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Research Article

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The Mobilization of Arachidonic Acid in Platelets Exposed to Thrombin or Ionophore A23187

EFFECTS OF ADENOSINE TRIPHOSPHATE DEPRIVATION

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ABSTRACT In studies conducted with human gel-filtered platelets, we have found: (a) that the release of serotonin and transfer of [³H]arachidonic acid from phosphatidylcholine and phosphatidylinositol to plasmalogen phosphatidylethanolamine which are associated with the activation of platelets by thrombin are both strongly dependent upon the presence of metabolic ATP; (b) that serotonin release and arachidonic acid mobilization in labeled phosphatides are promoted by the calcium ionophore A-23187 in media free of calcium ions; (c) that inhibitors of ATP synthesis, while leading to impairment of the release reaction induced by ionophore, do not inhibit ionophore-stimulated mobilization of arachidonic acid.

We conclude that the activation of phospholipase A₂ responsible for freeing arachidonic acid from platelet phosphatides is solely dependent upon the increased cytoplasmic levels of calcium ions promoted by either ionophore or, in an energy-dependent fashion by thrombin. Phospholipase activation is not a function of latent hydrolytic activity made available by the release reaction.

INTRODUCTION

Platelets deprived of metabolic ATP are unable to undergo the release and aggregation responses ordinarily inducible by potent effectors such as thrombin, collagen, and ADP (1-3). In this report we examine whether similar starvation leads to the impairment of thrombin-promoted mobilization of arachidonic acid (4, 5) to determine what link may exist between the release reaction and the activation of platelet phospholipase A₂. We have utilized the calcium ionophore

A23187 to determine further whether platelet phospholipase activation is comparably energy-dependent or is a direct consequence of calcium flux.

METHODS

Blood was drawn from normal donors and the platelet-rich plasma incubated with [5,6,8,9,11,12,14,15-³H]arachidonic acid (10 nM) or [¹⁴C]hydroxytryptamine (creatinine sulfate salt, 0.4 μM) as previously described (4). Platelet-rich plasma was then filtered through a 0.5-ml layer of 2 mM ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetate (EGTA)¹ on Sepharose 2B equilibrated with a calcium ion-free buffer (pH 7) described elsewhere (6). Gel-filtered platelets (GFP), free of unincorporated label (4), were used in all the experiments to be discussed.

Ionophore A23187 was dissolved in spectroquality dimethyl sulfoxide, and stored as a 42 mM solution at -20°C. Dilutions of ionophore were made in 12.5% ethanol with rapid mixing just before use. The final concentration of ethanol in incubation mixtures was never >0.3%. Appropriate controls containing dimethyl sulfoxide, and ethanol were included in each experiment. Ionophore (0-1,000 nM), GFP (4 × 10⁸ cells), and 1 mM EGTA, pH 7 in a total volume of 2.1 ml, were incubated for 5 min in a shaking water bath at 37°C. Incubations were terminated by the addition of 1.0 ml ice cold EDTA, pH 6.5, final concentration 5 mM, and were followed by rapid sedimentation of the cells at 4,000 g for 5 min. Release of radiolabel to the supernate was determined, and the lipids of the drained pelleted cells were extracted into chloroform/methanol (2:1). Supernatant lipids were also extracted into chloroform/methanol. Lipid chromatography and distribution of radiolabel among the lipid components were determined as previously described (4, 5).

In time-course experiments, ionophore (75 nM), GFP, and EGTA were incubated as above at intervals from 0 to 15

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¹ *Abbreviations used in this paper:* EGTA, ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetate; GFP, gel-filtered platelets; LDH, lactate dehydrogenase; PC, phosphatidylcholine.

min, and the release and distribution of radiolabel were determined.

GFP, prepared without dextrose in the gel-filtration buffer (pH 7), were incubated from 10 to 45 min at 37°C with 6 mM 2-deoxyglucose and 4 µg/ml antimycin A. ATP content was determined in all samples according to the method of Holmsen et al. (7). GFP were then incubated 5 min further with thrombin (2.5 U/ml, ionophore (75 nM), or buffer, and 1 mM EGTA or 4 mM CaCl₂. Incubations were terminated, and the release of radiolabel to the supernate, and the distribution of [³H]arachidonic acid, where appropriate, were determined as described above.

As a control for cellular damage, lactate dehydrogenase (LDH) assays were performed by the method of Leathwood et al. (8). LDH activity released to the medium during the above-described incubations was compared with that made available by sonication or freeze-thawing (dry ice-acetone bath) of platelet suspensions.

Radioisotopes were obtained from New England Nuclear (Boston, Mass.), and Amersham/Searle Corp. (Arlington Heights, Ill.). All solvents employed were spectrograde or re-distilled. Highly purified human α-thrombin (1 nM = 0.11 U/ml) was kindly provided by Dr. J. W. Fenton, II. Ionophore A23187 was the gift of Dr. Robert J. Hosley of Eli Lilly & Co. (Indianapolis, Ind.).

