Human group II 14 kDa phospholipase A₂ activates human platelets

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Recombinant human group II phospholipase A₂ (sPLA₂) added to human platelets in the low µg/ml range induced platelet activation, as demonstrated by measurement of platelet aggregation, thromboxane A₂ generation and influx of intracellular free Ca²⁺ concentration and by detection of time-dependent tyrosine phosphorylation of platelet proteins. The presence of Ca²⁺ at low millimolar concentrations is a prerequisite for the activation of platelets by sPLA₂. Mg²⁺ cannot replace Ca²⁺. Mg²⁺, given in addition to the necessary Ca²⁺, inhibits sPLA₂-induced platelet activation. Pre-exposure to sPLA₂ completely blocked the aggregating effect of a second dose of sPLA₂. Albumin or indomethacin inhibited sPLA₂-induced aggregation,

similarly to the inhibition of arachidonic acid-induced aggregation. Platelets pre-treated with heparitinase or phosphatidylinositol-specific phospholipase C lost their ability to aggregate in response to sPLA₂, although they still responded to other agonists. This suggests that a glycosphosphatidylinositol-anchored platelet-membrane heparan sulphate proteoglycan is the binding site for sPLA₂ on platelets. Previous reports have stated that sPLA₂ is unable to activate platelets. The inhibitory effect of albumin and Mg²⁺, frequently used in aggregation studies, and the fact that isolated platelets lose their responsiveness to sPLA₂ relatively quickly, may explain why the platelet-activating effects of sPLA₂ have not been reported earlier.

INTRODUCTION

Phospholipase A₂ (PLA₂; EC 3.1.1.4) hydrolyses the *sn*-2 fatty acyl ester bond of glycerophospholipids, producing non-esterified fatty acids and lysophospholipids. PLA₂s occur in almost every mammalian cell type and play an important role in the metabolism of phospholipids and in the production and regulation of prostaglandins, thromboxanes (TXs) and platelet-activating factor (reviewed in [1,2]). The non-esterified fatty acid liberated by PLA₂s is often arachidonic acid (AA). AA is converted by the cyclo-oxygenase pathway into a variety of prostaglandins and TXs [3,4], or by the lipoxygenase pathway to generate hydroxy-eicosatetraenoic acids and leukotrienes [5]. Based on their amino acid sequences, PLA₂s have been divided into main groups and several subgroups (reviewed in [6]). The three best characterized human PLA₂s are the pancreatic secretory PLA₂ (group I) [7,8], the non-pancreatic secretory PLA₂ (group II) [9,10] and the cytosolic PLA₂ (cPLA₂, group IV) [11,12].

Two types of PLA₂ have been described and characterized in platelets so far. Experimental evidence strongly suggests that the 85 kDa cPLA₂ is responsible for agonist-induced AA liberation in platelets [13]. Human group II PLA₂ (or non-pancreatic secretory PLA₂, group II secretory PLA₂ or platelet secretory PLA₂; sPLA₂), the subject of this paper, has a molecular mass of 14 kDa [9,10]. Low-molecular-mass PLA₂s were also found in various snake and bee venoms [14] and the platelet-aggregating effect of venom PLA₂s has been known for more than 15 years [15]. In spite of the strong similarity to venom PLA₂s, the platelet-activating effects of sPLA₂ have not previously been described. Notably, although sPLA₂ can be released from various human cells (reviewed in [16]) and platelets are postulated to be one of the potential sources of sPLA₂ in pathological situations [9], recent reports suggest that extracellular sPLA₂ has no effect

on platelets [2,17–19]. The original aim of our study was to investigate whether extracellular sPLA₂ affected platelet activation induced by various agonists. We were surprised to find that sPLA₂ itself induced platelet activation.

In this report, the platelet-activating effects of sPLA₂ are described, the different experimental conditions used here and in other reports, which found no effect of sPLA₂ on platelets, are discussed, and a model for the basis of sPLA₂-induced platelet activation is proposed.

