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Some aspects of rat platelet and serum phospholipase A_2 activities

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Rat platelet lysate contained appreciable phospholipase A_2 activity. In agreement with literature data this enzymatic activity eluted in the void volume of a Sephadex G-100 column. When the void volume peak was chromatographed over a Matrex gel blue A column, part of the phospholipase A_2 activity ran through, whereas the remainder was bound to the gel. The latter activity could be eluted with buffers containing a high salt concentration. In contrast, phospholipase A_2 activity solubilized from rat platelet lysates by treatment with high salt eluted from Sephadex G-100 columns with an apparent molecular weight of 10–15 kDa. This solubilized enzyme completely bound to Matrex gel blue A and, in the presence of Ca^{2+} also to an alkylphosphocholine-AH Sepharose affinity column. No indications were obtained for the presence of inactive phospholipase A_2 and activator proteins in rat platelet lysates as described by Etienne, J., Grüber, A. and Polonovski, J. ((1980) Biochim. Biophys. Acta 619, 693–698; (1982) Biochemie 64, 377–380). Phospholipase A_2 activity, both the associated form in platelet lysate and the monomeric form as eluted from Sephadex G-100 was slightly inhibited by trifluoperazine but calmodulin exerted no stimulation. Likewise, phospholipase A_2 activity from rat serum eluted in the void volume of a Sephadex G-100 column. Rather than indicating the presence of high molecular weight forms of the enzyme, this is apparently caused by association with lipids or other proteins, in that chromatography in the presence of high salt revealed a molecular weight similar to that found for solubilized platelet phospholipase A_2 activity.

Introduction

Free arachidonic acid is the precursor of prostaglandins, leukotrienes, thromboxanes and prostacyclin [1,2]. Phospholipids in membranes have a high content of esterified arachidonic acid and it is believed that liberation of this acid from the phospholipids is the rate-limiting step in the synthesis of eicosanoids [3]. Several mechanisms are described in the literature for the mobilization of this tetraenoic acid [4–6]. A straightforward way to liberate this fatty acid from the *sn*-2 position of phosphoglycerides is by the action of phospholipase A_2. By regulating this phospholipase A_2 activity, the amount of biologically active polyunsaturated fatty acid derivatives can be controlled. Several models to regulate membrane-bound phospholipase have been put forward in the literature (for a recent review, see Ref. 7). One of the possible regulating mechanisms is by interaction of phospholipase A_2 with non-enzymatic proteins. In recent years, the presence of natural inhibitor proteins for a large variety of enzymes has been reported [8–14].

Inhibitor proteins for phospholipase A_2 have been described by Flower and coworkers in guinea pig lungs [15], and rat peritoneal leukocytes [16], by Hirata et al. in rabbit neutrophils [17,18], and murine thymocytes [19], and by Rothut et al. [20] in rat renomedullary interstitial cells. The formation of these inhibitor proteins appears to be induced by glucocorticoids. Phospholipase A_2

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inhibitor proteins were also reported to be present in bovine plasma [21] and porcine [22] and human serum [23], whereas evidence for the presence of a lipidic inhibitor in human platelets was obtained [24].

Another model for the regulation of phospholipase A_2 postulated the existence of inactive enzymes that can be activated by association with activator proteins. Rat platelet lysates were reported to contain a factor that was able to activate phospholipase A_2 activity in rat plasma, human serum and plasma, rabbit plasma and human erythrocyte lysates from 6- to 35-fold [25]. Further experiments revealed that the phospholipase A_2 activity in both rat serum [26] and rat platelets [27] resulted from an association of inactive enzyme and an activator protein. These two components could be separated by blue Sepharose CL-6B chromatography to yield fractions virtually devoid of phospholipase A_2 activity. Full activity was reported to be restored by recombination of the components [26,27]. Rat platelet phospholipase A_2 behaved in an unusual manner in these experiments, in that it eluted in the void volume of a Sephadex G-100 column. Without exception, Ca^{2+}-dependent phospholipases A_2, including those from pancreas [28,29], snake venoms [30,31], sheep erythrocytes [32], rat liver mitochondria [33] and rabbit platelets [34], exhibit molecular weights of about 15 kDa. To clarify the unusual behaviour of rat platelet phospholipase A_2, we initiated experiments to partially purify the phospholipase A_2 activity from rat platelet lysates and from rat serum and to further characterize the activating factor. The results of these experiments are reported in this paper.

