Cholesterol relieves the inhibitory effect of sphingomyelin on type II secretory phospholipase A_2

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Secretory type II phospholipase A_2 ($sPLA_2$) is inhibited by sphingomyelin (SPH); cholesterol either mixed with the model glycerophospholipid substrate or added to the assay medium as separated liposomes counteracts this inhibition efficiently. The inhibition of fatty acid release assayed by quantitative gas chromatography–MS is observed when SPH is added to erythrocyte membranes as the substrate instead of a readily hydrolysable phosphatidylethanolamine/phosphatidylserine model mixture. Hydrolysis of SPH by *Staphylococcus aureus* sphingomyelinase suppresses its inhibitory potency. The addition of cholesterol to SPH liposomes with a 1:1 stoichiometry relieves completely the inhibition of $sPLA_2$ exerted by SPH. The mechanism of inhibition suggested by the binding assay is that $sPLA_2$ binds with affinity to the SPH interface, after either phase segregation at the assay temperature or on the pure SPH liposomes added to the incubation medium. Cholesterol is shown to suppress the binding affinity of the enzyme for the SPH interface. A model for inhibition is suggested in which binding of the sphingosine moiety is competitive for $sPLA_2$ (inhibition) or for cholesterol (release of the enzyme).

INTRODUCTION

For many years sphingomyelin (SPH) has been considered mostly as a structural component of biological membranes. The structural effects depend in part on the particular phase behaviour of SPH and the consequential effects on its interaction with cholesterol. Interest in this phospholipid was renewed when its role as a precursor of a variety of intracellular signalling derivatives was demonstrated [1]. The ceramide backbone, sphingosine and sphingosine 1-phosphate derived from SPH are now recognized as prominent regulatory effectors of cell growth, differentiation and apoptosis [2].

In addition to this recently recognized regulatory activity, SPH has a particular structural role among the phospholipids of biological membranes, especially in plasma membranes, where it is located preferentially in the outer lipid leaflet. The particularly close association of SPH with cholesterol was noted in early studies of Chapman et al. [3]. This is believed to result from particular features of SPH. The sphingosine hydroxy group at the C-3 position along the Δ4,5-*trans* double bond and the amide group nitrogen act as hydrogen-bonding sites whose interaction with the 3β-hydroxy group of cholesterol has been reported [4,5]. The 'condensing' effect of cholesterol is manifested by a decrease of 10–20% in the average molecular area occupied by the phospholipid at the aqueous interface of monomolecular films maintained at surface pressures representative of biomembranes. Van der Waals interactions between the long-chain base and cholesterol [6] are considered to have a role similar to that of the *sn*-1 saturated acyl chain of phosphatidylcholine (PtdCho) in enhancing the cohesive interaction with α-face of cholesterol. Because SPH forms a gel phase at physiological temperatures, there is a close alignment of the extended acyl chain and the planar sterol ring system, which favours van der Waals interactions between the two molecules. Molecular species of SPH are characterized by a high proportion of saturated very long acyl chains amide-bonded to the sphingosine moiety. This contrasts with the moderately long polyunsaturated chains at the *sn*-2 position of membrane glycerophospholipids. This results in a high temperature (T_m) for the transition from gel to liquid-crystal phase of sphingolipids, which forces them into separate gel phase domains within a fluid bilayer matrix of glycerophospholipids at physiological temperatures [7].

A consequence of the close association of SPH with cholesterol in segregated domains of the gel phase is that membrane fractions can be isolated from a number of eukaryotic cells that resist dissociation by mild detergents [8,9]. The detergent-insoluble fraction, commonly referred to as 'detergent-resistant membranes' (DRM), was found to contain a variety of membrane proteins involved in signal transduction or lipid trafficking [10,11].

