Ceramides increase the activity of the secretory phospholipase A_2 and alter its fatty acid specificity

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Modulation of human recombinant secretory type II phospholipase A_2 activity by ceramide and cholesterol was investigated using model glycerophospholipid substrates composed of phosphatidylethanolamine and phosphatidylserine dispersed in aqueous medium. Enzyme activity was monitored by measurement of released fatty acids using capillary GC-MS. Fatty acids from the *sn*-2 position of the phospholipids were hydrolysed by the enzyme in proportion to the relative abundance of the phospholipid in the substrate. Addition of increasing amounts of ceramide to the substrate progressively enhanced phospholipase activity. The increased activity was accomplished largely by preferential hydrolysis of polyunsaturated fatty acids, particularly arachidonic acid, derived from phosphatidylethanolamine. The addition of sphingomyelin to the substrate glycerophospholipids inhibited phospholipase activity but its progressive substitution by ceramide, so as to mimic sphingomyelinase activity, counteracted the inhibition. The presence of cholesterol in dispersions of glycerophospholipid-substrate-containing ceramides suppressed activation of the enzyme resulting from the presence of ceramide. The molecular basis of enzyme modulation was investigated by analysis of the phase structure of the dispersed lipid substrate during temperature scans from 46 to 20 °C using small-angle synchrotron X-ray diffraction. These studies indicated that intermediate structures created after ceramide-dependent phase separation of hexagonal and lamellar phases represent the most susceptible form of the substrate for enzyme hydrolysis.

Key words: cholesterol, lipid phase, sphingomyelin, X-ray diffraction.

INTRODUCTION

The existence of microdomains of sphingomyelin (SM)-cholesterol (Chol) in the plasma membrane, which are believed to be associated with transmembrane signal transduction, lipid sorting and protein trafficking [1,2], has focused attention on the influence of sphingolipids on the metabolism of membrane phospholipids. Huang et al. [3] have reported that ceramides (Cer) activate cobra venom phospholipase A_2 (PLA_2) by creating structural defects in the substrate bilayer. Recently, we reported that addition of SM to dispersed phospholipid substrates inhibits the hydrolytic activity of both secretory PLA_2 ($sPLA_2$) IIA and cytosolic PLA_2 ($cPLA_2$) [4–6]. The specific sequestration of the enzyme, apart from the glycerophospholipid substrate, on phase-separated domains of SM was said to be responsible for the inhibition [5]. This was consistent with the finding that Chol, which relieved the inhibitory effect of SM on $sPLA_2$, was shown to displace the enzyme from domains formed with SM [7,8].

Cer are hydrolytic products of SM attack by sphingomyelinases but can also arise from *de novo* synthesis. Plasma membrane microdomains (rafts) enriched in SM and Chol represent a hydrolysable pool of SM where Cer accumulate upon activation of sphingomyelinase in response to various stimuli [9]. The accumulated Cer are molecules with a small polar non-ionic headgroup thought to enhance the tight packing of membrane phospholipids by reducing the repulsion between their polar headgroups [10]. Due to the high saturation of the acyl moiety, Cer have a high transition temperature (T_m), favouring gel-phase separation at physiological temperatures. Holopainen et al. [11] showed that Cer exceeding 5 mol % formed phase-separated microdomains in bilayers of dioleoylphosphatidylcholine. It might be expected, therefore, for Cer to favour the formation of membrane defects at the boundaries surrounding the microdomains which, in turn, could activate various lipolytic enzymes. The present data show that $sPLA_2$ activity is influenced strongly by relatively low proportions of Cer in substrate liposomes consisting of mixtures of phosphatidylethanolamine (PE), phosphatidylserine (PS) and SM, in which phase separations have been described previously [12]. The substitution of SM by Cer enhanced the enzyme activity. Chol, which relieves the inhibition of SM on $sPLA_2$, acts differently in combination with Cer. Thus at Chol/Cer ratios of < 1 it activates the phospholipase but at Chol/Cer ratios of > 1 it is inhibitory.

To relate enzyme activity to the form of presentation of the substrate, small-angle synchrotron X-ray diffraction studies were performed. Inhibition or enhancement of hydrolytic activity was found to correlate with the phase structure of the dispersed substrate and indicated that structural intermediates existing at the boundaries separating lamellar (L) and hexagonal-II (H_{II}) phases were most susceptible to hydrolysis by $sPLA_2$. Analysis of the types of fatty acid released from the substrate in the presence of Cer indicated that polyunsaturated molecular species of PE rather than molecules containing a monoenoic fatty acid at the *sn*-2 position were the most likely to form these intermediate structures.

