Modulation of human type II secretory phospholipase A_2 by sphingomyelin and annexin VI

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Conjectural results have been reported on the capacity of inflammatory secreted phospholipase A_2 ($sPLA_2$) to hydrolyse mammalian membrane phospholipids. Development of an assay based on the release of non-esterified fatty acids by the enzyme acting on the organized phospholipid mixture constituting the membrane matrix has led to the identification of two prominent effectors, sphingomyelin (SPH) and annexin. Recombinant human type II $sPLA_2$ hydrolyses red-cell membrane phospholipids with a marked preference for the inner leaflet. This preference is apparently related to the high content of SPH in the outer leaflet, which inhibits $sPLA_2$. This inhibition by SPH is specific for $sPLA_2$. Cholesterol counteracts the inhibition of $sPLA_2$ by SPH, suggesting that the SPH-to-cholesterol ratio accounts *in vivo* for the variable susceptibility of cell membranes to $sPLA_2$. Different effects were observed of the presence of the non-hydrolysable D-α-dipalmitoyl phosphatidylcholine (D-DPPC), which renders the membranes rigid but does not inhibit $sPLA_2$. Annexin VI was shown, along with other annexins, to inhibit $sPLA_2$ activity by sequestering the phospholipid substrate. The present study has provided the first evidence that annexin VI, in concentrations that inhibit hydrolysis of purified phospholipid substrates, stimulated the hydrolysis of membrane phospholipids by $sPLA_2$. The activation requires the presence of membrane proteins. The effect is specific for type II $sPLA_2$ and is not reproducible with type I PLA_2. The activation by annexin VI of $sPLA_2$ acting on red cell membranes results in the preferential release of polyunsaturated fatty acids. It suggests that type II $sPLA_2$, in conjunction with annexin VI, might be involved in the final step of endocytosis and/or exocytosis providing the free polyunsaturated fatty acids acting synergistically to cause membrane fusion.

INTRODUCTION

Inflammatory type II secreted phospholipase A_2 ($sPLA_2$) is among a group of evolutionarily related enzymes [1]. They have in common a relatively small size (14 kDa) and a millimolar Ca^{2+} ion requirement. Of the four different $sPLA_2$ species cloned in mammals [2,3], two are well characterized. Type I $sPLA_2$ is referred to as the pancreatic type [1]; type II is secreted by a number of cells in which its expression is either constitutive, e.g. platelets [2] and neutrophils [4], or induced by inflammatory cytokines, e.g. renal mesangial cells [5], chondrocytes [6], vascular smooth-muscle cells [7] or astrocytes [8]. Cloned from arthritic cells [2], this enzyme is highly homologous to enzymes from Crotalidae or Viperidae [1]. Large amounts of type II $sPLA_2$ are found in plasma after various inflammatory [9] diseases.

The actual role of type II $sPLA_2$ in the onset of inflammation is considered controversial with regard to its apparent lack of specificity and modulation with the usual assay methods. Whether a high level of the enzyme is a consequence or a cause of the inflammation has not been established. For example, the role of $sPLA_2$ in the aetiology of arthritis had been inferred from experiments in which type II $sPLA_2$ was injected intra-articularly [10], but whether this is responsible for the abundant and specific release of arachidonic acid leading to the synthesis of prostaglandin E_2 observed in arthritis is debatable. A likely candidate, on this basis of specificity and activation, is cytoplasmic 85 kDa PLA_2.

Type II $sPLA_2$ was previously shown to hydrolyse phospholipids in the membranes of *Escherichia coli*, suggesting that it might participate in antimicrobial defence [4]. Conflicting results have been reported in regard to the activity of $sPLA_2$ acting on mammalian cell membranes. Type II $sPLA_2$ was found to induce arachidonic acid release and PGE_2 synthesis in mast cells [11], mesangial cells [12], neutrophils [4] and chondrocytes [13] and in murine fibroblasts overexpressing human type II $sPLA_2$ [14]. In contrast, platelet membranes were found to be refractory [15].

The mechanism by which $sPLA_2$ might participate in the generation of phospholipid-derived lipid mediators is not fully understood. It is thought that activation of the enzyme might be the result of membrane 'scrambling' or a synergistic activation of sphingomyelinase. Recently, Fourcade et al. [16] demonstrated that, although inactive on intact blood cells, $sPLA_2$ hydrolyses phospholipids in membrane microvesicles shed from Ca^{2+}-loaded erythrocytes and platelets or from whole blood cells challenged by inflammatory stimuli. Phospholipid asymmetry and lipid packing are known to be widely modified under these circumstances and to have a critical role in modulating the activity of $sPLA_2$ acting on biological membranes [17]. Kudo et al. [18] have suggested, for example, that the activity *in vivo* of $sPLA_2$ requires a 'membrane rearrangement' induced by tumour necrosis factor α (TNFα) or on cross-linking of IgE receptors.

