Sphingolipids and cholesterol modulate membrane susceptibility to cytosolic phospholipase A_2

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Abstract Modulation of cytosolic phospholipase A_2 ($cPLA_2$) activity by sphingomyelin (SPH), ceramide (Cer), and cholesterol (Chol) was investigated in CHO-2B cells activated by the calcium ionophore A23187 and epinephrine. Chol depletion of CHO-2B cells by treatment with methyl-β-cyclodextrin (5 mM) resulted in the inhibition of the release of arachidonic acid whereas the restoration of the level by Chol-loaded cyclodextrin relieved inhibition. Conversion of CHO-2B cellular SPH to Cer by *Staphylococcus aureus* sphingomyelinase enhanced endogenous $cPLA_2$ activation as well as uptake by cells of C2- and C6-ceramide analogs. These results were confirmed in vitro with purified human recombinant $cPLA_2$ acting on a model phospholipid substrate. The enzyme activity was inhibited by SPH but reactivated by Cer as well as by Chol added to glycerophospholipid liposomal substrates containing SPH. The results of this study, which combine in situ and in vitro experimental approaches, indicate that membrane microdomains enriched in SPH and Chol play a role in the modulation of the activity of $cPLA_2$ and in arachidonic acid-derived mediator production.—Klapisz, E., J. Masliah, G. Béréziat, C. Wolf, and K. S. Koumanov. **Sphingolipids and cholesterol modulate membrane susceptibility to cytosolic phospholipase A_2. *J. Lipid Res.* 2000.** 41: **1680–1688.**

Supplementary key words arachidonic acid • ceramide • CHO cells • cholesterol • phospholipase A_2 • sphingomyelin

Ceramides (Cer) have been shown to act as second messengers in cellular processes such as cell proliferation, differentiation, and apoptosis (1–4). Cer are generated after membrane sphingomyelin (SPH) hydrolysis by cellular sphingomyelinases or by de novo synthesis, depending on their membrane localization in the cell. Caveolae or microdomains are enriched in sphingolipids and cholesterol (Chol) (5) and are assumed to be the site where a hydrolyzable pool of SPH is located (6, 7).

Hydrolysis triggered by interleukin 1 (IL-1) or nerve growth factor has been reported to reside only in the caveolae or in the caveolae-like structures (5, 8). Cer in membranes of apoptotic cells can reach up to 10% of membrane phospholipids (9) and are supposed to accumulate in such microdomains.

The close association of SPH with Chol was noted in early studies by Chapman et al. (10). The sphingosine hydroxyl group at the C-3 position along the 4,5-*trans* double bond and the amide group nitrogen act as hydrogen-bonding sites, which interact with the 3β-hydroxy group of Chol (11, 12). In addition, because SPH forms a gel phase at physiological temperatures, the close alignment of the extended acyl chain and the planar sterol ring system favor van der Waals interactions between the two molecules.

Molecular species of SPH are characterized by a high content of saturated, very long acyl chains amide-bonded to the sphingosine moiety. This results in a high transition temperature (T_m) from a gel to liquid-crystalline phase of sphingolipids, which forces them into separated gel-phase domains within the fluid bilayer matrix of glycerophospholipids at physiological temperatures (13).

Holopainen et al. (14, 15) showed that Cer, at a concentration higher than 10 mol%, when mixed with phosphatidylcholine (PC), form microdomains. These microdomains were observed with some delay after dioleoylphosphatidylcholine (DOPC)/SPH vesicles were treated with an exogenous sphingomyelinase (15). In addition, structural defects in the lipid bilayer created by Cer act as positive effectors for several enzymes taking part in the mitogen-activated protein kinase (MAPK), protein kinase C ζ (4), or ceramide-activated protein kinase pathways (16).

The presence of SPH also regulates the hydrolytic activity of enzymes. Leslie and Channon (17) showed that SPH added at 20 mol% to PC-substrate vesicles inhibited cytosolic PLA_2 ($cPLA_2$) by 60%. The authors suggested that the inhibition was due to the packing effect of SPH. In other studies (18, 19) we have shown that SPH is an inhibitor of

Abbreviations: BEL, bromoenol lactone; Bodipy, dipyrrometheneboron difluoride; Cer, ceramide; Chol, cholesterol; $cPLA_2$, cytosolic phospholipase A_2; DOPC, dioleoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; GC-MS, gas chromatography-mass spectrometry; $iPLA_2$, calcium-independent PLA_2; MAPK, mitogen-activated protein kinase; PBS, phosphate-buffered saline; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PUFA, polyunsaturated fatty acids; SPH, sphingomyelin; SMase, sphingomyelinase; $sPLA_2$, secretory phospholipase A_2.

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human recombinant type II secretory PLA_2 ($sPLA_2$) but that hydrolysis of SPH with a bacterial sphingomyelinase (*Staphylococcus aureus*) removes the inhibitory effect. Huang, Goldberg, and Zidovetzki (20) have shown that Cer from bovine brain can activate PLA_2 from cobra venom when acting on PC vesicles by creating structural defects in the lipid bilayer.

$cPLA_2$, a highly selective enzyme, plays a key role in lipid mediator biosynthesis by hydrolyzing phospholipids containing arachidonic acid at position *sn*-2 (21–23). Maximal activation of $cPLA_2$ is obtained after phosphorylation of Ser-505 via the MAPK pathway and mobilization of intracellular calcium (24–26). Calcium activation is mediated by a calcium-dependent lipid-binding domain that triggers the translocation of the enzyme, which makes the catalytic domain accessible to the phospholipid substrate (27). However, other mechanisms might be involved in $cPLA_2$ activation.