MATERIALS AND METHODS

Materials

sPLA₂ [9], specific sPLA₂ inhibitor LY311727 [20], thrombin-receptor-activating peptide SFLRN-NH₂ [21] and low-molecular-mass thrombin inhibitor LY288570 [22], were produced as described earlier. Methyl arachidonyl fluorophosphonate (MAFP) [23] was obtained from Cayman Chemicals. Sodium arachidonate, indomethacin, heparinase III (heparitinase I; EC 4.2.2.8.), ionomycin, fura 2/AM, peroxidase-conjugated goat anti-mouse antibodies, 1-oleoyl-lysophosphatidic acid, egg yolk lysophosphatidylcholine (LPC) and fatty-acid-free human serum albumin (HSA) and BSA were from Sigma (St. Louis, MO, U.S.A.), phosphatidylinositol-specific phospholipase C (PI-PLC) was from Boehringer-Mannheim (Germany), Iloprost was kindly given by Schering (Zürich, Switzerland) and Sepharose 2B was from Pharmacia (Uppsala, Sweden). Anti-phosphotyrosine monoclonal antibody 4G10 was from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Monoclonal anti-p60^{c-src} (sc-19) and anti-p72^{syk} (4D10) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). The

Abbreviations used: PLA₂, phospholipase A₂; sPLA₂, group II human PLA₂; cPLA₂, cytosolic 85 kDa PLA₂; PI-PLC, phosphatidylinositol-specific phospholipase C; GPI, glycosphosphatidylinositol; AA, arachidonic acid; LPA, lysophosphatidic acid; LPC, lysophosphatidylcholine; TX, thromboxane; HSA, human serum albumin; PRP, platelet-rich plasma; MAFP, methyl arachidonyl fluorophosphonate; [Ca²⁺]_i, intracellular Ca²⁺ concentration.

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SuperSignal chemiluminescence detection system was from Pierce (Rockford, IL, U.S.A.) and autoradiography (Fuji RX) films were from Fujifilm.

Isolation of platelets from buffy coats

Human platelets were isolated from buffy coats, less than 20 h after blood collection (obtained from the Central Laboratory of the Swiss Red Cross Blood Transfusion Service) by washing or gel filtration. Isolation of washed platelets from buffy coats was performed as described previously [24]. Isolation of gel-filtered platelets from buffy coats was performed as follows. To one buffy coat was added 6 ml of 100 mM citrate, pH 6.5, and platelet-rich plasma (PRP) was prepared by centrifugation. PRP (5–7 ml) was loaded on to a 1.6 cm × 25 cm Sepharose 2B column, equilibrated with 3 mM Mes/4 mM KCl/140 mM NaCl/5.5 mM glucose, pH 6.5 (buffer A), and eluted with buffer A. The platelet count was adjusted to 4×10^8 – 1×10^9 platelets/ml by dilution with 20 mM Hepes/140 mM NaCl/4 mM KCl/5.5 mM glucose, pH 7.4 (buffer B). Samples were kept at room temperature until used for aggregation studies.

Isolation of platelets from freshly drawn blood

Blood (6 vol.) was drawn from apparently healthy volunteers, not taking any medication for at least 10 days previously, by venipuncture through 19-gauge butterfly needles, and was mixed gently by inversion with 1 vol. of 85 mM trisodium citrate/65 mM citric acid/110 mM glucose, pH 4.5. PRP was prepared by centrifugation and platelets were isolated by washing or gel filtration as described above with the following modification. The PRP was gently concentrated before gel filtration by centrifugation at 300 g for 3 min and removing the upper two-thirds of the sample.

Preparation of thrombin-inhibitor-anticoagulated PRP

LY288570, a low-molecular-mass direct thrombin inhibitor [22], was used at a final concn. of 20 µg/ml in whole blood and PRP was prepared in the standard fashion. This platelet preparation allowed study of platelet responses at physiological Ca^{2+} levels.

Platelet aggregation studies

Platelet aggregation was monitored by light transmission [25] with continuous stirring at 1000 rev./min and 37 °C. Platelets were preincubated in buffer containing 2 mM CaCl_2 at 37 °C before adding the agonists in a volume of less than 5% of the total. In some experiments, where indicated, MgCl_2 , HSA, BSA or indomethacin were also added to the samples during the preincubation.