MATERIALS AND METHODS

Reagents

L-α-PE (from egg yolk), L-α-PS (from bovine brain), Chol, SM, Cer (from bovine brain SM) and sphingomyelinase (from *Staphy-*

Abbreviations used: Cer, ceramides; Chol, cholesterol; PE, phosphatidylethanolamine; PS, phosphatidylserine; SM, sphingomyelin; PLA_2, phospholipase A_2; $sPLA_2$, secretory PLA_2; $cPLA_2$, cytosolic PLA_2; H_{II}, hexagonal-II.

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lococcus aureus) were purchased from Sigma (St. Louis, MO, U.S.A.). The fatty acid composition at the *sn*-2 position of the substrates L-α-PE and L-α-PS was checked by GC after complete hydrolysis with *Crotalus* venom PLA_2 (Sigma). The fatty acid profile of PE was as follows: 18:1, $n-9$, 59%; 18:2, $n-6$, 32%; 20:4, $n-6$, 8% and 22:6, $n-3$, 1%. The profile of PS was: 18:1, $n-9$, 72%; 18:2, $n-6$, 1%, 20:4, $n-6$, 1% and 22:6, $n-3$, 26%.

Preparation of substrates

Substrate dispersions were prepared by sonication of the various lipid mixtures in buffer as described previously [4]. Typically, the lipids in the desired proportions dissolved in chloroform were dried under a stream of oxygen-free dry nitrogen, hydrated in 100 mM Tris/HCl buffer, pH 8.6, at room temperature, and sonicated twice for 2 min with a tip-probe sonicator (20 kHz, approx. 100 W; MSE, Crawley, Surrey, U.K.) until clear dispersion was obtained.

Assay of $sPLA_2$ activity

The activity of human recombinant $sPLA_2$ (3 m-units) [4] was assayed *in vitro* using 400 μM glycerophospholipid liposomal substrate suspended in 100 mM Tris/HCl (pH 8.6)/5 mM $CaCl_2$ and fatty acid-free BSA (0.1 mg/ml). The mixture (final volume, 250 μl) was incubated at 37 °C for 15 min with rotational shaking. The fatty acids released by the enzyme were extracted by a modified procedure of Dole (see [13]), and methylated using diazomethane. The fatty acid methyl esters were separated and quantified by GC-MS using a weighted internal standard of heptadecanoic methyl ester. Results from control incubations carried out in the absence of enzyme were subtracted from those of fatty acids released in the presence of $sPLA_2$.

Quantitative measurement of free fatty acids with GC-MS

The extracted free fatty acids methylated for 5 min with a freshly prepared diazomethane diethyl ether solution at room temperature were separated by GC on a polar capillary column coated with a Supelcowax-10-bound phase (inner diameter, 0.32 mm; length, 30 m; film thickness, 0.25 μm; Supelco, Bellafonte, PA, U.S.A.). This was fitted in a Hewlett-Packard (Palo Alto, CA, U.S.A.) 5890 series II gas chromatograph.

Fatty acids were detected at picomolar sensitivity by MS (Nermag 10-10C; Quad-Service, Poissy, France) in the chemical ionization mode, with ammonia (10^4 Pa) as the reagent gas. The positive quasimolecular ions $[M+18]$ were selectively monitored and time-integrated. Quantification was achieved by normalization with the internal standard of heptadecanoic methyl ester and the response factors for the various fatty acids were calculated with methyl ester calibrators.

Synchrotron X-ray diffraction

The dry lipids were dissolved in chloroform and mixed in proportions given as molar ratios. The solvent was evaporated under a stream of oxygen-free dry nitrogen and traces of solvent removed by 24 h storage at 20 °C *in vacuo*. The dry lipid films were hydrated with 100% (w/w) of the aqueous buffer, 100 mM Tris/HCl (pH 8)/10 mM $CaCl_2$. To ensure the complete mixing and hydration of samples the hydrated lipid dispersions were cooled and heated repeatedly to 60 °C under an argon atmosphere before storage for several days at room temperature. The samples were then equilibrated at 4 °C prior to X-ray examination.

Synchrotron X-ray diffraction measurements were performed at station 8.2 of Daresbury Synchrotron Radiation Laboratory (Daresbury, Cheshire, U.K.). Small-angle X-ray scattering was recorded and normalized for the incident beam intensity. The detector response (512 channels) was calibrated with a static radioactive source of ^{59}Fe. Calibration of the small-angle X-ray scattering camera (2 m length) for *d*-spacings (*x*-axis) was obtained using hydrated rat tail collagen (first order of reflection at 65 nm). Data analysis was carried out with the OTOKO program, kindly provided by M. H. J. Koch [14].

