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Role of catalytic function in the antiplatelet activity of phospholipase A_2 cobra (*Naja naja naja*) venom

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Abstract

Three acidic phospholipases A_2 from Indian cobra (*Naja naja naja*) venom inhibited platelet aggregation in platelet rich plasma induced separately by ADP, collagen and epinephrine with different potencies. The order of inhibition was epinephrine > collagen > ADP. They did not inhibit platelet aggregation induced by arachidonic acid (10 μM). The inhibition was dependent on concentration of the protein and the time of incubation of the phospholipases A_2 with platelet rich plasma. Parabromophenacyl bromide modified PLA_2 enzymes lost their enzymatic activity as well as platelet aggregation inhibition activity suggesting the involvement of catalytic function in platelet aggregation inhibitory activity. (Mol Cell Biochem **219**: 39–44, 2001)

Key words: platelet aggregation inhibitors, acidic phospholipases, antiplatelet activity, catalytic function, phospholipases A_2

Introduction

Snake venom phospholipases A_2 (PLA_2) are known to exhibit a broad spectrum of pharmacological activities such as neurotoxicity, cardiotoxicity, cytotoxicity, effects on platelet aggregation, hemolytic, pro- and anticoagulant, edema inducing and hypotensive [1, 2]. Phospholipases A_2 exhibiting these activities, in synergism with other venom protein may help in the acquisition and/digestion of the prey under natural conditions. The venom PLA_2 provides useful tools for understanding molecular events in normal physiology as well as new targets of drug development for clinical use.

Platelet aggregation is an important cellular process in thrombosis and hemostasis [3]. Several snake venom PLA_2 potentiates initiation and/inhibition of platelet aggregation. Acidic phospholipases are also among the PLA_2, which interfere in platelet functions. The role of catalytic activity of the PLA_2 enzyme in the inhibition of agonist induced platelet aggregation has been addressed by several groups [2, 4–11]. The results are varying from enzyme to enzyme suggesting that the individual PLA_2 effect inhibition of platelet aggregation differently. Present paper deals with role of catalytic activity of phospholipases A_2 from *Naja naja naja* venom on platelet aggregation induced by agonists such as epinephrine, collagen, adenosine diphosphate (ADP) and arachidonic acid.

Materials and methods

Arachidonic acid, ADP, collagen and epinephrine were purchased from Sigma Chemical Co. St. Louis, USA. 1-nitroso-2-naphthol was purchased from Sigma Chemical Co. St. Louis, USA. Phosphatidyl choline (PC) was prepared from hen's egg yolk according to the method of Singleton *et al*. [12]. Human blood samples were from healthy volunteers from Department of studies in Biochemistry, Manasagangothri, Mysore, India and Central Food Research Institute (CFTRI) Mysore, India. All other chemicals used were of analytical grade.

Purification of the acidic phospholipases A_2 from Naja naja naja *venom*

The three acidic PLA_2 (NN-I_{2c}, NN-I_{2d} and NN-I_{2e}) were purified from *Naja naja naja* venom as described in our previ-

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ous paper [13]. Indian cobra venom was fractionated on a CM-Sephadex C-25 column according to the method of Bhat and Gowda [14]. This resolved the venom into 14 peaks. The non-retained protein was eluted, as peak I (NN-I-PLA_2). NN-I-PLA_2 was resolved into three peaks upon gel filteration through a Sephadex G-50 column. The peaks were named as NN-I_1, NN-I_2 and NN-I_3. The enzyme elution profile showed the presence of PLA_2 activity in NN-I_2 (NN-I_2-PLA_2). NN-I_2-PLA_2 gave several coomassie blue stained bands on native – PAGE. This fraction was resolved into five peaks on a QAE-Sephadex A-25 column chromatography. They were named NN-I_{2a}-PLA_2, NN-I_{2b}-PLA_2, NN-I_{2c}-PLA_2, NN-I_{2d}-PLA_2 and NN-I_{2e}- PLA_2 [13]. In the present study, NN-I_{2c}, NN-I_{2d} and NN-I_{2e}, PLA_2 were used to investigate their effect on platelet aggregation.

Phospholipase A_2 assay

Phospholipase A_2 activity was assayed by estimating the free fatty acid released as described by Bhat and Gowda [14], using egg phosphatidyl choline as substrate. The incubation mixture contained 1 μM of egg phosphotidyl choline, 0.2 ml of diethyl ether, 0.05 M Tris-HCl buffer (pH 7.5) 40 mM $Ca^{2+}$and 0.2 ml of enzyme (2–5 μg) in 1 ml. The free fatty acid released was extracted as cobalt soap and was complexed with 1-nitroso 2-naphthol and estimated colorimetrically. Activity is expressed as nmoles of free fatty acid released/min/mg of protein.