Materials and Methods

Methods

Preparation of rat platelet lysate and high salt extract. Platelet-rich plasma was prepared by low-speed centrifugation of freshly obtained citrated rat blood. This platelet-rich plasma was centrifuged once again for 15 min at $300 \times g$ at room temperature. The upper three-quarters of this centrifuged platelet-rich plasma were then spun for 15 min at $2600 \times g$. The pellet was washed twice with a procaine/NaCl solution (12.15 g and 1.0 g, respectively, per 500 ml), and the washed rat platelets were then resuspended in 50 mM Tris buffer (pH 7.4), freeze-thawed five times, and sonicated for two periods of 15 s each, using a Branson Sonifier B12 with an output of 50 W, while cooling in an ice bath. This platelet suspension was then centrifuged in a Sorvall SS-34 rotor for 5 min at $27\,000 \times g$, and the supernatant was designated platelet lysate.

A high salt extract was prepared by resuspending washed rat platelet sin 50 mM Tris/1 M KCl buffer (pH 7.4); it was freeze-thawed and sonicated as before, and stirred at 4°C for 18 h. The mixture was then centrifuged at $42\,000 \times g$ for 15 min to yield a high salt extract.

Assay of phospholipase A_2 activity. Phospholipase A_2 activity was assayed with 100 nmol of the appropriate phospholipid as a sonicated dispersion in water in the presence of 10 mM Ca^{2+}, 100 mM Tris-HCl (pH 8.5) in a final volume of 0.5 ml. After incubation at 37°C the liberated fatty acid was extracted according to a modified Dole extraction procedure as previously described [35].

To determine phospholipase A_1 and lysophospholipase activities, 300 nmol of 1-acyl-2-[1-^{14}C]linoleoylphosphatidylethanolamine or 26 nmol of phosphatidyl[*methyl*-^{14}C]choline was incubated as described for the phospholipase A_2 assay. After incubation at 37°C the reaction was terminated by extracting the lipids according to Bligh and Dyer [36]. The water/methanol layer was measured for radioactivity and the lipids in the chloroform phase were separated on thin-layer plates. Spots were detected by staining with iodine and, after evaporation of iodine, scraped into scintillation vials for determination of radioactivity.

Protein was determined by the procedure described by Bradford [37].

Materials

Substrates. 1-Acyl-2-[1-^{14}C]linoleoylphosphatidylcholine and 1-acyl-2-[1-^{14}C]linoleoylphosphatidylethanolamine were prepared as described previously [35].

Phosphatidyl[*Me*-^{14}C]choline was a product from New England Nuclear.

Affinity column. Synthesis of ligand and its coupling to AH-Sepharose have been published recently [29].

Matrex gel Blue A was used as purchased from both Amicon Corporation, Lexington, MA, U.S.A. and Pharmacia, Uppsala, Sweden (blue Sepharose).

Procaine hydrochloride was obtained from Aldrich Europe, Beerse, Belgium, and aquacide was a product from Calbiochem, La Jolla, CA, U.S.A.

Calmodulin from bovine brain and trifluoperazine were kindly donated by Dr. P.Y.-K. Wong, Valhalla, New York. Silica gel used to free extracted fatty acids in the heptane layer from contaminating substrates was Silic AR CC-4 as obtained from Mallinckrodt, St. Louis, MO, U.S.A.