Interleukin 1β- or TNFα-inflammatory stimuli have also been shown to activate the degradation of membrane sphingomyelin (SPH) [19]. Under conditions in which SPH was hydrolysed by exogenous sphingomyelinase, type II $sPLA_2$ could in turn degrade a large amount of the microvesicle phospholipids. Because

Abbreviations used: D-DPPC, D-α-dipalmitoyl phosphatidylcholine; GC–MS, gas chromatography–MS; LCAT, lecithin:cholesterol acyltransferase; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PLA_2, phospholipase A_2; PS, phosphatidyl-L-serine; SPH, sphingomyelin; $sPLA_2$, secreted phospholipase A_2; TNBS, 2,4,6-trinitrobenzenesulphonic acid; TNF, tumour necrosis factor.

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ceramides produced by SPH hydrolysis do not activate $sPLA_2$ [16], the content of SPH in cell membranes could therefore be the effector of $sPLA_2$. We have addressed this question in the present study by distinguishing the specific inhibition of $sPLA_2$ by SPH from its rigidifying effect on the membrane lipid matrix reproduced by the saturated non-hydrolysable D-α-dipalmitoyl phosphatidylcholine (D-DPPC).

Annexins are known to inhibit $sPLA_2$ activity on the phospholipid matrix [20] via so-called substrate-depletion inhibition. This inhibition is expected to protect the inner leaflet of the biological membrane where annexins complexed with Ca^{2+} bind tightly to membrane phospholipids and intercalated fatty acids [21–23]. The various annexins are also involved in the multiple membrane fusions associated with cell-cycle membrane traffic [23,24]. Annexin VI has been found in many secretory tissues [25] localized in the plasma membranes underlying specialized secretory regions [26]. Annexins have been described in most mammalian cells with the notable exeption of erythrocytes [27]. This prompted us to use erythrocyte membranes as the model for evaluation of exogenous annexin VI as a $sPLA_2$ modulator.

Annexin VI is a destabilizing factor during vesicle budding [28]. This raises the possibility that annexin VI might act by increasing the susceptibility of crucial lipids to enzyme hydrolysis during the process leading to cleavage of vesicles from the membrane. Because non-esterified fatty acids increase in turn the rate of annexin binding to membranes and the fusion process [29], the possibility that annexin VI might be involved in $sPLA_2$ regulation has been investigated. The concerted activities of both proteins, $sPLA_2$ and annexin VI, which have been currently revealed when acting on organized membranes, support a role for type II $sPLA_2$ in cellular membrane traffic.

The enzyme is apparently regulated by membrane-associated proteins as well as by membrane lipid composition. These conclusions are based on the monitoring of fatty acids released from membrane phospholipids instead of the hydrolysis of a single pure (radioactive or fluorescent) molecular species serving as the substrate of $sPLA_2$, a model of the natural substrate.

MATERIALS AND METHODS

Reagents

D-DPPC, L-α-phosphatidylethanolamine (PE) from egg yolk, L-α-phosphatidyl-L-serine (PS) from bovine brain, SPH from egg yolk, cholesterol, porcine pancreatic PLA_2, fluorescamine and 2,4,6-trinitrobenzenesulphonic acid (TNBS) were purchased from Sigma. Boron trifluoride–methanol complex was obtained from Merck–Schuchardt. Silica gel plates for TLC (TLC Ready Foils F1500, 0.25 mm thickness) were obtained from Schleicher & Schuell. Annexin VI purified from bovine liver was a gift from Dr. Rainteau (Faculté de Médecine St. Antoine, Université Pierre et Marie Curie, Paris, France). Recombinant type II $sPLA_2$, obtained from C127 mouse fibroblasts overexpressing the human enzyme [14], was a gift from Dr. Olivier (Faculté de Médecine St. Antoine, Université Pierre et Marie Curie, Paris, France).

Oriented erythrocyte membrane vesicles

Human erythrocytes were isolated from fresh citrated blood by the procedure of Steck et al. [30]. The blood was centrifuged for 10 min at 100 ***g***. Erythrocytes were washed several times with 5 vol. of PBS [150 mM NaCl/5 mM sodium phosphate (pH 8.0)] and haemolysed in 40 vol. of 5 mM sodium phosphate, pH 8.0. The ghosts were collected by centrifugation for 20 min at 22000 ***g*** (Beckman J2-HS), and were washed (two to four cycles) with 5 mM phosphate buffer until 'white ghosts' were obtained.

Sealed 'inside-out' vesicles were obtained in the absence of divalent cation at alkaline pH by diluting 1 ml of erythrocyte ghosts to approx. 40 ml with 0.5 mM phosphate buffer, pH 8.0. After incubation for 18 h at 4 °C, the membranes were pelleted at 28000 ***g*** for 30 min, resuspended in 1 ml and forced 3–5 times through a 27 gauge needle fitted to a syringe to complete vesiculation. This 'sheared' preparation, diluted 2–4-fold in 0.5 mM phosphate buffer, was layered over an equal volume of dextran barrier solution (density 1.03 g/ml) and centrifuged (Spinco SW41 rotor) for 2 h at 197000 ***g*** to resolve the mixture into a pellet and a band floating on top of the barrier. The top layer ('inside-out' purified vesicles) was washed with 40 ml of 0.5 mM phosphate buffer and pelleted (28000 ***g*** for 30 min).

