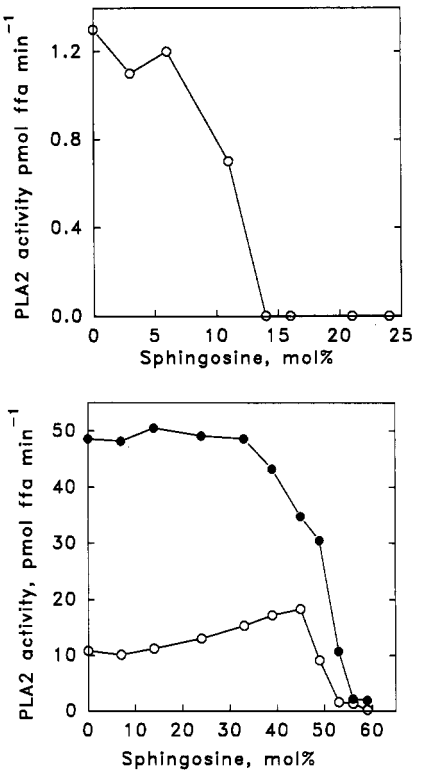

FIGURE 5: PLA2-catalyzed hydrolysis of 31 μM C_{28}-O-PHPC (panel A, top) and C_{28}-O-PHPM (panel B, bottom) as a function of sphingosine content. In panel B (○) only residual Ca^{2+} is present or (●) 4 mM $CaCl_2$ is included. The medium was 20 mM Tris-HCl, pH 7.4. Temperature was 37 °C. The amount of enzyme was 150 ng for C_{28}-O-PHPM and 4 μg for C_{28}-O-PHPC, and the incubation time was 20 min.

Penetration of Cyt c, Adr, and PLA2 into Lipid Monolayers. Monomolecular lipid films on an air/water interface were used to obtain complementary information on the effects of sphingosine on the membrane association of cyt *c*, Adr, and PLA2. An increase in surface pressure π of phospholipid monolayers due to cyt *c* (Quinn & Dawson, 1969), Adr (Mustonen & Kinnunen, 1991, 1992), or PLA2 (Zografi et al., 1971) is caused by the penetration of these molecules into the lipid films. At the initial surface pressures of 15, 20, and 25 mN/m of an eggPA monolayer, 1 μM cyt *c* and 2 μM Adr caused a rapid increase $\delta\pi$, which was complete within approximately 10 min. However, increasing the content of sphingosine in the lipid films progressively decreased the surface pressure increement due to cyt *c* and Adr (Figure 6, panels A and B). Interestingly, the critical content of sphingosine reversing the membrane penetration of cyt *c* was dependent on surface pressure, i.e., at lower initial surface pressure the increase in π was larger and more sphingosine was needed to reverse the membrane association of cyt *c*. This surface pressure-dependent efficiency of sphingosine to reverse monolayer association was not observed for Adr. Complete reversal of the monolayer penetration by Adr was observed at approximately a 3:1 [sphingosine]/[eggPA] molar ratio independently of the initial surface pressure.

For comparison we also studied the reversal of the penetration by sphingosine of cyt *c* and Adr into an eggPG monolayer at an initial surface pressure of 25 mN/m (Figure 7). Complete reversal of monolayer penetration was observed at an approximately equimolar [sphingosine]/[eggPG] molar ratio for both ligands.

The effect of sphingosine on the membrane penetration of PLA2 was studied using monolayers of the nonhydrolyzable