Isolation of platelets

Human blood was collected from the vein of healthy non-smoker volunteers who had not taken any medication for at least the previous 2 weeks. Nine volumes of blood was collected into one volume of acid citrate dextrose (93 mM Sodium citrate, 7 mM citric acid and 140 mM glucose, pH 6.5) and immediately centrifuged for 10 min at 90 × g at room temperature (25°C). The supernatant obtained was collected as platelet rich plasma (PRP). Homologous platelet poor plasma (PPP) was prepared by recentrifugation of the remaining blood for 15 min at 500 × g at 25°C. The platelet concentration of PRP was adjusted with PPP to 2,25,000 ± 25,000 platelets/μl. The PRP was kept at 37°C and was used within 3 h after preparation. All the above preparation was carried out using plasticware or siliconized glassware.

Platelet aggregation

Platelet aggregation was studied by the turbidometric method of Born [15], using a chronolog dual channel aggregometer connected to omniscribe dual pen recorder to record the light transmission as a function of time. Briefly, the action of agonist on platelets is measured by increased light transmission. When a platelet aggregating agent (agonist) is added to PRP, the platelet clump and PRP becomes progressively clear, showing increased light transmission with time. For concentration dependent platelet aggregation studies, different concentrations (0–24 μg/ml) of NN-I_{2c}-PLA_2, NN-I_{2d}-PLA_2 and NN-I_{2e}-PLA_2 were preincubated separately with PRP with constant stirring for 2 min. For time dependent platelet aggregation studies, aliquots of PRP were preincubated with each of the above mentioned enzymes (10 μg/ml) for different time intervals (2–60 min). Platelet aggregation of treated PRP was then initiated by addition of ADP (76 μM), collagen (~ 2 μg/ml), epinephrine (22 μM), and arachidonic acid (10 μM) separately. Percent inhibition was calculated assuming the values of control (without any aggregation inhibitor) to be 100%.

Chemical modification of phospholipases A_2 by p-bromophenacyl bromide

Chemical modification of PLA_2 by p-bromophenacyl bromide (p-BPB) was carried out as described by Condrea *et al*. [16]. One hundred microliters of 40 mM p-BPB in acetone were added to 3 ml of PLA_2 solution (0.5 mg/ml, in 0.05 M Tris-HCl buffer, pH 7.5). The reaction was allowed to proceed for 40 min, and then acidified with glacial acetic acid to pH 4.0 to stop the reaction. Excess of reagent was removed by dialysing against 0.05 M Tris-HCl buffer pH 7.5.

Determination of protein

Protein was estimated according to the method of Lowry *et al*. [17], using Bovine serum albumin as standard (0–75 μg).

Results

The three PLA_2 isoforms from *Naja naja naja* venom NN-I_{2c}, NN-I_{2d}, and NN-I_{2e} were purified to homogeneity using combinations of ion exchange and gel filteration chromatographies [13]. The PLA_2's showed high catalytic activity. The specific activity, K_m and V_{max} of the three PLA_2 isoforms are given in Table 1.

The effect of these PLA_2 isoforms in platelet aggregation was determined using PRP sample. All three PLA_2 isoforms inhibits platelet aggregation induced by each of the agonist epinephrine, collagen and ADP. Figure 1 represents a typical inhibition pattern of platelet aggregation induced by all three agonist with NN-I_{2e}-PLA_2. The inhibition is dose dependent for all the three agonist induced platelet aggregation; with ADP and collagen induced platelet aggregation, maximum inhibition was observed at 24 μg/ml, whereas with

Table 1. Comparative phospholipaseA_2 activity of NN-I_{2c}-PLA_2, NN-I_{2d}-PLA_2, NN-I_{2e}-PLA_2

	Specific activity*	K_m (μM)	V_{max} (μM)
NN-I_{2c}-PLA_2	9.0 ± 0.3	0.32	0.49
NN-I_{2d}-PLA_2	13.0 ± 0.5	0.42	0.74
NN-I_{2e}-PLA_2	16 ± 0.5	0.38	0.94

*Specific activity is expressed in terms of μM of fatty acid released/min/mg of protein. Data are mean values ± S.E.M. obtained with 6 experiments.

epinephrine induced platelet aggregation, maximum inhibition was observed even at 9 μg/ml. Similar pattern of dose dependent inhibition was observed for the other two PLA_2 isoforms. These PLA_2 isoforms inhibited platelet aggregation induced by epinephrine at low protein concentration on the other hand they required high protein concentration to inhibit platelet aggregation induced by ADP and collagen. Interestingly these three PLA_2 isoforms failed to inhibit platelet aggregation induced by arachidonic acid.