Measurement of intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$)

$[\text{Ca}^{2+}]_i$ was measured using fura 2/AM as described by Grynkiewicz et al. [26]. Briefly, 1 vol. of isolated platelets (1×10^9 /ml) was incubated in buffer B containing 2 mM CaCl_2 and 4 µM fura 2/AM at 37 °C for 20 min. After centrifugation (1200 g for 3 min), platelets were resuspended in 10 vol. of buffer B with 2 mM CaCl_2 . The measurement of $[\text{Ca}^{2+}]_i$ after adding various agonists was performed at 37 °C, and the samples were stirred at 1300 rev./min. The fluorescence of fully saturated fura 2 was obtained by adding 1 µM ionomycin. The fluorescence was fully quenched with 1 mM MnCl_2 . Data were converted into $[\text{Ca}^{2+}]$ by using a K_d value of 135 nM for fura 2 and Ca^{2+} .

Measurement of TXA_2 formation

Platelet TXA_2 formation was measured by specific radioimmunoassay of its stable metabolite TXB_2 as described previously [27].

Treatment of platelets with heparitinase and PI-PLC

Isolated platelets (6×10^8 /ml) in buffer B were stirred in the cuvette of the aggregometer at 1000 rev./min and 37 °C with continuous monitoring of light transmission. CaCl_2 (2 mM) and heparitinase I (0.1–2 units/ml) were added to one sample and CaCl_2 but no heparitinase I to another at the same time. After 10 min, 8 µg/ml sPLA_2 or 50 nM AA were added to both samples and platelet aggregation was monitored. The same procedure was performed using PI-PLC (0.01–0.2 unit/ml of enzyme; 2 min) instead of heparitinase.

Treatment of platelets with the specific PLA_2 inhibitor LY311727

Isolated platelets were preincubated (1 min) with various concentrations of LY311727 before stimulation with 8 µg/ml sPLA_2 . Platelet samples activated with the thrombin-receptor-activating peptide SFLLRN-NH₂ (1 µM) were used as the control in these experiments.

sPLA_2 -induced tyrosine phosphorylation of platelet proteins

Isolated platelets (745 µl, 6×10^8 /ml in buffer B containing 2 mM CaCl_2) in the absence and presence of 50 µM indomethacin or 0.2% (w/v) fatty-acid-free BSA, were stirred at 1000 rev./min and 37 °C for 2 min. A 45 µl portion from this sample was added to 5 µl of 10% (w/v) SDS/50 mM EDTA/20 mM Na_3VO_4 /10 mM *N*-ethylmaleimide/30 mM Hepes, pH 8.0. The sample was mixed vigorously and put on ice. After adding 2.8 µl of 2 mg/ml sPLA_2 to the remaining 700 µl sample, additional 45 µl probes were treated similarly at various times. These samples were centrifuged and the supernatants were prepared for electrophoresis on a 7–17% (w/v) gradient SDS/polyacrylamide gel by the method of Laemmli [28]. Proteins were transferred to PVDF membrane and phosphotyrosine-containing proteins were detected on the blot using 4G10 anti-phosphotyrosine antibody, peroxidase-conjugated goat anti-mouse second antibodies and a chemiluminescence detection system. $\text{p60}^{\text{c-src}}$ and p72^{syk} were identified using monoclonal antibodies sc-19 and 4D10 respectively.

RESULTS

sPLA_2 -induced platelet aggregation

sPLA_2 added to platelets induced aggregation. In preliminary experiments the optimal Ca^{2+} concentration for sPLA_2 -induced aggregation was found to be 1–3 mM (results not shown). Based on this the aggregation studies presented here were performed in the presence of 2 mM CaCl_2 . Different platelet samples exhibited different aggregation patterns with various concentrations of sPLA_2 . Figure 1 shows two representative platelet samples. No relationship could be found between the degree of aggregation induced by a particular concentration of sPLA_2 and the source of platelets, freshly drawn blood ($n = 10$) or buffy coats ($n = 22$), or isolation method, washing ($n = 21$) or gel filtration ($n = 11$). However, isolated platelets lost their ability to aggregate to sPLA_2 much faster than to other agonists, including AA, ristocetin–von Willebrand factor and thrombin. In the case of the sample shown in Figure 1(B), 4 µg/ml sPLA_2 , which induced a strong signal with freshly isolated platelets, induced an aggregation pattern similar to those with 2, 1 and 0.4 µg/ml sPLA_2 .