A major role for the lipid composition, which modulates the physical properties of the substrate, is expected to be the modulation of the docking of $cPLA_2$ to membranes. In the present study, to characterize modulation of $cPLA_2$ activation induced by modification of the membrane lipid composition, we carried out investigations in situ with CHO cells or in vitro with purified recombinant $cPLA_2$ acting on model bilayer membranes. The influence of SPH, Cer, and Chol was investigated in CHO cells overexpressing the G protein-coupled α_{2B}-adrenergic receptor (CHO-2B cells) after simultaneous stimulation with the calcium ionophore A23187 and epinephrine, which resulted in maximal arachidonic acid ($C_{20:4}$) release. This release was mainly mediated by $cPLA_2$, as shown by specific inhibitors (28). The similarity of the results obtained in vitro and in situ highlights a role for microdomains enriched in sphingolipids and Chol in the regulation of $cPLA_2$ in biological membranes.

MATERIALS AND METHODS

Reagents

L-α-Phosphatidylethanolamine (L-α-PE; from egg yolk), L-α-phosphatidylserine (L-α-PS; from bovine brain), L-α-phosphatidylcholine (L-α-PC; dipalmitoyl, β-oleoyl-γ-palmitoyl, β-linoleoyl-γ-palmitoyl, β-arachidonoyl-γ-palmitoyl, and β-docosahexaenoyl-γ-palmitoyl), cholesterol, sphingomyelin, ceramide (from bovine brain sphingomyelin), *N*-acetyl-D-sphingosine (C2-ceramide) and *N*-hexanoyl-D-sphingosine (C6-ceramide), methyl-β-cyclodextrin, epinephrine, and sphingomyelinase (from *S. aureus*) were purchased from Sigma (St. Louis, MO).

Bromoenol lactone (BEL) was obtained from Calbiochem (San Diego, CA) and calcium ionophore A23187 was obtained from Boehringer Mannheim France SA (Meylan, France).

Highly purified human recombinant cytosolic PLA_2 was generously provided by L. Fraisse (Groupement de Recherches de Lacq, Labège, France).

Cells

CHO cells stably expressing the rat α_{2B}-adrenergic receptor (CHO-2B cells) were established and donated by H. Gavras (Boston University, Boston, MA). Cells were cultured in MEMα medium (Sigma) without ribo- and deoxyribonucleosides, containing 10% dialyzed fetal calf serum (GIBCO, Grand Island, NY), penicillin (100 IU/ml), and streptomycin (100 μg/ml) (29).

Confluent cells (2×10^6 per dishes) were washed twice with 2 ml of phosphate-buffered saline (PBS) and preincubated for 30 min with 1 ml of serum-free medium containing 5 mM methyl-β-cyclodextrin, 5 mM methyl-β-cyclodextrin-0.6 mM Chol, or 30 mU of sphingomyelinase (from *S. aureus*). After washing with 2 ml of PBS (twice) the cells were stimulated for 15 min in 1 ml of serum-free medium containing 1 μM epinephrine and 1 μM calcium ionophore A23187.

Preparation of liposomes

Liposomes were prepared (19) by sonication of the variously composed lipid mixtures as indicated in the legends to figures. Typically, the lipids 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 (MSE, Crawley, Surrey, UK) (20 kHz, approximately 100 W) until a clear dispersion was obtained.

$cPLA_2$ activity assay

The phospholipase A_2 activity (0.5 μg of purified human recombinant $cPLA_2$) was assayed in vitro on 100 μM glycerophospholipid liposomal substrate suspended in 100 mM Tris-HCl, pH 8.6, 30% (v/v) glycerol, 1 mM $CaCl_2$, fatty acid-free bovine serum albumin (0.1 mg/ml). The mixture (final volume of 250 μl) was incubated at 37°C for 15 min with rotational shaking. The fatty acids released by the enzyme, extracted by a modified procedure of Dole (30), were methylated by diazomethane and quantified by gas chromatography-mass spectrometry (GC-MS). Control incubations in the absence of added $cPLA_2$ were used to correct for the level of fatty acids released by $cPLA_2$.

The endogenous $cPLA_2$ activity of CHO-2B cells was determined after 15 min of stimulation with the calcium ionophore A23187 (1 μM) and epinephrine (1 μM). Fatty acids released into the culture medium were extracted and quantified as described above. To eliminate the interference of calcium-independent PLA_2 activity ($iPLA_2$) experiments were carried out in the presence of 50 μM BEL.

Quantitative measurements with GC-MS

Free fatty acids. The extracted free fatty acids were methylated for 5 min with freshly prepared diazomethane; fatty acid methyl esters were gas chromatographed on a polar capillary column coated with Supelcowax-10-bound phase (i.d. 0.32 mm, length 30 m, film thickness 0.25 μm; Supelco, Bellafonte, PA) fitted in a Hewlett-Packard (Palo Alto, CA) 5890 Series II gas chromatograph. Fatty acids were detected with picomolar sensitivity by MS (Nermag 10-10C; QuadService, 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 an internal standard of heptadecanoic methyl ester and the response factors were calculated for the various fatty acid methyl ester calibrators.

Cholesterol. The cellular nonesterified Chol was assayed as described previously (31) in the form of trimethylsilyl ether prepared with *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA)-10% trimethylchlorosilane (TMCS) (CHROMPACK, Middelburg, The Netherlands). Chol trimethylsilyl ether was gas chromatographed on a medium polarity RTX-65 capillary column (i.d. 0.32 mm; length 30 m; film thickness 0.25 μm).

The positive fragment ion at $m/z = 329$ produced in the electron impact mode at 70 eV is used to quantify after normalization with an internal standard (epicoprostanol). Calibration was achieved by a weighted standard for Chol.