It is well known that phospholipases A_2 (PLA_2) must bind to the water/substrate interface to mount a hydrolytic attack on the susceptible *sn*-2 ester bond [12]. One of the explanations for the marked resistance of the outer layer of plasma membranes to extracellular phospholipases is that the high packing density of the bilayer leaflet enriched in SPH, PtdCho and cholesterol hinders the penetration of the enzymes into the substrate structure. The notion that SPH is the primary activity-limiting factor has come from studies that have shown a marked stimulation of PLA_2 activity against biological membranes subjected to pretreatment with bacterial sphingomyelinase [13,14]. The physiological significance that is usually given to the resistance of the outer layer of the cell membrane to the eventually high level of circulating PLA_2 during inflammatory syndromes could be a consequence of the lipid composition of the exposed leaflet comprising SPH.

Previously we have observed that enrichment of the rat liver plasma membranes with SPH was followed by a decrease in the membrane-bound PLA_2 activity [15]. This result is consistent with other studies on the effect of SPH on phospholipid hydrolysis. Dawson et al. [16], for example, showed that SPH at high concentrations (40 mol%) significantly inhibited the PLA_2

Abbreviations used: DPPC, D-α-dipalmitoylphosphatidylcholine; GC–MS, gas chromatography–MS; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdSer, phosphatidylserine; SPH, sphingomyelin; $sPLA_2$, secretory phospholipase A_2.

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hydrolysis of intestinal mucosal phosphatidylethanolamine (PtdEtn). Leslie and Channon [17] reported that SPH that had been co-dispersed with arachidonoyl-PtdCho substrate markedly inhibited macrophage PLA_2; they concluded that inhibition was due to an increase in the lipid packing density. Lobo and Wilton [18] observed a strong inhibition of both type I pig pancreatic and *Naja naja* PLA_2 when the PtdCho substrate was co-sonicated with 30 mol% SPH. In a study on lecithin:cholesterol acyltransferase, Bolin and Jonas [19] reported 'specific' competitive inhibition by SPH. For phospholipase C, Scarlata et al. [20] showed that inclusion of SPH in PtdCho/PtdIns(4,5)P_2 substrate inhibits phospholipase C-δ activity and suggested that it is due to increased interlipid hydrogen bonding, which decreases membrane hydration. The authors also found that SPH itself did not bind phospholipase C-δ; neither did it affect binding to the PtdIns(4,5)P_2 substrate.

A previous study from this laboratory [21] has reported that SPH inhibited human type II secretory PLA_2 ($sPLA_2$) acting on erythrocyte membranes. The action of SPH was not due to a non-specific 'packing density' or substrate dilution effect because replacement of SPH with the non-hydrolysable enantiomer of disaturated PtdCho, D-α-dipalmitoylphosphatidylcholine (DPPC), a membrane 'rigidifier', did not inhibit the enzyme. The enzyme has a putative role in the release of pro-inflammatory lipid mediators but its direct participation in the release of arachidonic acid is conjectural and could be influenced by the composition of target membranes [22,23]. The present study was undertaken to characterize the modulation of human $sPLA_2$ in membranes containing SPH and cholesterol. In particular, we wished to decipher whether SPH inhibits the enzyme in a specific competitive manner or via an alteration in the physical properties of the substrate. The strategy employed was to assay $sPLA_2$ activity with a highly susceptible substrate consisting of a mixed dispersion of PtdEtn/phosphatidylserine (PtdSer) in the presence of liposomes formed from SPH. In this approach, SPH does not alter the physical state of the glycerophospholipid substrate but can interact separately with the enzyme. We conclude that the enzyme binds to SPH liposomes and is thereby diverted from the substrate. The presence of cholesterol in the SPH liposomes decreased the binding affinity of the enzyme and released more enzyme to hydrolyse the substrate.

MATERIALS AND METHODS

Reagents

L-α-PtdEtn (from egg yolk), L-α-PtdSer (from bovine brain), SPH (from egg yolk), DPPC and cholesterol were purchased from Sigma (St. Louis, MO, U.S.A.). Recombinant type II secretory PLA_2 collected from C127 transfected mouse fibroblasts overexpressing the human enzyme [24] was generously provided by Dr. Olivier (Faculté de Médecine St. Antoine, Paris, France).