Exactly the same procedure was applied to prepare sealed 'rightside-out' vesicles except that they were formed by passing through the 27 gauge needle the 'white ghosts' in 0.5 mM phosphate buffer containing 100 μM $MgSO_4$ to provide a high concentration of the divalent cation.

Determination of PE distribution in oriented erythrocyte vesicles

The labelling of the PE amino group by a non-permeant reactant is based on the method of Gordesky and Marinetti [31]. The procedure checks the sidedness of the vesicles prepared as described above. The oriented vesicle suspension (3 mg of protein [32]) in 0.5 mM phosphate buffer, pH 8.0, was incubated for 30 min on ice in the presence of 2 mM TNBS (1.5 ml) and 5% (w/v) $NaHCO_3$ (0.4 ml). HCl (1 M) was then added to stop the reaction at pH 7.2. The vesicles were washed twice with phosphate buffer, pH 8.0, and centrifuged for 30 min at 28000 ***g*** (Beckman J2-HS). The phospholipids were extracted [33] and chromatographed on silica gel plates with chloroform/methanol/acetic acid/water (65:25:8:4, by vol.) as solvent. PE and PE-TNBS spots were scraped, saponified with 0.5 M KOH in methanol and methylated with boron trifluoride–methanol complex. The fatty acid methyl esters were extracted with hexane and quantified by gas chromatography–MS (GC–MS).

Preparation of liposomes

Liposomes were prepared by sonication in buffer of the appropriate mixture of phospholipids (PE, PS, SPH, D-DPPC or erythrocyte ghost phospholipid extract). When indicated, cholesterol was added to the phospholipids. The phospholipids stored in chloroform were dried under a stream of nitrogen and hydrated in Tris/HCl buffer, pH 8.6, before being sonicated (twice for 2 min) at 20 kHz with an MSE tip probe at approx. 100 W.

Fluorescamine assay

Fluorescamine labelling of PE [34] was used to assess the exposure of the aminophospholipids of sonicated liposomes to a non-permeant reagent. Fluorescamine (2% in acetone) was added to the vesicle suspension at a molar ratio of 2:1. After 30 min of incubation at room temperature the phospholipids were extracted with chloroform/methanol (2:1, v/v) and PE was separated by TLC on silica gel plates. Spots of labelled and non-labelled PE were scraped and quantified as described for TNBS labelling of membrane PE.

PLA_2 assay

PLA_2 activity assayed on erythrocyte membrane phospholipids

The assay mixture [100 mM Tris/HCl (pH 8.6)/5 mM $CaCl_2$, final volume 0.5 ml] contained 3 m-units (0.05 mkat) 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). Erythrocyte membranes (100 nmol of hydrolysable phospholipids) served as the substrate. Fatty acid-free BSA (0.1 %, w/v) was added initially to trap the enzyme reaction products. Incubations were performed for 15 min at 37 °C with translational shaking. Released fatty acids were extracted by the procedure of Dole [35], methylated by diazomethane and quantified by GC–MS. Control incubations of erythrocyte membranes in the absence of added $sPLA_2$ were performed and the amount of non-esterified fatty acids (accounting for less than 10 %) was subtracted from the amount released by $sPLA_2$.

Activity assayed on mixed phospholipid liposomes

The incubation was performed for 15 min at 37 °C in a medium containing 100 nmol of hydrolysable phospholipids sonicated as described above in 100 mM Tris/HCl (pH 8.6)/5mM $CaCl_2$/0.25 mg/ml BSA. Human recombinant type II $sPLA_2$ (3 m-units) was added to start the reaction in a final volume of 0.5 ml. The released non-esterified fatty acids were extracted by the procedure of Dole [35], methylated with diazomethane and quantified. Control incubations in the absence of $sPLA_2$ were also performed.

GC–MS measurements

The non-esterified fatty acids extracted by the procedure of Dole [35] were methylated with diazomethane and separated by gas chromatography on a capillary column of Supelcowax-10 (Supelco) bonded phase (diameter 0.32 mm, length 30 m) in a Hewlett Packard 5890 Series II gas chromatograph. Fatty acids were detected by mass spectrometry (Nermag 10-10C) in the chemical ionization mode with ammonia (10 kPa) as the reagent gas. The positive quasi-molecular ions were monitored and time-integrated. Quantification was with an internal standard of heptadecanoic methyl ester with response factors calculated for the various fatty acid methyl esters with calibrators.

RESULTS

Fatty acids are released from erythrocyte membranes incubated with human type II $sPLA_2$

Type II $sPLA_2$ hydrolyses phospholipids of erythrocyte non-oriented ghosts with both time and concentration dependence (Figure 1). When the activity of type II $sPLA_2$ was compared on inside-out and rightside-out vesicles prepared from erythrocyte membranes, the rate of fatty acid release was 3-fold higher from inside-out vesicles than from rightside-out vesicles (Table 1). The rate of fatty acid release from non-oriented ghosts (168 pmol/min) was within the range of values for rightside-out (124 pmol/min) and inside-out vesicles (375 pmol/min).

