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Secretory Phospholipase A_2 Generates the Novel Lipid Mediator Lysophosphatidic Acid in Membrane Microvesicles Shed from Activated Cells

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Summary

Nonpancreatic secretory phospholipase A_2 ($sPLA_2$) displays proinflammatory properties; however, its physiological substrate is not identified. Although inactive toward intact cells, $sPLA_2$ hydrolyzed phospholipids in membrane microvesicles shed from Ca^{2+}-loaded erythrocytes as well as from platelets and from whole blood cells challenged with inflammatory stimuli. $sPLA_2$ was stimulated upon degradation of sphingomyelin (SPH) and produced lysophosphatidic acid (LPA), which induced platelet aggregation. Finally, lysophospholipid-containing vesicles and $sPLA_2$ were detected in inflammatory fluids in relative proportions identical to those used in vitro. We conclude that upon loss of phospholipid asymmetry, cell-derived microvesicles provide a preferential substrate for $sPLA_2$. SPH hydrolysis, which is provoked by various cytokines, regulates $sPLA_2$ activity, and the novel lipid mediator LPA can be generated by this pathway.

Introduction

Phospholipase A_2 (PLA_2) is thought to play a key role in cell signaling by releasing arachidonic acid esterified at the sn-2 position of glycerophospholipids (van den Bosch, 1980; Irvine, 1982). The best known enzymes of this group are secretory PLA_2 ($sPLA_2$ or type II PLA_2) and cytosolic PLA_2 ($cPLA_2$) (reviewed by Kudo et al., 1993; Vadas et al., 1993). Although it is generally agreed that 85 kDa $cPLA_2$ is the best candidate to achieve receptor-coupled arachidonic acid liberation, the precise role of $sPLA_2$ still remains obscure. This 14 kDa protein is secreted by a number of cells in which its expression is either constitutive (e.g., platelets and neutrophils) or induced by inflammatory cytokines (e.g., as described in renal mesangial cells, chondrocytes, vascular smooth muscle, or astrocytes). $sPLA_2$, which displays proinflammatory properties, is also currently detected in various exudates and is considerably increased in the plasma of patients suffering from septic shock, strongly suggesting a possible involvement of this enzyme in the inflammatory reaction. Coupled to the fact that its catalytic activity requires millimolar Ca^{2+} concentrations, these observations have led to the concept of $sPLA_2$ becoming fully active upon release into the extracellular space.

Among various biological membranes susceptible to interact with $sPLA_2$, the plasma membrane of Escherichia coli has been the most studied, suggesting that this enzyme might participate in antimicrobial defense (Wright et al., 1990). As for the membrane of mammalian cells, somewhat controversial data are available. These apparent discrepancies actually reflect the use of different cell models such as platelets (Mounier et al., 1993; Riendeau et al., 1994; Bartoli et al., 1994), which appear refractory to the enzyme, or various proinflammatory cells, in which a role of $sPLA_2$ in arachidonic acid liberation was demonstrated (Hara et al., 1991; Murakami et al., 1991, 1993; Pernas et al., 1991; Pfeilschifter et al., 1994). However, opposite conclusions were also reported for macrophages (Barbour and Dennis, 1993; Hidi et al., 1993; Miyake et al., 1994). As recently discussed by Kudo et al. (1993), the action of $sPLA_2$ requires a "membrane rearrangement" induced by tumor necrosis factor (TNF) or upon cross-linking of immunoglobulin E receptors.

Phospholipid asymmetry as well as lipid packing have been shown to play a crucial role in modulating the activity of phospholipases toward biological membranes (Verkleij et al., 1973; Zwaal et al., 1975; Chap et al., 1977). For instance, pancreatic PLA_2, which is unable to degrade phosphatidylcholine (PC) in the external leaflet of erythrocyte or platelet membranes, readily hydrolyzes phosphatidylserine (PS) and phosphatidylethanolamine (PE) present in the internal layer. $sPLA_2$ has a strong preference for anionic phospholipids and for PE and is even less efficient than pancreatic PLA_2 at penetrating a monomolecular film of phospholipids (Ransac et al., 1992). Its failure to act on intact cells exposing choline phospholipids on their external surface thus appears as an obvious consequence of this behavior. However, the asymmetrical arrangement of phospholipids in the plasma membrane corresponds to a dynamic equilibrium susceptible to change dramatically under certain conditions (reviewed by Schroit and Zwaal, 1991; Zwaal et al., 1992; Zachowski, 1993; Devaux, 1993). This has been shown particularly in erythrocytes, platelets, and endothelial cells in response to various agents such as Ca^{2+} ionophore A23187, complement membrane-attack complex, a combination of thrombin and collagen, or α-toxin from Staphylococcus aureus. This results in the

†The first two authors contributed equally to this work.

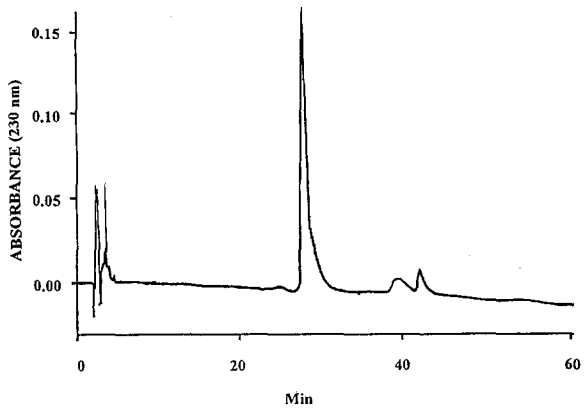

Figure 1. Reverse Phase HPLC Analysis of Recombinant $sPLA_2$

Renatured protein (100 μg) was loaded onto a Nucleosil C18 column as described in the Experimental Procedures. Elution was achieved using a linear gradient of acetonitrile (5%–60%) in 0.1% trifluoroacetic acid, which was started at the time of the injection. Protein was detected by continuous monitoring of optical density at 230 nm. The small peaks around 40 min correspond to unidentified impurities present in the buffer used for PLA_2 solubilization.