Materials

Centricon-100 filters were obtained from Amicon (Beverly, MA, U.S.A.) and were soaked in a 0.1% albumin solution to prevent non-specific protein adsorption, in accordance with the supplier's recommendations.

Preparation of the erythrocyte ghosts serving as the substrate

Human erythrocytes were isolated from fresh citrated blood [25]. All steps were performed at 4 °C. The blood was centrifuged for 10 min at 100 ***g***. Erythrocytes were collected and washed several times with 5 vol. of PBS, pH 8.0, and haemolysed in 5 mM sodium phosphate buffer, pH 8.0 (40 vol.). The ghosts were collected by centrifugation for 20 min at 22000 ***g*** (Beckman J2-HS), then washed with 5 mM phosphate buffer until 'white ghosts' were obtained (four cycles). Membrane proteins were measured by using the method of Bradford [26].

Preparation of liposomes

Liposomes were prepared by sonication of PtdEtn/PtdSer (80:20, mol/mol), SPH, SPH/cholesterol or DPPC. Typically, phospholipids were dissolved in chloroform, dried under a stream of oxygen-free dry nitrogen, hydrated in Tris/HCl buffer, pH 8.6, and sonicated (twice for 2 min) with a tip probe (MSE, Crawley, Surrey, U.K.) (20 kHz, approx. 100 W) until a clear dispersion had been obtained.

Binding and filtration procedures

Liposomes of non-hydrolysable phospholipids SPH, DPPC or SPH/cholesterol (1:1, mol/mol) and DPPC/cholesterol (1:1, mol/mol) were incubated with $sPLA_2$ (100 nmol of SPH or DPPC and 3 m-units of $sPLA_2$). After 10 min the incubations were spun down through a Centricon 100 filter, with the temperature maintained at 37 °C, and filtrates were collected for the determination of unbound $sPLA_2$. We confirmed independently that the filters were permeable to sPLA2 (14 kDa) but excluded SPH, DPPC or SPH/cholesterol and DPPC/cholesterol liposomes.

Phospholipase A_2 assay

PLA_2 activity assayed with erythrocyte membranes

The assay contained erythrocyte membranes representing 100 nmol of hydrolysable glycerophospholipids serving as the substrate in 0.5 ml of 100 mM Tris/HCl (pH 8.6)/10 mM $CaCl_2$/0.1% fatty acid-free BSA. The reaction was initiated with 3 m-units of human recombinant type II $sPLA_2$ (the activity of the enzyme preparation was calibrated by comparison with a reference activity of pancreatic PLA_2). Incubations were performed for 15 min at 37 °C with translational shaking. Released fatty acids, extracted by a modification of the procedure of Dole [27] and methylated by diazomethane, were quantified by gas chromatography–MS (GC–MS). Control incubations of erythrocyte membranes incubated in the absence of added $sPLA_2$ were used to correct the levels of fatty acids released by $sPLA_2$.

PLA_2 activity on phospholipid liposomes

The incubation was performed for 15 min at 37 °C in a final volume of 0.5 ml, containing 100 nmol of hydrolysable phospholipids [PtdEtn/PtdSer (80:20, mol/mol)] suspended in 100 mM Tris/HCl (pH 8.6)/10 mM $CaCl_2$/0.1% BSA. Human recombinant type II $sPLA_2$ (3 m-units) was added to start the reaction. The released free fatty acids were extracted, methylated with diazomethane and quantified by GC–MS. Control incubations were performed in the absence of $sPLA_2$; the values were used to correct the levels of enzyme activity.