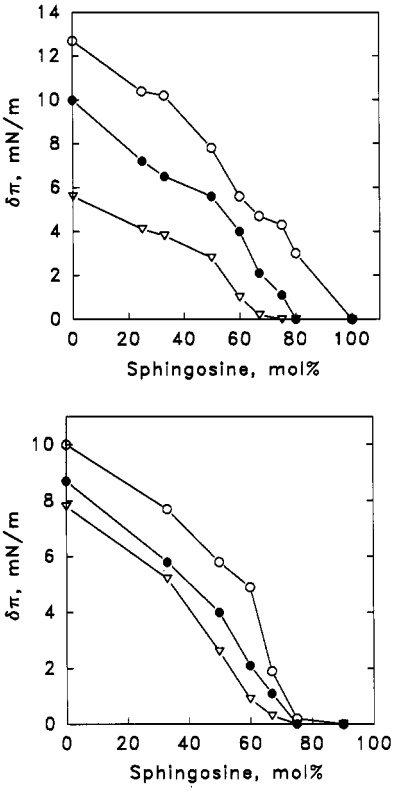

FIGURE 6: Effect of sphingosine on the change in the surface pressure ($\delta\pi$) of eggPA monolayers at initial pressures of 15 (○), 20 (●), and 25 (▽) mN/m induced by 1 μM cyt *c* (panel A, top) and 2 μM Adr (panel B, bottom). The magnetically stirred 50-mL subphase was 20 mM Tris-HCl and 0.1 mM EDTA, pH 7.4. Cyt *c* and Adr were injected through the lipid film after a stabilization time of 15 min.

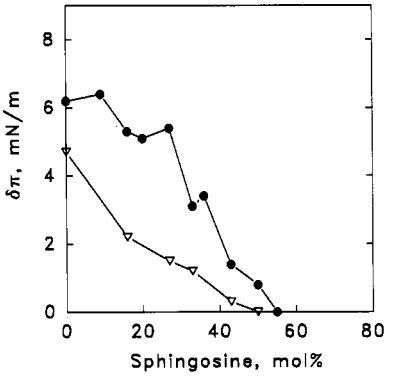

FIGURE 7: Reversal by sphingosine of the increase in the surface pressure ($\delta\pi$) of eggPG monolayers induced by 1 μM cyt *c* (●) and 2 μM Adr (▽). Initial pressure was 25 mN/m. Experimental conditions were identical to those described in the legend to Figure 6.

substrate analog dihexadecylphosphatidic acid (DHPA) instead of eggPA. In Figure 8 is shown the increase in π as a function of the sphingosine mol % in DHPA monolayers due to the penetration of PLA2 into the lipid film. At an initial surface pressure of 9 mN/m, PLA2 penetrated into pure DHPA as well as sphingosine monolayers. However, in mixed monolayers the presence of sphingosine enhanced the penetration, and a maximal increase in π was observed at a 2:1 sphingosine/DHPA molar ratio. Thus, unlike for Adr and cyt *c*, sphingosine does not prevent the association of PLA2 with the acidic phospholipid monolayers although loss of the enzymatic activity can be caused by this compound. However, it is important to note that in the monolayer penetration experiments we had to utilize the dialkyl-PA lacking the ester carbonyl present at the *sn*-2 ester bond of the alkyl–acyl

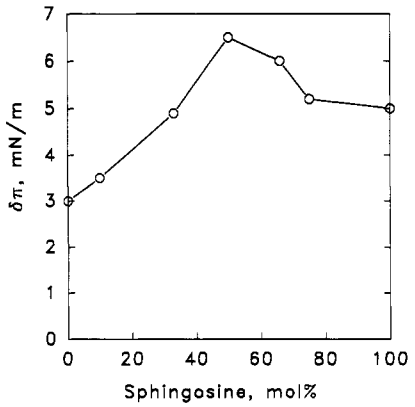

FIGURE 8: Effect of sphingosine on the membrane penetration of PLA2. Changes in the surface pressure ($\delta\pi$) due to the injection of 50 μg of PLA2 underneath DHPA monolayer at an initial pressure of 9 mN/m were monitored. The subphase was 20 mM Tris, pH 7.4; otherwise the experimental conditions were identical to those described in the legend to Figure 6.

phospholipids. Accordingly, the interpretation of this experiment warrants caution when conclusions on the association of PLA2 with its hydrolyzable substrates are made.

