Primary role of calcium ions in arachidonic acid release from rat platelet membranes

Comparison with human platelet membranes

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The liberation of arachidonic acid (AA) was investigated in platelet membranes prelabelled with [³H]AA. In rat platelet membranes, Ca²⁺ at concentrations over several hundred nanomolar induced [³H]AA release, with a concurrent decrease in ³H radioactivity of phosphatidylethanolamine and phosphatidylcholine. Some 4–6 % of total radioactivity incorporated into platelet membrane lipids was released at 1–10 μM-Ca²⁺, which is nearly equivalent to that attained in agonist-stimulated platelets. Formation of lysophospholipids in [³H]glycerol-labelled membranes and decrease in [³H]AA liberated by the phospholipase A₂ inhibitors mepacrine and ONO-RS-082 suggest that [³H]AA release is mainly catalysed by phospholipase A₂. In intact platelets agonist-stimulated [³H]AA release was markedly decreased in the absence of extracellular Ca²⁺ or in the presence of the intracellular Ca²⁺ chelator quin 2. These results indicate that in rat platelets the rise of intracellular Ca²⁺ plays a primary role in the activation of phospholipase A₂. In contrast, Ca²⁺ even at high millimolar concentrations did not effectively stimulate [³H]AA release in human platelet membranes. Thus factor(s) additional to or independent of Ca²⁺ is required for the liberation of AA in human platelets.

INTRODUCTION

In response to certain physiological or pharmacological stimuli, platelets release arachidonic acid (AA), which is then converted into biologically active prostaglandins, thromboxanes and 12-HETE. Because the intracellular concentration of free AA is low, the liberation of AA from its *sn*-2 linkage in membrane phospholipids is a necessary first step toward the production of thromboxane A₂ and other eicosanoids [1]. Two major pathways have been proposed for AA liberation; direct deacylation of phospholipids by phospholipase A₂ [2], and sequential action of phosphoinositide-specific phospholipase C followed by diacylglycerol (DG) lipase [3]. The former is thought to play a greater role for AA release in stimulated platelets [4–8].

Phospholipase A₂ has been purified from the membrane as well as the cytosolic fraction of platelets [9–15]. Although most investigators have reported that maximal activation of the enzyme system *in vitro* requires millimolar Ca²⁺ concentrations, some recent studies have described its activation at micromolar Ca²⁺ [13–15]. As for the regulatory mechanism(s) of phospholipase A₂ in receptor-stimulated platelets, several possibilities have been proposed: limited proteolysis [16], lipocortin [17], membrane physical state (substrate accessibility) [18] and Na⁺/H⁺ exchange [19,20]. However, the precise mechanism underlying the receptor-mediated phospholipase A₂ activation is still obscure. Our recent observations have suggested evidence for implication of GTP-

binding protein in phospholipase A₂ activation in permeabilized human platelets [21,22].

The present study was designed to examine phospholipase A₂ activation in [³H]AA-prelabelled platelet membrane preparations. It was found that the activity was critically dependent on the free Ca²⁺ concentration in rat platelet membranes, but not in human platelet membranes. In rat platelet membranes, phospholipase A₂ was effectively activated by Ca²⁺ at 0.5–10 μM, which corresponded to cytoplasmic Ca²⁺ concentrations observed in stimulated platelets.

EXPERIMENTAL

Preparation of [³H]AA-labelled platelets

Human blood was drawn from healthy young volunteers, who had no medication for at least 2 weeks [22]. Rat blood was removed from the abdominal aorta of overnight-fasted male Wistar rats, into plastic syringes with 1 vol. of anticoagulant (acid-citrate-dextrose) [22] for 6 vol. of blood. Platelet-rich plasma was obtained after centrifugation at 200 *g* for 10 min. The platelet pellet, obtained after centrifugation (1000 *g* for 10 min) of the platelet-rich plasma, was washed twice at room temperature in 25 ml of a modified Tyrode-Hepes buffer (134 mM-NaCl, 12 mM-NaHCO₃, 2.9 mM-KCl, 0.36 mM-NaH₂PO₄, 1 mM-MgCl₂, 5.9 mM-dextrose, 10 mM-Hepes, pH 7.40) containing 1 mM-EGTA. To incorporate [³H]AA, 5 ml of a modified Tyrode-Hepes solution containing [³H]AA (0.5 μCi/ml) complexed to fatty acid-