Type II $sPLA_2$ has been shown to express specificity for phospholipid head groups ranking in the following order: phosphatidylglycerol (PG) > phosphatidylethanolamine (PE) > phosphatidylcholine (PC) ≫ N(PS) [36]. As a result of membrane asymmetry [17], the internal leaflet of the membrane is expected to be enriched in polyunsaturated fatty acids predominantly associated with PE and PS. However, Table 1 shows little difference in the fatty acid composition of hydrolysates produced from the different membrane sides. This suggests that $sPLA_2$ has preferentially hydrolysed polyunsaturated molecular species of substrate phospholipids available in the outer leaflet.

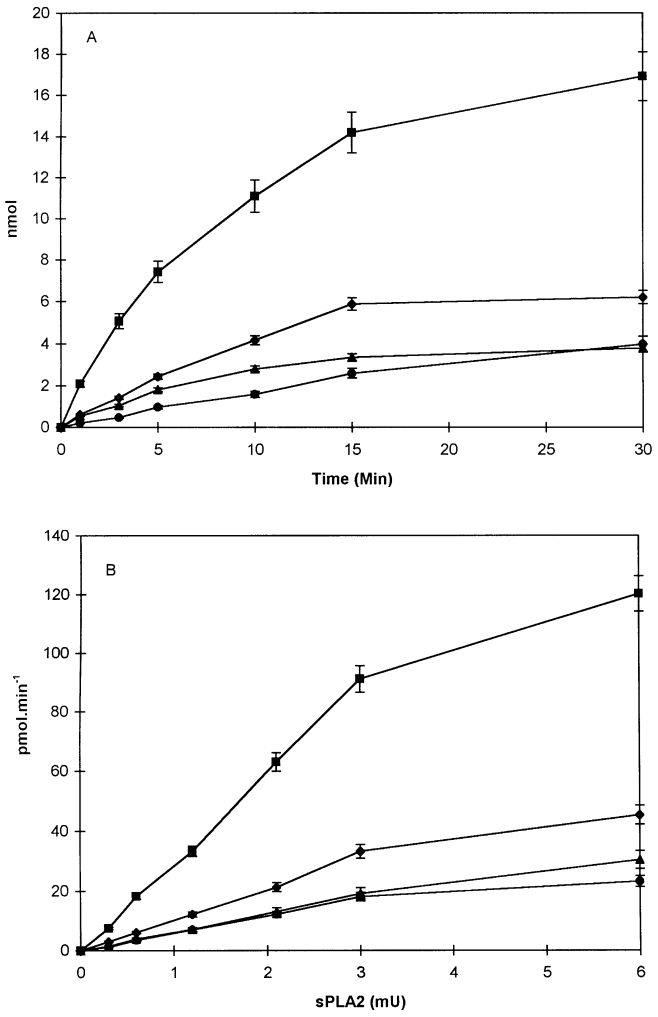

Figure 1 Erythrocyte membrane phospholipid hydrolysis by type II $sPLA_2$ as a function of time (A) and enzyme concentration (B)

Erythrocyte ghosts (100 nmol of phospholipids) serving as substrate in a final volume of 0.5 ml of Tris/HCl buffer (pH 8.6)/5 mM $CaCl_2$/0.1 % BSA were incubated at 37 °C. The time dependence was monitored in the presence of 3 m-units of $sPLA_2$ and the dependence on enzyme concentration was determined in 15 min incubations. Released fatty acids were extracted and quantified by GC–MS as described. Values are means ± S.D. for five determinations. Symbols: ■, $C_{18:1}$; ◆, $C_{20:4}$; ▲, $C_{18:2}$; ●, $C_{22:6}$. Abbreviation: mU, m-units.

Labelling by TNBS accounted for 81.4 % of inside-out and 18.6 % of the rightside-out vesicle PE. Even if the hydrolysis was restricted to PE (in addition to PE, one should consider that PC is also a substrate for $sPLA_2$), the substrate was in large excess on both oriented vesicles. Hydrolysis restricted to PE would represent only 23 % (of 24 nmol) or 31 % (of 6 nmol) of available PE on inside-out or rightside-out vesicles respectively.

SPH, but not PC, strongly inhibits phospholipid hydrolysis by type II $sPLA_2$

The resistance to hydrolysis of the rightside-out membrane vesicles has been suggested to result from its high SPH content in the leaflet exposed to the enzyme. The effect of SPH or

Table 1 $sPLA_2$-induced release of fatty acids from oriented erythrocyte membrane vesicles

Oriented erythrocyte vesicles (100 nmol of hydrolysable phospholipids) and 3 m-units of $sPLA_2$ were incubated in a final volume of 0.5 ml for 15 min at 37 °C in 100 mM Tris/HCl buffer (pH 8.6)/5 mM $CaCl_2$/0.1% BSA. Released fatty acids were quantified by GC–MS as described in the Materials and methods section. Values are means ± S.D. for triplicates in two experiments.