Results and Discussion

When a freeze-thawed and sonicated platelet suspension was centrifuged (see Materials and Methods section), 40–50% of the protein content and 20–60% of phospholipase A_2 activity ($n = 4$) remained in the supernatant designated platelet lysate. Optimal phospholipase A_2 activity in such a platelet lysate was found at pH 8.5 with 5 mM Ca^{2+}. In contrast to Etienne et al. [38] who showed optimal activity at 16 μM phosphatidylethanolamine, in our hands a substrate concentration of 0.5 mM was not yet saturating (results not shown). When rat platelet lysate or a high salt extract of platelets were incubated with 1-acyl-2-[1-^{14}C]linoleoylphosphatidylethanolamine, only radioactive free fatty acids were formed, whereas with [*choline-methyl*-^{14}C]phosphatidylcholine, only ^{14}C-labeled lysophosphatidylcholine was produced and no labeled glycerophosphocholine could be detected. This indicated that under the incubation conditions used, only phospholipase A_2 activity was measured.

When rat platelet lysate was chromatographed on a Sephadex G-100 column, phospholipase A_2 activity was mainly eluted in the void volume peak, as did most of the protein (Fig. 1). The large A_{280nm} peak in fractions 28–34 was caused by procaine and appeared to contain no protein. Recovery of the enzymatic activity varied from 20 to 40%, whereas protein recovery was more than 80%. The low recovery of enzymatic activity could not be improved by combining the void volume peak with other column fractions, indicating the absence of an activator in the eluate. Further chromatography of this G-100 breakthrough peak on Matrex gel blue column (Fig. 2) showed that enzymatic activity was eluted both in the low salt peak (fractions 1–5) and in the high salt peak (fractions 8–12). Here again, the recovery of enzymatic activity was low, varying from 5 to 40% ($n = 3$). Recombination experiments using peak I (fractions 2–5), peak II (fractions 8–12), fractions 6–7 and fractions 13–20 did not reveal any improvement of the recovery. However, the phospholipase A_2 activity is apparently tightly bound to the dye column and enzymatic activity could still be eluted from the column after fraction 20.

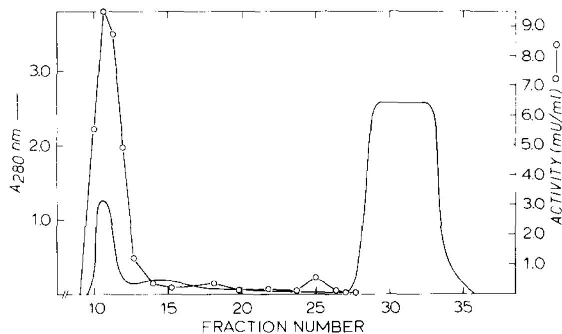

Fig. 1. Sephadex G-100 filtration of rat platelet lysate. Elution buffer: 50 mM Tris-HCl (pH 7.4) (column size 1.9×56 cm). Fractions of 20 min were collected at a flow rate of 12 ml/h.

Collectively, the results of Figs. 1 and 2 show that at low ionic strength the phospholipase A_2 activity in rat platelet lysate either is a high molecular weight protein or that the enzyme is still present in an aggregated form. The results ob-

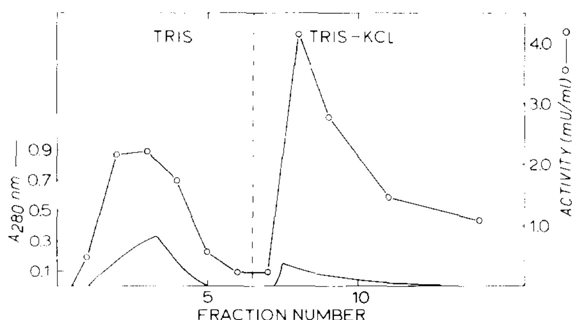

Fig. 2. Matrex gel blue A chromatography of phospholipase A_2 activity. The void volume peak of the Sephadex G-100 column was chromatographed on a Matrex gel blue A column (1.0×10 cm) at a flow rate of 10 ml/h. Fractions of 20 min were collected. After fraction 7, the 50 mM Tris-HCl buffer (pH 7.4) was replaced by the same Tris buffer containing 1 M KCl.

