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BIPHASIC EFFECT ON PLATELET AGGREGATION BY PHOSPHOLIPASE A PURIFIED FROM *VIPERA RUSSELLII* SNAKE VENOM

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A basic phospholipase A was isolated from *Vipera russellii* snake venom. It induced a biphasic effect on washed rabbit platelets suspended in Tyrode's solution. The first phase was a reversible aggregation which was dependent on stirring and extracellular calcium. The second phase was an inhibitory effect on platelet aggregation, occurring 5 min after the addition of the venom phospholipase A without stirring or after a recovery from the reversible aggregation. The aggregating phase could be inhibited by indomethacin, tetracaine, papaverine, creatine phosphate/creatine phosphokinase, mepacrine, verapamil, sodium nitroprusside, prostaglandin E_1 or bovine serum albumin. The venom phospholipase A released free fatty acids from synthetic phosphatidylcholine and intact platelets. *p*-Bromophenacyl bromide-modified venom phospholipase A lost its phospholipase A enzymatic and platelet-aggregating activities, but protected platelets from the aggregation induced by the native enzyme. The second phase of the venom phospholipase A action showed a different degree of inhibition on platelet aggregation induced by some activators in following order: arachidonic acid > collagen > thrombin > ionophore A23187. The longer the incubation time or the higher the concentration of the venom phospholipase A, the more pronounced was the inhibitory effect. The venom phospholipase A did not affect the thrombin-induced release reaction which was caused by intracellular Ca^{2+} mobilization in the presence of EDTA, but inhibited collagen-induced release reaction which was caused by Ca^{2+} influx from extracellular medium. The inhibitory effect of the venom phospholipase A and also lysophosphatidylcholine or arachidonic acid could be antagonized or reversed by bovine serum albumin. It was concluded that the first stimulatory phase of the venom phospholipase A action might be due to arachidonate liberation from platelet membrane. The second phase of inhibition of platelet aggregation and the release of ATP might be due to the inhibitory action of the split products produced by this venom phospholipase A.

Introduction

Phospholipase A_2 is an important cellular endozyme for initiating the synthesis of prostaglandins. Because the concentration of free arachidonic acid is extremely low in cells, the availability of this precursor is a rate-limiting step for the cellular responses mediated by prostaglandins [1,2].

There have been some papers discussing the effects of the venom phospholipases A on platelets. Most authors reported their effects on the profiles of the fatty acids released from platelet membranes [3–5], and their anticoagulant effects by inactivating the procoagulant activity of platelet factor 3 [6–10]. Boffa and Boffa [11] reported that the phospholipase A of *Vipera aspis* venom inhibits ADP-induced platelet aggregation. We also reported the inhibitory effect of two phospholi-

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pases A isolated from *Trimeresurus gramineus* and *Agkistrodon halys* venoms [12,13].

We have isolated the coagulant and anticoagulant principles from *Vipera russellii* venom [14]. The anticoagulant activity was related to its phospholipase A activity. We report in this paper the biphasic effect of this phospholipase A on platelet aggregation and try to elucidate its action mechanism.

Materials and Methods

Materials. Vipera russellii venom was obtained from Sigma, U.S.A. Bovine thrombin was purchased from Parke & Davis, U.S.A. and dissolved in 50% glycerol to give a stock solution of 100 NIH units/ml. Ionophore A23187 was obtained from Calbiochem-Behring Co. U.S.A., and was dissolved in dimethyl sulfoxide. Collagen (Type 1, bovine achilles tendon), sodium arachidonate, *p*-bromophenacyl bromide, EDTA, EGTA, indomethacin, tetracaine, verapamil, creatine phosphate, creatine phosphokinase, adenosine triphosphate, sodium nitroprusside, theophylline, apyrase, synthetic phosphatidylcholine, lysophosphatidylcholine, lysophosphatidylethanolamine, serum albumin (bovine), prostaglandin E_1 and purified luciferase-luciferin were purchased from Sigma, a radioimmunoassay kit for thromboxane B_2 was obtained from New England Nuclear, U.S.A. Collagen was homogenized in 25 mM acetic acid and stored at $-70\,^{\circ}C$ at a concentration of 1 mg/ml.

Purification of phospholipase A. The venom of *V. russellii* was fractionated by DEAE-Sephadex A-50 column chromatography as previously described [14]. The anticoagulant phospholipase A was further purified by gel filtration on Sephacryl S -200. Homogeneity was confirmed by disc polyacrylamide gel electrophoresis and isoelectrofocusing.

Chemical modification of phospholipase A. p-Bromophenacyl bromide (1%) was used to modify the histidine residue of this venom phospholipase A according to the method described previously [10]. Modified phospholipase A lost more than 99% of its enzymatic activity.