	Inside-out vesicles		Rightside-out vesicles	
Fatty acid	Release (pmol/min)	Release (mol%)	Release (pmol/min)	Release (mol%)
$C_{18:1}$	131 ± 11	51.0	62 ± 11	50.3
$C_{18:2}$	36 ± 5	9.5	15 ± 6	12.2
$C_{20:4}$	102 ± 21	27.3	33 ± 3	26.9
$C_{22:6}$	46 ± 5	12.3	13 ± 2	10.6
Total	375 ± 5	100	124 ± 9	100

saturated D-DPPC on $sPLA_2$ activity was tested with liposomes comprising constant proportions of hydrolysable phospholipids PE and PS (80:20, mol/mol). The substrate was gradually enriched in non-hydrolysable choline-containing phospholipids (SPH or D-DPPC). SPH enrichment at relative concentrations mimicking that normally found in biomembranes induced a marked inhibition of fatty acid release (Table 2). The extent of inhibition was similar for all fatty acids. In contrast, enrichment of liposomes by D-DPPC, which is not a substrate of the enzyme [37], slightly increased the rate of phospholipid hydrolysis at high concentrations.

Fluorescamine labelling to monitor the PE distribution in liposomes showed that SPH or D-DPPC added to PE/PS did not change significantly its availability for enzyme action (remaining between 31% and 35% of the total PE). This also confirms the similarity in the morphologies of the vesicles serving as substrate.

Addition of SPH to liposomes prepared by sonication of the total lipids extracted from erythrocytes also inhibited fatty acid release by $sPLA_2$ (Table 3). No inhibition was observed when the lipid dispersion was enriched in D-DPPC. The extent of inhibition of $sPLA_2$ by SPH was less (Table 3) on lipid extracts of erythrocyte membranes than on PE/PS liposomes (Table 2).

Table 2 Effect of SPH and D-DPPC enrichment of PE/PS liposomes on $sPLA_2$ activity

Incubation medium (0.5 ml) contained mixed phospholipid substrate (100 nmol) in Tris/HCl buffer (pH 8.6)/5 mM $CaCl_2$/0.1% BSA, with 3 m-units of $sPLA_2$. The substrate was PE/PS (80:20, mol/mol) enriched with SPH or with D-DPPC (43–400 nmol). Incubations were performed for 15 min at 37 °C. Released fatty acids were extracted and quantified by GC–MS as described. The values are means ± S.D. for triplicates of two independent experiments.

		$sPLA_2$ activity (pmol of fatty acids released/min)					
Fatty acid	SPH (nmol) ...	43	67	100	150	233	400
$C_{18:1}$		152 ± 15	115 ± 7	103 ± 3	88 ± 2	81 ± 4	69 ± 10
$C_{18:2}$		117 ± 11	78 ± 7	59 ± 2	45 ± 3	34 ± 2	22 ± 5
$C_{20:4}$		66 ± 6	38 ± 3	26 ± 2	16 ± 2	12 ± 2	6 ± 1
$C_{22:6}$		8 ± 1	5 ± 1	3 ± 1	2 ± 0.09	1 ± 0.003	1 ± 0.002
Total		343 ± 31	235 ± 18	190 ± 32	150 ± 5	129 ± 8	98 ± 18
		$sPLA_2$ activity (pmol of fatty acids released/min)					
Fatty acid	D-DPPC (nmol) ...	43	67	100	150	233	400
$C_{18:1}$		188 ± 8	177 ± 5	177 ± 2	195 ± 7	205 ± 10	245 ± 4
$C_{18:2}$		88 ± 2	75 ± 1	77 ± 4	89 ± 6	119 ± 4	149 ± 10
$C_{20:4}$		63 ± 2	49 ± 1	51 ± 1	57 ± 1	73 ± 4	82 ± 3
$C_{22:6}$		28 ± 2	25 ± 2	22 ± 1	26 ± 3	26 ± 2	28 ± 1
Total		367 ± 5	327 ± 3	327 ± 7	367 ± 17	423 ± 15	504 ± 7

Table 3 Effect of SPH and D-DPPC enrichment of erythrocyte ghost phospholipids liposomes on $sPLA_2$ activity

Incubation medium (0.5 ml) contained 100 nmol of hydrolysable phospholipid substrate in Tris/HCl buffer (pH 8.6)/5 mM $CaCl_2$/0.1% BSA, with 3 m-units of $sPLA_2$. The substrate was composed of ghost phospholipids enriched with SPH or with D-DPPC. Incubations were performed for 15 min at 37 °C. Released fatty acids were extracted and quantified by GC–MS as described. The values are means ± S.D. for triplicates of two experiments.