Abbreviations used: AA, arachidonic acid; DG, 1,2-diacylglycerol; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; BSA, bovine serum albumin; [Ca²⁺]_i, intracellular free Ca²⁺ concentration; BW755C, 3-amino-1-[*m*-(trifluoromethyl)phenyl]-2-pyrazoline; 12-HETE, 12-hydroxy-5,8,10,14-eicosatetraenoic acid; ONO-RS-082, 2-(*p*-amylcinnamoyl)amino-4-chlorobenzoic acid; RHC 80267, 1,6-di[*O*-(carbamoyl)cyclohexanone oxime]hexane.

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free bovine serum albumin (BSA) (100 mg/ml) [23] was added to 5 ml of platelet suspension in a modified Tyrode-Hepes buffer. After incubation for 120 min at 37 °C in a shaking incubator bath, platelets were washed twice in a modified Tyrode-Hepes buffer containing 1 mM-EGTA. To obtain quin 2-loaded platelets, quin 2 acetoxymethyl ester was added during the last 30 min of the [3 H]AA-labelling period. In some experiments, platelets were incubated with 10 μ Ci of [3 H]glycerol/ml for 120 min at 37 °C to obtain [3 H]glycerol-labelled platelets.

[3 H]AA liberation in rat intact platelets

Platelets were suspended in a modified Tyrode-Hepes buffer at a concentration of 5×10^9 cells/ml. The standard reaction was carried out in a 250 μ l volume containing 200 μ l of platelet suspension in the presence of BW755C (100 μ M), an inhibitor of both cyclo-oxygenase and lipoxigenase activities [24], to obtain accurate recovery of released [3 H]AA [25], since released AA is rapidly converted into its metabolites. In the presence of 100 μ M-BW755C, more than 95% of released [3 H]AA was recovered as unmetabolized [3 H]AA (results not shown). Incubations were carried out in a shaking incubator bath at 37 °C for 5 min. The reaction was terminated by addition of 2 ml of chloroform/methanol (1:2, v/v). After the reaction was terminated, the samples were mixed with 250 μ l of the buffer and then vortex-mixed.

Membrane preparation and measurement of its phospholipase A_2 activity

Washed platelet pellet was suspended in 10 ml of ice-cold relaxation buffer (50 mM-KCl, 1 mM-EGTA, 100 mM-Tris/HCl, pH 7.40). The suspension was kept at 4 °C. The cells were essentially lysed by nitrogen cavitation [8.3 MPa (1200 lb/in 2) for 20 min]. Where indicated, the cells were lysed by three other methods; homogenized with a motor-driven Teflon pestle in a glass homogenizer (30 strokes), sonicated with a micro-probe (Branson Sonifier, B-12) for 4 $\times$ 15 s, or frozen and thawed twice with liquid N $_2$. The lysate was centrifuged at 1000 g for 10 min to remove unbroken cells, and the resulting supernatant was then centrifuged at 105 000 g for 60 min at 4 °C. The pellet was suspended in buffer A (75 mM-KCl, 1 mM-EGTA, 50 mM-Tris/HCl, pH 7.40) and washed once. The final membrane pellet was suspended in buffer A. Protein concentration was measured by the Bio-Rad protein assay.

Incubations were carried out in a shaking incubator bath at 37 °C for 20 min. The assay, in a final volume of 500 μ l, contained 50 μ g of membrane protein, 75 mM-KCl, 1 mM-EGTA, 0.4% BSA and 50 mM-Tris/HCl, pH 7.40. The free Ca $^{2+}$ concentration was modified in the reaction buffers as required by varying the amount of CaCl $_2$ added [26]. The reaction was stopped by 2 ml of chloroform/methanol (1:2, v/v).