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RESULTS

Modulation of $cPLA_2$ activation in CHO-2B cells

Effect of cholesterol. CHO-2B cells overexpress the rat α_{2B}-adrenergic receptor (29) and therefore maximally release [^{3}H]arachidonic acid ($C_{20:4}$) in response to A23187 and epinephrine (28). We found that these cells under stimulation released mainly $C_{20:4}$ and linoleic acid ($C_{18:2}$). The release of oleic acid ($C_{18:1}$), despite its high content in membrane phospholipids, is not increased under stimulation. Incubation of cells with BEL, a specific inhibitor of calcium-independent PLA_2 ($iPLA_2$), has no effect on the release of $C_{20:4}$ under basal and stimulated conditions (**Fig. 1A**). In contrast, BEL decreases the release of $C_{18:2}$ and, to a lesser extent, of $C_{18:1}$. These results are in accordance with the involvement of $cPLA_2$ in $C_{20:4}$ release by CHO-2B cells (28). The effect of Chol depletion on $cPLA_2$ activation in CHO-2B cells was studied after methyl-β-cyclodextrin extraction. CHO-2B cells pretreated for 30 min with 5 mM methyl-β-cyclodextrin (Fig. 1B) showed a strong decrease in the Chol level (by about 50% as compared with the control). Chol could be restored to 85% of the initial level after subsequent incubation in the presence of the cyclodextrin/cholesterol complex (5 mM/0.6 mM).

Treatment of CHO-2B cells with bacterial sphingomyelinase (Smase) also provoked a slight (20%) decrease in the nonesterified membrane Chol content. This decrease is probably due to a partial redistribution of Chol within the intracellular membranes, which increases esterification in the endoplasmic reticulum after extensive SPH hydrolysis by SMase (32, 33).

$cPLA_2$ activation was significantly increased by an alteration in the cell cholesterol content (Fig. 1C). A 30% decrease in total fatty acid release accompanied Chol depletion and the activation of $cPLA_2$ could be reversed when the Chol level was restored. The highest decrease was obtained for the release of $C_{18:2}$ (about 50% compared with the nonpretreated cells). The release of $C_{20:4}$ was reduced by 20%.

A sharp increase (200% for $C_{20:4}$) in the release of fatty acids was observed after SMase treatment while the Chol level decreased only slightly (−20%) (Fig. 1B). This increase has been assigned to SPH hydrolysis, which was accompanied by an accumulation of Cer, the activating properties of which are shown below.

Effect of SPH. The effect of SMase pretreatment on fatty acid release and on the SPH content is shown in **Fig. 2**. During the first 10 min up to 40% of the cell SPH was hydrolyzed. Hydrolysis continued for 40 min at a slower rate (52% SPH was hydrolyzed). The decrease in the SPH level of the cell paralleled a strong increase in the release of unsaturated fatty acids, which reached after 40 min 4-, 2-, and 2-fold for $C_{18:2}$, $C_{20:4}$, and $C_{18:1}$, respectively. At its maximum the hydrolysis of SPH was accompanied by a 60 and 37% release of $C_{20:4}$ and $C_{18:2}$, respectively, from the *sn*-2 position of cell phospholipids. These results show an inverse correlation between the SPH content of the cells and activation of $cPLA_2$ by agonists.

Effect of cell-permeable C2- and C6-ceramide. To mimic SPH hydrolysis and ceramide formation CHO-2B cells were incubated with cell-permeable C2- or C6-ceramide (20 μM) for 30 min. **Table 1** shows that both ceramides increased the release of polyunsaturated fatty acids when pretreated cells were stimulated with A23187-epinephrine. C6-ceramide induced a 2-fold higher release of $C_{20:4}$ than the C2 analog. Remarkably, the release of $C_{20:4}$