The inhibition of agonists induced platelet aggregation by inhibitors depends on the duration of preincubation time of platelets with inhibitors before inducing aggregation with agonist. Figure 2 shows the effect of preincubation time with platelets at low concentration of all PLA_2 isoforms. Preincubation effect was observed with ADP induced platelet aggregation. The inhibition of platelet aggregation increased with preincubation time. Among all three isoforms, NN-I_{2e} showed higher inhibition. Time dependent aggregation studies are not presented for the other two agonist as similar patterns were observed.

All three PLA_2 isoforms inhibits platelet aggregation in a dose dependent manner induced by ADP (Fig. 3A), collagen (Fig. 3B) and epinephrine (Fig. 3C). Maximum inhibition of 20–30% was observed at 24 μg/ml of PLA_2 isoforms with ADP induced platelet aggregation (Fig. 3A), whereas collagen induced platelet aggregation was inhibited to 70–100% at the same concentration. Much stronger inhibition of 60–100% was observed with 9 μg/ml of PLA_2 isoforms with epinephrine induced platelet aggregation. NN-I_{2d} PLA_2 inhibited platelet aggregation induced by ADP to a greater extent. NN-I_{2e} PLA_2 inhibited platelet aggregation induced by collagen and epinephrine to a greater extent than NN-I_{2c} and NN-I_{2d} PLA_2.

All PLA_2 isoforms exhibit/showed similar affinity for phospholipid and its hydrolysing activity. The specific activity was ranging from 9–16 μM of fatty acid released/min/mg of protein. The maximum concentration of protein used in ADP and collagen induced platelet aggregation inhibition studies was 24 μg and less in epinephrine induced platelet aggregation. At these protein concentrations the amount of

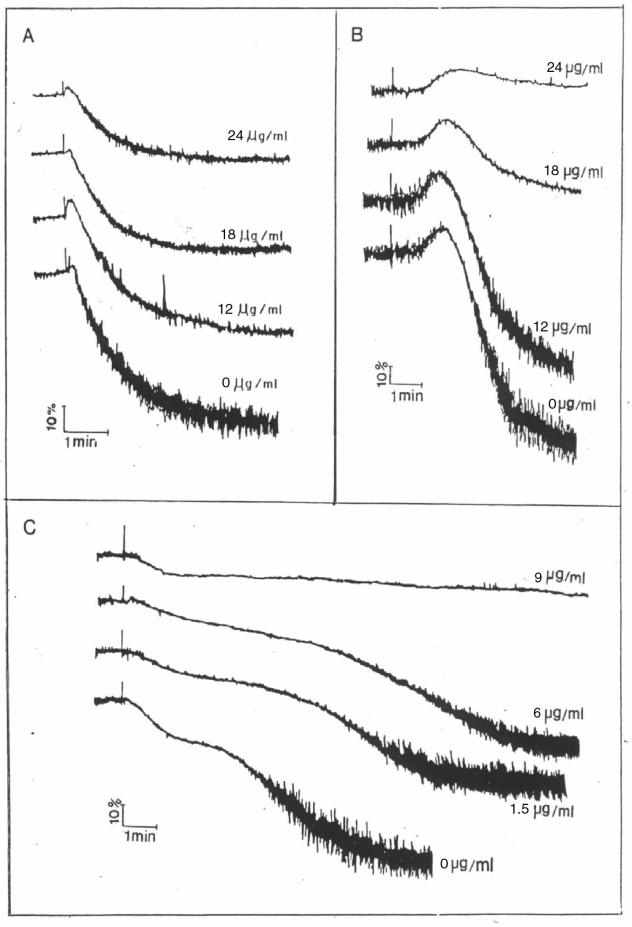

Fig. 1. Inhibition of ADP (A), collagen (B) and epinephrine (C) induced platelet aggregation by NN-I_{2e}–PLA_2. PRP was incubated with NN-I_{2e}–PLA_2 (0–24 μg/ml) for 2 min at 37°C. Inhibition of platelet aggregation initiated by the addition of ADP (76 μM), collagen (~ 2 μg/ml), epinephrine (22 μM) was recorded.