Table 1. Hydrolysis of Microvesicle Phospholipids by Pancreatic and Recombinant Secretory PLA_2

	$sPLA_2$	Pancreatic PLA_2
PC	19.4 ± 1.8 ($p < 0.01$)	26.5 ± 3.1 ($p < 0.001$)
PE	22.2 ± 2.4 ($p < 0.01$)	28.2 ± 6.5 ($p < 0.01$)
PS	15.1 ± 5.0 ($p < 0.10$)	21.0 ± 1.5 ($p < 0.001$)

Microvesicles were incubated for 60 min at 37°C in the absence or in the presence of 10 μg/ml of recombinant human $sPLA_2$ (four experiments) or pig pancreatic PLA_2 (six experiments). Phospholipids were analyzed as described in the Experimental Procedures. Data (means ± SEM) correspond to percentages of hydrolysis, taking into account the production of LPC (or LPE), or the decrease of PS. p, probability of significance according to Student's t test.

exposure of procoagulant phospholipids such as PS. In red cells, A23187 was shown to promote shedding of microvesicles displaying a progressive loss of membrane asymmetry with surface exposure of aminophospholipids (Allan and Michell, 1975; Raval and Allan, 1984; Scott et al., 1984; Chandra et al., 1987; Comfurius et al., 1990; Bevers et al., 1992). Similar observations have been extended to platelets and endothelial cells exposed to complement proteins C5b-9, which promote Ca^{2+} entry into their target cells (Chang et al., 1993; Hamilton et al., 1990). This prompted us to consider the possibility that microvesicles formed under these conditions could provide a suitable substrate for $sPLA_2$, with Ca^{2+}-loaded erythrocytes appearing as the most convenient model to perform this study.

In human erythrocytes, Ca^{2+} ionophore also stimulates phosphoinositide-specific phospholipase C (PLC), which produces diacylglycerol, a large part of which is rapidly converted into phosphatidic acid (PA) (Allan and Michell, 1975; Allan and Thomas, 1981). There is now increasing evidence that lysophosphatidic acid (LPA) is a novel phospholipid mediator with various biological effects, although the metabolic pathways responsible for its biosynthesis are not yet clearly established (reviewed by Moolenaar, 1994). A second aim of this study was thus to investigate whether $sPLA_2$ could hydrolyze PA present in microvesicles shed from Ca^{2+}-loaded erythrocytes. Our data indicate that PLA_2 secreted during inflammation could be actively involved in the generation of LPA.

Results

Properties of Recombinant Human $sPLA_2$

All the experiments were performed with a human recombinant $sPLA_2$ displaying a Met-8→Leu mutation. This allows the production of the enzyme in E. coli as a fusion protein, which is then selectively cleaved with cyanogen bromide (CNBr), Met-8 being unique in the sequence of the mature enzyme. As shown by Franken et al. (1992), the mutation does not influence the activity of $sPLA_2$, which is recovered after renaturation under oxidative conditions. After two successive purification steps on SP–Sephadex, $sPLA_2$ appeared homogenous upon electrophoresis on polyacrylamide gels in the presence of sodium dodecylsulfate and displayed an apparent molecular mass of 17 kDa, i.e., a slightly higher size than expected (14 kDa). This abnormal migration of both type I and type II PLA_2 was previously reported by Aarsman et al. (1989). The most crucial point in the preparation of recombinant $sPLA_2$ is to obtain proper refolding of the protein, which requires the formation of six disulfide bridges. This led us to explore further the physicochemical properties of the enzyme by reverse phase high pressure liquid chromatography (HPLC). As shown in Figure 1, the protein was eluted within a single peak at 33.09% acetonitrile, where all the PLA_2 activity was recovered with a yield of 80%–90%. Upon reduction and carboxymethylation, elution occurred at 34.63% acetonitrile (data not shown), reflecting exposure of internal hydrophobic amino acid residues. Using didodecanoyl-PC as a substrate, pure $sPLA_2$ displayed a specific activity of 89 U/mg protein, which is comparable to the value of 115 U/mg reported by Franken et al. (1992). Altogether, these data indicated that recombinant human type II PLA_2 displayed all the properties of the native enzyme and could thus be used for further experiments on membrane vesicles.

Hydrolysis of Erythrocyte Microvesicle Phospholipids by Pancreatic and Secretory PLA_2

In preliminary experiments using intact erythrocytes, neither pig pancreas PLA_2 nor recombinant $sPLA_2$ were able to hydrolyze phospholipids, as already shown for pancreatic PLA_2 (Zwaal et al., 1975). On the contrary, in microvesicles shed from Ca^{2+}-loaded erythrocytes, both pancreatic PLA_2 and $sPLA_2$ hydrolyzed PC, PE, and PS to a similar extent (Table 1). Only PS degradation by $sPLA_2$ was not significant, mainly for technical reasons. Indeed, whereas lysophosphatidylcholine (LPC) and lysophosphatidylethanolamine (LPE) were quantitatively recovered, lysophosphatidylserine (LPS) is not extracted by current procedures. This implies the determination of PS hydrolysis by measuring its decrease. Since incubations were performed in the absence of lysis, these data indicated that