DISCUSSION

Cyt *c* associates only weakly with zwitterionic phosphatidylcholine membranes (Nichols, 1974). However, the affinity is greatly increased when acidic phospholipids are included (Nichols & Malviya, 1973). The membrane association of cyt *c* with acidic phospholipid-containing membranes can be prevented by increasing the concentration of salt and is thus mainly ionic in nature. Binding of cyt *c* to liposomes appears to require a critical negative surface charge density which results in a sigmoidal dependency on the content of the acidic lipid in vesicles (Mustonen et al., 1987; Rytömaa et al., 1992). Similarly to cyt *c*, electrostatic interactions are involved in the membrane association of the currently widely used cytotoxic compound Adr (Duarte-Karim et al., 1976). Accordingly, this drug has a high affinity to acidic lipids (Goormaghtigh et al., 1980). Two different binding sites for Adr have been suggested to be constituted by acidic phospholipid-containing membranes (Henry et al., 1985; Dupou-Cezanne et al., 1989; Mustonen & Kinnunen, 1991). Binding of Adr to mitochondrial cardiolipin has been suggested to be involved in the mechanisms of the cardiotoxicity of this drug (Goormaghtigh et al., 1990). Adriamycin has been reported to reverse the binding of cyt *c* to cardiolipin by interacting with the negatively charged phosphates of the lipid at equimolar drug–lipid concentrations (Goormaghtigh et al., 1982).

Membrane association of cyt *c* and Adr was enhanced as the molar fraction of eggPA in eggPC liposomes was increased. Binding of these ligands to liposomes containing a fixed amount of an acidic phospholipid is reversed upon increasing the content of the cationic amphiphile sphingosine in membrane and thus suggests charge neutralization (Figure 3, panel B). Interestingly, even though the affinity of Adr to acidic phospholipids is high (De Wolf et al., 1991) it appears to be exceeded by the affinity between sphingosine and eggPA. At ≈2:1 [spingosine]/[eggPA] molar ratio, the quenching by Adr was approximately the same as for eggPC liposomes lacking eggPA. Higher sphingosine contents (up to 40 mol %) in the presence of 15 mol % eggPA gave similar results as the presence of 30 mol % sphingosine, thus indicating that excess sphingosine has no further effect on the membrane association of cyt *c* and Adr. In accordance, the presence of up to 10 mol % sphingosine in eggPC liposomes lacking acidic lipids had insignificant effects on the membrane association of cyt *c* and Adr (Figure 4).

Additional information was obtained by monitoring the penetration of cyt *c* and Adr into monomolecular phospholipid films on an air/water interface. Notably, increasing concentrations of sphingosine prevented in a progressive manner the penetration of both cyt *c* and Adr into eggPA monolayers. In accordance with our fluorescence quenching data, these results suggest that the presence of sphingosine in the monolayer reverses the electrostatic interaction of cyt *c* and Adr with the acidic lipids. Notably, as qualitatively essentially similar results on the effects due to sphingosine were evident both in monolayers and liposomes, sphingosine-induced phase changes in liposomes are unlikely to be the underlying mechanism. In addition, our present results strongly indicate an effective partitioning of sphingosine into both types of model membranes used.

The above results do suggest that the association of cyt *c* and Adr with acidic lipids involves similar mechanisms. However, the concentration dependency of the reversal by sphingosine of the binding of cyt *c* to monolayers and liposomes differ. In brief, higher concentrations of sphingosine are required to prevent the penetration of cyt *c* into monolayers than to detach this protein from liposomes. As both hydrophobic as well as electrostatic interactions are involved, it is probable that the excess sphingosine which has to be introduced into the monolayers (especially at lower initial surface pressures) is required to counterbalance for the hydrophobic free energy of association of cyt *c* with lipids. At surface pressures above 40 mN/m the association of cyt *c* with eggPA monolayer has been suggested to be purely electrostatically controlled (Quinn & Dawson, 1969). Compared with cyt *c*, hydrophobicity appears to contribute less to the membrane association of Adr, and an almost surface pressure-independent reversal of the monolayer penetration as well as detachment of Adr from liposomes by sphingosine occurred at approximately 2:1 [spingosine]/[eggPA] molar ratio.