Figure 1 Aggregation pattern of human platelets induced by various concentrations of sPLA₂

Isolated platelets were preincubated with 2 mM CaCl₂ at 37 °C for 2 min before adding the agonist at the time points indicated by arrows. All of the platelet samples isolated from freshly drawn blood ($n = 10$) and buffy coats ($n = 22$) by gel filtration ($n = 11$) or washing ($n = 21$) were analysed with 8 µg/ml sPLA₂, and several samples were tested with various other concentrations of sPLA₂ as well. (A) and (B) show two representative platelet samples. (A) A sample which gave a weak response to sPLA₂; (B) a sample which gave a strong response to sPLA₂. No relationship could be found between the degree of aggregation induced by a particular concentration of sPLA₂ and the source of platelets, freshly drawn blood or buffy coats, or isolation method, washing or gel filtration. The inset shows the scales: on the ordinate 20% of the maximum change in light transmission, and a 1 min interval on the abscissa.

Table 1 Specific PLA₂ inhibitor LY311727 inhibits sPLA₂-induced platelet aggregation

Isolated platelets were preincubated with 2 mM CaCl₂ and various concentrations of LY311727 at 37 °C for 1 min before stimulation with 8 µg/ml sPLA₂ or 1 µM SFLLRN-NH₂; nd, not determined. Results are given as percentage aggregation induced by sPLA₂ or SFLLRN-NH₂ and are means $\pm$ S.D. ($n = 3$).

[LY311727] (µM)	Aggregation (%)	
	sPLA ₂	SFLLRN-NH ₂
0	45 $\pm$ 7	54 $\pm$ 7
0.03	48 $\pm$ 6	nd
0.1	36 $\pm$ 7	nd
1	9 $\pm$ 3	nd
10	0	50 $\pm$ 6

in fresh platelets when the sample was stored at room temperature for 60, 90 and 120 min respectively. Storage of the samples at 37 °C rather than room temperature and/or the presence of 2 mM CaCl₂ during the storage resulted in faster 'desensitization' of platelets to sPLA₂. In general, when platelets no longer responded to 4 µg/ml sPLA₂, even 20 times more, 80 µg/ml sPLA₂ was also completely ineffective, although these platelets still reacted to other agonists.

Specific PLA₂ inhibitor LY311727 prevents sPLA₂-induced platelet aggregation

LY311727, a specific inhibitor of sPLA₂ [20], inhibited sPLA₂-induced platelet aggregation in a concentration-dependent manner (Table 1). The use of highly purified recombinant PLA₂ and the fact that LY311727 could block completely the effect of

Figure 2 sPLA₂ induces Ca²⁺ influx in human platelets

Isolated platelets were loaded with fura 2 (see the Materials and methods section) and treated with sPLA₂ (sPLA₂) and/or thrombin as indicated.

sPLA₂ on platelets (Table 1), clearly show that it is sPLA₂ which is responsible for the platelet activation presented here.

In addition to the detection of platelet aggregation, a platelet-activating effect of sPLA₂ was demonstrated by measurement of influx of [Ca²⁺]_i and by detection of tyrosine phosphorylation of platelet proteins.

sPLA₂-induced elevation in [Ca²⁺]_i

sPLA₂ induced Ca²⁺ influx in fura 2-loaded platelets (Figure 2). The maximum Ca²⁺ influx induced by 4 µg/ml sPLA₂ was 40% of that induced by 1 nM thrombin. After a transient rise in [Ca²⁺]_i, a second dose of sPLA₂ did not induce Ca²⁺ influx, although thrombin was still effective (Figure 2A). The thrombin-induced signal was slightly inhibited in platelet samples previously exposed to sPLA₂ (Figure 2A). As in aggregation experiments, discussed below, even a small first dose of sPLA₂, inducing only a minor Ca²⁺ influx, blocked the effect of a second dose of sPLA₂, which otherwise induced a strong signal (Figure 2B).

sPLA₂ induces tyrosine phosphorylation of platelet proteins

A transient rise in phosphorylation levels of bands corresponding to p72^{syk} and several other proteins with molecular masses from 14 to 120 kDa, over the time scale of the reversible aggregation/Ca²⁺ elevation, was observed (Figure 3A). In the presence of 50 µM indomethacin, which inhibited platelet aggregation completely, the signalling was delayed and the pattern was somewhat different (Figure 3B). However, no sPLA₂-dependent tyrosine phosphorylation was observed in the presence of 0.2% fatty-acid-free BSA (results not shown).