		$sPLA_2$ activity (pmol of fatty acids released/min)							
Fatty acid	SPH (nmol) ...	0	10	30	50	D-DPPC (nmol) ...	10	30	50
$C_{18:1}$		93 ± 4	86 ± 1	84 ± 3	74 ± 3		79 ± 3	96 ± 5	82 ± 5
$C_{18:2}$		41 ± 3	38 ± 1	35 ± 1	27 ± 3		36 ± 2	47 ± 1	36 ± 4
$C_{20:4}$		71 ± 4	71 ± 2	62 ± 1	39 ± 4		65 ± 3	74 ± 4	59 ± 8
$C_{22:6}$		32 ± 1	33 ± 1	29 ± 1	14 ± 1		29 ± 2	31 ± 1	24 ± 4
Total		237 ± 9	228 ± 5	210 ± 6	154 ± 6		209 ± 7	248 ± 10	202 ± 20

Table 4 Influence of cholesterol on type II $sPLA_2$ activity acting on PE/PS/SPH substrates

The incubation medium (0.5 ml) contained 100 nmol of hydrolysable phospholipid liposomes [PE/PS (80:20, mol/mol)] enriched with 43 nmol (column A) or 233 nmol (column B) of SPH. Cholesterol (hydrolysable phospholipid/cholesterol 2:1, mol/mol) was added to the liposomes enriched with 233 nmol of SPH (C). The incubations were performed in Tris/HCl buffer (pH 8.6)/5 mM $CaCl_2$/0.1% BSA in the presence of 3 m-units of $sPLA_2$ for 15 min at 37 °C. Released fatty acids were extracted and quantified by GC–MS as described. The values are means ± S.D. ($n = 4$).

	$sPLA_2$ activity (pmol of fatty acids released/min)		
Fatty acid	A	B	C
$C_{18:1}$	261 ± 13	131 ± 6	191 ± 15
$C_{18:2}$	109 ± 1	27 ± 3	101 ± 18
$C_{20:4}$	75 ± 3	17 ± 6	63 ± 11
$C_{22:6}$	36 ± 4	14 ± 5	27 ± 5
Total	482 ± 6	194 ± 19	382 ± 45

The modulating role of cholesterol on the inhibition brought about by SPH was investigated in the context of the SPH/cholesterol association's playing a probable role in the structure–function relationships in biological membranes [38,39]. Enrichment of liposomes with cholesterol counteracted the inhibition of $sPLA_2$ by SPH (Table 4). The effect is specific for SPH-enriched substrate but not for D-DDPC-enriched liposomes, which retain the same susceptibility to $sPLA_2$ in the presence of cholesterol (results not shown).

Whereas SPH is known to increase the order of membrane phospholipids acyl chains [38], annexin VI is known to destabilize the membrane bilayer arrangement [21]. To investigate whether 'scrambling' can facilitate the availability of phospholipids to $sPLA_2$, we have assessed the effect of exogenous annexin VI on the fatty acid released by the enzyme from erythrocyte membranes normally devoid of this protein.

Annexin VI at low concentrations stimulates fatty acid release by type II $sPLA_2$ from erythrocyte membranes

In a preliminary control experiment, we confirmed the inhibitory effect of annexin VI previously described for the 'substrate depletion' model [20]. With human type II $sPLA_2$ acting on the fluorescent phosphatidic acid micellar substrate, the IC_{50} was 4 nM (results not shown). At this low concentration, no inhibition of activity was detected when acting on erythrocyte ghosts. In contrast, an activation was observed that was obvious at a concentration as low as 1 nM and reached a maximum at 5 nM (Figure 2A). The release of fatty acids (especially arachidonic and docosahexaenoic acids) was stimulated, indicating a preference of the enzyme for polyunsaturated phospholipid molecular species. When annexin VI concentrations were increased above 5 nM, the inhibitory effect described above with liposomes also became apparent on membranes.

The activation by annexin VI is not a direct effect on membrane phospholipids because it cannot be reproduced on liposomal substrate, including the sonicated total lipid extract prepared from membranes (Figure 2B). The inhibitory effect of annexin VI can be observed only for $sPLA_2$ acting on such a substrate (Figure 2B). The influence of annexin VI is specific for type II $sPLA_2$ because type I pancreatic PLA_2 is neither inhibited nor stimulated by annexin when acting on membranes or a dispersion of membrane lipid extracts (results not shown).