Preparation of platelet suspension. Blood was collected from the rabbit marginal ear vein and was mixed with EDTA to a final concentration of 6 mM. It was centrifuged for 10 min at $90 \times g$ and room temperature, and the supernatant was obtained as platelet-rich plasma. The latter was further centrifuged at $500 \times g$ for 10 min. The platelet pellets were washed with Tyrode's solution (Ca^{2+}-free)/2 mM EDTA/0.1 mg $\cdot$ ml^{-1} apyrase/3.5 mg $\cdot$ ml^{-1} serum albumin, and centrifuged at $500 \times g$ for 10 min. Then the pellets were washed with the above Tyrode's solution without EDTA. After centrifugation at the same condition, the platelet pellets were finally suspended in Tyrode's solution of the following composition (mM): NaCl (136.8), KCl (2.8), $NaHCO_3$ (11.9), $MgCl_2$ (1.1), NaH_2PO_4 (0.33), $CaCl_2$ (1.0) and glucose (11.2).

Platelet aggregation and ATP release reaction. Aggregation was measured by a turbidimetric method [15,16]. ATP released from platelets was detected by the bioluminescence method described by DeLuca and McElory [17]. Both aggregation and release reaction were simultaneously and continuously measured by a Lumi-aggregometer (Model 1020, Payton, Canada) connected to two dual-channel recorders. All glassware was siliconized. Just 1 min before the addition of the aggregation inducer, the platelet suspension was stirred at 900 rpm. ATP of known concentration was used to calibrate the intensity of bioluminescence. The percent of aggregation was calculated as follows (A, absorbance):

$$\text{Aggregation (\%)} = \frac{\text{initial } A - \text{final } A \text{ after aggregation}}{\text{initial } A - A \text{ of suspending medium}}$$

Percent inhibition was expressed in some figures assuming the value of control (without any inhibitor) to be 100%.

Thromboxane B_2 assay. 6 min after the challenge of platelets with inducers, 2 mM EDTA and 30 μM indomethacin were added. After centrifugation in an Eppendorf Centrifuge (Model 5414) for 5 min, thromboxane B_2 was assayed using the radioimmunoassay kit according to the procedure described by the manufacturer.

Agglutination of platelets. Washed platelets were mixed with freshly prepared formaldehyde (2%)/0.9% NaCl and incubated for 2 h at room temperature. After standing overnight at 4°C and

centrifugation at 500 × *g* for 10 min, platelet pellets were washed twice with Tris-HCl/saline buffer (pH 7.4) and resuspended in this buffer. Agglutination was triggered by polylysine and monitored with the same aggregometer.

Phospholipase A activity. The enzymatic hydrolysis of synthetic phosphatidylcholine or intact platelets by phospholipase A was determined by the pH-stat titration method of Strong et al. [44]. Sodium deoxycholate of concentration equimolar (2.5 mM) to phosphatidylcholine was used as emulsifier in 0.2 mM EDTA. Intact platelets prepared as described above were finally suspended in saline and 0.2 mM EDTA without any buffer. Calcium (2 mM) was added just before the addition of venom phospholipase A. The release of fatty acids from phosphatidylcholine or intact platelets at 37 °C was continuously titrated at pH 7.3 with 2 mM NaOH.

Results

Purified phospholipase A of *V. russellii* venom induced aggregation of washed platelets. As shown in Fig. 1, low concentrations of venom phospholipase A induced small aggregation followed by disaggregation (reversible aggregation). At higher concentrations, up to 50 μg/ml, aggregation was more pronounced. However, the extent of aggregation decreased and the disaggregation was more rapid if the venom concentrations were higher than 100 μg/ml. Thus, venom phospholipase A induced biphasic effect on platelet aggregation. The aggregation phase needed stirring and the minimal concentration for inducing aggregation of platelets varied in different batches in a range from 0.01 to 1 μg/ml. The inhibitory effect occurred at higher concentrations (over 2 μg/ml), it did not need stirring, but required incubation and the sensitivity was quite similar in different batches of platelets.

Platelet-aggregating activity of the venom phospholipase A

The aggregating phase of the venom phospholipase A action on platelets was dependent on extracellular calcium. The optimal concentration of Ca^{2+} for aggregating activity of the venom phospholipase A was about 0.5 to 1.0 mM (Fig. 2).