RESULTS

The effect of Cer on the susceptibility of phospholipid dispersions to hydrolysis by $sPLA_2$ was investigated by adding increasing amounts of Cer to a substrate mixture consisting of PE/PS in a molar ratio of 4:1. The results are presented in Figure 1 and show that Cer acts as a highly effective activator of $sPLA_2$, inducing a proportionate increase in the hydrolysis of *sn*-2 fatty acids by the enzyme. This was observed from the lowest molar ratio investigated (2.5% Cer relative to the total lipids). The basal activity recorded in the assay illustrated in Figure 1 was 11.53 nmol of fatty acids released/15 min. The maximum activation was observed upon the addition of 20 mol% Cer, when the total fatty acids released during the assay represented about 40% of the hydrolysable phospholipids. Higher concentrations of Cer did not induce any further increase in phospholipid hydrolysis.

Cer-triggered activation of $sPLA_2$ was accompanied by a marked alteration in the substrate specificity. Under the standard assay conditions $sPLA_2$ IIA, unlike $cPLA_2$, does not exhibit any preference for the type of fatty acid esterified to the *sn*-2 position of the glycerophospholipid substrate [15]. Analysis of the fatty acids released as a function of the proportion of Cer in the substrate dispersion (Figure 2) showed that the enhanced enzyme activity was due almost entirely to the hydrolysis of polyun-

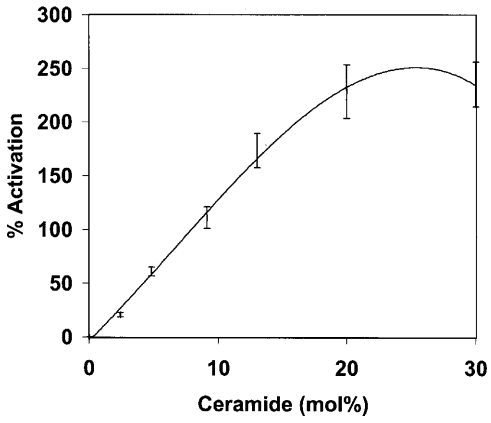

Figure 1 Stimulation of $sPLA_2$-induced fatty acid release by Cer

Substrate phospholipids (100 nmol; PE/PS, 4:1 mol/mol) were dispersed in 0.3 ml of 100 mM Tris/HCl (pH 8.6)/5 mM $CaCl_2$/0.1% (w/v) fatty acid-free BSA and incubated at 37 °C for 15 min in the presence of 0.3 m-units of human recombinant $sPLA_2$ (the activity of the enzyme preparation was calibrated by comparison with a reference activity of pancreatic PLA_2). Various quantities of Cer prepared from bovine brain were added to the substrate dispersions. The released fatty acids were extracted by a modification of Dole's procedure (see [13]), methylated by diazomethane and quantified by GC-MS. The percentage of released fatty acids was calculated relative to control incubations performed in the absence of $sPLA_2$ and detected fatty acids subtracted from the values obtained in reaction mixtures containing enzyme. Values represent means ± S.D. from six determinations.

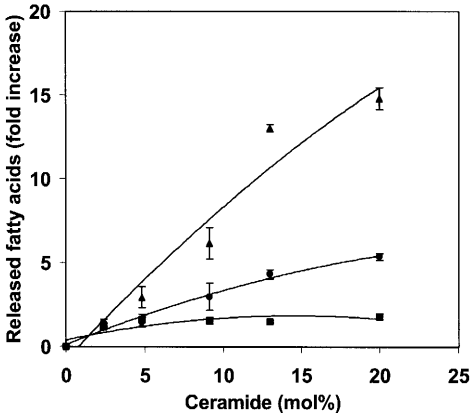

Figure 2 Fatty acid specificity of Cer-activated $sPLA_2$

The incubation conditions were the same as in Figure 1. Substrate dispersions consisting of PE/PS (4:1, mol/mol) containing 0–20 mol% Cer were incubated with $sPLA_2$ for 15 min at 37 °C. Activation of $sPLA_2$ was calculated as the activity relative to that on substrate dispersions of PE/PS (4:1, mol/mol) prepared in the absence of Cer. Values are means ± S.D. ($n = 6$). The fatty acids produced were $C_{18:1}$, $n-9$ (■), $C_{18:2}$, $n-6$ (●) and $C_{20:4}$, $n-6$ (▲).

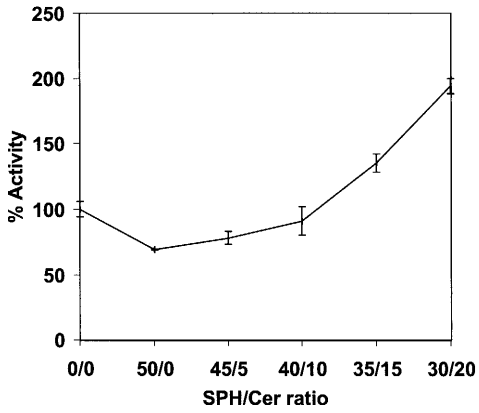

Figure 4 Modulation of $sPLA_2$ activity by variation of the SM/Cer ratio added to PE/PS (4:1) substrate

The incubation conditions were the same as described in Figure 1. The total amount of sphingolipids [SM (SPH) + Cer] was kept constant at 30 mol% of total lipids. The activity was expressed relative to the $sPLA_2$ hydrolysis of PE/PS (4:1) substrate in the absence of sphingolipid. Values are means ± S.D. ($n = 6$).