tained on Matrex gel blue A column deviate from those reported by Etienne et al. [27]. These authors have claimed the presence in rat platelet lysate of activator protein(s) capable of enhancing the activity of phospholipase A_2 from various other sources, including human platelets and human serum and plasma [25]. Subsequently, they were able to separate an inactive phospholipase A_2 and an excess of activator protein from rat platelet lysate [27]. By recombination of peak I (inactive phospholipase) and peak II (activator), the original phospholipase A_2 activity applied to the column could be recovered completely [27].

In order to establish the presence of an activator in rat platelet lysate, its effect on the phospholipase A_2 activity in human platelet lysate was investigated. The results (Table I) show that the phospholipase activity in both human and rat platelet lysate is about 2.5-fold more active at pH 9 than at pH 7, and that on protein basis the rat platelet enzyme is about 300-fold more active than that of human platelet lysate. Neither at pH 7 and at pH 9 was a many-fold increase of human platelet phospholipase A_2 activity by addition of rat platlet lysate observed (Table I). This lack of activation was confirmed in other experiments in which the ratio of rat platelet lysate to human platelet lysate protein was varied from 1 : 1000 to 1 : 10 (not shown). Also, when human serum or human plasma were preincubated with rat platelet lysate, no indications for phospholipase A_2 activation were obtained.

To verify the possibility that the phospholipase A_2 activity in rat platelet lysate was still present in an aggregated form (compare Fig. 1), rather than representing a high molecular weight enzyme, we extracted a freeze-thawed and sonicated platelet suspension with buffer containing 1 M KCl according to Kannagi and Koizumi [34]. Following this procedure, 60–80% of both protein and phospholipase A_2 activity was recovered in the supernatant. Indeed, when such a high salt extract was chromatographed on Sephadex G-100, enzymatic activity eluted with an apparent molecular weight of 10000–15000 (Fig. 3). Fractions with phospholipase A_2 activity were pooled, concentrated in a dialysis bag in aquacide, and dialyzed against 50 mM Tris-HCl buffer (pH 7.4) containing 0.1 M KCl. The dialyzed protein solution was then percolated through a Matrex gel blue A column. The column was rinsed with the same buffer until the eluate appeared free of protein. Enzymatic activity was then eluted with buffer containing 1 M KCl (Fig. 4). Compared to the applied activity, a recovery of about 150% was found. Chromatography of the Matrex gel blue A fraction, after concentration and dialysis, on an affinity column as previously described [29], showed that enzymatic activity was bound to the immobilized ligand in the presence of Ca^{2+} and that it could be eluted with buffers containing EDTA (Fig. 5).

Recoveries of the solubilized phospholipase A_2 activities from these columns were all slightly over 100%, and no indications for separation into an

TABLE I

EFFECT OF RAT PLATELET LYSATE ON PHOSPHOLIPASE A_2 ACTIVITY IN HUMAN PLATELET LYSATE

Human or rat platelet lysate (75 μg and 6 μg protein, respectively) were incubated either separately or in combination. Results of two experiments at the indicated pH values are expressed as mU per assay.

pH	Phospholipase A_2 activity		
	human	rat	human + rat
7	0.008	0.25	0.17
	0.011	0.27	0.16
9	0.030	0.68	0.58
	0.025	0.68	0.62

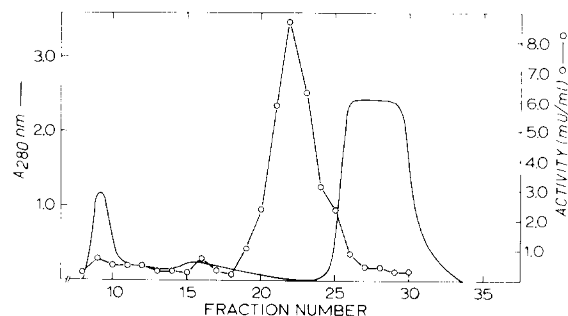

Fig. 3. Sephadex G-100 filtration of 1 M KCl extract of rat platelet lysate. Elution buffer: 50 mM Tris-HCl (pH 7.4), 1 M KCl. Fractions of 20 min were collected at a flow rate of 13 ml/h.