Lipid extraction and analysis

Lipids were extracted essentially by the method of Bligh & Dyer [27]. The phases were separated by the addition of 0.6 ml of chloroform and 0.6 ml of 0.2 M-KCl/5 mM-EDTA [26]. After centrifugation, the lower organic phase was removed and evaporated under a stream of N $_2$ gas. Neutral lipids were separated on silica-gel 60 plates impregnated with 0.4 M-boric acid in the solvent system chloroform/acetone (24:1, v/v) [26]. Phospholipids were separated by two-dimensional

t.l.c. on silica-gel 60 plates impregnated with 2.5% (w/v) magnesium acetate, in chloroform/methanol/aq. 13.5 M-NH $_3$ (65:35:6, by vol.) in the first dimension and chloroform/acetone/methanol/acetic acid/water (6:8:2:2:1, by vol.) in the second [26]. Individual lipids were observed by exposure of the plates to I $_2$ vapour and identified by co-migration with authentic standards. The areas corresponding to individual lipid fractions were scraped into vials and the radioactivity was determined in a liquid-scintillation counter [26].

Chemicals

[5,6,8,9,11,12,14,15- 3 H]AA (230 Ci/mmol) was purchased from Amersham, and [1,2,3- 3 H]glycerol (38.2 Ci/mmol) was from New England Nuclear. BW755C was from Teikokuzoki Co. Collagen was from Horm. Silica-gel 60 plates were obtained from Merck. BSA and A23187 were obtained from Sigma. Thrombin was from Mochida Pharmaceutical Co. Quin 2 acetoxymethyl ester was from Dojindo Lab. ONO-RS-082 [20] was provided from Ono Pharmaceutical Co. RHC 80267 [28] was generously given by Dr. P. W. Majerus (Washington University School of Medicine). All other chemicals were of analytical grade.

RESULTS

[3 H]AA release in human and rat platelet membranes

[3 H]AA-labelled membrane preparations were incubated for 20 min at pH 7.40 with BSA and various concentrations of Ca $^{2+}$. Since BSA binds non-esterified fatty acid, which is reported to attenuate the activity of phospholipase A_2 [12,29], BSA was included in the incubation medium. Ca $^{2+}$ did not effectively stimulate AA release in [3 H]AA-labelled human platelet membranes (Fig. 1). To disrupt human platelets, four different methods were employed; sonication, freezing-thawing, homogenization in a glass homogenizer, and nitrogen cavitation. None of the methods was successful to obtain the membrane preparations which liberate [3 H]AA in response to Ca $^{2+}$. In contrast, in rat platelet membranes the liberation of [3 H]AA was dose-dependently enhanced by Ca $^{2+}$ (Fig. 1). In general, the assay of phospholipase A_2 *in vitro* was performed in the presence of millimolar Ca $^{2+}$, by using exogenously added substrate. However, in endogenously [3 H]AA-labelled rat platelet membranes, micromolar Ca $^{2+}$ (the concentration attained intracellularly in agonist-stimulated platelets) induced the liberation of a considerable amount of AA. Thus the AA release in response to Ca $^{2+}$ was markedly different between two types of platelets. Therefore the role of Ca $^{2+}$ in the regulation of AA liberation was further examined in rat platelets.

[3 H]AA release in rat intact platelets

In response to ionophore A23187, thrombin and collagen, rat platelets released [3 H]AA. However, the release of AA was dependent on extracellular Ca $^{2+}$ (Table 1). Omission of exogenously added Ca $^{2+}$ was sufficient to decrease the liberation of AA drastically. Owing to its high Ca $^{2+}$ buffering capacity, quin 2 is often used as a potent intracellular Ca $^{2+}$ chelator [30,31]. Loading platelets with increasing concentrations of quin 2 did not affect [3 H]AA incorporation into their phospholipids, compared with control unloaded platelets (results not shown). As shown in Fig. 2, AA release was dose-

Fig. 1. Effect of Ca^{2+} concentration on $[^3\text{H}]\text{AA}$ release in $[^3\text{H}]\text{AA}$ -labelled membranes prepared from human or rat platelets

Washed platelets were incubated with $[^3\text{H}]\text{AA}$ for 120 min at 37°C and were lysed by nitrogen cavitation [8.3 MPa (1200 lb/in²) for 20 min]. The reaction mixture contained membrane (50 μg of protein), BSA and various concentrations of Ca^{2+} , and incubation was carried out at 37°C for 20 min. The details are described in the Experimental section. The results are expressed as a percentage of the total $[^3\text{H}]\text{AA}$ incorporated into the membrane lipids. The values are means of triplicate determinations. Six other experiments gave similar results. Key: $\square$, human; $\square$ (stippled), rat.