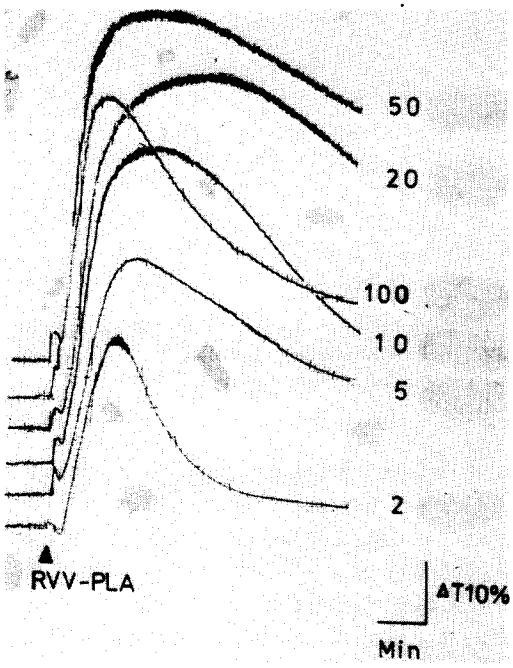

Fig. 1. Platelet aggregation induced by the purified venom phospholipase A. Washed rabbit platelets were warmed at 37°C and stirred for 1 min before the addition of venom phospholipase A (RVV-PLA) at the various concentrations (μg/ml) shown on the right of the figure. The variation of the aggregation recording was between 5 and 10% in transmission.

Lactate dehydrogenase could not be detected in the supernatant after the incubation of platelets with the venom phospholipase A.

Indomethacin (10 μM) could completely inhibit the aggregation induced by the venom phospholipase A (20 μg/ml). In the absence of indomethacin, the venom phospholipase A (20 μg/ml) caused the increase of thromboxane B_2 from 15.8 ± 1.4 ng/ml (n = 9) of resting platelets to 216.1 ± 23.2 ng/ml ($n = 3$). However, in the presence of indomethacin, the level of thromboxane B_2 increased by the venom phospholipase A was suppressed to 14.7 ng/ml ($n = 2$), which was close to the basal level.

It is well known that platelet aggregation could be inhibited by agents increasing cAMP either by activating adenyl cyclase (prostaglandin E_1) or by inhibiting phosphodiesterase (papaverine, theophylline), or by agents inhibiting Ca^{2+}-mobilization (verapamil, tetracaine, sodium nitroprusside), or by phospholipase A inhibitor (mepacrine) or by

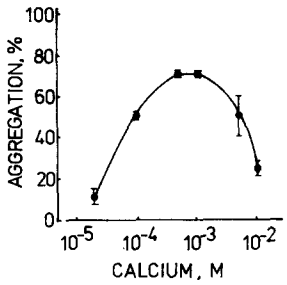

Fig. 2. Relationship between calcium concentration and platelet aggregation induced by venom phospholipase A (10 μg/ml). Mean ± S.E. is presented in each datum ($n = 5-7$).

ADP-scavenger (creatine phosphate/creatine phosphokinase). We tested these agents on the aggregation induced by the venom phospholipase A. Prostaglandin E_1 could completely inhibit the platelet aggregation induced by the venom phospholipase A (Fig. 3). It also disintegrated the aggregated platelets. The earlier the addition of prostaglandin E_1, the more rapidly the disaggregation occurred. The aggregation of platelets induced by the venom phospholipase A (20 μg/ml) could be completely inhibited by tetracaine (0.5 mM), papaverine (50 μM), mepacrine (50 μM) verapamil (55 μM), sodium nitroprusside (0.1 mM), creatine phosphate/creatine phosphokinase (2.5 mM/4U) and bovine serum albumin (3.5 mg/ml). All of the above reagents, including prostaglandin E_1, also inhibited aggregation of platelets induced by arachidonic acid (10 μM).

Using a pH-titration method, the phospholipase A activity of the venom enzyme was estimated to be 540 ± 30 μmol/min per mg using phosphatidylcholine as substrate. The venom phospholipase A caused only limited hydrolysis of membrane phospholipids of intact platelets. The fatty acid release was 0.85 ± 0.20 μmol/min per mg, which was equivalent to 40 μM of fatty acid released in 3 min by 10 μg/ml of the venom phospholipase A.

The histidine residue of the venom phospholipase A was modified by using *p*-bromophenacyl bromide. More than 99% of the enzymatic activity of this modified phospholipase A was lost. It did not induce platelet aggregation at a concentration of 50 μg/ml. However, this modified phospholipase A inhibited the aggregating activity of the native enzyme. As shown in Fig. 4, the higher the ratio of modified phospholipase A to native phospholipase A, the more the aggregating activity was suppressed. When the ratio was 10 : 1, a complete protection was obtained.