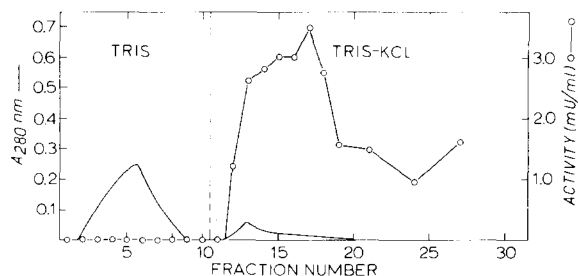

Fig. 4. Matrex gel blue A chromatography of phospholipase A_2 activity of a rat platelet KCl extract after Sephadex filtration. Elution buffer: 50 mM Tris-HCl (pH 7.4), 0.1 M KCl. After fraction 10 this buffer was replaced by the same buffer containing 1 M KCl. Fractions of 15 min were collected at a flow rate of 7 ml/h.

inactive phospholipase A_2 and its activator protein were obtained. Furthermore, the solubilized phospholipase A_2 activity from rat platelets exhibited a molecular weight comparable to that of other purified membrane-bound phospholipases A_2 [32–34,39]. In addition, the enzyme eluted from Matrex gel blue A with a high salt buffer, and in this respect behaved like phospholipase A_2 from rat liver mitochondria [33], and cobra venom [31]. Likewise, the behaviour of the monomeric platelet phospholipase A_2 on an affinity column, i.e., binding in the presence of Ca^{2+} and elution with buffers containing EDTA, is completely comparable to that of phospholipase A_2 activities from cobra venom [29,40], porcine pancreas [29], sheep erythrocytes [32] and rat liver mitochondria [29]. The combined results of Figs. 3–5 make it extremely unlikely that the partially purified rat platelet phospholipase A_2 activity still consists of a complex of an inactive phospholipase A_2 together with an activator.

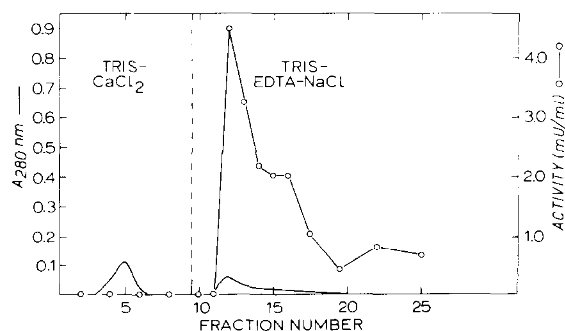

Fig. 5. Alkylphosphocholine-AH Sepharose affinity chromatography of the Matrex gel blue A phospholipase A_2 activity peak. Column size: 1.5×3.5 cm; elution buffer: 50 mM Tris-HCl, 1 mM EDTA, 200 mM NaCl and 10 mM $CaCl_2$ (pH 7.4). After fraction 9 this buffer was replaced by a buffer containing 50 mM Tris, 50 mM EDTA and 1 M NaCl (pH 7.4).

In view of the small amount of material available, the above experiments did not aim to obtain a complete purification of rat platelet phospholipase A_2. In addition, too little protein was left after the affinity chromatography step to allow for an accurate specific activity determination.