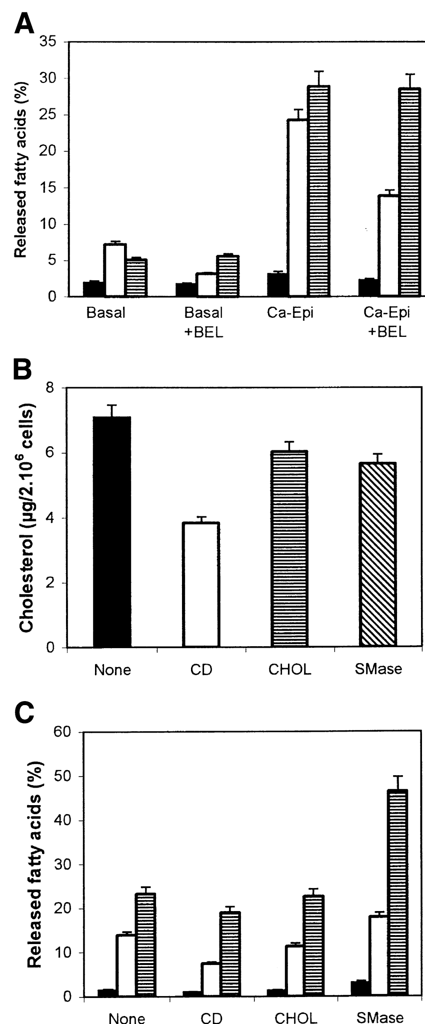

Fig. 1. Effect of cholesterol depletion of CHO-2B cells on $cPLA_2$ activation. (A) Comparison of the basal and A23187 and epinephrine (Ca-Epi)-stimulated $cPLA_2$ activation of CHO-2B cells in the presence and absence of 50 μM BEL: $C_{18:1}$ (filled columns), $C_{18:2}$ (open columns) and $C_{20:4}$ (striped columns) fatty acids released from the *sn*-2 position. Values represent means ± SD of six independent determinations. (B) CHO-2B cells were treated for 30 min with 5 mM methyl-β-cyclodextrin (CD) (open column), methyl-β-cyclodextrin/cholesterol (CHOL) (5 mM/0.6 mM) (striped column), or *Staphylococcus aureus* sphingomyelinase (SMase, 30 mU/ml) (hatched column). Nontreated cells (filled column) served as a control. The cholesterol level was determined as described in Materials and Methods by GC-MS. (C) Released $C_{18:1}$ (filled columns), $C_{18:2}$ (open columns), and $C_{20:4}$ (striped columns) fatty acids from the *sn*-2 position of CHO-2B cell phospholipids (2.4×10^6 cells) before and after pretreatment with CD, CHOL, and SMase and stimulation for 15 min with A23187 (1μM) and epinephrine (1 μM). The released fatty acids were extracted and assayed by GC-MS as described.

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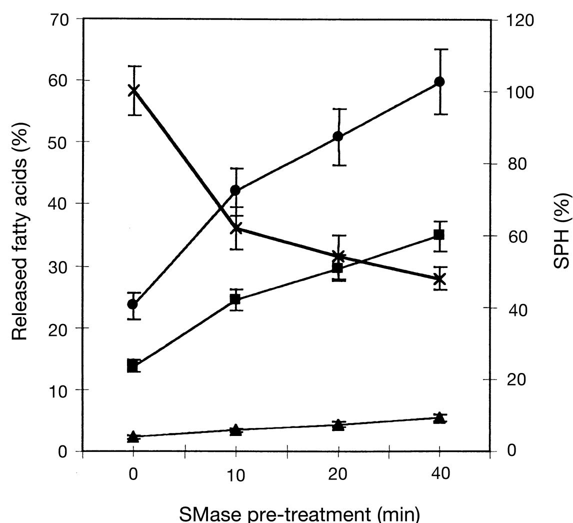

Fig. 2. The cell SPH level correlates with fatty acid release. CHO-2B (2.4×10^6) cells were treated for 10, 20, and 40 min with *S. aureus* sphingomyelinase (30 mU/ml), washed twice with PBS, and stimulated for 15 min with A23187 and epinephrine. The SPH level (×) and fatty acids released from the *sn*-2 position of cell phospholipids were assayed as described in Materials and Methods. Values represent means ± SD of six independent determinations. $C_{20:4}$ (solid circles); $C_{18:2}$ (solid squares); $C_{18:1}$ (solid triangles).

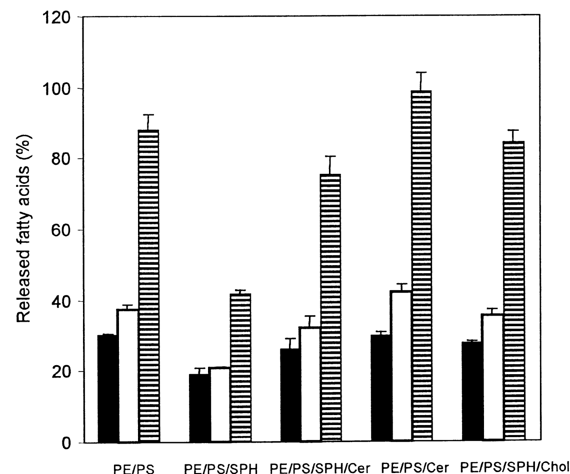

Fig. 3. Effect of SPH, Cer, and Chol on recombinant $cPLA_2$ acting on PE/PS liposomes. Recombinant $cPLA_2$ was assayed with PE/PS (4/1 mol:mol), PE/PS/SPH (4:1:2), PE/PS/SPH/Cer (4:1:1:1), PE/PS/Cer (4:1:2), and PE/PS/SPH/Chol (4:1:2:2) liposomes. $cPLA_2$ (0.5 μg) was incubated with 100 μM glycerophospholipid substrate liposomes in Tris-HCl (pH 8)–1 mM $CaCl_2$–BSA (2 mg/ml)–30% glycerol for 15 min at 37°C. Determination of released fatty acids ($C_{18:1}$, filled columns; $C_{18:2}$, open columns; $C_{20:4}$, striped columns) was performed by GC-MS as described in Materials and Methods. Values represent means ± SD of three determinations of two independent experiments.

was 2-fold higher than the release of $C_{18:2}$ and 18-fold higher than that of $C_{18:1}$.

Effects of SPH, Cer, and Chol observed in activated CHO-2B cells could be reproduced with purified recombinant $cPLA_2$ acting on model lipid mixtures

Three different glycerophospholipid mixtures supplemented with SPH, Cer, or Chol were studied.

PE/PS (4/1 molar ratio). Aminoglycerophospholipids (which contain at the *sn*-2 position about 60% $C_{18:1}$, 25% $C_{18:2}$, and 6% $C_{20:4}$) are substrates for human recombinant $cPLA_2$, which is presumed to act at the cytoplasmic face of the subcellular membranes. As shown in **Fig. 3**, purified recombinant $cPLA_2$ (0.5 μg) released 30, 37, and 87% of fatty acids present at the *sn*-2 position (for $C_{18:1}$, $C_{18:2}$, and $C_{20:4}$, respectively). Addition of 30 mol% SPH to PE/PS inhibited $cPLA_2$ activity, especially the release of $C_{20:4}$ (50% inhibition), while Cer restored $cPLA_2$ activity.