The major uncertainty concerning the interpretation of the present results is due to the lack of information on the protonation states of the acidic phospholipids and sphingosine under the experimental conditions employed. The pK_a values for these compounds may in addition depend on the membrane composition as well as on parameters such as monolayer surface pressure. In mixed micelles with Triton X-100, values of 6.7 and 7.7 have been measured for the pK_a of sphingosine (Merrill et al., 1989; Bottega et al., 1989), whereas an intrinsic pK_a of 8.5 for sphingosine as such has been reported (Bottega et al., 1989). Somewhat similarly, the membrane association and degree of protonation of the amphiphilic drug propranolol has been shown to depend on the presence of acidic phospholipids and cationic amphiphiles in the membrane (Schlieper & Steiner, 1983). At neutral pH, phosphatidic acid should be half protonated with a net negative charge of one per lipid (Träuble & Eibl, 1974; van Dijck et al., 1978). However, complete deprotonation of eggPA could result in the presence of sphingosine. Thus, at equimolar sphingosine/eggPA ratio (15 mol % of each in the vesicles), the efficiencies of fluorescence quenching by cyt *c* and Adr were similar to those using liposomes containing 7.5 mol % eggPA and no sphingosine. To obtain further information on the role of the net charge of the acidic lipid, we used eggPG instead of eggPA as the former has only one protonating group. In brief, our results from studies on both monolayer penetration and liposome fluorescence quenching by cyt *c* and Adr would be

compatible with charge neutralization at [sphingosine]/[eggPA] and [sphingosine]/[eggPG] molar ratios of approximately 2:1 and 1:1 respectively, to result in the detachment of these cationic peripheral ligands from membrane acidic phospholipids. In our preliminary studies, we observed a 1.2-fold increase in the excimer/monomer emission intensity ratio for PPDPA/eggPC liposomes (5:95 molar ratio) due to the inclusion of sphingosine. This change in fluorescence saturated at 5 mol % of the cationic amphiphile and is likely to result from phase separation in liposomes. Similar phase separation of the fluorescent acidic phospholipid could be induced by 0.1 mM Ca^{2+}. Notably, above an equimolar sphingosine/PA molar ratio, 0.5 mM $CaCl_2$ had no further effect on the pyrene fluorescence (data not shown). To conclude, our findings are consistent with an electrostatically controlled complex formation by PA and sphingosine and subsequent phase separation of these complexes in eggPC.

Phospholipase A_2 is currently taken as a paradigm for Ca^{2+}-activated lipolytic enzymes (van den Bosch, 1980; Waite, 1987). Similarly to PKC and other amphitropic proteins (Burn, 1988; Klee, 1988), cytosolic PLA2 has been shown to be translocated from the cytoplasm to the plasma membrane by Ca^{2+} (Channon & Leslie, 1990; Yoshihara & Watanabe, 1990). Unlike most enzymes acting on soluble substrates, a distinct feature of lipases is the high degree of dependency of the measured reaction rates on the physicochemical properties of the substrate/water interface where the enzyme catalysis occurs. For instance, PLA2 is most active toward phospholipids at the phase transition temperature (Op den Kamp et al., 1975; Menasche et al., 1981), and it prefers acidic phospholipids as substrates (Verheij et al., 1981). Pancreatic phospholipase A_2 contains a specific region nominated as the interfacial recognition site. Unmasking of this site by proteolytic cleavage of the proenzyme causes a marked increase in the lipid association of this protein, and the catalytic rate of the active enzyme is greatly enhanced when the critical micellar concentration of the substrate lipid is exceeded. However, other factors also appear to be involved, and several mechanisms for the interfacial activation of PLA2 have been proposed [for a review, see Waite (1987)]. An additional approach to the membrane effects of sphingosine was obtained by studying the hydrolysis of pyrene-labeled alkyl–acyl phospholipids by porcine pancreatic PLA2, a thoroughly investigated and widely employed model enzyme for PLA2's (Waite, 1987; Volwerk & deHaas, 1982).