Inhibitory effect of the venom phospholipase A on platelet aggregation

After a reversible aggregation induced by a low concentration of arachidonic acid (0.5 μM), aggre-

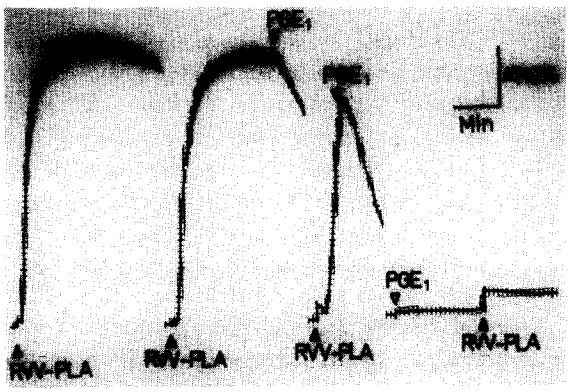

Fig. 3. Effect of prostaglandin E_1 on platelet aggregation induced by venom phospholipase A. Prostaglandin E_1 (PGE_1, 10^{-7} M) was added before or at various time intervals after the aggregation triggered by venom phospholipase A (RVV-PLA, 20 μg/ml).

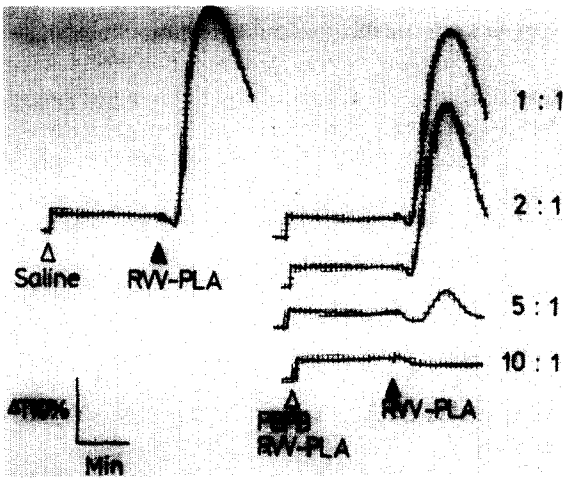

Fig. 4. Protection of venom phospholipase A (RVV-PLA)-induced platelet aggregation by *p*-bromophenacyl bromide-modified RVV-PLA. Salilne or *p*-bromophenacyl bromide-modified RVV-PLA in different ratios to RVV-PLA was added 3 min before the challenge with native RVV-PLA (2 μg/ml).

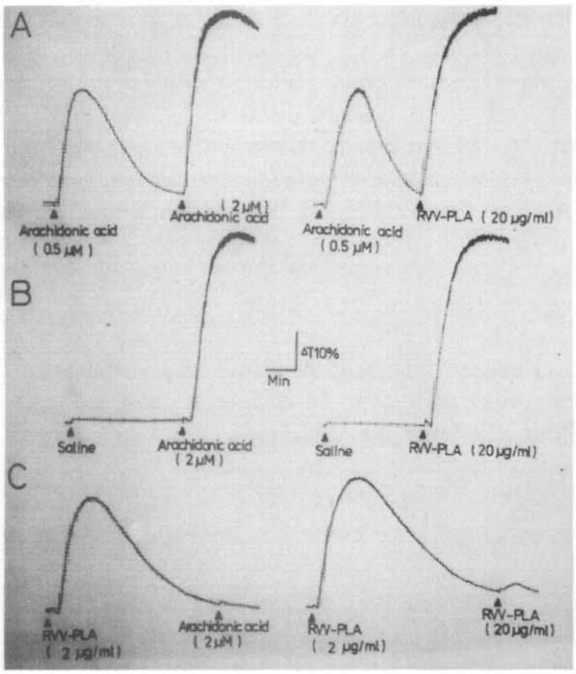

Fig. 5. Effect of previous reversible aggregation on the second challenge with arachidonic acid or venom phospholipase A (RVV-PLA). Reversible aggregation of platelets was induced by 0.5 μM arachidonic acid (A) or 2 μg/ml RVV-PLA (C). After disaggregation, second challenge was induced by arachidonic acid (2 μM) or RVV-PLA (20 μg/ml). Platelet aggregation without any pretreatment (saline cntrol) is compared in B.

gation still could occur in a second challenge of arachidonic acid (2 μM) or the venom phospholipase A (20 μg/ml) (Fig. 5A). However, second aggregation could not occur by these inducers if the first inducer was the venom phospholipase A (2 μg/ml) (Fig. 5C). This inhibitory effect also happened in platelets without stirring during incubation with the venom phospholipase A.