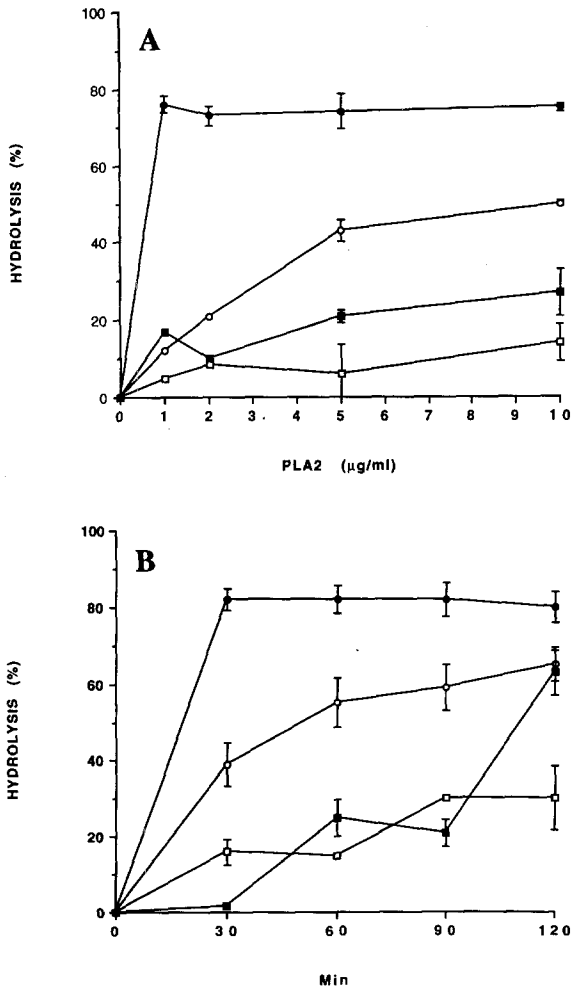

Figure 2. Effect of Sphingomyelinase on the Hydrolysis by $sPLA_2$ of Phospholipids from Erythrocyte Microvesicles

In (A), microvesicles were incubated for 60 min at 37°C in the presence of sphingomyelinase (0.1 U/ml) and various concentrations of recombinant human $sPLA_2$. In (B), incubation was performed for indicated times in the presence of sphingomyelinase (0.1 U/ml) and $sPLA_2$ (2 μg/ml). Phospholipids were analyzed as described in the Experimental Procedures and in Table 1. Data (means ± SEM, three different experiments) correspond to SPH (closed circles), PC (open circles), PE (closed squares), and PS (open squares).

the three main classes of glycerophospholipids were present on the external surface of microvesicles, in agreement with previous reports (Raval and Allan, 1984; Scott et al., 1984; Chandra et al., 1987; Comfurius et al., 1990). However, both PLA_2 certainly did not degrade all the glycerophospholipids present in the outer layer of the membrane.

Effect of Sphingomyelinase on the Hydrolysis of Erythrocyte Microvesicle Phospholipids by $sPLA_2$

In the presence of sphingomyelinase, a number of phospholipases were previously shown to degrade phospholipids in intact cells much more efficiently (Verkleij et al., 1973; Zwaal et al., 1975; Chap et al., 1977). The same behavior was observed with $sPLA_2$. Under conditions in which almost 80% of sphingomyelin (SPH) was hydrolyzed, $sPLA_2$ could degrade 50% of PC, 25% of PE, and 12% of PS present in microvesicles (Figure 2A). Hydrolysis of PC, PE, and PS could be extended to 60%, 60%, and 30%, respectively, by increasing the incubation time up to 2 hr (Figure 2B). These values are quite similar to those previously obtained with other more penetrating enzymes such as Naja naja PLA_2 (Scott et al., 1984), indicating that in the presence of sphingomyelinase, $sPLA_2$ acquires the capacity to degrade virtually all the glycerophospholipids present on the external surface of microvesicles.

To determine whether the effect of sphingomyelinase was related to the accumulation of ceramide or to the decrease of SPH, microvesicles were incubated with $sPLA_2$ in the presence of the membrane permeant ceramide acetyl-D-sphingosine. No significant effect could be observed at up to 100 μM C2-ceramide (data not shown).

Conversion of PA into LPA in Microvesicles Incubated with $sPLA_2$

As previously shown, accumulation of PA in erythrocyte-derived microvesicles can easily be followed by ^{32}P labeling (Allan et al., 1976; Allan and Thomas, 1981). Using this procedure, we observed a progressive hydrolysis of [^{32}P]PA into [^{32}P]LPA by $sPLA_2$. In the absence of sphingomyelinase, this did not exceed 10% (Figure 3). Here again, SPH degradation greatly stimulated this effect, PA hydrolysis attaining 35% within 60 min in the presence of 2–10 μg/ml PLA_2 (Figure 3). Finally, during the incubation period, the sum of the radioactivity associated with PA and LPA did not change, indicating a relative metabolic stability of the two compounds.

In additional experiments, microvesicles were incubated with bovine serum albumin (10 mg/ml) after phospholipase treatment. Within 1 min, 63% of [^{32}P]LPA was extracted into the supernatant and reached a maximal value of 74% after 30 min. Finally, to check that the accessibility of microvesicle phospholipids to $sPLA_2$ was not due to the use of a low protein medium, we repeated the phospholipase incubations in the presence of a physiological concentration of albumin (40 mg/ml). Under these conditions, PA hydrolysis by $sPLA_2$ alone (2 μg/ml, 60 min) was increased from 6.7% ± 1.4% to 12.7% ± 3.8% (means ± SEM, three experiments). The stimulating effect of albumin was no longer observed when $sPLA_2$ was used together with sphingomyelinase, corresponding values of PA hydrolysis then being 22.8% ± 2.0% and 21.1% ± 2.1%.