Quantitative measurements with GC–MS

The extracted free fatty acids were methylated for 5 min with fresh diazomethane; methyl esters were separated by gas chromatography on a capillary column wall-coated with Supelcowax-10 bonded phase [internal diam. 0.32 mm, length 30 m, film thickness 0.25 μm (Supelco, Bellafonte, PA, U.S.A.)] fitted in a

Hewlett Packard 5890 Series II gas chromatograph. Fatty acids were detected with picomolar sensitivity by MS (Nermag 10-10C; Nermag, Reuil, France) in the chemical ionization mode with ammonia (10^4 Pa) as the reagent gas. The positive quasi-molecular ions were selectively monitored and time-integrated. Quantification was achieved by reference to an internal standard of heptadecanoic methyl ester with response factors calculated for the various fatty acid methyl ester calibrators.

RESULTS

Inhibitory activity of SPH on $sPLA_2$

The effect of SPH on the activity of $sPLA_2$ is illustrated in Figure 1. In this experiment the inhibitory effects of SPH on the $sPLA_2$ activity were compared when the sphingolipid was added either co-dispersed with the glycerophospholipid substrate or when added separately as liposomes of pure SPH. In both cases the presence of SPH caused inhibition of the release by $sPLA_2$ of the various fatty acids from the *sn*-2 position of the glycerophospholipids. Inhibition reached a plateau (50 % when added separately; 70 % when co-dispersed) over a molar ratio of 0.5 for SPH/glycerophospholipid. The experiment showed that a 10 min preincubation of the enzyme with SPH before addition of the substrate liposomes reinforced the extent of inhibition for low concentrations of SPH when compared with incubations in which SPH liposomes were added simultaneously with the substrate glycerophospholipids. As discussed below, this is consistent with the binding of the enzyme to SPH liposomes and subsequent progressive release to attack the substrate. In addition, when the enzyme is acting on the mixed liposomes the inhibition is influenced by the potential SPH-binding sites formed.

To test the possibility that $sPLA_2$ interacts transiently with the substrate and the SPH surface throughout the incubation an experiment was performed in which SPH liposomes were added at different intervals after initiation of the reaction (final SPH-to-glycerophospholipid molar ratio, 1:1). The results in Figure 2 show that there was an inhibition of hydrolysis of the glycerophospholipid that depended on the length of time that the SPH liposomes were present in the reaction mixture. The magnitude of inhibition was greatest if SPH liposomes were added within 2 min of initiation of the reaction; the inhibition decreased as the reaction progressed.

The effect of SPH on the activity of $sPLA_2$ acting on a natural substrate was examined next. Such assays are possible with the GC–MS method, which monitors the fatty acids produced by the hydrolytic activity from any (unlabelled) substrate with great sensitivity. Figure 3 shows that the addition of SPH liposomes

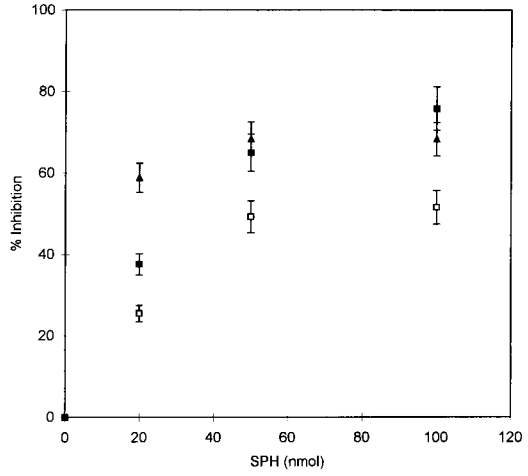

Figure 1 Inhibition of PLA_2 by SPH

The activity was estimated as the quantity of fatty acids released after 15 min (33.06 ± 1.80 nmol in the absence of SPH) extracted and quantified by GC–MS as indicated in the Materials and methods section. Incubations were performed at 37 °C in 0.5 ml of 100 mM Tris/HCl (pH 8.6)/10 mM $CaCl_2$/0.1 % BSA in the presence of 3 m-units of $sPLA_2$. PtdEtn/PtdSer (80:20, mol/mol; 100 nmol) was co-sonicated with different amounts of SPH (■); alternatively, SPH vesicles were added separately to the incubation 10 min before the addition of substrate (▲) or together with the substrate on addition of the enzyme (□). Values are means ± S.D. for three independent determinations.