Rat serum also contains relatively high phospholipase A_2 activity. As in platelets, this activity has been ascribed to the presence of an inactive phospholipase A_2 in rat plasma which can then be activated by an activator protein released from platelets [25]. Chromatography of a 30–55% ammonium sulfate fraction from rat serum on blue Sepharose CL 6B has been claimed [26] to separate the inactive enzyme, which was eluted with low salt from the activator protein eluted with high salt

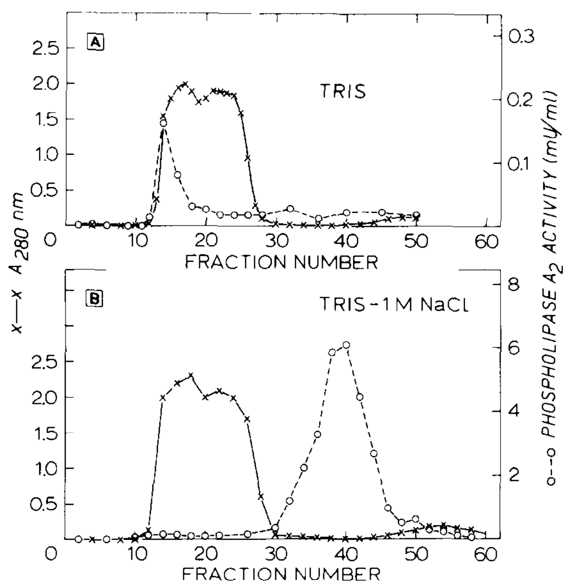

Fig. 6. Sephadex G-100 filtration of rat serum. Rat serum (2 ml) was chromatographed over a Sephadex G-100 column (1.9×56 cm) at a flow rate of 10.8 ml/h. Fractions of 2.7 ml were collected. A, elution with 50 mM Tris-HCl (pH 7.4). Recovery of protein and phospholipase A_2 activity amounted to 99% and 2%, respectively. B, rat serum was made 1 M with respect to NaCl and the column was eluted with 50 mM Tris-HCl (pH 7.4), containing 1 M NaCl. Recovery of protein and phospholipase A_2 activity was 94% and 114%, respectively.

TABLE II

EFFECT OF SODIUM DEOXYCHOLATE AND TRIFLUOPERAZINE ON PLATELET PHOSPHOLIPASE A_2 ACTIVITY

The indicated substrates (100 nmol) were incubated for 15 min with partially purified rat platelet phospholipase A_2 preparation (Sephadex G-100 peak of high salt extract) in the presence or absence of 2 mM sodium deoxycholate and trifluoperazine as indicated. Values of two experiments are given as nmol fatty acid released in 15 min per 10 μl Sephadex G-100 fraction. TFP, trifluoperazine.

	Phospholipase A_2 activity					
	− deoxycholate			+ deoxycholate		
	−TFP	+TFP (25 μM)	+TFP (50 μM)	−TFP	+TFP (25 μM)	+TFP (50 μM)
Phosphatidylcholine	0.44	0.42	0.36	0.84	0.74	0.66
	0.56	0.50	0.32	0.96	0.84	0.74
Phsophatidylethanolamine	3.61	2.41	1.98	18.8	14.0	13.9
	3.38	2.50	2.22	17.1	15.8	12.8

in much the same way as described for platelet lysates [27]. In agreement with our earlier results which failed to detect activator proteins' in rat platelet lysate (compare Table I), chromatography of the 30–55% ammonium sulfate fraction from rat serum on Cibacron blue 3GA columns, either Matrex gel blue A (Amicon) or blue Sepharose CL 6B (Pharmacia), did not reveal such proteins in rat serum. Just like other phospholipases A_2, the rat serum phospholipase A_2 activity was only eluted from these columns with buffer containing high salt, although at low yields varying from 1 to 10% ($n = 4$). Subsequent exhaustive elution with high salt buffer indicated considerable tailing of the activity but recombination of eluted fractions gave no additional increase in recovery. The low recovery of rat serum phospholipase A_2 was also encountered upon chromatography over Sephadex G-100. As can be seen in Fig. 6A, activity eluted in the void volume peak with a recovery of less than 10% while protein recoveries ranged from 85 to 100%. This behaviour changed by the addition of 1 M NaCl to total rat serum prior to Sephadex G-100 filtration (Fig. 6B). This not only increased the recovery of phospholipase A_2 activity to 85–100% while leaving protein recovery unaffected, but it also shifted the elution volume of the phospholipase A_2 to that expected for a protein with a molecular weight of about 15 kDa. We would like to suggest from these experiments that the phospholipase A_2 activity as detectable in both rat platelets and rat serum is not caused by the presence of inactive enzymes in conjunction with activator proteins. Rather, the enzymatic activity seems to reside in a low molecular weight protein as is the case for most other Ca^{2+}-requiring phospholipases A_2.