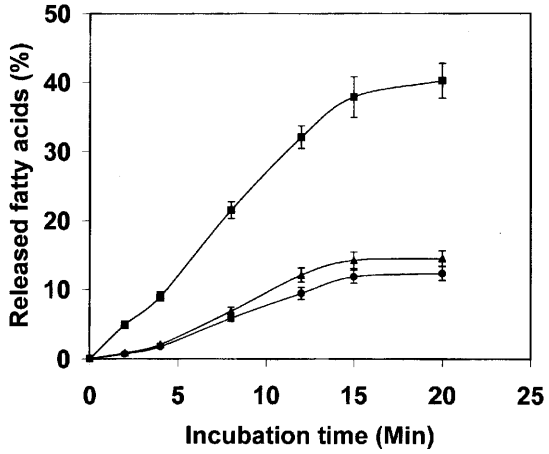

Figure 3 Time dependence of $sPLA_2$ activity in the presence of Cer

Substrate dispersions consisting of PE/PS (4:1, mol/mol) were incubated in the presence of 0.3 m-units of $sPLA_2$, as in Figure 1, for various times with 20 mol% Cer (■), 2.5 mol% Cer (▲) or without Cer (●). Values are means ± S.D. ($n = 6$).

saturated molecular species of the glycerophospholipid, particularly those containing arachidonic acid ($C_{20:4}$) acylated at the *sn*-2 position. There was virtually no enhanced hydrolysis of the monoenoic molecular species, with the amount of oleoyl ($C_{18:1}$ $n-9$)-containing species hydrolysed remaining constant with increasing amounts of Cer in the reaction mixture. The hydrolysed molecular species was almost entirely PE, since the content of arachidonic acid in brain PS is extremely low (approx. 1%). The effect of Cer was apparent from the lowest concentration investigated. The addition of 5 mol% Cer enhanced $C_{20:4}$ release by 2-fold compared with the basal activity, 5-fold was reached with 10 mol% Cer and a maximum of approx. 15-fold was reached upon the addition of 20 mol% Cer. The hydrolysis of linoleic acid ($C_{18:2}$, $n-6$)-containing molecular species was enhanced to a lesser extent than those containing arachidonic acid. As a result of the altered profile of fatty acids released by Cer-activated $sPLA_2$, the molecular species hydrolysed by the enzyme resemble more closely that released by $cPLA_2$, which is known to be highly specific to $C_{20:4}$ molecular species of PC [16,17].

A study of the time dependence of substrate hydrolysis by $sPLA_2$ in the presence of 2.5 or 20 mol% Cer showed that this remained approximately linear for 15 min (Figure 3), at which point about 40% of the substrate had been hydrolysed. The addition of 2.5 mol% Cer had a similar effect to 20 mol%, although at such low concentrations were not suspected to promote the formation of microdomains detectable by biophysical observation [11]. A plateau of activity was also observed after 15 min in substrate dispersions without Cer, but in these reactions less than 10% substrate was transformed.

Previous studies [4,5] have shown that SM, a precursor of Cer in the plasma membrane subjected to hydrolysis by sphingomyelinase, inhibits $sPLA_2$ strongly. Accordingly, the treatment of SM-containing substrate liposomes with bacterial sphingomyelinase results in relief from the inhibitory effect of SM and, moreover, leads to hyperactivation of $sPLA_2$ [4]. Since Cer are products of the sphingomyelinase pathway, the gradual substitution of SM with Cer was mimicked *in vitro* in SM-containing substrate (30 mol%; Figure 4). Whereas 30 mol% SM brought about 30% inhibition of basal $sPLA_2$ activity, the presence of Cer in proportions as low as approx. 9 mol% activated the enzyme. The enzyme activity restored by Cer in the presence of SM (Figure 4) was similar to the direct activation achieved in the absence of SM (Figure 1). This observation suggests that Cer, in proportions greater than 10 mol%, relieves the inhibition of PLA_2 due to the presence of SM and activates the enzyme by independent mechanisms. By contrast, the earlier study on the effect of SM and Chol on hydrolytic activity showed that Chol counteracted only the enzyme inhibition brought about by SM but did not stimulate hydrolytic activity above the basal level.