Platelet Aggregation Induced by Microvesicles

As shown in Figure 4 (trace a), LPA induced an extensive aggregation of human platelets, as previously described (Simon et al., 1982). Under the same conditions, microvesicles had a very slight effect (trace b), which hardly increased upon treatment with $sPLA_2$ (trace c). However, microvesicles incubated for 1 hr with a combination of sphingomyelinase and $sPLA_2$ induced significant platelet aggregation (trace d), which was dose dependent (data not shown). In further experiments, microvesicles were incubated with 10 mg/ml bovine serum albumin, and the

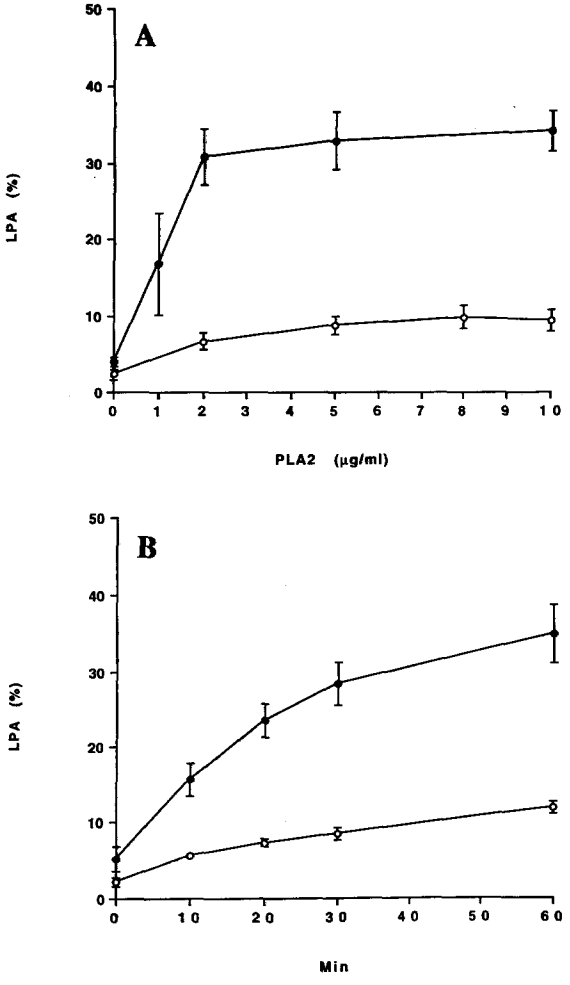

Figure 3. Conversion of [^{32}P]PA into [^{32}P]LPA by sPLA$_2$ in Erythrocyte Microvesicles

In (A), ^{32}P-labeled microvesicles were incubated for 60 min at 37°C with various concentrations of recombinant human sPLA$_2$, in the absence (open circles) or in the presence (closed circles) of sphingomyelinase (0.1 U/ml). In (B), the same procedure was applied except that sPLA$_2$ was used at 2 μg/ml for various incubation times as indicated. PA and LPA were determined for ^{32}P radioactivity as described in the Experimental Procedures. Data (means ± SEM, five and three different experiments in the absence and in the presence of sphingomyelinase, respectively) correspond to the percentages of radioactivity present in LPA, taking as 100% the sum of the radioactivity determined in PA and LPA, which remained constant.

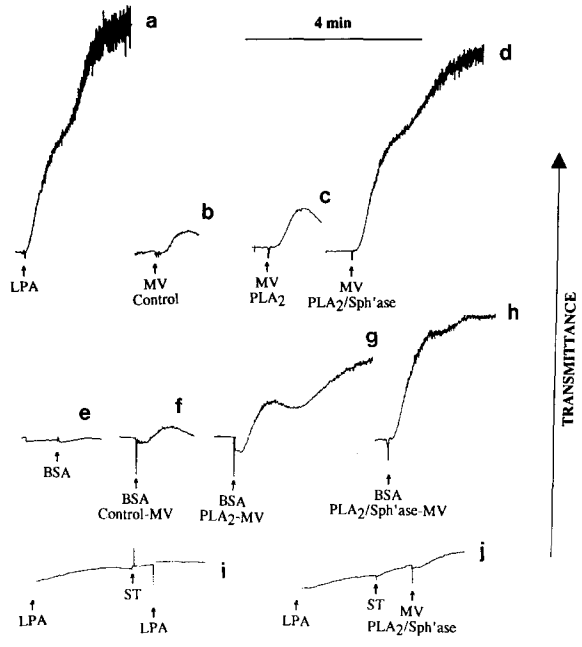

Figure 4. Aggregation of Human Platelets by LPA and by Erythrocyte Microvesicles Treated with sPLA$_2$

Aggregation was determined under stirring (1,000 rpm) by measuring absorbance at 600 nm of platelet suspensions in Tyrode's buffer (300 μl) as described previously (Simon et al., 1982). In trace (a), 1 μl of a 300 μM ethanolic solution of 1-oleoyl-LPA was added at the time indicated by the arrow. Microvesicles (MV, 400 μl) were incubated for 60 min without any addition in traces (b) and (f), in the presence of sPLA$_2$ (2 μg/ml) in traces (c) and (g), or in the presence of sPLA$_2$ (2 μg/ml) plus sphingomyelinase (Sph'ase, 0.1 U/ml) in traces (d) and (h). Microvesicles were then centrifuged at 12,000 × g for 10 min and suspended in 30 μl of Tyrode's buffer. Aggregation was measured upon addition of 3 μl of this final suspension at the times indicated by arrows in traces (b), (c), and (d). In traces (f), (g), and (h), microvesicles were centrifuged at the end of the 60 min incubation, and suspended in 30 μl of Tyrode's buffer containing 10 mg/ml of bovine serum albumin. After equilibration for 10 min at room temperature, microvesicles were sedimented by centrifugation at 12,000 × g for 10 min, and 3 μl of the supernatant were added to the platelet suspension at the times indicated by arrows. Trace (e) shows the lack of effect of 3 μl of the albumin solution (BSA). In trace (i), LPA (1 μM, final concentration) was added as described for trace (a), but without stirring. Stirring (ST) was started as indicated, followed by a second addition of LPA (1 μM, final concentration). In trace (j), the same procedure was used, except that the second addition (MV) corresponded to 3 μl of microvesicles treated with PLA$_2$ and sphingomyelinase as described for trace (d). Data are representative of three independent experiments with identical results.