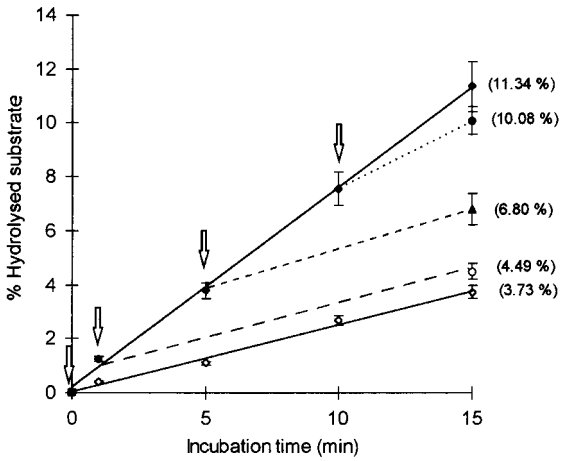

Figure 2 Time-dependent inhibition of PLA_2 by SPH

Incubations were performed as described in the legend to Figure 1. SPH liposomes (100 nmol) were introduced into the incubation medium containing 100 nmol of glycerophospholipid (PtdEtn/PtdSer) substrate at different time points (arrows) after addition of the enzyme (3 m-units of $sPLA_2$). Determination of the total released fatty acids was made as described in the legend to Figure 1 after incubation for 15 min at 37 °C. Values are means ± S.D. for three independent determinations.

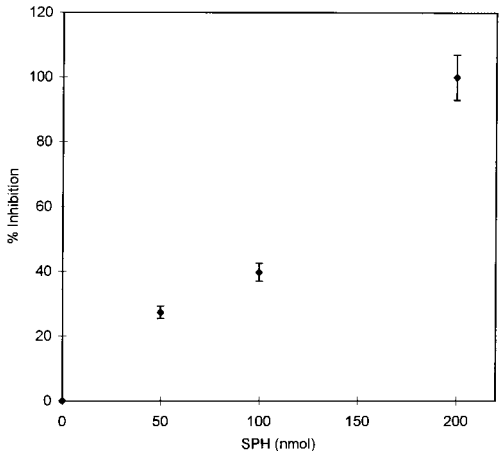

Figure 3 Inhibition by SPH of $sPLA_2$ acting on erythrocyte membranes

Erythrocyte membranes (100 nmol of glycerophospholipids) were incubated in the presence of 3 m-units of $sPLA_2$. Various quantities of SPH liposomes were present during the 15 min incubation at 37 °C, after which the released fatty acids were quantified by GC–MS as described in the legend to Figure 1. Values are means ± S.D. for six independent determinations.

Table 1 Cholesterol reverses the inhibition of $sPLA_2$ by SPH

The substrate mixture consisting of 100 nmol of PtdEtn/PtdSer (80:20, mol/mol) was assayed in the presence of 3 m-units of $sPLA_2$ for 15 min at 37 °C by performing a GC–MS assay of the hydrolysed fatty acid products (100% activity). Parallel assays were performed with substrate (100 nmol) co-sonicated with 10, 50 or 100 nmol of cholesterol as indicated and with 100 nmol SPH (Expt. A). Another series of substrate mixtures was prepared with cholesterol but SPH liposomes (100 nmol) were added separately to the assay mixtures (Expt. B). Values are means ± S.D. for three independent determinations.