Figure 3 sPLA₂ induces tyrosine phosphorylation of platelet proteins

Isolated platelets were exposed to the effect of 8 µg/ml sPLA₂ in the absence (A) and presence (B) of 50 µM indomethacin for the times indicated at the top of the lanes. Phosphotyrosine-containing proteins (p72^{syk} and p60^{c-src} shown on the right) were detected by SDS/PAGE/immunoblotting (see the Materials and methods section). Molecular-mass markers are shown on the left.

Experimental conditions inhibiting sPLA₂-induced platelet aggregation

The finding that sPLA₂ aggregates platelets is different from earlier reports and calls into question the generally accepted idea that, without any stimulus inducing membrane rearrangements, sPLA₂ itself has no effect on platelets [2,17,18]. In order to assess the possible reasons for negative results with sPLA₂ [17–19] and why, in spite of the availability of recombinant sPLA₂ for more than 6 years [9,10], platelet aggregation induced by sPLA₂ has not been described previously, the experimental conditions used for platelet aggregation studies were evaluated. MgCl₂, at concentrations of 0.5 mM and 1 mM, in the presence of 2 mM CaCl₂, inhibited the aggregation by 68(±9)% and 91(±6)% respectively (means ± S.D., *n* = 3). So, in the presence of 1 mM MgCl₂, which is frequently used in buffers for aggregation studies [25], the inhibition was almost complete. Indomethacin (50 µM) blocked aggregation to sPLA₂ completely. The presence of 0.2 %

Table 2 Inhibition of cPLA₂ does not affect TX generation induced by sPLA₂

Platelets were stirred with either no addition or with 8 µg/ml sPLA₂. After 6 min, 10 µM indomethacin was added, platelets were removed by centrifugation and the supernatant was collected. In subsequent experiments, platelet samples were preincubated with 5 µM MAFP or 0.1% (v/v) DMSO for 5 min at 37 °C before adding sPLA₂. TXB₂ was measured in all samples by radioimmunoassay (means ± S.D.; *n* = 3–6).

Addition	TXB ₂ (pg/10 ⁵ platelets)
No addition	0.92 ± 0.10
sPLA ₂	7.19 ± 1.09
sPLA ₂ , DMSO	5.53 ± 0.52
sPLA ₂ , DMSO, MAFP	5.62 ± 0.92

(w/v) HSA or BSA, commonly used in buffers for aggregation studies, also completely blocked the aggregating effect of sPLA₂.

sPLA₂-induced TXA₂ generation is not effected by inhibition of cPLA₂

The inhibitory effect of indomethacin and serum albumin suggests that, as with venom PLA₂s [15,17,29], AA liberation and metabolism play an important role in sPLA₂-induced platelet activation. As shown in Table 2, sPLA₂ directly induced TXA₂ generation by platelets. We have tested the effect of MAFP, an irreversible inhibitor of cPLA₂ that has no effect on sPLA₂, on sPLA₂-induced platelet TXA₂ formation. At 5 µM, a concentration previously reported to inhibit ionophore-induced platelet cPLA₂ activity [30], we found no effect on sPLA₂-induced platelet TXA₂ generation. At higher concentrations of MAFP (25–100 µM, concentrations that we found necessary to inhibit TRAP-induced platelet TXA₂ generation) we noted an apparently non-specific promotion of platelet TXA₂ formation in response to sPLA₂ (results not shown). Thus our data would suggest that platelet TXA₂ synthesis induced by sPLA₂ is not secondary to the actions of intracellular cPLA₂.

Does sPLA₂ induce platelet aggregation in PRP?