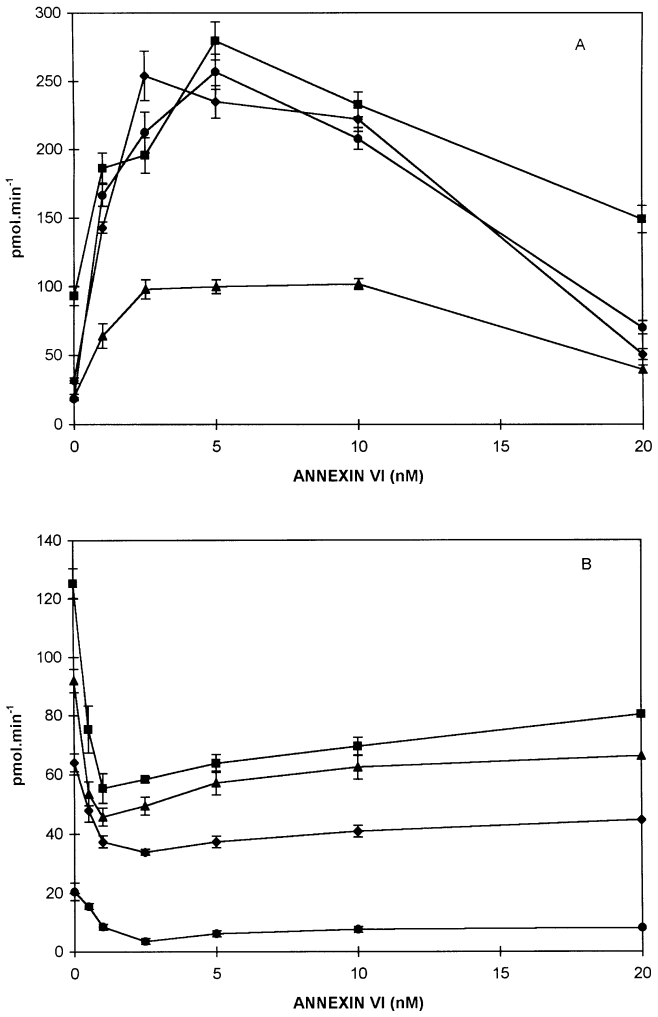

Figure 2 Effect of annexin VI on type II $sPLA_2$ activity on erythrocyte membranes

Incubation medium (0.5 ml) contained erythrocyte ghosts (100 nmol of phospholipids) (**A**) or sonicated total phospholipids extracted from erythrocytes (100 nmol) (**B**) in Tris/HCl buffer (pH 8.6)/5 mM $CaCl_2$/0.1% BSA. Annexin VI was preincubated with the substrate for 5 min. $sPLA_2$ (3 m-units) was added and the mixture incubated for 15 min at 37 °C. Released fatty acids were extracted and quantified as described. Values are means ± S.D. for triplicates in two experiments. Symbols: ■, $C_{18:1}$; ◆, $C_{20:4}$; ▲, $C_{18:2}$; ●, $C_{22:6}$.

The discrepancy between the effect of annexin VI on the hydrolysis of erythrocyte membranes and liposomal phospholipid substrate suggests that membrane-associated proteins might take part in the specific modulation of type II $sPLA_2$ by annexins, as discussed below.

DISCUSSION

Type II $sPLA_2$ is secreted by different cells in response to various stimuli [2,4–8]. The precise physiological substrate of the enzyme is conjectural. It hydrolyses membrane phospholipids of Gram-negative bacteria after the action of bactericidal-permeability-increasing protein (BPI) [4]. Although only indirect evidence has been published to indicate that type II $sPLA_2$ can hydrolyse mammalian cell phospholipids *in vivo* to generate lipid mediators [11–14,16], direct evidence is still lacking for the site of this hydrolytic action. The enzyme is synthesized and exported from

cells via the secretory pathway [2,40]. During this process it remains tightly bound to membranes via interactions with glycoconjugates [41], from which it may be released by heparin or high ionic strength. Mechanisms must therefore exist *in vivo* to protect membranes of the secretory system and plasma membranes from untimely hydrolysis. Inhibition has been suggested to result from co-secretion of inhibitory molecules such as uteroglobin [42] or from the resistance to the enzyme of the luminal leaflet of the secretory system and the external leaflet of the plasma membrane. This last hypothesis is addressed by the experiments performed in the present study where hydrolysable substrates, whether PE/PS mixture or membrane lipid extract, were enriched in SPH.

Plasma membranes are a highly asymmetrical phospholipid bilayer. The asymmetric distribution exposes choline-containing phospholipids (PC and SPH) to the luminal or outer surface of the membrane. It is known that in platelets and in erythrocytes pretreated with sphingomyelinase, the cleavage of the phosphocholine moiety of SPH is associated with a susceptibility of plasma membrane phospholipids to the attack of venom or pancreatic PLA_2 species previously inactive on intact cells [17]. We found that phospholipids of rightside-out erythrocyte vesicles, which expose a leaflet highly enriched in PC and SPH, are less susceptible to type II $sPLA_2$ than inside-out vesicles (Table 1). The difference of hydrolysis observed (124 compared with 375 pmol/min) did not parallel PE enrichment of the leaflet exposed to the enzyme (6 compared with 24 nmol). The availability of PE (a major substrate of $sPLA_2$) due to the asymmetric distribution of membrane phospholipids exceeds the difference of hydrolysis between the two sides recorded presently. An estimate of the hydrolysis (less than 31 %) relative to the amount of PE exposed on the rightside-out vesicles to the $sPLA_2$ has shown that accessibility of the substrate is probably not the limiting factor for the enzyme activity. In agreement with these observations favouring an inhibitory role for SPH when added in liposomal models composed of hydrolysable PE and PS mixtures or lipid extracts from erythrocytes, we found SPH to be a potent inhibitor of phospholipid hydrolysis by type II $sPLA_2$ (Tables 2 and 3). This effect is specific for SPH because the non-hydrolysable analogue of PC, D-DPPC, another choline-containing phospholipid, does not inhibit type II $sPLA_2$ activity (Tables 2 and 3). Remarkably, D-DPPC shares with SPH a similar rigidifying effect on plasma membranes [43], which rules out a simple inhibitory effect due to the physical state of phospholipid substrate. In addition, fluorescamine labelling has now shown that the accessibility of PE for $sPLA_2$ remains identical (within the range 31–35 %) when SPH or D-DPPC was added. This supports the observation that not only does the orientation of PE remain constant, but the presentation of the substrate is also identical for the various liposomes used. The inhibitory effect of SPH is lower on liposomal phospholipids extracted from erythrocytes than on PE/PS liposomes (compare Tables 2 and 3). This can be explained by the presence of cholesterol in the lipid extract from erythrocyte ghosts, as reflected by the suppression of inhibition by the addition of cholesterol (Table 4). It was established that SPH strongly binds cholesterol in biomembranes [38], which counteracts its ability to inhibit $sPLA_2$. The amide bond with the acyl chain has been shown to enhance the interaction of SPH with cholesterol [39]. Whether the amide bond of SPH also has a role similar to amide bond-containing $sPLA_2$ synthetic inhibitors [44] is questionable. Recent studies on the regulation of lecithin:cholesterol acyltransferase (LCAT) [45] suggested that SPH competes with PC for binding to the active site. The authors showed that the phosphorylcholine group is required and that ceramide is not an inhibitor of LCAT. Results were also obtained that show a competitive inhibition of LCAT by SPH [46].