The partially purified platelet phospholipase A_2 hydrolyzed phosphatidylethanolamine 7-fold more rapidly than phosphatidylcholine (Table II). Apparently, this preference is not merely an effect of the aggregated phospholipid structures, i.e., hexagonal H_{II} for phosphatidylethanolamine and lamellar for phosphatidylcholine. The preference for phosphatidylethanolamine hydrolysis sustained when both lipids were used in mixed micellar form with sodium deoxycholate. Hydrolysis of the two substrates both in the absence and presence of sodium deoxycholate was slightly inhibited by trifluoperazine (Table II).

Table III shows that rat platelet phospholipase A_2, both in crude lysate and in partially purified form after Sephadex G-100 filtration (compare Fig. 3), is completely Ca^{2+}-dependent. Although phospholipase A_2 activity in the presence of 100 μM $CaCl_2$ was slightly inhibited by trifluoperazine, this did not appear to be caused by its specific action as calmodulin antagonist. Trifluoperazine inhibition could not be overcome by calmodulin addition and calmodulin alone did not stimulate phospholipase A_2, either in the absence or in the presence of added $CaCl_2$ (Table III). We conclude from these experiments that the Ca^{2+} requirement of rat platelet phospholipase A_2 is

TABLE III

EFFECT OF VARIOUS ADDITIONS ON PLATELET PHOSPHOLIPASE A_2 ACTIVITY

Rat platelet lysate or Sephadex G-100 fractions obtained after chromatography of a 1 M KCl extract of platelet lysate were assayed for phospholipase A_2 activity. Indicated additions were as follows: EDTA, 10 mM; $CaCl_2$, 100 μM; calmodulin, 5 μM and trifluoperazine, 50 μM. Values of two experiments are expressed in mU/ml.

Addition	Phospholipase A_2 activity	
	lysate	Sephadex G-100 fraction
None	0.36; 0.41	0.49; 0.45
EDTA	0.00; 0.00	0.00; 0.00
Calmodulin	0.32; 0.32	0.32; 0.40
$CaCl_2$	1.27; 1.22	0.65; 0.73
$CaCl_2$ + calmodulin	0.90; 0.78	0.49; 0.53
$CaCl_2$ + trifluoperazine	1.00; 0.90	0.62; 0.64
$CaCl_2$ + calmodulin + trifluoperazine	0.72; 0.60	0.42; 0.41

not mediated by calmodulin. This conclusion contrasts with the original suggestion made by Wong and Cheung [41], that stimulation of human platelet phospholipase A_2 activity by Ca^{2+} is regulated by calmodulin, but is in good agreement with the more recent results of Withnall et al. [42,43]. These authors also concluded hat calcium regulation of rat and human platelet phospholipase A_2 is independent of calmodulin. It should be mentioned in this respect that Ballou and Cheung [24], in reevaluating the calmodulin effect on human platelet phospholipase A_2, have reported this effect to be variable in crude enzyme preparations, whereas partially purified enzyme was affected neither by calmodulin, nor by trifluoperazine. Thus, platelet phospholipase A_2 appears to be activated directly by Ca^{2+} without calmodulin mediation. Similar conclusions were reached for pancreatic [42] and for rat liver mitochondrial phospholipase A_2 [44,45].

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