RESULTS

The ionophore A23187 proved to be a very potent activator of platelet phospholipase A, at concentrations one to two orders of magnitude less than those which begin to cause damage to cell membranes as measured by nonspecific leakage of cytoplasmic constituents (9). We found that ionophore A23187 (500 nM for 15 min) caused no more leakage of LDH in our incubation systems, than was found in controls (2.4% of total LDH), while having no inhibitory effects on LDH per se. Fig. 1 illustrates the abrupt release of [³H]arachidonic acid from phosphatidylcholine, (PC) and phosphatidylinositol, and corresponding increased content of radioisotope in plasmalogen phosphatidylethanolamine observed for GFP exposed to increasing doses of ionophore. The remaining radioactivity released from PC and phosphatidylinositol was present in the supernatant of the incubation medium. Of this, only 1.6% was bound to phospholipid, and >90% chromatographed as free arachidonic acid and cyclo-oxygenase, and lipoxidase metabolites. Thus, the loss of radioactivity observed from PC could not be attributed to a solubilizing effect of ionophore on phosphatides at these concentrations, and points to the activation of phospholipase A. Maximum activation appeared to occur at a final concentration of 150 nM ionophore. Aggregation of GFP was not observed under our conditions of incubation, nor was any effect of ionophore solvent observed as compared with buffer.

Fig. 2 represents the time-course of release, and transfer of [³H]arachidonic acid in the three phosphatides which underwent a major change in radio-

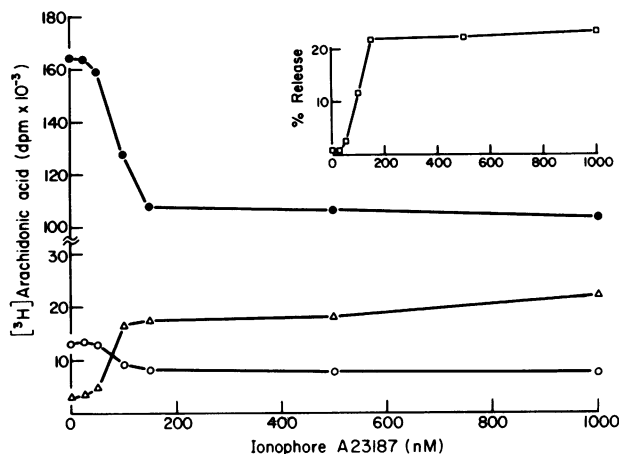


FIGURE 1 Mobilization of [³H]arachidonic acid in phosphatides of GFP exposed to varied concentrations of ionophore A23187. GFP (2×10^8 cells/ml) were incubated with 1 mM EGTA and ionophore or 0.20% ethanol for 5 min at 37°C, and the radioactivity of platelet phosphatides was determined. Inset shows the percent of total radioactivity released from GFP to the medium with ionophore. ●, PC; ○, phosphatidylinositol; △, plasmalogen phosphatidylethanolamine.

activity. At an ionophore concentration of 75 nM, half-maximal release and transfer of [³H]arachidonic acid occurred in 30 s. The release of radioisotope to the medium as neutral lipid (Fig. 2, inset) proved

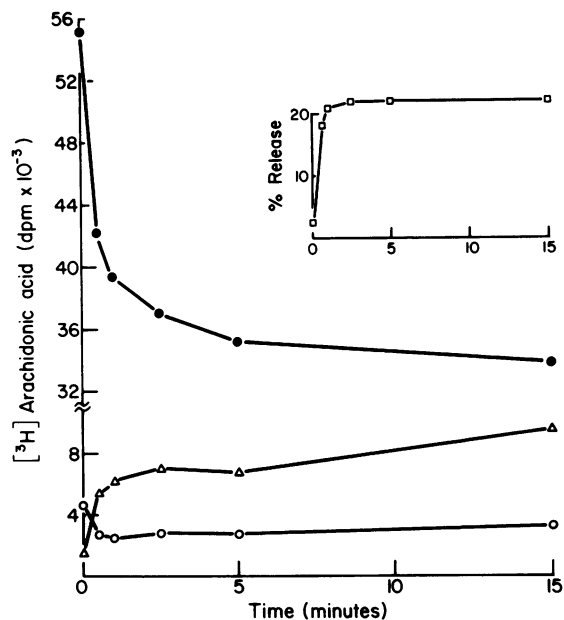


FIGURE 2 Mobilization of [³H]arachidonic acid in phosphatides of GFP exposed to ionophore A23187 for different periods. GFP were incubated with 1 mM EGTA and 75 nM ionophore at 37°C for the times indicated. Inset shows the kinetics of total release of [³H]arachidonic acid to medium. ●, PC; ○, phosphatidylinositol; △, plasmalogen phosphatidylethanolamine.

to be kinetically similar to the course of loss from PC and phosphatidylinositol and to the transfer to plasmalogen phosphatidylethanolamine.