The venom phospholipase A inhibited platelet aggregation induced by some stimulants to different degrees. After incubation of platelets with the venom phospholipase A for 15 min, arachidonic acid-induced aggregation was inhibited most extensively, the collagen-induced aggregation was the second, while those by thrombin and ionophore A23187 were the least affected (Fig. 6). Increasing the concentration of arachidonic acid could not overcome the inhibition, while an increase in collagen concentration could. However, concentra-

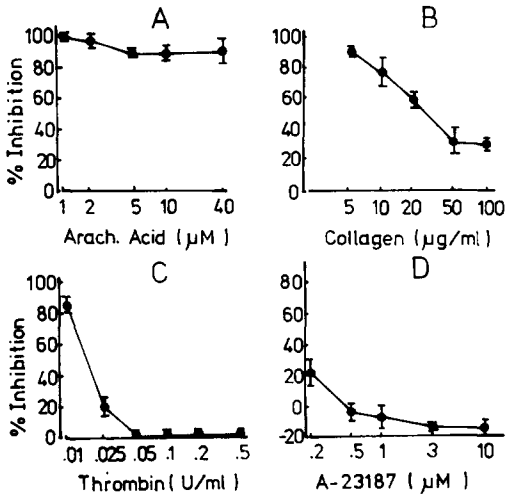

Fig. 6. Inhibitory effect of venom phospholipase A on platelet aggregation induced by some inducers. Platelets were incubated with venom phospholipase A (RVV-PLA, 20 μg/ml) for 15 min at 37°C before arachidonic acid (A), collagen (B), thrombin (C) or ionophore A23187 was added at various concentrations. Each datum is expressed by mean ± S.E. (n = 5–8).

tions of collagen higher than 50 μg/ml still could not reverse this inhibition completely. The aggregation induced by thrombin and ionophore A23187 could be inhibited by the venom phospholipase A only when the concentrations of the inducers were below that needed for maximal effect. The longer the incubation of platelets with the venom phospholipase A and the higher the concentrations of

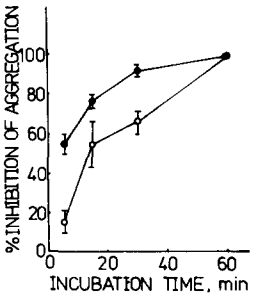

Fig. 7. Relationship between the incubation time and the inhibitory effect of venom phospholipase A on collagen-induced platelet aggregation. Platelets were incubated with venom phospholipase A at concentration of 20 μg/ml (●) or 2 μg/ml for various time intervals, then collagen of 20 μg/ml was added to trigger the aggregation. Each datum is expressed by mean ± S.E. (n = 3–6).

the venom phospholipase A, the more pronounced was the inhibition. Collagen-induced platelet aggregation was taken as a typical example and is presented in Fig. 7. The inhibition reached its maximal peak after 60 min incubation when the concentration of the venom phospholipase A was either 2 or 20 μg/ml (Fig. 7). *p*-Bromophenacyl bromide-modified venom phospholipase A did not possess inhibitory activity on platelet aggregation. The venom phospholipase A did not inhibit agglutination of formaldehyde-fixed platelets induced by polylysine even after incubation with platelets for 15 min at 50 μg/ml.

Reversal of the inhibitory effect of the venom phospholipase A by serum albumin

The venom phospholipase A did not cause aggregating or inhibitory action on washed platelets if bovine serum albumin was present in platelet suspension. In Fig. 8, we show that bovine serum albumin could reverse the inhibitory effect of the venom phospholipase A even after its incubation with platelets for 15 min (without stirring). Complete reversal could be obtained with bovine serum albumin at a concentration of 3.5 mg/ml. The enzymatic hydrolysis of membrane phospholipid of platelets by the venom phospholipase A would produce fatty acid and lysophospholipid, so we studied the effects of both products on platelet aggregation. As shown in Fig. 9, collagen-induced aggregation could be inhibited by lysophosphatidylcholine (lyso-PC) and arachidonic acid. Inhibition caused by 30 μM lyso-PC did not need incubation, while that by 20 μM arachidonate did. Both inhibitions could be reversed by bovine serum

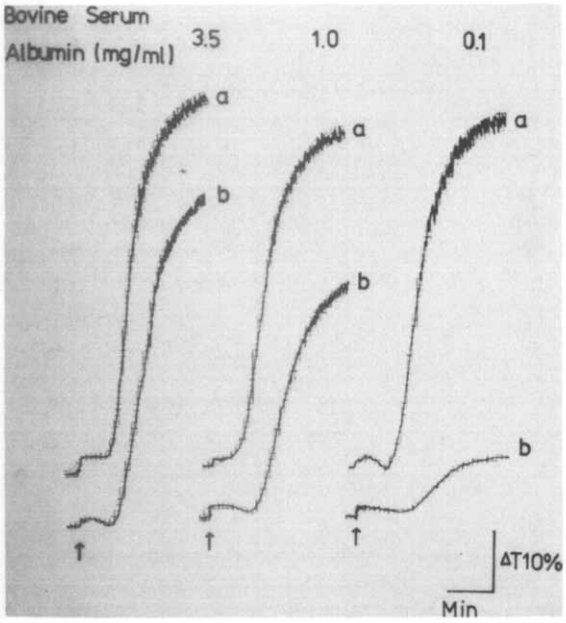

Fig. 8. Effect of bovine serum albumin on the inhibitory action of venom phospholipase A in collagen-induced platelet aggregation. Platelets were incubated with venom phospholipase A (20 μg/ml, curve b) or saline (curve a). After 15 min, various concentrations of bovine serum albumin was added. Then collagen (20 μg/ml) was added at arrow to trigger the aggregation.