The addition of 10 mol% Chol to substrate spiked with 10 mol% Cer (Cer/Chol, 1:1, mol/mol) induced a weak activation (+15%) of $sPLA_2$ when compared with the major activation produced by Cer (+75%, assessed by $C_{20:4}$ release; Figure 5). Further increases in Chol (Cer/Chol increased to 1:2, 1:3 and 1/5, mol/mol) suppressed the activation effect of Cer and

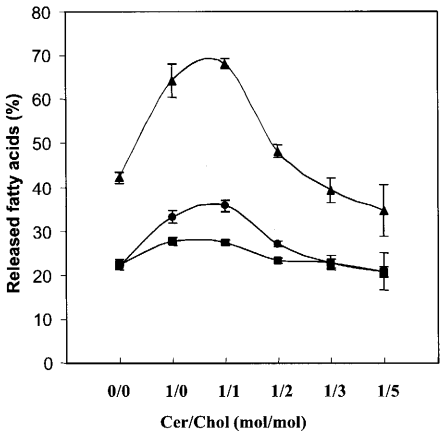

Figure 5 The effect of Chol on Cer-activated $sPLA_2$

Cer (30 mol%) were added to substrate dispersions consisting of PE/PS (4:1, mol/mol). Chol was also added in different proportions relative to Cer. Values are means ± S.D. ($n = 6$). The fatty acids released were $C_{18:1}$ (■), $C_{18:2}$ (●) and $C_{20:4}$ (▲).

restored $sPLA_2$ activity to its basal level in the absence of Cer. This result contrasted with earlier observations that Chol relieved the inhibition brought about by SM, and is discussed below in terms of the distinct influences of Cer and SM on the arrangements of the subtrate as revealed by X-ray diffraction.

In control experiments, sphingosine, the long-chain base common to sphingolipids including SM and Cer, was found to be without any regulatory effect on the hydrolytic activity of the enzyme (results not shown).

A small-angle X-ray diffraction study of the substrates used to examine the activity of $sPLA_2$ in the presence of sphingolipids was undertaken to establish a correlation between susceptibility to hydrolysis and the structure of substrate dispersion. Small-angle scattering is indicative of the long-range ordering (several nm) of lipids and accordingly is especially sensitive to the separation of phase domains that takes place in complex lipid mixtures.

A previous study by our group reported that in the temperature range 44–18 °C the binary mixture PE/PS (4:1, mol/mol) consisted of two phase-separated domains, an inverted H_{II} phase and a lamellar phase [12]. Evidence for traces of a cubic phase in the mixture was also obtained. The structural heterogeneity in the binary mixture was considerably reduced by addition of SM to the glycerophospholipid mixture. In a ternary mixture, PE/PS/SM, a lamellar phase dominated the phase diagram, consistent with the strong lamellar preference imposed by SM [12]. Unlike SM, addition of Cer to the binary phospholipid mixture PE/PS (Figure 6A) did not maintain the lamellar arrangement at temperatures higher than 28 °C. Above this temperature an inverted H_{II} phase was demonstrated by three strong reflections of periodicity $1:1/\sqrt{3}:1/2$, with a *d*-spacing of 6.64 nm (measured for 10 mol% Cer at 37 °C). The abundance of the H_{II} phase was associated with a corresponding decrease in the intensity of the lamellar reflections, indicating that the lipids of the mixture participated in a transition as a function of the temperature.

The phase behaviour observed in the presence of both SM and Cer was particularly interesting. This case was encountered during the conversion of SM by sphingomyelinase, a situation that has been shown to enhance the $sPLA_2$ susceptibility of glycerophospholipids (Figure 4). The results presented in