	Expt. A		Expt. B	
Addition	Activity (%)	$C_{20:4}/C_{18:1}$	Activity (%)	$C_{20:4}/C_{18:1}$
Substrate (PtdEtn/PtdSer)	100	1.42		
+ SPH	31.5 ± 2.1	1.26		
+ SPH/cholesterol (1:0.1)	36.7 ± 3.6	1.50	33.6 ± 2.7	1.45
+ SPH/cholesterol (1:0.5)	69.4 ± 7.3	1.66	42.6 ± 5.1	1.53
+ SPH/cholesterol (1:1)	99.1 ± 9.6	2.06	67.1 ± 7.9	2.90

Table 2 Binding of $sPLA_2$ to SPH liposomes

The relative binding affinity of $sPLA_2$ was judged by hydrolytic activity measured in the filtrate of enzyme (3 m-units) incubated with sonicated liposomes of SPH (100 nmol), SPH (100 nmol)/cholesterol (10 or 100 nmol), DPPC (100 nmol) or DPPC (100 nmol)/cholesterol (100 nmol). Unbound enzyme was separated by centrifugation through a Centricon-100 filter at 37 °C. The filtrate was assayed for $sPLA_2$ activity as described in the Materials and methods section. The recovered activity of unbound enzyme was calculated relative to the filtrate collected in the absence of liposomes (100% activity, as all the enzyme was recovered in the filtrate under the experimental conditions used). Values are means ± S.D. for three independent determinations.

Preincubation with liposomes	Activity (nmol)	Recovery (%)
None	32.55 ± 3.15	100
SPH	18.25 ± 2.85	57
SPH/cholesterol (1:0.1)	22.78 ± 1.43	70
SPH/cholesterol (1:1)	33.52 ± 2.91	103
DPPC	37.75 ± 5.86	116
DPPC/cholesterol (1:1)	30.27 ± 1.78	93

gave a strong inhibition of $sPLA_2$ acting on biomembranes. The enzyme was completely inhibited at an estimated SPH-to-hydrolysable phospholipid ratio of 2:1, taking into account the SPH content of human erythrocyte membranes (20 mole % of total membrane phospholipids).

The effect of hydrolysis of SPH in substrate mixtures was investigated next. Pretreatment of substrate/SPH liposomes with sphingomyelinase from *Staphylococcus aureus* essentially restored $sPLA_2$ activity to levels observed in substrate dispersed in the absence of SPH (100 % released fatty acids in the absence of SPH; 31 % in the presence of SPH; 83 % after pretreatment with sphingomyelinase). The ceramide products that resulted from hydrolytic cleavage of the phosphocholine group were likely to remain in the glycerolipid substrate [28].

A number of studies have indicated that SPH binds more strongly to cholesterol than do most of the glycerophospholipids [29]. Because a physiological significance is inferred from this molecular association in detergent-resistant biomembrane fractions, we examined the inhibitory effect of SPH on $sPLA_2$ in the presence of cholesterol. The results presented in Table 1 show that the inclusion of cholesterol in the SPH/glycerophospholipid substrate mixture decreased the inhibitory effect of SPH on the enzyme. When cholesterol was present in equimolar amounts with SPH, no inhibition was detected. A control experiment showed that cholesterol co-sonicated with PtdEtn/PtdSer alone did not influence $sPLA_2$ susceptibility in the absence of SPH. Relief of the inhibition was also observed if cholesterol was initially co-dispersed with the glycerolipid substrate and SPH liposomes were added separately. The effect of cholesterol in this case was much decreased, suggesting that progressive transfer of cholesterol from the substrate to the SPH liposomes might be required for cholesterol to prevent inhibition of the enzyme by SPH.

An interesting observation in these experiments was that the composition of the hydrolysate was enriched in polyunsaturated fatty acids compared with mono-unsaturated oleic acid when cholesterol was added to SPH/glycerophospholipid liposomes. The shift in the composition of the released fatty acids was consistent with decreased protection by cholesterol of the saturated and mono-unsaturated molecular species of glycerophospholipid initially associated with SPH into domains with 'detergent-resistant membrane' properties, as suggested by previous physical studies [30]. It could be envisaged that $sPLA_2$ has a lowered binding affinity for these cholesterol/glycerophospholipid/SPH domains and therefore the enzyme is available for hydrolysis of the polyunsaturated molecular species of the surrounding glycerophospholipids.