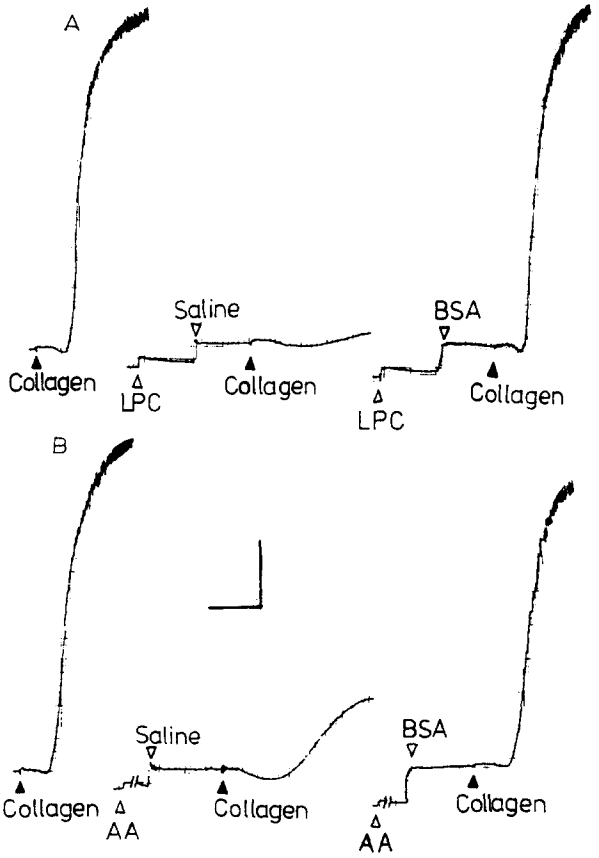

Fig. 9. Inhibitory effects of lysophosphatidylcholine (A) and arachidonic acid (B) on the platelet aggregation induced by collagen. Platelets were preincubated with lysophosphatidylcholine (LPC, 30 μM) for 1 min or arachidonic acid (AA, 20 μM) for 15 min. Bovine serum albumin (BSA, 3.5 mg/ml) or saline was added and then collagen (10 μg/ml in A, 5 μg/ml in B) was used to trigger the aggregation.

albumin added just before the challenge of platelets by collagen. Lyso PC inhibited thrombin- or A23187-induced aggregation only at submaximal concentrations of the inducers. Arachidonate was ineffective against the aggregation induced by thrombin or A23187.

Effect of the venom phospholipase A on the ATP release in the presence or absence of extracellular calcium

It is well known that thrombin can induce release of ATP by mobilizing intracellular calcium, while collagen is more dependent on the extracellular calcium for this process [18–20]. However, both require calcium influx from extracellular medium to induce aggregation. Using this difference in the requirement of calcium, we studied the inhibitory effect of the venom phospholipase A on the release reaction in the presence or absence of extracellular calcium. Platelets were incubated with the venom phospholipase A (20 μg/ml) for 15 min in the presence of 0.5 mM Ca^{2+} which was essential for the enzymatic reaction of the venom phospholipase A to take place. EGTA (2 mM) was then added to chelate Ca^{2+}. Under this condition, both thrombin- and collagen-induced ATP release were unaffected by the venom phospholipase A. However, when extracellular Ca^{2+} (3 mM) was added to trigger the aggregation and second release of ATP, collagen-induced aggregation and ATP release were inhibited (Fig. 10A). The extent of this inhibition was similar in both the aggregation (39.3 ± 9.8%) and the release reaction (39.2 ± 3.8%). Second release of ATP caused by thrombin was only one-sixth that of the first release with or without the venom phospholipase A preincubation (Fig. 10B).

Thromboxane B_2 level of platelets and bovine serum albumin

The thromboxane B_2 level of resting platelets without stirring in the absence of bovine serum albumin (15.8 ± 1.4 ng/ml) was much higher than that in the presence of bovine serum albumin (1.0 ± 0.5 ng/ml). When platelets were challenged with arachidonic acid, the thromboxane B_2 level of platelets without bovine serum albumin was also much higher than that with bovine serum albumin. For example, thromboxane B_2 formation caused