The inhibitory effects of Mg²⁺ and albumin predicted that sPLA₂ probably does not induce platelet aggregation in PRP. Indeed, no aggregation could be detected with 4–10 µg/ml sPLA₂ in thrombin-inhibitor-anticoagulated PRPs of apparently healthy volunteers (*n* = 5). All these PRPs aggregated normally with 1–3 µM ADP, showing the sensitivity of the platelets in these samples. LY288570, at 20 µg/ml, used as anticoagulant in these experiments to maintain physiological levels of bivalent cations, had no effect on sPLA₂-induced aggregation of isolated platelets (results not shown).

Comparison of the platelet-aggregating effect of sPLA₂ and AA

In order to establish whether AA production alone could be responsible for all effects observed with isolated platelets that are exposed to sPLA₂, platelet-aggregation responses induced by AA and sPLA₂ were compared. Figure 4(b) shows that after reversible aggregation induced by sPLA₂, a second dose of sPLA₂, which otherwise induced a strong signal (d), was not able to aggregate

Figure 4 Comparison of the platelet aggregation effect of sPLA₂ and AA on human platelets

Isolated platelets were preincubated with 2 mM CaCl₂ at 37 °C for 2 min before adding the first agonist. The inset scale is explained in Figure 1.

platelets; however, AA was still effective. After a larger but still reversible change in the light transmission induced by somewhat larger concentrations of sPLA₂ (c), the effect of AA was inhibited but not completely blocked. When AA was added first (a), in a concentration which induced a small reversible change similar to the effect of the first dose of sPLA₂ shown in (b), sPLA₂, added in a concentration that otherwise induced a strong signal (d), gave a smaller signal but was not completely blocked. It was also found that when AA was added in a concentration that induced reversible aggregation, platelets responded, to a smaller extent, to the second or even to the third dose of AA as well (results not shown). In contrast, even a very small first dose of sPLA₂, inducing a virtually undetectable change in light transmission, blocked the aggregating effect of the second dose of sPLA₂ completely, independent of the concentration of the second dose of sPLA₂, but platelets still responded to AA. Similarly, platelets that no longer responded to sPLA₂ after storage (discussed above) still aggregated to AA. The differences found between sPLA₂- and AA-induced platelet aggregation suggest that although AA liberation and metabolism seem to have important roles in the activation process, additional components also have to be considered as critical elements of the mechanism of sPLA₂-induced platelet activation/aggregation.

LPC inhibits sPLA₂-induced platelet aggregation

In platelets, AA can be released from any of the four phospholipids, phosphatidylinositol, phosphatidylserine, phosphatidylethanolamine or phosphatidylcholine (reviewed in [31]). However, in resting platelets phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol are present predominantly

Table 3 LPC inhibits sPLA₂-induced platelet aggregation

Isolated platelets were preincubated with 2 mM CaCl₂ and various concentrations of LPC at 37 °C for 1 min before stimulation with 8 µg/ml sPLA₂ or 1 nM thrombin; nd, not determined. Results are given as percentage aggregation induced by sPLA₂ or thrombin and are means ± S.D. (*n* = 3).

[LPC] (µM)	Aggregation (%)	
	sPLA ₂	Thrombin
0	49 ± 7	59 ± 3
0 [1% (v/v) ethanol]	37 ± 5	57 ± 6
1	38 ± 6	nd
5	34 ± 5	nd
10	14 ± 3	nd
50	0	42 ± 7

in the inner leaflet of the plasma membrane, whereas phosphatidylcholine is preferentially found in the outer leaflet [32]. Accordingly, LPC may be the major split product, in addition to AA, produced by the enzymic action of sPLA₂ on resting platelets. LPC has been shown to inhibit platelet aggregation induced by a wide variety of agonists [29,33]. Table 3 shows that LPC inhibited sPLA₂-induced platelet aggregation.

Is the formation of lysophosphatidic acid (LPA) involved in sPLA₂-induced platelet activation?