resulting supernatant was added to platelet suspensions. Although albumin by itself had no effect (trace e), the platelet-aggregating activity associated with microvesicles was recovered in albumin supernatants, in agreement with data reported above for [^{32}P]LPA. The extent of platelet aggregation increased progressively using supernatants from control microvesicles (trace f), from sPLA$_2$-treated microvesicles (trace g), and from microvesicles incubated with sphingomyelinase and sPLA$_2$ (trace h). Finally, LPA did not induce platelet aggregation in the absence of stirring. If platelets were then stirred after 1 min, a second addition of LPA failed to aggregate platelets (trace i). Under these conditions, platelets remain fully responsive to other agonists such as platelet-activating factor, indicating a specific desensitization process (Simon et al., 1982; Sugiura et al., 1994). When microvesicles treated with sphingomyelinase and sPLA$_2$ were added to platelets previously incubated with LPA in the absence of stirring, again aggregation was abolished (trace j). This strongly suggested that LPA present in microvesicles was the main factor involved in their platelet-aggregating activity.

Comparison of Microvesicles Derived from Ca^{2+}-Loaded Erythrocytes with Microvesicles Shed from Aged Red Cells

Previous studies reported that vesicles released from

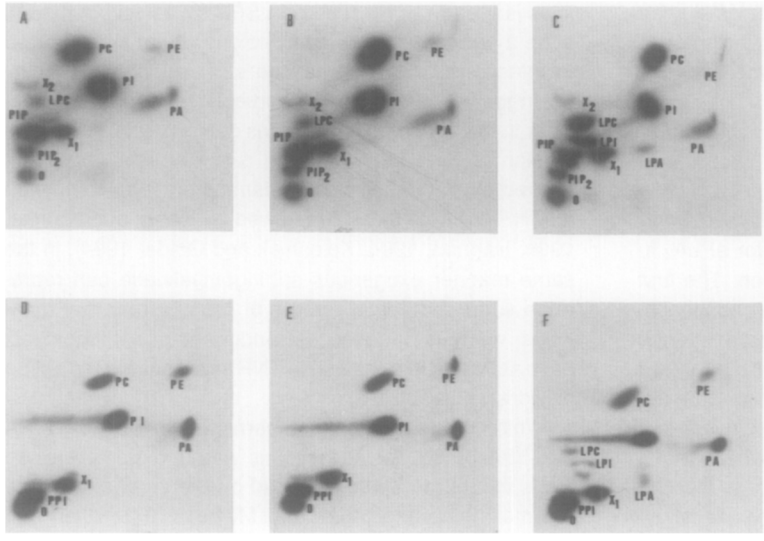

Figure 5. Effects of $sPLA_2$ on ^{32}P-Labeled Phospholipids Present in Vesicles Shed from Platelets Treated with S. aureus α-Toxin or from Whole Blood Cells Incubated with E. coli Lipopolysaccharide

Vesicles shed from platelets treated with S. aureus α-toxin (A, B, and C) or from whole blood cells incubated with E. coli lipopolysaccharide (D, E, and F) were isolated as described in the Experimental Procedures. They were then incubated for 60 min without any addition (A and D), in the presence of 2 μg/ml $sPLA_2$ (B and E), or in the presence of 2 μg/ml $sPLA_2$ plus 0.1 U/ml sphingomyelinase (C and F). Phospholipids were then extracted under acidic conditions, separated by two-dimensional thin-layer chromatography, and detected by autoradiography.

Abbreviations not used in the text: PIP, PI 4-phosphate; PIP2, PI 4,5-bisphosphate; PPI, polyphosphoinositides; LPI, lysoPI; X1 and X2 are unidentified compounds (see for instance Mauco et al., 1978).

aged red cells displayed increased sensitivity to PLC (Shukla et al., 1978). To examine whether this more physiological model would lead to the same effects triggered by $sPLA_2$, vesicles isolated from blood stored for 4 weeks under transfusion conditions were treated with $sPLA_2$ and sphingomyelinase. However, no platelet aggregation was then observed. In agreement with these negative data, aged vesicles were found to contain much less PA (1.1% ± 0.4%, mean ± SEM, four determinations) than vesicles obtained with calcium ionophore (5.7% ± 0.7%, eight determinations). Indeed, a similar difference between the two types of vesicles was also reported by Allan et al. (1976). This suggested that production of LPA by $sPLA_2$ might be restricted to pathological conditions, which are also circumstances under which $sPLA_2$ accumulates in biological fluids.

Effects of $sPLA_2$ on Microvesicles Shed from Platelets or from Whole Blood Cells Treated with S. aureus α-Toxin or with E. coli Lipopolysaccharide

To define more biological systems likely to react to $sPLA_2$ in a similar way as vesicles shed from Ca^{2+}-loaded erythrocytes, ^{32}P-labeled platelets were treated with S. aureus α-toxin. This toxin is thought to play a major role in pathological events accompanying sepsis (Bhakdi et al., 1994). Phospholipids from vesicles isolated under these conditions displayed the same susceptibility to $sPLA_2$ as red cell microvesicles. In the presence of sphingomyelinase, $sPLA_2$ achieved a significant degradation of most labeled phospholipids, i.e., PC, phosphatidylinositol (PI), and PA, which were converted into the corresponding lysophospholipids (Figures 5A–5C).