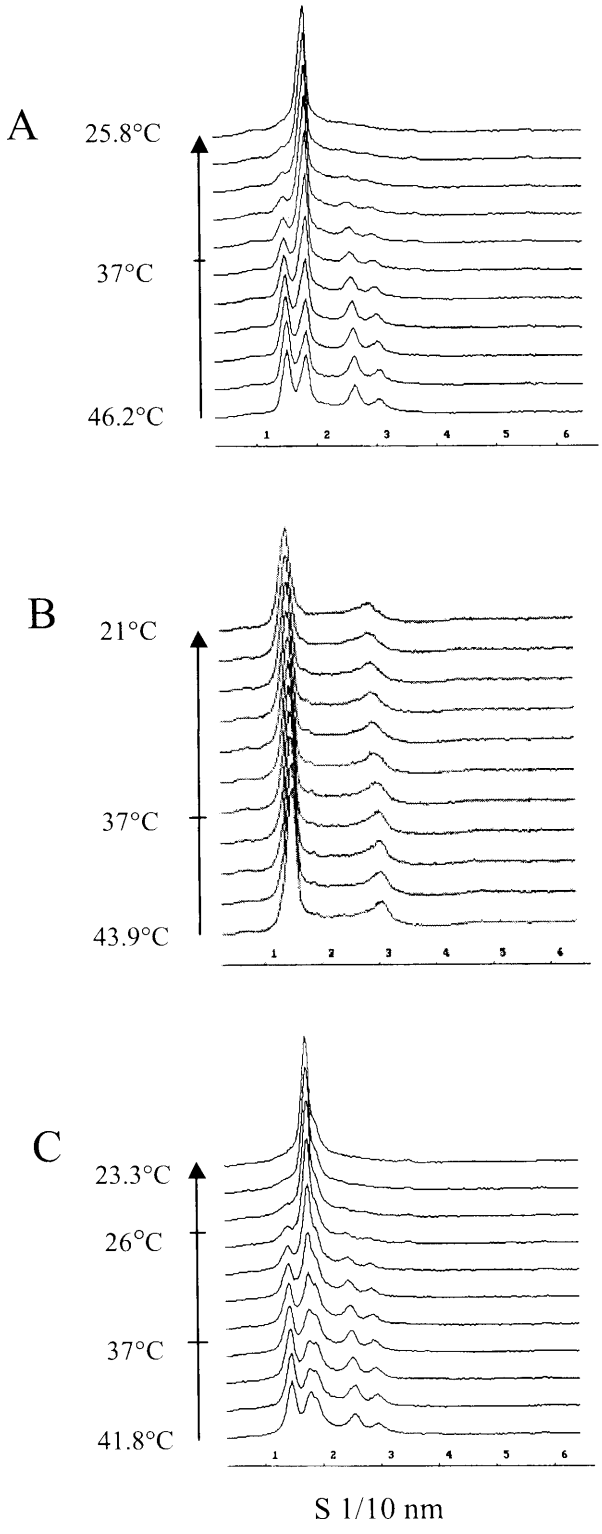

Figure 6 Small-angle X-ray diffraction patterns of substrate lipid dispersions

Small-angle X-ray scattering intensity patterns were recorded for 20 mg of hydrated lipid dispersions of (**A**) PE/PS/Cer (72:18:10, by mol), (**B**) PE/PS/Cer/SM (60:15:12.5:12.5, by mol) and (**C**) PE/PS/Cer/Chol (40:10:25:25, by mol). The dry lipid mixtures were hydrated (100%; w/w) with 100 mM Tris/HCl buffer (pH 8)/10 mM $CaCl_2$ and annealed for several days prior to X-ray examination. Scattering patterns are plotted with the *x*-axis (given in 10 nm^{-1}) during a temperature cooling scan from 45 to 20 °C at 2 °/min. Each pattern is the scattered intensity accumulated for 5 s.

Figure 6(B) show that SM in equimolar proportion with Cer (12.5 mol %) maintains a unique lamellar structure for the quaternary mixture PE/PS/SM/Cer at temperatures up to 44 °C. By contrast, in the quaternary mixture PE/PS/Cer/Chol (40/10/25/25, by mol; Figure 6C) Chol did not influence the lamellar/H_{II} phase transition observed for the ternary mixture PE/PS/Cer (Figure 6A). Nevertheless, in the presence of Chol, two lamellar arrangements with distinct d_{repeat}-spacings of 5.72 and 5.32 nm (at 37 °C) could be resolved by the long (2 m) camera length. This additional splitting of the lamellar arrangements was assigned to the separation of the liquid ordered lamellar arrangement of Cer/Chol-enriched domains.

DISCUSSION

Previous investigations by our group showed that addition of SM to glycerophospholipid dispersions serving as substrates for $sPLA_2$ inhibited the enzyme activity [4,5]. Treatment of SM-containing substrate dispersions with bacterial sphingomyelinase not only relieved the inhibition but also markedly activated $sPLA_2$ above basal levels [4,5]. This finding suggested that the Cer product of SM hydrolysis itself was responsible for the hyperactivation. Indeed, the addition of Cer, in amounts that mimic concentrations recorded after cell activation, induced a marked activation of $sPLA_2$ [18]. This activation occurred at the outset of the enzyme reaction (in less than 2 min, as shown in Figure 3) and was constant throughout the incubation.

To mimic sphingomyelinase activity we showed that the simultaneous reduction of SM and elevation of Cer in glycerophospholipid substrate dispersions caused a corresponding relief from the SM-induced inhibition of $sPLA_2$ and activation when Cer exceeded 10 mol %. The effect on phospholipase activity was consistent with the ability of Cer to form membrane microdomains when present in proportions exceeding 5 mol % [11]. The phase separation of Cer-enriched domains may result in the creation of membrane defects favourable to the activation of various lipolytic enzymes [11].