The involvement of LPA generation in sPLA₂-induced platelet activation was examined by aggregation experiments upon addition of 1 µM 1-oleoyl-LPA to isolated platelets from five different donors. We observed no significant change in light transmittance ($\Delta T < 5\%$) in three of them, whereas two samples showed bell-shaped, reversible aggregation patterns with a maximum ΔT of only 11% and 17% respectively. In contrast, all platelet samples gave a strong response to 8 µg/ml sPLA₂ or 50 nM AA, similar to those indicated in Figure 4, (d) and (e) (results not shown). In the two samples that gave a small, reversible signal to LPA, a second dose of LPA was completely ineffective, corresponding to the desensitization previously observed for LPA [18,34,35]. When suboptimal concentrations of sPLA₂ were added to platelets responding slightly to LPA, after the reversible signal induced by LPA, sPLA₂-induced platelet aggregation was only slightly inhibited. Similarly, after reversible aggregation induced by low concentrations of sPLA₂, the aggregatory effect of LPA was slightly inhibited but not completely blocked (results not shown). It is also noteworthy that the presence of serum albumin abolishes the aggregatory effect of sPLA₂, whereas albumin-bound LPA is biologically active and induces platelet aggregation [18,35]. Taken together these results suggest that LPA does not play a significant role in sPLA₂-induced platelet activation.

Platelets pretreated with heparitinase I or PI-PLC do not aggregate with sPLA₂

Preincubation of platelets with heparitinase I or PI-PLC resulted in concentration-dependent inhibition of sPLA₂-induced platelet aggregation (Table 4). These results strongly suggest the involvement of a glycosphosphatidylinositol- (GPI-) anchored heparan sulphate proteoglycan in sPLA₂-induced platelet activation. Complete inhibition could be achieved both with heparitinase I and PI-PLC. Platelets preincubated with 2 units/

Table 4 Pretreatment of platelets with heparitinase I or with PI-PLC inhibits sPLA₂-induced platelet aggregation

Platelets were preincubated with 2 mM CaCl₂ and various concentrations of heparitinase I for 10 min or PI-PLC for 2 min at 37 °C before stimulation with 8 µg/ml sPLA₂ or 50 nM AA; nd, not determined. Results are given as percentage aggregation induced by sPLA₂ or AA and are means ± S.D. (*n* = 3).

Addition	Aggregation (%)	
	sPLA ₂	AA
No addition	51 ± 6	53 ± 7
Heparitinase (units/ml)		
0.1	48 ± 7	nd
0.5	43 ± 7	nd
1	13 ± 6	nd
2	0	49 ± 6
PI-PLC (unit/ml)		
0.01	50 ± 5	nd
0.03	43 ± 5	nd
0.05	29 ± 4	nd
0.1	8 ± 5	nd
0.2	0	40 ± 6

ml heparitinase I for 10 min or 0.2 unit/ml PI-PLC for 2 min did not aggregate with sPLA₂, although they responded normally to AA (Table 4).

DISCUSSION

We demonstrate that sPLA₂ activates platelets and that a GPI-anchored platelet-membrane heparan-sulphate proteoglycan, AA metabolism, protein tyrosine phosphorylation and Ca²⁺-mobilization are implicated in this process. Our finding that sPLA₂ induces platelet activation contrasts with earlier reports [2,17–19]. However, according to the data presented here, there are several reasons for the failure of others to observe sPLA₂-induced platelet activation. Notably, isolated platelets lose their sensitivity to sPLA₂ relatively rapidly and the responsiveness of platelets to sPLA₂ is inhibited by BSA and Mg²⁺. Accordingly, it is not surprising that in the study of Bartoli et al. [19] platelets subjected to lengthy preincubations failed to respond to sPLA₂. Similarly, the experimental conditions of Fourcade et al. [18] and Mounier et al. [17] differed from ours in several respects, including the presence of Mg²⁺, the fact that a mutant (Met⁸-Leu) recombinant PLA₂ [36] was used in both cases, and that the supernatant of the resting rabbit platelets used in the latter study contained about 6% of total secretory PLA₂ [37].