Very similar data (see Figures 5D–5G) were obtained upon incubation of whole blood cells with lipopolysaccharide, which also plays a central role in septic shock, including direct cellular effects (Vadas et al., 1993) and activation of the alternative complement pathway (Minta and Gee, 1983). Other pathological in vitro models were also examined, including treatment of whole blood cells with S. aureus α-toxin or with zymosan, and they gave almost identical results (data not shown).

Isolation of Microvesicles from Inflammatory Fluids

To have at least one example of similar vesicles occurring under in vivo pathological conditions, inflammatory fluids obtained from patients suffering from arthritis were examined for the presence of vesicle material. As shown in Table 2, vesicles particularly rich in LPC and LPE could be isolated, strongly suggesting the contribution of $sPLA_2$ detected in supernatants. Vesicles were present at a concentration of 7.4 nmol of phospholipids/ml, which is 10-fold lower than the concentrations used in our in vitro incubations. However, the concentration of $sPLA_2$ could be estimated at 1.2 μg/ml, since pure recombinant $sPLA_2$ displayed a specific activity of 60 mU/μg protein using the same assay. This indicated that the ratio $sPLA_2$ to vesicle phospholipids in the inflammatory fluids was in the same range as that used during our in vitro experiments (10 μg/ml $sPLA_2$ for 80–120 nmol/ml phospholipids). To verify that the presence of $sPLA_2$ and vesicles reflected an inflamma-

Table 2. PLA_2 Activity and Vesicle Phospholipid Analysis in Inflammatory Fluids from Patients Suffering from Arthritis

PLA_2 activity	71.3 ± 29.6 mU/ml
Total phospholipids	7.4 ± 1.8 nmol/ml
Phospholipid composition	
LPC	11.3% ± 2.5%
LPE	13.5% ± 0.5%
SPH	20.4% ± 4.9%
PC	24.8% ± 2.3%
PS	4.8% ± 4.8%
PE	22.5% ± 4.6%
Others	2.7% ± 1.6%

Inflammatory fluids were drawn from knees of patients suffering from arthritis (two rheumatoid arthritis, one Churg and Strauss syndrome). Vesicles were isolated and analyzed for phospholipids as described in the Experimental Procedures. The 200,000 × g supernatant was determined for PLA_2 activity according to Radvanyi et al. (1989). Data are means ± SEM of three values.

tory process, 1 ml of fluid was collected from the knee of a patient suffering from a small arthrosis lesion on a meniscus. PLA_2 activity was only 1 mU/ml, whereas vesicles were absent.

Discussion

The present investigation led to three interesting observations, allowing us to propose a novel role for $sPLA_2$ in cell signaling, particularly during inflammation. The first concerns the ability of the enzyme to degrade phospholipids in vesicles shed from Ca^{2+}-loaded erythrocytes. Since internal phospholipids such as PE and PS were hydrolyzed under nonlytic conditions, this promotes the view that the loss of membrane phospholipid asymmetry plays a key role in the capacity of $sPLA_2$ to penetrate into the external layer of the vesicle membrane. We thus propose that the "membrane rearrangement" suggested by Kudo et al. (1993) to allow interaction of $sPLA_2$ with mammalian cells could involve the movement of phospholipids from the internal to the external leaflet of the membrane.

From a functional point of view, the loss of membrane phospholipid asymmetry has mainly been considered in relation to blood coagulation (Zwaal et al., 1992) and to recognition by monocytes/macrophages of cells undergoing in vivo clearing (Schwartz et al., 1985; Connor et al., 1994; Fadok et al., 1992). Our finding suggests a new meaning for the loss of membrane phospholipid asymmetry, which might regulate the accessibility of cell membranes to $sPLA_2$. So now a main question is to know whether similar vesicles can be formed under physiological or pathological conditions allowing a possible contact with $sPLA_2$.

As a first attempt to evaluate the biological relevance of our experimental model, we were able to isolate from inflammatory fluids microvesicles displaying large amounts of lysophospholipids bathing in a medium containing a relative proportion of $sPLA_2$ very close to that used during in vitro incubations. Also, in an effort to outline possible pathological processes responsible for microvesicle shedding, S. aureus α-toxin and lipopolysaccharide were found to generate from platelets and from whole blood cells, respectively, microvesicles displaying the same sensitivity to $sPLA_2$ as that previously observed using Ca^{2+}-loaded erythrocytes. Whereas the role of lipopolysaccharide in E. coli–induced septic shock is well understood, the model using α-toxin is particularly interesting for three reasons. First, S. aureus α-toxin is an example of a pore-forming protein promoting Ca^{2+}-dependent shedding of microvesicles and loss of membrane phospholipid asymmetry by a mechanism identical to that of the complement membrane-attack complex (Arvand et al., 1990; Chang et al., 1993; Hamilton et al., 1990; Stein and Luzio, 1991). Second, a recent review outlined the possible role of this and other similar bacterial exotoxins in the pathogenesis of sepsis syndrome and septic shock (Bhakdi et al., 1994). Third, S. aureus also secretes β-toxin, which is identical to sphingomyelinase C used in the present study.

This should lend more importance to our second conclusion that the activity of $sPLA_2$ toward membrane microvesicles is greatly increased upon SPH hydrolysis. Although such a dual effect of α- and β-toxins would be restricted to infections by S. aureus, the stimulation of $sPLA_2$ by sphingomyelinase might be particularly important to consider in the light of recent studies demonstrating that conversion of SPH into ceramide is a key signaling event evoked by a number of proinflammatory cytokines such as TNF or interleukin-1 (reviewed by Heller and Krönke, 1994; Hannun, 1994; Kolesnick and Golde, 1994). In the same manner exogenous sphingomyelinase can reproduce some biological effects of proinflammatory cytokines, we thus suggest that endogenous sphingomyelinase activated in response to TNF might enable the action of extracellular $sPLA_2$.