TABLE 1. Effect of C2- and C6-ceramide on release of unsaturated fatty acids by CHO-2B cells

Fatty Acids	None	C2-Ceramide	C6-Ceramide
$C_{18:1}$	2.27 ± 0.23	3.08 ± 0.47	2.83 ± 0.32
$C_{18:2}$	13.83 ± 0.83	16.45 ± 1.27	26.29 ± 1.65
$C_{20:4}$	28.53 ± 2.57	38.89 ± 2.19	51.52 ± 3.86

Confluent CHO-2B cells washed with PBS were preincubated for 30 min with serum-free medium containing 20 μM C2- or C6-ceramide and 50 μM BEL and then stimulated with the calcium ionophore A23187 (1 μM) and epinephrine (1 μM) for 15 min. Released fatty acids were quantified as described in Materials and Methods. The results are expressed as percent released fatty acids from the *sn*-2 position of cell substrate phospholipids. Values represent means ± SD for six determinations of two independent experiments.

The addition of Chol to PE/PS/SPH vesicles at an SPH/Chol 1:1 ratio also restored completely the $cPLA_2$ activity inhibited by SPH.

PE/PS/PC vesicles. Hydrolysis by recombinant $cPLA_2$ of liposomes composed of PE/PS/PC (4:2:4 mol/mol) as substrate, which mimicks the internal leaflet composition of the cell plasma membrane, is reported in **Fig. 4**. $cPLA_2$ released about 30% of the $C_{20:4}$ at the *sn*-2 position in 15 min. A significant quantity of $C_{18:2}$ (18%) was released but much less of $C_{18:1}$ (5%), the major fatty acid (65%) at the *sn*-2 position of this substrate. Compared with PE/PS, this substrate was less susceptible to hydrolysis by $cPLA_2$. The incorporation of 30% SPH again caused a sharp decrease in the release of fatty acids. Replacement of part of the SPH with 5 and 10 mol% of Cer, which mimicks SMase hydrolysis (the overall level of sphingolipids was kept at 30 mol%), led to reactivation of $cPLA_2$. Above 10 mol% Cer, the rate exceeded the activity in the absence of SPH.

Chol added to the substrate containing SPH (SPH/Chol 1:1) relieved completely the inhibitory effect of SPH and a slight reactivation of the enzyme occurred.

PC vesicles. Vesicles (small unilamellar vesicles, SUV) containing the same quantities of five different molecular species of PC (dipalmitoyl-, 1-palmitoyl-2-oleoyl-, 1-palmitoyl-2-linoleoyl-, 1-palmitoyl-2-arachidonoyl-, and 1-palmitoyl-2-docosahexaenoyl-PC) were prepared to investigate the effect of SPH and Cer on the fatty acid specificity and activity of $cPLA_2$. Recombinant $cPLA_2$ (0.5 μg) released 5, 7, 14, and 4% of $C_{18:1}$, $C_{18:2}$, $C_{20:4}$, and $C_{22:6}$, respectively, from the *sn*-2 position after incubation for 15 min (**Fig. 5A**). Compared with the previous complex substrates (PE/PS

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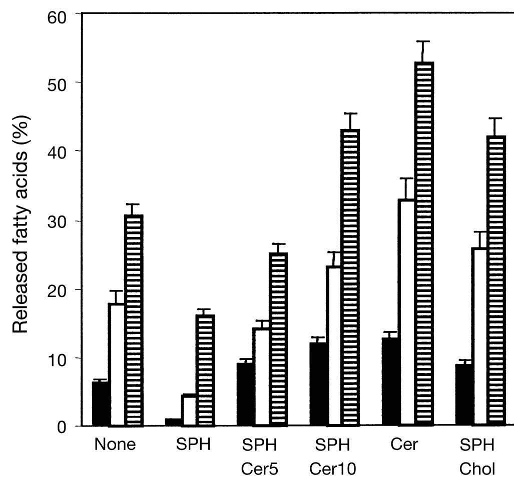


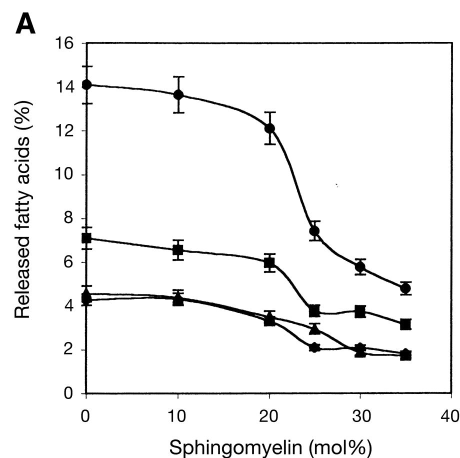
Fig. 4. Effect of SPH, Cer, and Chol on recombinant $cPLA_2$ acting on PE/PS/PC. PE/PS/PC (4:2:4 mol:mol) liposomes were supplemented with SPH (30 mol%); SPH (25 mol%) and Cer (5 mol%) (SPH/Cer5); SPH (20 mol%) and Cer (10 mol%) (SPH/Cer10); Cer (30 mol%); and SPH (30 mol%) and Chol (30 mol%) (SPH/Chol) and the activity of $cPLA_2$ (0.5 μg) was assayed as described in Fig. 3. Valus represent means ± SD of three determinations of two independent experiments. $C_{18:1}$, filled columns; $C_{18:2}$, open columns; $C_{20:4}$, striped columns.

and PE/PS/PC), PC vesicles were more resistant to $cPLA_2$ hydrolysis. SPH incorporation (between 20 and 35 mol%) resulted in a sharp inhibition of the enzyme activity. This inhibition was particularly strong for the hydrolysis of 1-palmitoyl-2-arachidonoyl- and 1-palmitoyl-2-linoleoyl-PC, which indicated that the inhibition by SPH shifted the specificity of $cPLA_2$ to less saturated molecular species.