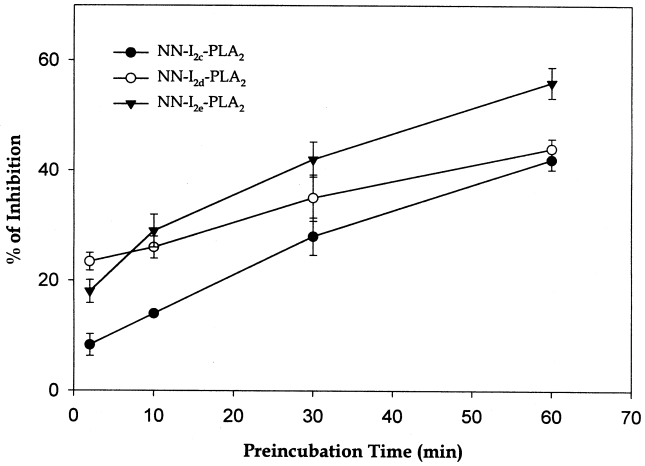

Fig. 2. Inhibition of platelet aggregation by phospholipase A_2 NN-I_{2c} (●), NN-I_{2d} (O) AND NN-I_{2e} (▼) with time. Each PLA_2 (10 μg/ml) was preincubated with PRP for different time intervals at 37°C. Platelet aggregation was initiated by the addition of ADP (76 μ M). Each value represents mean ± S.E.M. of 6 experiments.

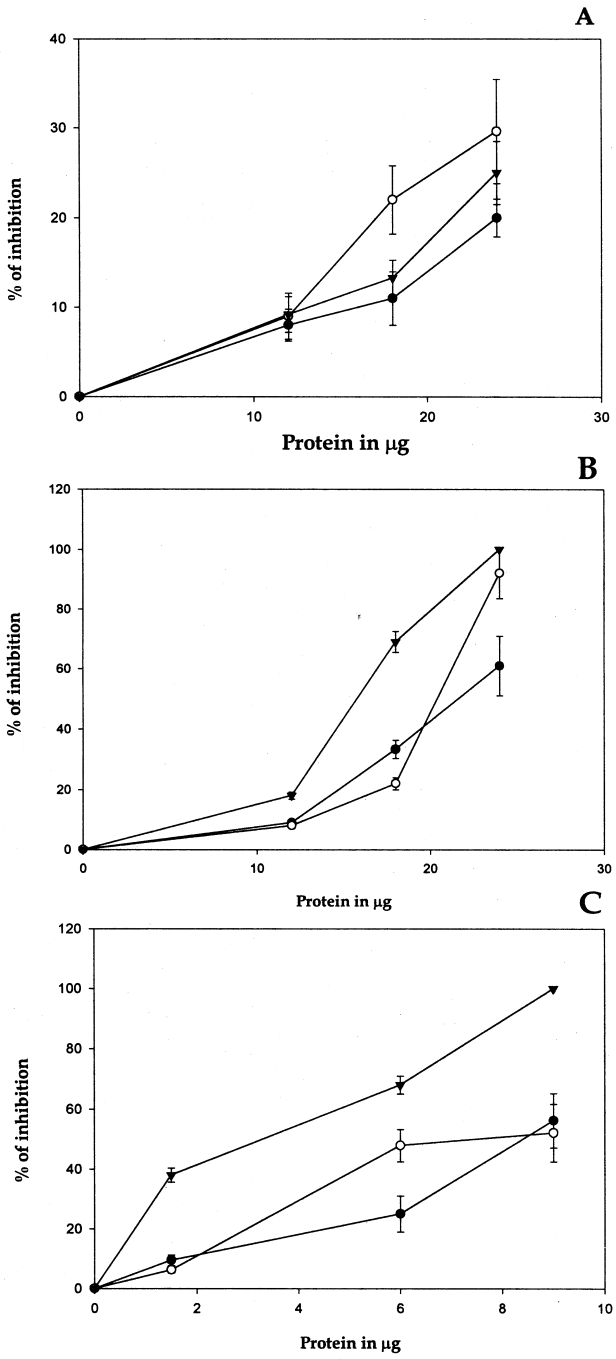

Fig. 3. Effect of phospholipase A_2 NN-I_{2c} (●), NN-I_{2d} (O) AND NN-I_{2e} (▼) concentration on (A) ADP (B) collagen (C) epinephrine induced platelet aggregation. Different concentrations of PLA_2 were separately incubated with PRP for 2 min at 37°C. The platelet aggregation initiated by addition of ADP (76 μM), Collagen (~ 2 μg/ml), epinephrine (22 μM) separately was recorded. Each value represents mean ± S.E.M. of 6 experiments.

fatty acid released was less than 1 μM when phosphatidyl choline was used as the substrate. An equivalent amount of lysophospholipid was also expected to be released.