To test the suggestion that phase-separated domains of SPH (or pure SPH liposomes) might compete with glycerophospholipid for the binding of $sPLA_2$, enzyme binding studies were performed. Initially, liposomes of SPH were incubated with $sPLA_2$ to assess the binding capability of the sphingolipid. After liposomes had been mixed with the enzyme, the dispersions were centrifuged through Centricon 100 filters, which exclude $sPLA_2$ bound to liposomes. The results presented in Table 2 show that 40 % of the $sPLA_2$ activity was retained on the SPH liposomes under the experimental conditions used. A control experiment showed that all of the $sPLA_2$ activity could be recovered after filtration through an albumin-soaked filter and that no SPH was detectable in the filtrate. When SPH/cholesterol liposomes were substituted for pure SPH liposomes, the affinity of the enzyme for the liposomes was decreased and virtually all of it was recovered in the filtrate for liposomes consisting of an equimolar ratio of SPH and cholesterol. DPPC and DPPC/cholesterol liposomes did not seem to bind the enzyme either.

DISCUSSION

The present study was instigated in view of previous results indicating that the inhibition of secretory PLA_2 by SPH was apparently not due to any alteration in the fluidity of the phospholipid matrix serving as substrate [21]. The increase in packing density and viscosity of the phospholipid hydrocarbon domain due to the effect of SPH cannot explain the low accessibility or activity of the enzyme on the *sn*-2 ester bond of the substrate at the lipid/water interface. Experiments performed with a non-hydrolysable enantiomeric disaturated PtdCho, DPPC, that is able to rigidify glycerophospholipid bilayers to about the same extent as SPH had no effect on activity of $sPLA_2$ [21]. Because a non-specific effect of SPH seemed to be ruled out, it follows that the inhibition of $sPLA_2$ results from a more specific effect of SPH on the reaction.

A simple experiment to investigate the nature of specific inhibition is to add SPH as pure liposomes to the incubation during the assay of $sPLA_2$ rather than as a co-dispersion with the glycerophospholipid substrate. The substrate mixture PtdEtn/PtdSer (80:20, mol/mol) was selected for the present experiments because of its high susceptibility to hydrolysis. This susceptibility

is assumed to explain the attack of biomembranes by the hydrolytic enzyme after the 'scrambling' of phospholipid asymmetry associated with cell activation [31]. The susceptibility relies on the anionic character of PtdSer, which is claimed to favour the binding of $sPLA_2$.

We observed that SPH inhibits $sPLA_2$ irrespectively of whether the sphingolipid is co-dispersed with the substrate before the incubation or is added separately as pure liposomes. Moreover inhibition is immediate as soon as SPH is present in the assay incubation. This seems to exclude fusion of the stable SPH liposomes with the PtdEtn/PtdSer substrate dispersion as a prerequisite for inhibition of $sPLA_2$, given the short duration of the assay. Small-angle X-ray diffraction of SPH co-dispersed with PtdEtn/PtdSer has shown a lamellar phase (C. Wolf, K. Koumanov and P. J. Quinn, unpublished work). Under the experimental conditions used here (37 °C), mixed liposomes or pure SPH liposomes probably offer similar phase-segregated gel SPH ($T_m \approx 40$ °C) interfaces available for interaction with $sPLA_2$ [32]. We suggest that the PtdEtn/PtdSer substrate is altered to an arrangement of two distinct lamellar phases by the addition of SPH. In the absence of cholesterol, the interface created by pure SPH could compete with the substrate glycerophospholipids for binding of $sPLA_2$.