To study the nature of the inhibitory effect of SPH on $cPLA_2$ activity, two incubation procedures were used: *i*) incubation of $cPLA_2$ with mixed PC/SPH (70:30 mol/mol) vesicles, and *ii*) preincubation of $cPLA_2$ with the SPH vesicles for 3 min before addition of the substrate PC vesicles (PC vesicles/SPH vesicles, 70:30). In the later case the physical state of PC substrate vesicles could not be changed by the presence of SPH added as separate vesicles.

SPH (30 mol%) incorporated into the PC substrate decreased by 70% the release of $C_{20:4}$ and $C_{18:2}$ (Fig. 5B). A similar SPH inhibitory effect was observed when $cPLA_2$ was successively preincubated with SPH vesicles and then added to substrate PC vesicles. The release of polyunsaturated fatty acid (PUFA) was also decreased by half. These observations showed clearly the specific $cPLA_2$-SPH interaction that led to enzyme sequestration and resulted in the decrease in $cPLA_2$ fatty acid release. This was confirmed by the observation that preincubation of $cPLA_2$ with SPH/Chol vesicles (1:1 mol/mol) had no inhibitory effect on the $cPLA_2$ activity (Fig. 5B), which is in agreement with the experiments conducted with mixed substrates. Apparently, the Chol association with SPH prevented enzyme-SPH binding, which led to inhibition.

Experiments carried out after substitution of SPH with Cer gave quite different results. Cer added to PC liposomes caused activation of substrate hydrolysis (**Fig. 6**). Cer at 30 mol% resulted in a 50% increase in $C_{20:4}$ release but only a small change in the release of other fatty acids. Surprisingly, preincubation of $cPLA_2$ with Cer before addition of the PC substrate liposomes resulted in a sharp inhibition of the $cPLA_2$ activity. The inhibition affected the hydrolysis of all the PC molecular species. The release of $C_{20:4}$ was decreased by about 60% as compared with the control. This inhibition was relieved by Chol when added to Cer (mol ratio 1:1) vesicles preincubated with $cPLA_2$. There was a striking similarity to the effect of SPH or SPH/Chol vesicles preincubated separately with $cPLA_2$.



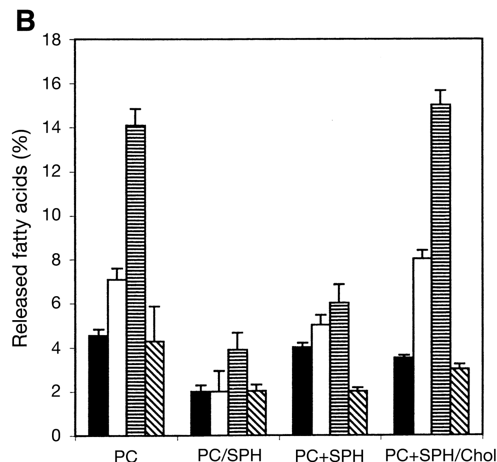


Fig. 5. Effect of SPH on recombinant cPLA2 acting on PC liposomes. (A) PC liposomes were enriched with SPH (from 10 to 35 mol%) and the $cPLA_2$ activity was assayed as described in Fig. 1. Released fatty acids (percentage of their *sn*-2 position content) were quantified by GC-MS. Values represent means ± SD of six independent determinations. $C_{18:1}$ (solid diamonds); $C_{18:2}$ (solid squares); $C_{20:4}$ (solid circles); $C_{22:6}$ (solid triangles). (B) The $cPLA_2$ activity was assayed on PC liposomes, PC/SPH (70/30 mol:mol) liposomes, or $cPLA_2$ was preincubated with SPH (PC + SPH) or SPH/Chol (PC + SPH/Chol) liposomes and then added to incubation medium containing PC liposomes. Determination and quantification of released fatty acids were performed as described in Fig. 3. $C_{18:1}$, filled columns; $C_{18:2}$, open columns; $C_{20:4}$, striped columns; $C_{22:6}$, hatched columns. Values represent means ± SD of three determinations in two independent experiments.

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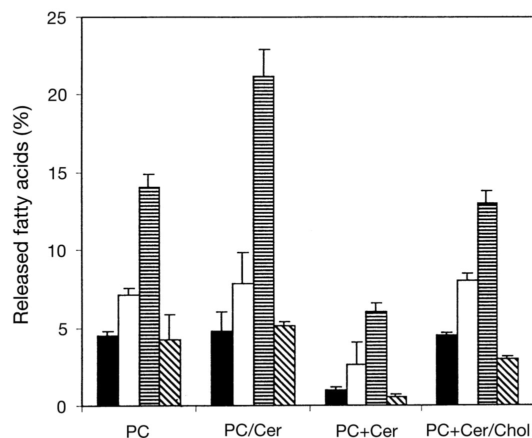

Fig. 6. Effect of Cer on recombinant $cPLA_2$ acting on PC liposomes. The $cPLA_2$ activity was assayed on PC liposomes, PC/Cer (70/30 mol:mol) liposomes or $cPLA_2$ was preincubated with a Cer (PC + Cer) or Cer/Chol (PC + Cer/Chol) suspension and then added to incubation medium containing PC liposomes. Determination and quantification of released fatty acids were performed as described in Fig. 3. Valus represent means ± SD of three determinations in two independent experiments. $C_{18:1}$, filled columns; $C_{18:2}$, open columns; $C_{20:4}$, striped columns; $C_{22:6}$, hatched columns.