Since the proportion of Cer in the membrane required to cause activation is relatively low the mechanism of activation can best be understood if Cer associates with other lipid molecules of the substrate to facilitate phase separation. As mentioned, the presence of 2.5 or 20 mol % Cer in sPLA2 substrate liposomes did not change the time course of the reaction. It was not possible to detect the formation of microdomains in mixtures containing 2.5 mol % Cer under the experimental conditions reported earlier [11], but significant activation was observed in the present study in which a complex mixture of lipids of biological origin was used. This argues for a facilitated phase separation of Cer-enriched domains susceptible to hydrolysis by sPLA2.

The creation of Cer-rich domains in dispersions of pure PEs has been characterized by ^{31}P-NMR and IR spectroscopy and the effect has been correlated with instability of the respective lamellar and H_{II} phases of the phospholipid [19]. The effect of relatively low proportions of Cer on transition from lamellar to H_{II} phase was of particular interest. Cer destabilized the lamellar liquid-crystal phase of PE such that domains enriched in Cer underwent lamellar-to-H_{II}-phase transitions at temperatures considerably less than observed in the pure phospholipid dispersion. Transition from lamellar to H_{II} phases of the phospholipid excluded from the Cer-rich domains took place at an even lower temperature than that of the Cer-rich domains. An additional observation was revealed by the sensitive enzyme assay method used in the present study which showed that Cer domain formation takes place at an even lower concentration. This effect was observed irrespective of differences in fatty acid composition between brain and egg yolk sources of Cer. From this it may be concluded that the Cer-rich domains themselves not only perturb the stability of the bilayer but also exert an even greater perturbation on the remaining phospholipid phase. The creation of phase-separated mixed domains facilitated by the presence of low amounts of Cer is likely to explain the effect of less than 5 mol % Cer on phospholipase activity.

Predictions of the likely phase separations that would result from addition of Cer to the substrate dispersion examined in the present work must take into account two factors: (i) two phospholipid classes are represented in the dispersion, and (ii) each phospholipid class is composed of different molecular species defined by the particular fatty acids acylated at the *sn*-1 and *sn*-2 positions of the glycerol moiety. The small-angle X-ray study demonstrated clearly that addition of Cer to the phospholipid substrate caused phase co-existence of lamellar and non-lamellar phases around the temperature used to assay enzyme activity (37 °C). The question of relevance to activation of the enzyme is where the different components of the mixture partition themselves. Wide-angle X-ray scattering recorded simultaneously with the small-angle scattering (results not shown) indicated a sharp diffraction peak at about 0.41 nm at temperatures up to 46 °C for the three-component mixture PE/PS/Cer. This indicates the formation of a gel phase due to the presence of a complex including Cer. The gel phase must be formed by components contributing to the lamellar phase. The Cer-rich phase is therefore inferred to be lamellar, which coexists with a non-lamellar liquid-crystal phase. From previous wide-angle X-ray studies of binary PE/PS mixtures the PS would be expected to contribute also to the gel-phase domain [12]. We also assume that the more saturated molecular species of PE, of which the *sn*-2 oleoyl derivative represents more than 50 % in phospholipid obtained from egg yolk, will also tend to segregate into the lamellar Cer-rich domains. The tendency of Cer to undergo segregation would give rise to an aggregate that behaves as a nucleation point around which other molecules are recruited on the basis of interaction between saturated acyl chains. The phospholipid excluded from the Cer-rich lamellar phase is most probably the polyunsaturated molecular species of PE. Analysis of the fatty acids released by the enzyme acting on substrates containing Cer indicates that it is the phospholipid excluded from the Cer-rich domains that is most susceptible to enzyme attack.

Most $sPLA_2$ enzymes do not exhibit any particular preference for fatty acids under optimal assay conditions, with the exception of $sPLA_2$ from groups X and V [20,21]. However, clearly the addition of Cer to the substrate converted $sPLA_2$ IIA from a non-specific to a highly specific enzyme for $C_{20:4}$-containing phospholipids. In this respect, the profile of the fatty acid released in the presence of Cer becomes similar to $cPLA_2$, which is known to be highly specific for $C_{20:4}$ [16,17]. A direct effect of Cer as an allosteric activator of the phospholipase is unlikely and its action is more probably indirect so that polyunsaturated molecular species of phospholipid are presented to the enzyme in a way more favourable to hydrolysis. Because hydrolytic activity is stimulated by the presence of Cer, the possibility that Cer specifically inhibits hydrolysis of the more saturated molecular species in the substrate dispersion can be excluded, although it is true that the susceptibility of the monoenoic phospholipids remains low, but constant, in the presence of various proportions of Cer. It is also noteworthy that the effect of Cer on hydrolytic activity of $sPLA_2$ is only manifest if it is co-dispersed with the glycerophospholipid substrate [6]. This evidence lends support to the suggestion that Cer does not exert a direct effect on the

enzyme but rather that its action is directed towards the manner of presentation of the substrate.