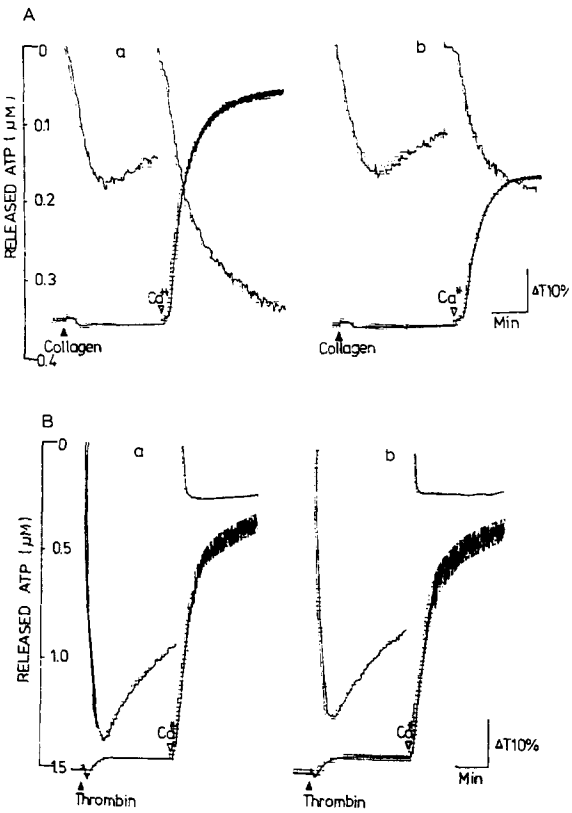

Fig. 10. Effect of venom phospholipase A on collagen and thrombin-induced aggregation and ATP release. Platelets were incubated with saline (a) or venom phospholipase A (b, 20 μg/ml) in the presence of 0.5 mM Ca^{2+}. After 15 min, EGTA (2 mM) and collagen (A panel, 20 μg/ml) or thrombin (B panel, 0.5 U/ml) were added, and ATP was released (downward tracings). 3 min later, Ca^{2+} (3 mM) was added to trigger further ATP release and aggregation (upward tracings).

by arachidonate (10 μM) without bovine serum albumin (447.7 ± 80.9 ng/ml) was 7 times that caused by a 10-fold concentration of arachidonate (100 μM) with bovine serum albumin (60.3 ± 3.3 ng/ml).

Discussion

In our previous paper [14], we have reported the purification of a basic phospholipase A from *V. russellii* venom. It was an anticoagulant through the hydrolysis of phospholipid which was essential for blood coagulation serving as surface on which the interaction of clotting factors takes place. In this study, a biphasic effect on platelet aggregation

caused by this venom phospholipase A has been investigated.

Platelet aggregating activity of the venom phospholipase A

The venom phospholipase A induced a reversible aggregation of washed rabbit platelets. When the concentration was higher than optimum it induced aggregation of lesser extent and caused more rapid disaggregation (Fig. 1). Thus the dose-response relationship as similar to that of arachidonic acid [21]. The aggregation phase of the venom phospholipase A was dependent on stirring and the concentration of calcium. This was not an agglutination because it occurred only in vital platelets. The venom phospholipase A-induced platelet aggregation could be inhibited completely by indomethacin at a concentration (10 μM) which abolished arachidonate-induced aggregation but not significantly inhibited aggregation induced by collagen or thrombin. The aggregation induced by arachidonate was proved to be due to thromboxane A_2 or protaglandin endoperoxides [22,23]. Recently, 15-hydroxyperoxythromboxane A_2 was also claimed to be another active metabolite [24]. Most authors suggested that thromboxane A_2 is the main mediator for aggregation induced by the venom phospholipase A. However, prostaglandin endoperoxides will become predominant if thromboxane A_2 synthetase is inhibited [25,26]. The involvement of thromboxane A_2 in the venom phospholipase A-induced aggregation was also confirmed by the assay of thromboxane B_2, which is a stable metabolite of thromboxane A_2. In the presence of indomethacin, both aggregation and thromboxane B_2 formation were suppressed.

The venom phospholipase A-induced aggregation could be inhibited by tetracaine. Local anesthetics might inhibit platelet aggregation through the inhibition of Ca^{2+} influx [20] or intracellular Ca^{2+} mobilization [27]. Recently, Volpi et al. [28] proposed that local anesthetics could inhibit calmodulin, which is important for the activation of endogenous phospholipase A [29,30]. The Ca^{2+} requirement of the venom phospholipase A was shown in a bell-shape dose-response curve (Fig. 2). The optimal concentration was about 0.5–1.0 mM. A lower concentration of Ca^{2+} was not sufficient for inducing maximal aggregation, while higher concentration of Ca^{2+} might stabilize the cell membrane and then decrease the aggregation. The venom phospholipase A-induced platelet aggregation was also inhibited by verapamil and sodium nitroprusside, which were reported to inhibit Ca^{2+} influx and prevent or reverse Ca^{2+} mobilization from intracellular stores, respectively [31,32].