DISCUSSION

The mechanism by which $cPLA_2$, a key enzyme in the inflammatory cascade of arachidonic acid, binds to its phospholipid substrate has been reported (34–37). It is widely accepted that $cPLA_2$ hydrolyzes preferentially aggregated phospholipids within the membrane/water interface rather than a monomeric form (27). The critical role of calcium in membrane penetration of $cPLA_2$ after binding has been demonstrated (34–37). The remarkable arachidonate specificity of $cPLA_2$ has also been tentatively assigned to the fully open active site in the presence of calcium (34). $cPLA_2$ binds tightly to vesicles containing anionic phospholipids such as PS in the present mixtures (38, 39), which results in the continuous activation of the enzyme on the interface without departure from the vesicle surface ("scooting mode"). In addition, the multiple phase defects that are present in the heterogenous substrate PE/PS should explain its high activity. X-ray diffraction (data not shown) reveals that at physiological temperature and in the presence of 10 mM calcium the coexistence of two phase-separated domains is observed. The lamellar domain is assumed to be PS enriched and the inverted hexagonal phase H_{II} is composed of PE. Dessen et al. (37) reported that "$cPLA_2$ can change from a soluble, closed conformation to an open, hydrophobic form stabilized by membrane binding" to emphasize the so-called interfacial activation in the presence of calcium, which indicates the importance of the physicochemical properties of the membrane, which acts as a natural substrate for PLA_2, and of the appropriate enzyme structure for activity in situ.

In contrast, little information is available concerning the influence of nonsubstrate membrane components on the hydrolysis of phospholipids by $cPLA_2$. Leslie and Channon (17) showed that the incorporation of SPH into PC vesicles inhibited strongly $cPLA_2$. They suggested that penetration of the enzyme into the lipid bilayer was hindered because of the packing effect of SPH. On the other hand the activation of cobra venom PLA_2 acting on dipalmitoylphosphatidylcholine (DPPC) liposomes enriched with Cer was reported by Huang, Goldberg, and Zidovetzki (20). These results designate sphingolipids (SPH and Cer) and Chol as a pivot in the regulation of the $cPLA_2$ activity in natural membranes.

SPH and Chol are present in almost all subcellular membranes. The balance of their opposite regulatory effects was observed in this study in a whole cell system using CHO-2B cells, thus demonstrating its physiological relevance. Chol depletion by treatment of CHO-2B cells with methyl-β-cyclodextrin caused a moderate but physiologically meaningful decrease in the release of arachidonic acid by $cPLA_2$. We suggest that Chol reduction provoked a relative enrichment of membranes in phase-separated domains of SPH; this would inhibit $cPLA_2$-mediated release of lipid derivatives. Restoration of the Chol level in depleted cells reversed the SPH inhibitory effect (Fig. 1B), indicating that this modulation could be relevant to the release of $C_{20:4}$ under pathologically high Chol levels.

The inhibitory effect of phase-separated SPH was confirmed in vitro with purified $cPLA_2$. The incorporation of 30 mol% SPH into glycerophospholipid liposomes (Figs. 3, 4, and 5A) induced a sharp inhibition of the release of all fatty acids. The inhibition is consistent with the occurrence of SPH domains whereupon the enzyme is sequestered apart from the fluid glycerophospholipid substrate. When SPH is partially intermixed with the glycerophospholipids we observed (X-ray data not shown) that the lamellar/hexagonal separated phases formed initially by PE/PS had been transformed to a single lamellar system composed of PE/PS/SPH. Within this lamellar system two distinct periodicities can be detected below the transition temperature of SPH (T_c >40°C). The long periodicity has been assigned to pure SPH in the gel state and accordingly it was erased after the addition of Chol (data not shown). This is to say that the suppression by Chol of phase-separated gel domains composed of SPH is also the cause of the suppression of the inhibitory influence of SPH on the enzyme activity. Alternatively, it was suggested (19) that hydrogen bonding of the enzyme to the SPH interface acceptor group competed with the hydroxyl group of Chol. It is noteworthy that the level of SPH can reach 80% of total phospholipids of red blood cell membranes of particular animal species (goat, sheep) in contrast to most cell plasma membranes, where it is about 20 mol%. This might explain why the high circulating PLA_2 activity observed in these animals does not hydrolyze cell membranes (40). The mechanism of "inactive docking" onto SPH, which inhibits $cPLA_2$, has been demonstrated in this study with vesicles added to the incubation medium separately from the substrate vesicles. These observations suggest that the inhibition exerted by SPH was due to a specific effect rather than to a packing effect. Indeed, the packing effects for SPH or D-DPPC are similar (17, 19)

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and Cer was known to increase further the packing effect of SPH (15). In spite of a packing effect shared by different derivatives, the present study supports the first demonstration of a specific inhibitory effect only for SPH. We assume that SPH, because of its particular structure, binds the enzyme by hydrogen donor/acceptor groups of the interfacial area in addition to the hydrophobic interactions of particular amino acid sequences with the lipid core. This binding to gel-phase separated areas removes the enzyme from the hydrolysis-susceptible glycerophospholipids. Alternatively, when the liquid ordered domain boundaries are extended by the fractionation of gel SPH domains by Chol, the activity of the enzyme into the boundary defects should be favored. Similar effects of SPH and Chol were described previously for the different type II $sPLA_2$ (19), which suggests that the nonsubstrate membrane lipids modify similarly the activities of numerous PLA_2 types.