A third important result concerns the conversion of PA into LPA by $sPLA_2$. At first, this was unexpected, since PA is thought to be synthesized on the inner side of the plasma membrane (Allan and Michell, 1975; Allan and Thomas, 1981). The fact that hydrolysis levels as high as 35% could be measured under nonlytic conditions shows unambiguously that PA itself undergoes an outward movement in the membrane of microvesicles. Thus, $sPLA_2$ could play a key role in the generation of LPA. It will be important to identify which vesicles other than those investigated herein would display loss of membrane asymmetry, SPH hydrolysis, and accumulation of PA as a consequence of cell activation. Vesicles derived from cells treated by TNF or complement membrane-attack complex appear as interesting potential candidates, since both agents were previously shown to activate SPH hydrolysis and the degradation of PC by a specific PLC (Schutze et al., 1991; Niculescu et al., 1993).

Finally, although PLA_2-treated microvesicles promoted platelet aggregation, albumin was found to extract almost totally both LPA and platelet-aggregating activity, in agreement with previous observations (Eichholtz et al., 1993). This suggests that albumin is likely to be a major presenter of LPA during inflammatory responses. Also, despite the results of specific desensitization, it is not certain that all the platelet-aggregating activity is supported by LPA alone, since other lipid derivatives such as arachidonic acid liberated by $sPLA_2$ could also be involved.

In conclusion, the use of a model system of microvesicles shed from Ca^{2+}-loaded erythrocytes allowed us to propose some new concepts concerning the possible mechanism and role of $sPLA_2$, which have remained obscure until now. The fact that loss of membrane phospholipid asymmetry and sphingomyelin hydrolysis regulate the activity of $sPLA_2$ should not mask a complementary role of cofactor proteins such as phospholipase-activating protein or PLAP (Clark et al., 1991). Finally, although we have mainly focused our study on LPA, other lysophospholipids generated by $sPLA_2$ should be considered, since LPC can activate some isoforms of protein kinase C (Asaoka et al., 1993), whereas LPS stimulates mast cell degranulation (Murakami et al., 1992).

Experimental Procedures

Materials

Calcium ionophore A23187, dilauroyl-PC, D-sphingosine from bovine

brain SPH, PLA_2 from pig pancreas, sphingomyelinase and α-toxin from S. aureus, lipopolysaccharide from E. coli K235, 1-oleoyl-LPA, and bovine serum albumin, essentially fatty acid free, were obtained from Sigma. [^{32}P]o-phosphate was from Amersham. Silica gel 60 plates (0.25 mm thick) were obtained from Merck. N-acetyl-D-sphingosine (C2-ceramide) was synthesized by acetylation of D-sphingosine with acetic anhydride as described (Gaver and Sweeley, 1966).

Preparation of Recombinant Human $sPLA_2$

This was adapted from two previous studies as follows (Pernas et al., 1991; Franken et al., 1992). Total cDNA was prepared from human placenta mRNA by reverse transcriptase in the presence of poly(dT) primer using the Superscript kit from BRL. The cDNA coding for human $sPLA_2$ was amplified by PCR using Taq DNA polymerase (Beckman) and a Perkin Elmer thermal cycler. Primers were synthesized by the solid phase phosphoramidite method on an Applied Biosystem 391 A automated oligonucleotide synthesizer. Upstream primer (5'-GCGGCGAATTCATGAATTTGGTGAATTTCCACAGACTGATC-3') contained an XbaI restriction site and a Met-8→Leu mutation of the unique methionine residue present in the sequence of human $sPLA_2$, the nucleotide modification being underlined in the sequence of the primer given above. Downstream primer (5'-AAAAGGGTCGACAGGGAAGAGGGGACTCAAGT-3') included a stop codon and a SalI restriction site. With the exception of the Met-8→Leu mutation, the amplified cDNA thus coded for the sequence covering from Asn-1 to the C-terminus of mature human $sPLA_2$ (Seilhammer et al., 1989; Kramer et al., 1989). The PCR product was purified, sequenced, and inserted into the polylinker of the expression plasmid pGEX-2T (Pharmacia) using XbaI and SalI restriction sites. The resulting fusion plasmid (pGEX-PLA_2) coding for a fusion protein glutathione S-tranferase–$sPLA_2$ was used to transform E. coli strain XL 1 Blue (Stratagene). Expression of the fusion protein was induced by isopropyl-β-D-thiogalactoside, inclusion bodies isolated after cell lysis were solubilized in 50 mM Tris–HCl (pH 8.0) containing 6 M guanidine, proteins were S-sulfonated according to Thannhauser and Scheraga (1985), and hydrolyzed with a 70-fold molar excess of CNBr in 75% formic acid (v/v). After dialysis against 5 mM acetic acid, soluble material was freeze dried, the resulting powder being solubilized in 3 M guanidine. Disulfide bridges were formed upon dialysis against 5 mM $CaCl_2$, 5 mM cysteine, and 0.8 M guanidine. Active PLA_2 was recovered by dialysis against 50 mM acetate buffer (pH 4.5) containing 150 mM NaCl. The renatured enzyme was purified by two successive runs on an SP–Sephadex C-25 column (Pharmacia) and eluted by a linear NaCl gradient (0.15–2 M) in 50 mM acetate buffer (pH 6.0).

Reverse Phase HPLC

This was performed as previously described (Rugani et al., 1992) using a 4 × 120 mm Nucleosil C18 column equilibrated at room temperature with 0.1% (v/v) trifluoroacetic acid in water and developed at 1 ml/min with a linear gradient of acetonitrile (5%–60%) in 0.1% trifluoroacetic acid. In each run, 100 μg of purified PLA_2 was injected. Alternatively, PLA_2 was previously reduced and carboxymethylated before HPLC analysis. This was achieved by incubating protein with 0.033 M dithiothreitol for 5 min at 96°C, followed by alkylation with 0.166 M iodoacetate for 30 min at 37°C.