We examined the role of catalytic activity in the agonists induced platelet aggregation. p-BPB inhibits PLA_2 activity by modifying Histidine-48 residue which is essential for the catalytic function of PLA_2 [18]. In the present investigation modification of all the PLA_2 isoforms by p-BPB resulted in the complete loss of enzyme activity (Table 2). The chemically modified PLA_2 isoforms failed to inhibit platelet aggregation induced by the three agonist. Figure 4 represents the effect of modified NN-I_{2e} PLA_2 on the inhibition of platelet aggregation induced by ADP, collagen and epinephrine.

Discussion

Platelet aggregation is induced by large number of physiological agents such as ADP, serotonin, epinephrine, collagen, thrombin and A23187 ionophore [18]. The platelets interact through a specific receptor mechanism, which mediates shape change, aggregation and release reaction. In the present in-

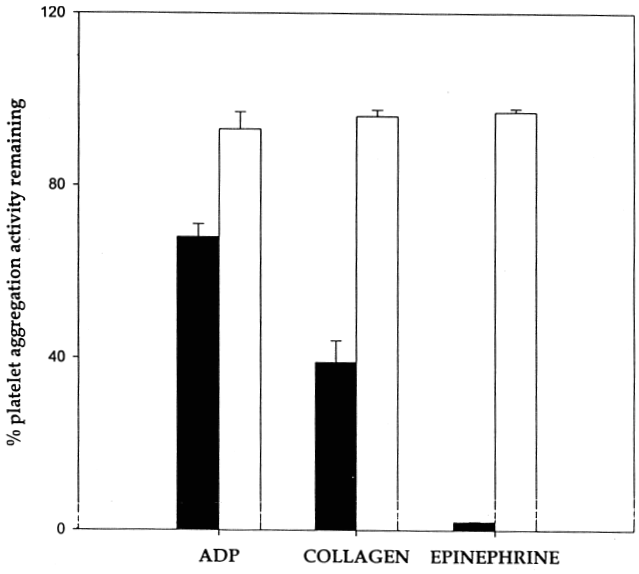

Fig. 4.

Table 2. PLA_2 activity of native and p-BPB modified PLA_2 isoenzymes

Isoenzymes	Specific activity*	
	Native	p-BPB modified
NN-I_{2c}-PLA_2	8.7 ± 0.4	0.1 ± 0.06
NN-I_{2d}-PLA_2	12.8 ± 0.6	0.3 ± 0.05
NN-I_{2e}-PLA_2	16.5 ± 0.3	0.5 ± 0.1

*Specific activity is expressed in terms of μM of fatty acid released/min/mg of protein. Data are mean values ± S.E.M. obtained with 6 experiments.

vestigation the effect of PLA_2 isoforms on platelet aggregation induced by agonist such as ADP, collagen, epinephrine and arachidonic acid was carried out. Platelet aggregation in response to ADP and epinephrine release serotonin and nucleotides mostly ADP, which further initiates aggregation [19–21]. When collagen is the stimulating agent, aggregation is caused by adhesion to platelets followed by release of platelet serotonin and ADP [22–23]. Arachidonic acid induced platelet aggregation is due to the inhibition of thromboxane synthase by its metabolites of the lipoxygenase pathway [24], or due to interference with agonist receptor interaction [25].

Snake venoms are a source of toxins which interfere/modify the process of hemostasis. [3, 26]. The phospholipases of the venoms are one of the major components associated with this function. Based on their effects on the platelet function, venom PLA_2 enzymes can be divided into three major classes: (a) initiate platelet aggregation [8, 11], (b) inhibit platelet aggregation [6, 26–28] and (c) exhibit biphasic effects (initiate aggregation at low concentration or with short incubation time but inhibit aggregation at high concentration or with long incubation time) [11, 29].