The reversal of the inhibition of the $cPLA_2$ activation is achieved by hydrolysis of SPH to Cer in CHO-2B cells treated with *S. aureus* sphingomyelinase. The arachidonic acid release parallels the sharp increase in Cer after 10 min of exogenous SMase pretreatment (Fig. 2). Whether only the SPH of the outer membrane leaflet was hydrolyzed has not been examined but it can be assumed that Cer were rapidly redistributed. There are no data concerning the flip-flop rate of Cer in membranes, but the flip-flop rate for a rather similar molecule, diacylglycerol, has been estimated to be in the range of milliseconds (41). Holopainen, Subramanian, and Kinnuinen (15) showed that the formation of Cer in SMase-treated DOPC-SPH liposomes was followed by their rapid (<3 min) redistribution between the outer (formation site) and inner liposome leaflets. The authors (15) also demonstrated by a fluorescence study that the formation of Cer from dipyrrometheneboron difluoride (Bodipy)-SPH was followed by their rapid internalization in giant liposomes formed by l-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine-SPH 16:0-Bodipy SPH. It is also known that Cer formed in the plasma membrane could by internalized by endocytosis (42). Alternatively, the suppression of transbilayer hydrocarbon interdigitation participating in the phase separation of SPH has also been evoked, after sphingomyelinase attack of the outer leaflet (43), to explain the fractionation of extended SPH domains.

The sharp enhancement of the activity suggests that both the hydrolysis of SPH and the accumulation of Cer are responsible for the activation of $cPLA_2$. The activating effect of Cer on $cPLA_2$ was demonstrated in vitro in liposomes containing a low amount of Cer (5 or 10 mol%) (Fig. 4). We would like to stress that these concentrations probably do not refer to those observed in the overall membrane but to restricted domains where enrichment in sphingolipids and Chol is expected.

Two mechanisms, based on data in the literature, by which Cer influences $cPLA_2$ activity can be proposed: *i*) Cer accumulation in the membranes may activate binding of the enzyme. The alkyl chain protrusion model proposed by Krönke (44) may be considered if one takes into account the exposed hydrophobic pocket of the enzyme; *ii*) Cer could interact with the enzyme and modify directly its activity. Cer have been shown to induce phase defects in DPPC bilayers (20). Holopainen, Lehtonen, and Kinnuinen (14) demonstrated the occurrence of ceramide-enriched microdomains in gel-state or fluid bilayers of DMPC. They showed that the enzymatic generation of Cer from SPH induced microdomains in DOPC vesicles (15) starting from 10 mol% Cer. To investigate the possibility that Cer rearrangement occurs in the formation of microdomain Cer were added in the present study to the enzyme assay using two protocols: *i*) Mixed liposomes were prepared just before the assay, a condition favorable to obtain the formation of Cer microdomains within the PC substrate at 37°C. Under these conditions an increase in the recombinant $cPLA_2$ activity was observed with only 5% Cer (Fig. 4). Accordingly, Holopainen, Subramanian, and Kinnuinen (15) reported that Cer below 5 mol% disperse into POPC membranes, while above 5 mol% Cer they separate into microdomains; *ii*) Cer were preincubated with $cPLA_2$ and then added to the assay medium containing substrate. Under such conditions we observed a completely different effect compared with codispersion of Cer/substrate. Cer preincubated with $cPLA_2$ inhibited the enzyme activity. The same inhibitory effect was observed with SPH and Cer when vesicles were preincubated with $cPLA_2$. These sphingolipids were able to bind $cPLA_2$, separating the enzyme from its substrate, which results in a decrease in hydrolysis. In both cases, Chol, which shows a preference for binding to sphingolipids, displaces the $cPLA_2$, which relieves the inhibition exerted by SPH or Cer. Therefore, the differences between the effects of SPH and Cer on $cPLA_2$ activity were observed only when these sphingolipids were intermixed with substrate glycerophospholipids and thus were in a position to influence the phase behavior of the substrate. Our X-ray data indicate that whereas SPH imposes a strong lamellar preference for the overall substrate, Cer reinforces the hexagonal/lamellar separation of the substrate PE/PS (data not shown).

Removal of the SPH head group (phosphocholine) by SMase induces an imbalance of the hydrophobic moiety relative to the polar head group in Cer. We observed in the substrate mixture as previously reported (44) a favored transition to a hexagonal arrangement. The heterogeneity of the substrate and the multiple boundary defects expected are probably the cause of the increased enzyme activity in the presence of Cer.

The participation of SPH, Cer, and Chol in the modulation of the $cPLA_2$ activity highlights the role of rafts and/or caveolae in PLA_2 regulation. It is largely accepted (45) that these membrane domains are composed mainly of sphingolipids and Chol organized in a liquid-ordered phase (l_o) in which many proteins are anchored. A chimeric $cPLA_2$ constitutively anchored to the plasma membrane by the 10 N-terminal amino acids of the protein kinase Lck was shown to interact with caveolin (46).

We now assume that a self-regulated cycle of SPH and phospholipid hydrolysis could occur at the time of cell activation in caveolae (8). On the other hand, it was shown that PUFA mediate the stimulation of SPH hydrolysis in

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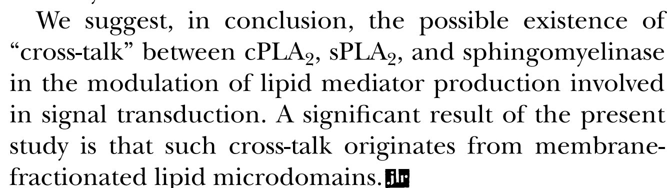

HL-60 cells or in neutrophils (47, 48). Murakami et al. (49) showed that secretory PLA$_2$-IIA was present in caveolae of cytokine-stimulated cells.

We suggest, in conclusion, the possible existence of "cross-talk" between cPLA$_2$, sPLA$_2$, and sphingomyelinase in the modulation of lipid mediator production involved in signal transduction. A significant result of the present study is that such cross-talk originates from membrane-fractionated lipid microdomains.

Elsa Klapisz is a recipient of a fellowship from CNRS-Elf and the Fondation pour la Recherche Médicale. We are grateful to Corinne Brachet-Ducos for technical assistance.

Manuscript received 21 December 1999 and in revised form 18 April 2000.

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