Our findings, that the specific sPLA₂ inhibitor LY311727 inhibited sPLA₂-induced platelet aggregation, and that TXA₂ generation, induced by sPLA₂, was not inhibited by the cPLA₂ inhibitor MAFP, support a direct enzymic activity of sPLA₂ on platelets as a prerequisite for its platelet-activating effect. This is consistent with previous results with *p*-bromophenacyl bromide-modified venom PLA₂ [29]. The present results also show that pretreatment of platelets with heparitinase or PI-PLC prevent the platelet-aggregating effect of sPLA₂. Based on these results the following model can be postulated to explain sPLA₂-induced platelet activation. sPLA₂ binds to platelets via a GPI-anchored heparan-sulphate proteoglycan and liberates platelet agonist(s) from membrane phospholipids by enzymic lipolysis. AA is the main split product that is responsible for platelet activation.

However, AA is not responsible for the desensitization of

platelets to the second dose of sPLA₂. Other products like LPC, found to inhibit sPLA₂-induced platelet aggregation, are potential candidates for this. Down-regulation of the binding site for sPLA₂ might also be involved in the desensitization process. LPA generation does not seem to play any role in either the activation or the desensitization process. Our studies showed only a weak aggregatory effect of LPA, which differs from a recent report [18] but agrees with other studies [34,38].

Various proteins are anchored to the platelet plasma membrane via GPI anchors [39] but GPI-anchored heparan-sulphate proteoglycans have not been reported on platelets. Our result, that a GPI-anchored heparan sulphate proteoglycan is the putative binding site for sPLA₂ on platelets, agrees with very recent reports showing that sPLA₂ binds to proteoglycans [40] and that, probably, C-terminal heparin-binding lysine residues are involved in docking sPLA₂ on cell surfaces [41]. Since first described [42], there is increasing evidence that GPI-anchored proteins associate with membrane tyrosine kinases and so participate in signal transduction [43–47]. The fact that no sPLA₂-dependent tyrosine phosphorylation could be detected in the presence of BSA argues against a possible direct signalling through the binding site, in parallel with the enzymic action of sPLA₂ on platelets, but does not completely rule out such a mechanism.

The reason why platelets lose their ability to respond to sPLA₂ more rapidly than to other agonists and how the first, threshold, dose of sPLA₂ desensitizes platelets to the second dose of sPLA₂ is not known. sPLA₂ is not the only such platelet agonist. The first dose of venom PLA₂s [15,29] and LPA [18,34,35] also desensitizes platelets to the second dose of these agonists, and it is known that platelets become 'refractory' to ADP-induced aggregation if they have been exposed to ADP previously [48].

Even though sPLA₂ does not aggregate platelets in normal PRP, a possible (patho)physiological significance of sPLA₂-mediated platelet effects *in vivo* cannot be disregarded. Not even 1 mM Mg²⁺, approximately double the physiological concentration of Mg²⁺ in plasma, completely inhibited sPLA₂-induced platelet aggregation. This suggests that HSA rather than Mg²⁺ is responsible for complete loss of the platelet-aggregating effect of sPLA₂ in PRP. The inhibitory effect of HSA is probably due to the sequestering of AA [49–51]. Hypoalbuminaemia reduces the threshold concentration of aggregating agents, and platelets of patients with nephritic syndrome produce more TXB₂ than controls when stimulated with AA or ADP [52]. So, in hypoalbuminaemia the inhibitory effect of HSA on sPLA₂-induced platelet activation may be significantly diminished. It has been shown recently that LPC is liberated from high-density lipoproteins by snake and bee venom and bovine-pancreas PLA₂s [33]. Whereas with isolated platelets the activating effect of sPLA₂ is dominant, it seems that in plasma this is overcompensated by both the sequestering effect of HSA, and by LPC generation from lipoproteins. It is possible that the platelet activation effect of sPLA₂ might overcome these potent inhibitory systems in pathological conditions with local, elevated sPLA₂ levels.

Various human cells, including platelets, are able to release sPLA₂ in response to various stimuli, and significantly elevated serum or plasma levels of sPLA₂ have been described in various pathological conditions (reviewed in [16]). Although the question whether sPLA₂ causes or is merely associated with these conditions is open [53], it is believed that inhibition of this enzyme will be important in a wide variety of inflammatory diseases. It will be of great interest to explore further the possible effects of sPLA₂ on platelets *in vivo* and contributions of the platelet effects of PLA₂ to the severity and complications in disease states.

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