Some vasodilators, such as papaverine or theophylline could inhibit platelet aggregation induced by the venom phospholipase A. These phosphodiesterase inhibitors increased cAMP level [33]. Prostaglandin E_1 also caused cAMP increase by activating adenyl cyclase [34]. Prostaglandin E_1 might inhibit aggregation induced by the venom phospholipase A (Fig. 3) through this mechanism.

The venom phospholipase A could not induce platelet aggregation in the presence of bovine serum albumin. Bovine serum albumin was routinely added to platelet suspension [35] to maintain the normal shape of platelets. It needed much higher concentrations of arachidonate to induce the same extent of aggregation in the presence of bovine serum albumin than in its absence. Furthermore, arachidonate caused much lower thromboxane B_2 production in the presence of bovine serum albumin. Bovine serum albumin might inhibit aggregation and thromboxane B_2 production by its high binding activity to arachidonate [36].

p-Bromophenacyl bromide-modified venom phospholipase A lost its aggregating activity together with the enzymatic activity of phospholipase A. However, this modified venom protein could protect the aggregating activity of the native enzyme. The modification of the histidine residue(s) of phospholipase A might cause the loss of its enzymatic activity but it still retains the binding activity. The necessity of enzymatic hydrolysis of membrane phospholipid of intact platelets was confirmed by pH titration of fatty acid released from platelets at the same condition of performing aggregation study, i.e., pH, temperature, stirring speed, platelet counts and amount of the venom phospholipase A. The fatty acid release in 3 min by the venom phospholipase A built up to a concentration (40 μM) about 4-times that of arachidonate (10 μM) needed to produce the same extent of aggregation. From these results,

it was concluded that the venom phospholipase A-induced platelet aggregation required binding and enzymatic activities, and the latter activity might produce arachidonate which then triggered the aggregation of platelets. However, we could not rule out the possibility that endogenous phospholipase A might further produce arachidonate during activation by venom phospholipase A.

Antiplatelet activity of the venom phospholipase A

The inhibitory phase of the venom phospholipase A action occurred 5 min after incubation without stirring or after the recovery from the reversible aggregation. The inhibitory effect of the venom phospholipase A was dependent on its concentration and incubation time with platelets. The higher the concentration, the shorter the incubation time required to produce maximal inhibition. The degree of this inhibitory effect was shown to be dependent on the kind of platelet aggregation inducers. The order of susceptibility for inhibition was: arachidonate > collagen > thrombin > ionophore A23187.

Exogenous phospholipases A and C can hydrolyze the phospholipids of platelet membranes [37,38]. If we assume that phospholipase A cannot penetrate into cell membrane, then the phospholipid in the outer leaflet of membrane which will be most susceptibly hydrolyzed will be phosphatidylcholine (PC) [39,40]. It was also reported that arachidonate was mostly incorporated in PC [41]. We observed that lysophosphatidylcholine at 20 μM inhibited thrombin-induced aggregation to 50% of the control, while lysophosphatidylethanolamine at 200 μM could not. The inhibition of aggregation by lyso-PC was immediate after addition. Prolonged incubation did not enhance the extent of inhibition. Inhibition of both venom phospholipase A and lyso-PC could be overcome by bovine serum albumin. A similar inhibitory effect of lyso-PC was also reported by Vincent and Zijlstra [42].

In addition to lyso-PC, another possible factor involved in the inhibitory phase of the venom phospholipase A action was arachidonate. At concentrations higher than 10 μM, arachidonate caused an inhibition on the aggregation induced by collagen (Fig. 9). This inhibition required incubation with platelets. Arachidonate-induced inhibition of aggregation also could be reversed after incubation or inhibited by the pretreatment with bovine serum albumin. The inhibitory effect of arachidonate might be due to the inhibition of thromboxane synthetase by its metabolite(s) of the lipoxygenase pathway [43] or due to interference with the agonist-receptor interaction at high concentrations [21].

The venom phospholipase A did not affect the agglutination of formaldehyde-fixed platelets induced by polylysine. Thus the venom phospholipase A might not inhibit the cohesion of platelet membrane in the final step of aggregation. Using an experiment which could differentiate the extracellular Ca^{2+} influx from intracellular Ca^{2+} mobilization, we found the venom phospholipase A inhibited more specifically the ATP release mediated by extracellular Ca^{2+} influx, while that by intracellular Ca^{2+} mobilization was least attacked. That thrombin could mobilize intracellular Ca^{2+} while collagen was more dependent on extracellular Ca^{2+} influx explained why aggregation induced by collagen was more susceptible than that induced by thrombin to the effect of the venom phospholipase A. It was concluded that the inhibitory phase of venom phospholipase A action on platelet aggregation and release reaction might be due indirectly to the inhibitory actions of lyso-PC and arachidonate.

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