The data in Table I compare the release of [³H]-arachidonic acid and its metabolites to that of [¹⁴C]-serotonin, under control conditions, and after depletion of metabolic ATP by incubation for 15 min with 2-deoxyglucose and antimycin A. Under these conditions, ATP was found to be depressed 30–40% (corresponding to a depression of metabolic ATP of 60–80%; [10]) by the presence of inhibitors whereas leakage of LDH was as yet only 3.7% of total cellular activity. Depression of metabolic ATP caused similar inhibition of thrombin- and ionophore-induced release of serotonin (74–77%). However, only thrombin-induced release of [³H]arachidonic acid to the medium was inhibited (81–86%). The ionophore-induced mobilization remained at control levels.

DISCUSSION

Our data indicate that the calcium ionophore A23187, in the absence of external calcium ions, promotes the activation of platelet phospholipase A₂. Several authors (9, 11, 12) have shown that the release reaction is promoted by ionophore A23187, and implied the existence of a contractile process. Feinman, and Detwiler (13), and White et al. (12) suggested that the critical event triggering the secretory process in human platelets is an increase in the cytoplasmic concentration of calcium ions, possibly arising from depots in the dense tubular system (12). Our data indicate that a calcium ion flux promotes phospholipase A₂ activity in such cells as well, in agreement with the findings of Pickett et al. (14), who did not examine platelets under circumstances in which the release reaction would be

inhibited. We propose that when platelets are activated by thrombin in the presence or absence of calcium ions, a contractile or some other ATP-dependent process is required to liberate internal calcium ions before phospholipase A₂ can hydrolyze arachidonic acid maximally from PC and phosphatidylinositol. Therefore, when metabolic pools of ATP are reduced, the release reaction is inhibited (Table I), in keeping with the results of Holmsen et al. (15), as is the increase in cytoplasmic calcium ions required by phospholipase A₂. Since depletion of metabolic ATP impairs thrombin-mediated phospholipase activation, even when calcium ions are present in the medium, thrombin alone does not render the membrane sufficiently permeable to calcium ions to trigger activation. We did find, however, that starvation of [³H]-arachidonic acid-labeled GFP led to higher levels of released radioactivity in the absence of the effectors thrombin or ionophore (Table I), than were found for cells whose metabolic ATP was maintained with dextrose. The effect became more pronounced with increasing periods of incubation with metabolic inhibitors. This finding may contribute to the evidence that the maintenance of low cytoplasmic levels of calcium ions in resting cells (perhaps to be pumped into the dense tubular system [12]) requires ATP. During starvation, some passive leakage of calcium ions to the cytoplasm may occur, resulting in the low levels of released [³H]arachidonic acid observed.

The addition of the calcium ionophore A23187 circumvents the need for a highly energy-dependent process to promote a sudden elevation in cytoplasmic calcium ions. Even when the release reaction is inhibited, activation of phospholipase A₂ is normal. Since ionophore A23187 is a specific carrier for divalent cations (16), it is unlikely that any platelet constituent

TABLE I
Release of [³H]Arachidonic Acid or [¹⁴C]Serotonin to the Incubation Medium*

Effector	³ H			¹⁴ C		
	Control	With inhibitors	% of Control†	Control	With inhibitors	% of Control†
		%			%	
Thrombin – Ca ⁺²	5.4±1.2	1.0±0.7	19	78±5	18±2	23
Thrombin + Ca ⁺²	10 ±1	1.4±0.6	14	72±3	16±1	22
Ionophore – Ca ⁺²	12 ±2	12 ±1	100	53±3	14±3	26

* GFP were incubated with dextrose (6 mM), or 2-deoxyglucose (6 mM) and antimycin A (4 μg/ml), for 15 min at 37°C, and for 5 min further with the effectors indicated: buffer + 1 mM EGTA, thrombin (2.5 U/ml) + 1 mM EGTA, thrombin + Ca⁺² (4 mM), or ionophore A23187 (75 nM) + 1 mM EGTA. The radioactivity (disintegrations per minute) of the cell-free medium was divided by that of the whole suspension and multiplied by 100. The values for control cells incubated with buffer and dextrose were subtracted (1.9±0.6% for ³H; 4.2±1.2% for ¹⁴C). Slightly higher release of ³H (3.3±0.4%) and comparable release of ¹⁴C (3.8±1.0%) were observed for cells incubated with buffer and metabolic inhibitors. Results are the means ± standard error of four determinations.

† The figures are arrived at by dividing the percent release under conditions inhibiting ATP synthesis by release under control (+dextrose) conditions.

other than calcium ions is moved from sequestered sites by this effector. Therefore, we conclude that platelet phospholipase A₂ is not latent in the classic lysosomal sense, but requires only a rise in cytoplasmic concentrations of calcium ions for full activity.

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