Table 1. Effects of extracellular Ca^{2+} on $[^3\text{H}]\text{AA}$ release in $[^3\text{H}]\text{AA}$ -labelled rat platelets

$[^3\text{H}]\text{AA}$ -labelled rat platelets were incubated for 5 min at 37°C with the indicated agents in the absence or presence of 1 mM- Ca^{2+} . The reaction was terminated and the lipids were analysed after separation by t.l.c. as described in the Experimental section. The results shown are mean values $\pm$ S.D. (d.p.m.) of triplicate determinations from one of three similar experiments: * $P < 0.001$ compared with 1 mM- Ca^{2+} .

Addition	$[^3\text{H}]\text{AA}$ release (d.p.m.)	
	1 mM- Ca^{2+}	No Ca^{2+}
Control	4095 $\pm$ 256	2662 $\pm$ 189*
A23187 (2 μM)	24503 $\pm$ 1689	4714 $\pm$ 576*
Thrombin (2 units/ml)	15309 $\pm$ 875	4191 $\pm$ 348*
Collagen (10 $\mu\text{g}/\text{ml}$)	15396 $\pm$ 1256	2749 $\pm$ 268*

Fig. 2. Effect of quin 2 concentration on $[^3\text{H}]\text{AA}$ release in rat platelets

Platelets were loaded with increasing concentrations of quin 2 acetoxymethyl ester (AM) as described in the Experimental section. The platelets were incubated for 5 min with thrombin ($\blacktriangle$, $\triangle$) or A23187 ($\bullet$, $\circ$) in the absence ($\triangle$, $\circ$) or presence ($\blacktriangle$, $\bullet$) of 1 mM- Ca^{2+} . The results are expressed as percentages of the values determined for quin 2-non-loaded platelets. Each point is the mean of triplicate determinations. Two other experiments gave similar results.

independently suppressed by quin 2. The inhibitory effect could be largely overcome by the addition of 1 mM- Ca^{2+} , when $\sim 50 \mu\text{M}$ quin 2 acetoxymethyl ester was used. However, at 200 μM quin 2 acetoxymethyl ester, almost complete inhibition of AA liberation was observed, regardless of the presence or absence of extracellular Ca^{2+} .

The phospholipid source(s) of AA was examined by two-dimensional t.l.c. (Table 2). Addition of 2 μM -A23187 to $[^3\text{H}]\text{AA}$ -prelabelled rat platelets induced loss of $[^3\text{H}]\text{AA}$ from phosphatidylcholine (PC) and phosphatidylethanolamine (PE). There was no decrease in ^3H radioactivity in phosphatidylinositol (PI), but rather a small increase. Thrombin (2 units/ml) elicited a loss of $[^3\text{H}]\text{AA}$ from PC and PI, with a slight increase in ^3H radioactivity of PE. Also the agent caused the formation of 1,2-diacylglycerol (DG) and phosphatidic acid (PA) after the breakdown of inositol phospholipids. The decrease in $[^3\text{H}]\text{PI}$ (989 $\pm$ 232 d.p.m.) was small compared with the increases in $[^3\text{H}]\text{DG}$ (1223 $\pm$ 213 d.p.m.) and $[^3\text{H}]\text{PA}$ (2387 $\pm$ 211 d.p.m.) (results not shown), suggesting that the increases in the last two lipids may be due to polyphosphoinositide hydrolysis, and also that the phospholipase C-DG lipase pathway plays a small, if any, role in AA liberation. The loss of ^3H radioactivity from PC (18932 $\pm$ 2200 d.p.m.) was essentially equal to the gain of that in PE (5457 $\pm$ 873 d.p.m.) and free AA (12215 $\pm$ 2172 d.p.m.). When rat platelets were activated