It is tempting to speculate that SPH content in the external leaflet of plasma membrane regulates $sPLA_2$ susceptibility *in vivo*. Various amounts of SPH and cholesterol in the plasma membranes could explain contradictory results reported in the literature concerning the ability of type II $sPLA_2$ to release arachidonic acid or produce eicosanoids in different cells. Differences in culture conditions could account for the variable amounts of cholesterol in plasma membranes. Furthermore it might be important to consider the influence of SPH in the light of recent studies demonstrating that hydrolysis of SPH is a key signalling event evoked by a number of proinflammatory cytokines such as TNFα or interleukin 1β [18,19]. Accordingly, during infection by Gram-positive bacteria, a condition known to stimulate strongly type II $sPLA_2$ secretion, bacterial sphingomyelinase could play a role in priming the membrane to the action of type II $sPLA_2$.

The involvement of type II $sPLA_2$ in secretory vesicles [2,40] or at the surface of the plasma membrane [41] participating in events leading to exocytosis/endocytosis has also been questioned in the present study because of the large differences in the effect of annexin on membranes or sonicated purified phospholipids. Annexin VI, at concentrations less than 5 nM, stimulates the release of arachidonic acid and other *cis*-polyunsaturated fatty acids required to achieve membrane fusion [29]. This stimulatory effect of annexin VI was found for concentrations at which inhibition was observed on a purified phospholipid substrate [47]. The inhibition of $sPLA_2$ acting on membranes by an annexin VI 'coating' of the water/substrate interface resulted from Ca^{2+}-induced tight association with the phospholipid head groups (substrate depletion) [48,49]. Saturating Ca^{2+} levels and a low annexin-to-vesicle ratio are the conditions used in the present study (5 mM Ca^{2+} and 25 pmol of annexin VI per mg of membrane protein), under which dissociation is slow and enzyme–substrate complexes are regarded as stable [50]. Despite a strong interaction of annexin VI with membrane phospholipids, no stimulatory effect of type II $sPLA_2$ was observed when liposomes were used as the substrate. An involvement of membrane protein has been suggested, in agreement with previous observations that annexins induce the fusion of PC vesicles with biological membranes. The fusion process was decreased when native biological membranes were replaced by sonicated lipids extracted from biological membranes [51]. In line with these results, we suggest that the activation of $sPLA_2$ by annexin VI at low concentration results from direct protein–protein interaction.

The role of annexin VI in type II $sPLA_2$ activity might be physiologically relevant because it occurs in concentrations found in cells [27]. It is also known that annexin binding to the surface induces disorder on both sides of the membrane [52]. A synergy between $sPLA_2$ and annexin VI could be envisaged because annexin has been found to release Ca^{2+} from chromaffin granules [53]. In a model of exocytosis, Ca^{2+}-dependent binding of secretory granules with the plasma membrane is the final stage of secretion. The formation of a ternary complex between secretory granules, plasma membrane and annexin VI could lead to the activation of $sPLA_2$. It is therefore tempting to speculate that type II $sPLA_2$, stored in secretory granules, is activated to produce non-esterified fatty acids required in the fusion process and in the binding of additional annexins to membranes [21–23].

Annexin VI has been found to participate in the severing of the stalk during the budding process of endocytosis [28]. Stimulation of PLA_2 activity by annexin VI could therefore participate in membrane destabilization and fusion. It would be reasonable to address whether receptors for type II $sPLA_2$ recently identified

and cloned [54,55] are the proteins that co-operate with annexin VI to activate $sPLA_2$, because the receptor could relocate the $sPLA_2$ on the internal side of the cell membrane along with the annexins.

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