Attempts to define the structure of the susceptible substrate from X-ray diffraction profiles in the present study were unsuccessful, although the binary mixture of PE/PS has already been shown to form an intermediate cubic phase at the region of coexistence between lamellar and H_{II} phases [12]. The contribution of such a cubic phase was shown to depend widely on the thermal history of the lipid sample, and the corresponding diffraction peaks were not detected in the present set of experiments [22].

Similar studies of binary mixtures of Cer and pure PE using NMR and Fourier-transform IR spectroscopy [19] were also unable to identify structures other than lamellar and H_{II} phases. One consistent feature in both studies was that the presence of Cer considerably broadened the range of temperature at which lamellar and H_{II} phases coexisted. This is in agreement with other reports that phospholipases are most active against substrates when assayed at temperatures where lamellar gel and fluid phases [23] or lamellar and H_{II} phases [24] coexist. Substrate dispersions under these conditions are dominated by boundary effects in the region that separates the two structural domains in the mixture. It is the region of the phase diagram where intermediate structures may form, such as isotropic phases [24], but the size or coherence of such domains was not sufficient to be detectable from the present low-angle X-ray scattering experiments. Indeed, cubic geometry is not expected for such an PLA_2-susceptible isotropic phase, since in a series of recent experiments abundant cubic phase of the Pn3m space grouping was shown in the PLA_2-resistant mixture PE/PS/SM (C. Wolf and K.S. Koumanov, unpublished work). It may be inferred from the data presented here that packing defects arising from the boundary regions between the Cer-rich compact lamellar domains and H_{II} structure, populated predominantly by molecular species of PE containing arachidonic acid, represent the form of presentation of the substrate that is most susceptible to hydrolysis. PE containing arachidonic acid has a large interfacial area and is likely to be 'squeezed out' from the ordered lipid domains. Monolayer studies have recently illustrated the phase separation of *sn*-2 docosahexaenoyl PE within mixed monolayers with egg yolk SM [25].

Chol strongly favours the squeezing out of polyunsaturated PE but does not influence the hexagonal/lamellar phase separation brought about by Cer (compare Figures 6A and 6C). Chol is known to form a lamellar liquid-ordered phase (Lo) with saturated sphingolipids, either SM or Cer. Lo has been shown to separate from fluid lamellar Lα phases [1] and the present X-ray diffraction evidence shows that it also phase separates from inverted hexagonal phase. The two distinct lamellar phases detected in Figure 6(C) probably reflect the separation of the Lα and Lo phases in the mixture comprising Chol. Such phase separations have previously been reported by other methods [1], but not characterized directly by X-ray diffraction. Finally, three separated phases, Lα/Lo/H_{II}, are expected in a substrate mixture containing Cer and Chol in addition to the glycerophospholipids. On the basis of enzyme assay it appears that the separation of Lo/Lα phases favoured by Chol would be less efficient for enzyme penetration than the separation lamellar/H_{II} phase favoured by Cer. In agreement with the 'structural defect' hypothesis, this means that less-disordered boundaries will occur at the Lo/Lα interface compared with at the lamellar/H_{II} interface.

The physiological relevance of these results may be put in the context of the role of Cer in signal-transduction mechanisms in cell membranes. The proportion of Cer in cell membranes can reach up to 10% of the total phospholipids [18]. The creation of susceptible substrate for $sPLA_2$ which releases arachidonic acid may augment the triggering of the eicosanoid cascade initiated by $cPLA_2$ during the inflammatory process. It is noteworthy that the level of the circulating secretory enzyme, also known as non-pancreatic inflammatory PLA_2, is markedly increased during an inflammatory reaction under the influence of interleukin-1 [26]. On the other hand, the PLA_2 product, $C_{20:4}$, is one of the activators of the neutral sphingomyelinase [27], a major enzyme in Cer production in the plasma membrane. In HL-60 and L929 cells, for instance, the activation of $cPLA_2$ and $C_{20:4}$ accumulation precede SM hydrolysis triggered by interferon-γ and tumour necrosis factor [28]. According to this observation and to our findings, a rapid deacylation of $cPLA_2$- and $sPLA_2$-susceptible molecular species could be triggered by the intermediary production of Cer by sphingomyelinase if the enzymes act synergistically.

Finally, the results support the suggestion that local alterations in the physical organization of the membrane can be highly significant for regulation of PLA_2 *in vivo* [29]. The present data illustrate the possibility that sphingomyelinase induces such a physical alteration of the structure and underscore its synergistic activity with PLA_2.

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Received 3 September 2001/7 December 2001; accepted 17 January 2002

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