Determination of PLA_2 Activity

Recombinant $sPLA_2$ was determined for enzyme activity using dilauroyl-PC as a substrate (Franken et al., 1992). Incubation was performed for 15 min at 37°C, lipids were extracted and separated by thin-layer chromatography, and the amount of LPC formed during the reaction was measured as described elsewhere (Gassama-Diagne et al., 1992). Alternatively, PLA_2 activity was determined using a substrate fluorescent assay (Radvanyi et al., 1989).

Preparation of Microvesicles Shed from Ca^{2+}-Loaded Erythrocytes

This was achieved essentially according to Allan et al. (1980). Fresh blood obtained from normal volunteer donors was drawn onto 0.129 M buffered sodium citrate (1 part to 9 parts of blood, Becton Dickinson Vacutainer System). Red cells were isolated by centrifugation at 200 × g (room temperature) and washed three times by centrifugation at 400 × g in buffer A containing 140 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 5 mM glucose, 10 mM HEPES (pH 7.4). Erythrocytes were suspended in buffer A at 5 × 10^6 cells/ml, $CaCl_2$ (1 mM, final concentration) was then added, followed by 2 μM A23187, and incubation was performed at 37°C for 45 min under magnetic stirring. Intact cells were eliminated by centrifugation at 1,500 × g for 15 min, and the supernatant was centrifuged at 13,000 × g for 20 min, allowing it to obtain a microvesicle pellet. In preliminary experiments, a relationship was established between the hemoglobin level of the vesicles lysed in water and their phospholipid content. In this way, vesicle concentration in buffer A was adjusted in order to contain 80–120 nmol of phospholipid/ml, lipid phosphorus content being determined accurately at the end of the various incubations performed afterward. In those experiments dealing with the production of PA and LPA, red cells were incubated for 1 hr in buffer A containing 15 μCi/ml of [^{32}P]o-phosphate, and ^{32}P-labeled vesicles were isolated exactly as described.

Preparation of Microvesicles from Platelets or Whole Blood Cells Treated with α-Toxin or Lipopolysaccharide

Human blood platelets (5 × 10^8 cells) isolated according to Ardlie et al. (1970) were labeled with [^{32}P]o-phosphate (200 μCi/ml, 1 hr, 37°C) and, after washing, were suspended at the same concentration in buffer A containing 1 mM $CaCl_2$. After incubation for 30 min at 37°C with 2 μg/ml α-toxin, platelets were sedimented at 1,500 × g for 15 min, and vesicles were isolated at 13,000 × g for 15 min.

Heparinized human blood (15 ml) was incubated with [^{32}P]o-phosphate (500 μCi/ml), diluted with 35 ml RPMI, and incubated under sterile conditions for 19 hr at 37°C in the presence of lipopolysaccharide (10 μg/ml). Cells were then discarded by centrifugation at 1,500 × g for 30 min, and vesicles were sedimented at 200,000 × g for 30 min.

Incubation of Microvesicles with Phospholipases

Suspensions of microvesicles in buffer A containing 1 mM $CaCl_2$ were incubated at 37°C for various times under magnetic stirring, in the absence or in the presence of sphingomyelinase (0.1 U/ml), and various concentrations of recombinant $sPLA_2$ or pig pancreas PLA_2 (1–10 μg/ml). At the end of the incubation, 5 mM EDTA (final concentration) was added, followed by 2 vol of chloroform/methanol (1/1 by vol). In some experiments, the suspension was centrifuged at 12,000 × g for 10 min and the hemoglobin content of the supernatant was estimated from the absorbance at 540 nm. This indicated the absence of lysis during the various treatments of the microvesicles.

Phospholipid Analysis

Lipids were extracted according to Bligh and Dyer (1959) or by a modified procedure including acidification with concentrated HCl (60 μl/ml suspension) when LPA was analyzed (Mauco et al., 1978). After phase separation, the organic layer was dried under nitrogen and dissolved in chloroform/methanol (1/1 by vol). One aliquot was used for phosphorus determination (Böttcher et al., 1961), major phospholipids present in the remaining extract being separated by two-dimensional thin-layer chromatography using chloroform/methanol/20% NH_3 (65/25/5, by vol) and chloroform/methanol/acetic acid/water (45/20/6/1) as solvents. The various spots were detected upon exposure to iodine vapor, scraped off, and determined for phosphorus content. When [^{32}P]PA and [^{32}P]LPA were analyzed, lipids were separated by one-dimensional thin-layer chromatography on silica gel 60 plates impregnated with 0.25 M oxalic acid as described (Mauco et al., 1978), using chloroform/methanol/12 M HCl (87/13/0.5, by vol) as a solvent. Radioactive phospholipids were detected by autoradiography, scraped off, and determined for ^{32}P radioactivity by scintillation counting.

Isolation of Microvesicles from Inflammatory Exudates

Inflammatory fluids were obtained by direct punction into the knees of patients suffering from arthritis (two rheumatoid arthritis, one Churg and Strauss syndrome) and anticoagulated with EDTA. Cells were eliminated by centrifugation at 1,500 × g for 30 min; vesicles were pelleted at 200,000 × g for 30 min and washed once in buffer A before lipid extraction. The first 200,000 × g supernatant was used for determination of PLA_2 activity.

Miscellaneous

Platelet aggregation was measured as previously described using sus-

pensions of human platelets (Simon et al., 1982). Protein was determined according to Bradford (1976) using bovine serum albumin as a standard. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was performed according to Laemmli (1970) using 15% polyacrylamide.

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