In our previous studies we have shown that in the presence of low (≈50 nM) concentrations of Ca^{2+} different polyamines, PAF, Adr, and phorbol 12-myristoyl 13-acetate all inhibit the PLA2-catalyzed hydrolysis of phosphatidylcholines yet enhance the reaction with acidic phospholipids (Thurén et al., 1986, 1990; Mustonen & Kinnunen, 1991, 1992). Both effects are by large abrogated in the presence of molar concentrations of Ca^{2+}. In accordance with the observed effects of electric fields imposed over subtrate phospholipid monolayers (Thurén et al., 198b), we have suggested modulation of PLA2 activity to be due to changes in the substrate surface potential caused by the above compounds (Mustonen & Kinnunen, 1991, 1992). In the presence of the residual calcium concentration of ≈50 nM, sphingosine in a concentration of 5 μM (corresponding to a [sphingosine]/[C_{28}-O-PHPC] molar ratio of 1:6) inhibited the hydrolytic reaction completely. Increasing the content of sphingosine in C_{28}-O-PHPM vesicles had a slight activating effect on the PLA2-catalyzed hydrolysis followed by an abrupt inhibition upon exceeding 45 mol % sphingosine and finally resulting in a nearly complete loss of activity at >55 mol %. Due to the headgroup-dependent differences, a direct effect of sphingosine on PLA2 is unlikely. Accordingly, the modulation by sphingosine of the expression of the catalytic activity of PLA2 is likely to be due to changes in the substrate.

The presence of 4 mM $CaCl_2$ activated the hydrolysis of C_{28}-O-PHPM approximately 4-fold, and no effect due to the addition of sphingosine was observed up to 1:2 [sphingosine]/[C_{28}-O-PHPM] molar ratio. At higher sphingosine concentrations, an inhibition followed, and at 56 mol % sphingosine almost complete loss of activity was evident. Half-maximal inhibition was obtained at equimolar [sphingosine]/[C_{28}-O-PHPM] concentration both in the presence and absence of added $CaCl_2$. Accordingly to our studies with DHPA monolayers, sphingosine does not reverse the binding of PLA2 to the membrane. The inhibition by sphingosine could be due to a positive surface charge of the vesicle and subsequent dissociation of the Ca^{2+} from the substrate/enzyme complex. Analogously, inhibition of PKC by sphingosine has been reported to be competitive with Ca^{2+} (Hannun et al., 1986) and to correlate with the presence of a positive charge (Bottega et al., 1989). However, half-maximal inhibition in the presence of both residual (≈50 nM) and 4 mM $CaCl_2$ was observed at ≈1:1 [sphingosine]/[C28-O-PHPM], thus suggesting that displacement of Ca^{2+} from the active site of PLA2 may not be the underlying mechanism producing inhibition of the enzyme. Another possibility is a conformational and/or orientational change in the lipid-bound enzyme molecule due to the presence of sphingosine. The possible physiological significance of the above in vitro results on PLA2 and sphingosine is uncertain. Yet, in this context, it might be of interest to note that thrombin-induced arachidonate release in human platelets was strongly reduced in the presence of sphingosine in spite of the elevated levels of intracellular Ca^{2+} (Krishnamurthi et al., 1989). Sphingosine also blocked arachidonic acid release and PLA2 activity in neutrophils (McIntyre et al., 1987) and smooth muscle cells (Chakraborti et al., 1991). Franson et al. (1992) reported inhibition of phospholipases A_2 and D by sphingosine in vitro.

Several peripherally membrane-associating proteins appear to be involved in the cellular signal transmission mechanisms. Accordingly, there is considerable current interest in the determinants for the lipid binding of proteins such as protein kinase C (Bell & Burns, 1991), calpactins, and synexins (Klee, 1988). Both acidic phospholipids and Ca^{2+} seem to be required. However, membrane association and the expression of the catalytic activity may, under proper conditions, be disconnected. An example is provided by the present results on PLA2. Our results also emphasize the similarity of the electrostatic determinant regulating the membrane association of cyt *c* and Adr. Continuing this line of research further, we wish to be able to get more rational insight on the role of specific lipid structures regulating the membrane association and, when applicable, also the catalytic activity of peripheral membrane proteins as well as the effects of drugs on such molecular associations.

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