Table 2. Effects of A23187, thrombin and collagen on hydrolysis of [³H]AA-labelled phospholipids in rat platelets

[³H]AA-labelled rat platelets were incubated for 5 min at 37 °C in the presence of 1 mM-Ca²⁺ with the indicated stimulant. Lipid extraction and determination of [³H]AA radioactivity were performed as described in the Experimental section. The results shown are mean values $\pm$ S.D. (d.p.m.) of triplicate determinations from one of four similar experiments: **P* < 0.005, ***P* < 0.01, ****P* < 0.05 compared with control.

Addition	[³ H]AA radioactivity (d.p.m.)		
	PC	PE	PI
Control	71 028 $\pm$ 1342	35 306 $\pm$ 834	7624 $\pm$ 208
A23187 (2 μ M)	51 414 $\pm$ 1115*	28 589 $\pm$ 1837*	8144 $\pm$ 178***
Thrombin (2 units/ml)	52 096 $\pm$ 1724*	40 763 $\pm$ 1214*	6635 $\pm$ 314***
Collagen (10 μ g/ml)	62 703 $\pm$ 1092*	32 149 $\pm$ 989*	6254 $\pm$ 337**

by collagen (10 μ g/ml), [³H]AA was lost from PC, PE and PI. The amount of [³H]DG and [³H]PA produced was more than the decrease in [³H]PI, as was the case with thrombin stimulation. The loss of ³H radioactivity from PC (7825 $\pm$ 1425 d.p.m.) plus PE (3157 $\pm$ 1046 d.p.m.) correlated well with the increase in that of free AA (11 300 $\pm$ 1542 d.p.m.). Other lipids, such as phosphatidylserine and triacylglycerol, showed no remarkable changes in radioactivity when incubated with either collagen or thrombin.

Ca²⁺-dependent [³H]AA liberation in rat platelet membranes

The resting [Ca²⁺]_i in platelets was calculated to be 80–120 nM by using the fluorescent dye quin 2 [32] or fura 2 [33]. Thrombin caused a rapid elevation in [Ca²⁺]_i to a peak value of $\sim$ 2 μ M measured by fura 2 [33], or $\sim$ 10 μ M measured by aequorin [34]. In membranes prepared from [³H]AA-labelled rat platelets, $\sim$ 200 nM-Ca²⁺ (resting value of [Ca²⁺]_i) did not stimulate the liberation of [³H]AA. Maximal AA release was observed at 5 mM-Ca²⁺ and reached a plateau. However, even at micromolar concentrations, Ca²⁺ caused considerable (more than 40 % of maximum) AA release: 1–3 μ M-Ca²⁺ liberates 5–7 % of the total [³H]AA radioactivity incorporated into membrane lipids (Fig. 1), which

corresponded to the amount liberated in intact platelets in response to thrombin.

When [³H]AA-labelled membranes were stimulated with Ca²⁺ alone, decreases in radioactivity were observed in PE and PC fractions, but no measurable change was seen in PI (Table 3). There was no loss of ³H radioactivity from other lipid fractions (results not shown). In [³H]glycerol-labelled membranes, hydrolyses of PC and PE were observed (Table 4). The formation of [³H]lysophospholipids (lysoPE plus lysoPC) indicates phospholipase A₂-mediated phospholipid breakdown. To examine the pathway of AA release, lipase inhibitors were employed (Table 5). The DG lipase inhibitor RHC 80267 [28] was without effect on AA release. In contrast, the phospholipase A₂ inhibitors mepacrine [12,26,35] and ONO-RS-082 [20] effectively inhibited the release of AA, indicating phospholipid hydrolysis via phospholipase A₂.