When SPH liposomes are added after the substrate during enzyme assay, the $sPLA_2$ activity is decreased very rapidly, indicating that the enzyme can be readily displaced from the interface of the hydrolysable substrate by presentation of non-hydrolysable sphingolipid. Phase-segregated SPH within mixed liposomes or pure SPH liposomes might serve similarly to bind and detain the enzyme on its interface. This could provide a mechanism whereby enzyme activity is regulated by the partition of $sPLA_2$ between the SPH interface and PtdEtn/PtdSer. The affinity of the enzyme for a pure SPH interface has been demonstrated by filtration: significant amounts of $sPLA_2$ seem to be adsorbed on non-filtrating liposomes under the experimental conditions used for the enzyme assay. The fact that cholesterol is able to restore the filterability of $sPLA_2$ (14 kDa) suggests a mechanism for the dynamic control of the affinity of the enzyme for SPH surfaces.

The reason for the lack of affinity of $sPLA_2$ for SPH/cholesterol lamellae remains unclear. It could be that the sphingosine moiety of SPH acts specifically because it could be bound (possibly through hydrogen-bonding of interfacial residues) by either $sPLA_2$ residues at the lipid/water interface or competitively by cholesterol. Because the phosphocholine groups of PtdCho and SPH are identical it cannot be the only cause of the binding of $sPLA_2$, but the suppression of the inhibitory activity of SPH after cleavage of the polar head group by treatment with sphingomyelinase suggests that there is a requirement for the phosphocholine group to bind the enzyme to the interface.

A number of studies with a variety of different substrates have established the inhibitory activity of the long chain base sphingosine. For example, Franson et al. [33] showed that sphingosine inhibits Ca^{2+}-dependent phospholipase A_2 from *Naja naja*, pig pancreas, *Crotalus adamanteus* and human neutrophils in a dose-dependent manner, with the IC_{50} ranging from 5 to 40 μM, with the use of [1-^{14}C]oleate-labelled autoclaved *Escherichia coli* or *Candida albicans*, or 1-acyl-2-[1-^{14}C]linoleoylglycerophosphoethanolamine or 1-acyl-2-[1-^{14}C]linoleoylglycerophosphocholine as the substrate. Subbaiah et al. [34] showed that lyso-SPH, which lacks the N-acyl group amidifying SPH, is as effective as SPH itself in inhibiting lecithin:cholesterol acyltransferase. On the basis of this observation we suggest that a co-operation involving the particular orientation of the phosphocholine group of SPH and the two hydrogen-bonding sites of the long-chain base probably constitutes a site of special affinity for $sPLA_2$ and various lipolytic enzymes. The relief by cholesterol of SPH inhibition points to a defined stoichiometry of SPH and cholesterol. A specificity confined to the inhibitory action of SPH is evident from the absence of any activating effect of cholesterol when the enzyme is assayed with the glycerophospholipid PtdEtn/PtdSer or PtdEtn/PtdSer/PtdCho mixtures (results not shown).

Physical heterogeneity of the substrate has been judged to be a favourable condition for PLA_2 activity [35]. Segregation of SPH/cholesterol in the substrate mixture could be an additional cause of increased susceptibility of the substrate. This condition is satisfied by most of the highly saturated molecular species of SPH at physiological temperatures and is thought to be relevant in biological membranes.

Finally, the present observations support the view that the structural default between SPH/cholesterol 'rafts' and surrounding fluid glycerophospholipids could be the target for the enzyme in biological membranes. The interface between phase-separated bilayers of SPH and fluid glycerophospholipid could not be hydrolysed when the enzyme was bound to SPH domains. We assume that SPH exerts its inhibitory potency via specific interactions with the interfacial binding site of $sPLA_2$. This interaction shows a stronger affinity than the interaction with glycerophospholipids. Hydrolytic activity was restored after cholesterol released $sPLA_2$ from its association with SPH.

The present study paves the way for studies *in vivo*, where inflammatory cytokines tumour necrosis factor α and interleukin 1β have been shown to induce simultaneous synthesis and secretion of $sPLA_2$ and sphingomyelinase [36]. A forward cross-regulation can thereby be inferred.

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