The three acidic PLA_2's purified from Indian cobra *Naja naja naja* venom, are catalytically highly active when compared to the basic PLA_2's. So far no pharmacological property associated with the acidic PLA_2 have been reported. All the three isoforms of PLA_2 ($NN\text{-}I_{2c}$, $NN\text{-}I_{2d}$, and $NN\text{-}I_{2e}$) did not induce aggregation of platelets but actively inhibited the platelet aggregation induced by the agonists epinephrine, collagen and ADP. The inhibition of platelet aggregation by PLA_2 isoforms is concentration dependent. Hence these PLA_2 isoforms do not belong to either group (a) or (c) therefore they may be placed under group (b).

An acidic PLA_2 from *Agkistrodon acutus* venom initiates aggregation by enzymatic hydrolysis of platelet phospholipids and inhibits platelet aggregation in PRP which was not dependent on protein concentration [4]. Platelet aggregation inhibition exhibited by *Agkistrodon halys* PLA_2 is independent of PLA_2 activity [2]. However, the inhibition exhibited by the three isoforms of PLA_2 isolated from *Naja naja naja* venom is dependent on concentration and the PLA_2 activity of the enzyme.

The PLA_2 may cause a limited hydrolysis of membrane phospholipids and release arachidonic acid [30–31]. The arachidonate is an agonist, which can induce platelet aggregation/enhance the aggregation induced by the agonist mentioned above [32]. In contrast, arachidonate and lysophosphatidylcholine can also, serve as antagonist of platelet aggregation induced by collagen [31]. The lysophosphatidylcholine formed by the digestion of phospholipids by the PLA_2 enzymes also have effects on the platelet [33]. Therefore the PLA_2 should have served as agonist of platelet aggregation as their action results in the formation of arachidonate and lysophosphotides [32]. However all the isoforms of PLA_2 investigated in this study inhibited the platelet aggregation induced by the agonists epinephrine, collagen and ADP.

The pattern of inhibition brought about by PLA_2 isoforms appears to differ depending on the agonists responsible for platelet aggregation. When identical concentrations of PLA_2 isoforms are used against the platelet aggregation induced by ADP and collagen, a dose dependent platelet aggregation inhibition was observed (Figs 3A and 3B). The extent of inhibition was not comparable especially at higher concentrations of the isoforms of PLA_2. The present study indicates the result of PLA_2 action on platelets which may not be due to release of agonists such as arachidonate and lysophosphotides, but due to modification brought about on the surface of the platelets. The inhibition could be due to the reduced pseudopod induction in the presence of the inhibitor [34].

The platelet aggregation induced by the third agonist in this study, epinephrine is also inhibited by the PLA_2 isoforms from *Naja naja naja* venom. The amount of PLA_2 isoforms present to inhibit platelet aggregation is much lower when compared to the other two agonists used ADP and collagen (Figs 3A–3C). Therefore the amount of arachidonate and lysophosphatides that may be released by these PLA_2 isoforms from the platelets should be lower and should enhance platelet aggregation. Since arachidonate can bring about the platelet aggregation at a concentration range of 0.1–2 μM [32], the PLA_2 concentration used in the study are expected to release arachidonate in this range. It can be seen from the platelet aggregation inhibition by the PLA_2 isoforms when ADP (Fig. 3A) is the agonist, it is about 10% at a PLA_2 concentration of 12 μg. On the other hand the same inhibitory effect is observed at half the concentration of the PLA_2 when epinephrine (Fig. 3C) is used as the agonist. But the extent of inhibition of aggregation observed was markedly higher when compared to the inhibition of platelet aggregation induced by collagen and ADP. This indicates that the platelet aggregation inhibited by the PLA_2 isoforms is not due to the release of arachidonate and lysophosphotides but due to specific modification of the platelets by the catalytic activity of the PLA_2 isoforms. Further, if the fluidity of the membrane is decreased it can lead to reduced interaction among the platelets [35, 36].

Chemical modification of the PLA_2 by p-BPB results in the modification of histidine-48 of the PLA_2 enzymes. Such a modification has been demonstrated to abolish the PLA_2 activity of the enzyme [16, 37]. In our study the chemical modification of all the three isoforms of PLA_2 by p-BPB resulted in the loss of catalytic activity. The chemically modified PLA_2 isoforms lost their platelet aggregation activity induced by epinephrine, collagen and ADP. This further strengthens the involvement of catalytic function in platelet aggregation process. The evidences present in this paper clearly indicate that (a) PLA_2 alters the platelets by their catalytic function, (b) this modification is differentially rec-

ognized in the aggregation process induced by the different agonists.

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