DISCUSSION

The release of AA from stimulated platelets is a rate-limiting step in the synthesis of biologically active eicosanoids [1]. Studies of AA metabolism in platelets indicate two major mechanisms for release of AA, one mediated by phospholipase A₂ [2] and the other via phospholipase C followed by DG lipase [3]. The former pathway is considered to play a greater role [4–8]. It has generally been thought that the membrane-bound phospholipase A₂ would be responsible for the generation of AA upon platelet activation. Membrane-bound phospholipase A₂ has recently been purified from rat platelets [10]. Thus in the present study AA liberation was investigated in platelet membrane preparations. The results obtained here demonstrated that marked AA liberation was induced by Ca²⁺ in rat platelets. Formation of lysophospholipids (Table 4) and inhibitory effects of phospholipase A₂ inhibitors (Table 5) suggest that AA release is mainly due to phospholipase A₂ activity. From our [³H]AA-prelabelling study, the DG lipase pathway plays little, if any, role in AA liberation in rat platelets.

Phospholipase A₂ has usually been reported to require millimolar concentrations of Ca²⁺ for its maximal activity in a system *in vitro* [9,11,36]. However, this unphysiologically high Ca²⁺ requirement has been questioned for platelet activation [37], since [Ca²⁺]_i is known to increase to $\sim$ 2 μ M, as monitored by quin 2 or fura 2 fluorescence [32,33], or to $\sim$ 10 μ M measured with aequorin [34].

Table 3. Ca²⁺-induced changes in ³H radioactivity distribution in rat platelet membrane lipids labelled with [³H]AA

[³H]AA-labelled rat platelet membranes were incubated with the indicated concentration of Ca²⁺ for 20 min at 37 °C. Lipid extraction and determination of [³H]AA radioactivity were carried out as described in the Experimental section. The results shown are mean values $\pm$ S.D. (d.p.m.) of triplicate determinations from one of three similar experiments: **P* < 0.005 compared with EGTA alone.

Lipid	[³ H]AA radioactivity (d.p.m.)		
	EGTA alone	1.32 μ M-Ca ²⁺	1 mM-Ca ²⁺
AA	4813 $\pm$ 652	12 380 $\pm$ 1514*	24 856 $\pm$ 2314*
PC	39 322 $\pm$ 896	35 613 $\pm$ 1023*	29 772 $\pm$ 1314*
PE	25 384 $\pm$ 912	19 972 $\pm$ 985*	13 259 $\pm$ 1252*
PI	6827 $\pm$ 528	7627 $\pm$ 412	7032 $\pm$ 492

Table 4. Ca^{2+} -induced changes in ^3H radioactivity distribution in rat platelet membrane phospholipids labelled with ^3H glycerol

^3H Glycerol-labelled rat platelet membranes (50 μg) were incubated with the indicated concentration of Ca^{2+} for 20 min at 37 °C. Other experimental conditions are described in Table 3. The results shown are mean values $\pm$ s.d. (d.p.m.) of triplicate determinations from one of two similar experiments: * $P < 0.005$, ** $P < 0.05$ compared with EGTA alone.

Lipid	^3H Glycerol radioactivity (d.p.m.)		
	EGTA alone	1.32 μM - Ca^{2+}	1 mM- Ca^{2+}
PC	12972 $\pm$ 867	10372 $\pm$ 1322**	8892 $\pm$ 625*
PE	25198 $\pm$ 982	13827 $\pm$ 1205*	7853 $\pm$ 1620*
PI	20472 $\pm$ 644	20991 $\pm$ 521	20105 $\pm$ 872
Lysophospholipids	1992 $\pm$ 108	9872 $\pm$ 946*	15781 $\pm$ 1651*

Table 5. Effects of lipase inhibitors on ^3H AA release in ^3H AA-labelled rat platelet membranes

^3H AA-labelled rat platelet membranes (50 μg) were incubated with the indicated lipase inhibitors for 20 min at 37 °C. Other experimental conditions are described in Fig. 1. The results shown are mean values $\pm$ s.d. (d.p.m.) of triplicate determinations from one of two similar experiments: * $P < 0.001$, ** $P < 0.005$ compared with control.

Additions		^3H AA release (d.p.m.)		
		EGTA alone	1.32 μM - Ca^{2+}	1 mM- Ca^{2+}
Control		1162 $\pm$ 125	7781 $\pm$ 153	15275 $\pm$ 346
RHC 80267	(10 μM)	952 $\pm$ 192	7438 $\pm$ 256	14925 $\pm$ 423
	(30 μM)	1032 $\pm$ 162	7725 $\pm$ 324	14527 $\pm$ 682
Mepacrine	(150 μM)	1021 $\pm$ 182	4192 $\pm$ 450**	10238 $\pm$ 863**
	(600 μM)	1023 $\pm$ 215	2614 $\pm$ 421*	4799 $\pm$ 359*
ONO-RS-082	(1 μM)	1087 $\pm$ 125	4831 $\pm$ 782**	10731 $\pm$ 723**
	(4 μM)	955 $\pm$ 202	2396 $\pm$ 374*	5987 $\pm$ 651*

Recent reports have described that purified platelet phospholipase A_2 is active with sub-micromolar concentrations of Ca^{2+} and with exogenously added substrate [13–15]. Our present study clearly demonstrated that rat platelet membrane phospholipase A_2 was effectively activated by Ca^{2+} alone in the range 0.5–10 μM , which corresponded to cytoplasmic Ca^{2+} concentrations observed in stimulated platelets (Fig. 1). Omission of extracellular Ca^{2+} (Table 1) or chelation of intracellular Ca^{2+} by quin 2 (Fig. 2) resulted in a considerable inhibition of AA release, indicating that the rise in $[\text{Ca}^{2+}]_i$ plays an essential role in phospholipase A_2 activation.

When rat intact platelets were stimulated by A23187, AA was mainly released from PE and PC (Table 2). Similarly Ca^{2+} stimulated the hydrolysis of PE and PC in isolated membranes (Table 3). Ca^{2+} -dependent hydrolysis of PI was not observed. On the other hand, ^3H PI decreased in response to collagen or thrombin (Table 2), suggesting that PI hydrolysis is mainly catalysed by phospholipase C. PI has been reported to take a minor part in AA release [8,24]. If PI was hydrolysed by phospholipase A_2 , the enzyme should be regulated by factor(s) other than Ca^{2+} . Thrombin induced a large decrease in ^3H PC, but ^3H PE rather increased to some extent (Table 2). This may be due to the transacylation of PC to plasmalogen-PE as previously reported [38,39]. Although data from experiments with ^3H AA labelling may not show the net amount of released AA, recent reports indicate that a metabolically active AA pool(s), which rapidly incorporates AA, serves as a major source

of released AA [40,41]. Our present results were consistent with the previous observations by Colard *et al.* [36,41] that phospholipase A_2 preferentially hydrolysed PE and PC in rat platelets. Thus it is reasonable to consider that PC and PE serve as major AA sources.

On the other hand, Ca^{2+} -induced AA liberation was not observed in human platelet membranes under the same experimental conditions (incubation for 20 min at 37 °C in pH 7.4) as for rat platelet membranes (Fig. 1). Although the release of AA from human platelet membranes has been demonstrated [42,43], it requires high concentrations of Ca^{2+} (10 mM), alkaline pH (8.5–10, optimal at 9.5) and longer incubation periods (more than 1 h). Thus it appears to be likely that Na^+/H^+ exchange, which leads to cytoplasmic alkalinization, promotes the activation of phospholipase A_2 [19,20]. In contrast, van den Bosch and co-workers [9] have demonstrated that human platelet phospholipase A_2 is about 300-fold less active than that of rat platelet at both pH 7 and 9 in the presence of 10 mM- Ca^{2+} . According to the recent observations [4–8], AA liberation is thought to be mainly catalysed by phospholipase A_2 in stimulated human platelets. Thus the human platelet enzyme may be activated by mechanism(s) additional to or independent of $[\text{Ca}^{2+}]_i$ and pH. Recently, GTP-binding protein [22,26,44–46] and DG [47,48] have been suggested to be implicated in phospholipase A_2 activation. However, the precise mechanism of phospholipase A_2 activation in human platelets still requires further investigation.

In summary, the rise in $[\text{Ca}^{2+}]_i$ plays a primary role

in the activation of rat platelet phospholipase A_2 . In contrast, other regulatory factor(s) additional to or independent of Ca^{2+} is required for human platelet phospholipase A_2 .

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