

## Differential Activation of Platelet Phospholipases by Thrombin and Ionophore A23187\*

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Although exposure of platelets to ionophore A23187 causes some activation of phospholipase C, ionophore is an inefficient stimulus for this enzyme. A23187 induces the formation of one-fourth to one-sixth as much diglyceride as does thrombin when comparable amounts of phosphatidylinositol are hydrolyzed. We have shown previously that in the presence of indomethacin thrombin-treated platelets accumulate significant quantities of diglyceride via inhibition of diglyceride lipase. However, a similar accumulation of diglyceride does not occur when ionophore is used as a stimulus in the presence of indomethacin. Ionophore does not appear to be stimulating the catabolism of diglyceride, since the simultaneous addition of ionophore and thrombin does not impair the formation and metabolism of diglyceride which is promoted by thrombin alone.

Further, whereas indomethacin exerts no inhibitory effects upon phospholipase C or the formation of diglyceride in platelets responding to either stimulus, indomethacin does inhibit 1) the loss of arachidonic acid from phosphatidylcholine in response to thrombin and 2) the loss of arachidonic acid from phosphatidylcholine and phosphatidylinositol in response to A23187.

We conclude that in A23187-activated platelets, phosphatidylinositol is hydrolyzed primarily by an enzyme other than phospholipase C. This indomethacin-inhibitable enzyme is probably a phospholipase A. Therefore, the full expression of phospholipase C in platelets requires more than a general flux in intracellular calcium.

The rapid stimulation of phosphatidylinositol-specific phospholipase C (EC 3.1.4.10) in human platelets exposed to thrombin appears to be a potentially important stage of platelet activation. We have shown that diacylglycerol is produced from the PI<sup>1</sup> which is hydrolyzed by Ca<sup>2+</sup>-dependent PI-specific phospholipase C (1, 2). Arachidonic acid-rich DG is then metabolized by two enzymes. It is hydrolyzed by a lipase (3,

4) to yield free arachidonic acid and acted upon by a kinase to yield phosphatidic acid (2, 5, 6). If we are to understand the role which PI-specific phospholipase C plays in the stimulated platelet, further clarification of the factors which regulate the activity of PI-specific phospholipase C is desirable. We have observed in earlier studies that a flux in intracellular Ca<sup>2+</sup> caused by exposure of platelets to the divalent cationophore A23187 leads to hydrolysis of PC and PI and liberates arachidonic acid (7), the precursor of biologically active endoperoxides, hydroperoxides, and hydroxyacids. In the present report, we have explored further the effects of A23187 and, by implication, the effects of a shift in calcium stores upon the metabolism of PI. In addition, we have examined the effects of indomethacin upon lipid metabolism in platelets stimulated by thrombin or A23187.

### MATERIALS AND METHODS

Fresh human platelets or platelets obtained within 15 h of venipuncture were incubated in plasma for 30 min with [5,6,8,9,11,12,14,15-<sup>3</sup>H]arachidonic acid (New England Nuclear, 72 Ci/mmol, 10 nM) and washed twice as described previously (2). Final suspensions of platelets were prepared in a calcium-free Tris/citrate/bicarbonate buffer, pH 6.9 (8).

Labeled platelet suspensions (final concentration  $2.0 \times 10^9$  platelets/ml) were incubated at 37 °C for 0–120 s with 0–5 units/ml of human  $\alpha$ -thrombin (1 nM = 0.11 unit/ml, kindly provided by Dr. John Fenton, II, New York State Department of Health) or ionophore A23187 (0–10  $\mu$ M). Two different batches of ionophore were the gift of Dr. Robert J. Hosley of Eli Lilly Co. (Indianapolis, IN). Ionophore was stored in dimethyl sulfoxide in the dark at –20 °C and diluted just prior to use with 50% ethanol. The final concentrations of dimethyl sulfoxide and ethanol in these assays were 0.02 and 0.23%, respectively. Preparations of solvents at these concentrations were without effect on phospholipid metabolism in control studies. In some experiments, both A23187 and thrombin were incubated with cell suspensions; thrombin was added within 2 s of the addition of A23187. Indomethacin was dissolved in 0.5 N NaOH, diluted within 10 s with buffer (final pH = 7.0), and added at a final concentration of 0–100  $\mu$ g/ml to the platelet suspensions 2 min prior to the addition of thrombin or ionophore. All incubations were terminated by the addition of 3.75 volumes of chloroform/methanol (1:2). In most studies, two solvent phases were formed by the addition of 1 volume each of chloroform and H<sub>2</sub>O. However, in some experiments directed at examining the metabolism of phosphatidylinositol 4-monophosphate and phosphatidylinositol 4,5-diphosphate, 1 volume of 2 M KCl/0.1 M EDTA was substituted for H<sub>2</sub>O to facilitate the extraction of these phosphoinositides into the chloroform phase (9). The lower phase was then removed in both procedures and the upper phase washed three times with 2 volumes of chloroform. Thromboxane B<sub>2</sub> was quantitated in the upper methanol/H<sub>2</sub>O phase by high pressure liquid chromatography (4). Polar lipids in the chloroform extracts were resolved on silica-impregnated paper (10), and neutral lipids separated on Silica G plates (4) or Silica H plates containing (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, included to allow quantitative charring (2). Radiolabel in resolved lipids was quantitated by scintillation spectrophotometry in H<sub>2</sub>O-Hydrofluor (1:10, v/v; National Diagnostics, Somerville, NJ) and the counts obtained were adjusted for any quenching (10). Lipid phosphorus on the silica papers was assayed after digestion of parallel samples (10) and charred DG was quantitated by densitometry as described in a prior publication (2).

A procedure described previously, which utilizes [<sup>3</sup>H]PI labeled on myo-inositol, was employed for the assay of PI-specific phospholipase C (2). Phospholipase C activity was measured in both sonicated platelets and the 100,000  $\times$  g for 60 min supernatants of platelet sonicates in the presence of varied concentrations of indomethacin (0–100  $\mu$ g/ml). Indomethacin (two batches), phosphatidylinositol 4-monophosphate, and phosphatidylinositol 4,5-diphosphate were purchased from Sigma.

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<sup>1</sup> The abbreviations used are: PI, phosphatidylinositol; DG, diacylglycerol; PC, phosphatidylcholine.

## RESULTS

As shown in Fig. 1, thrombin and A23187 both promoted the hydrolysis of PI in human platelets. This effect could be measured either as a loss of [ $^3\text{H}$ ]arachidonic acid (Fig. 1) from PI, or as a decrease in PI phosphorus. In 15 s, PI phosphorus decreased by 30% in response to 5 units/ml of thrombin and by 32% in response to 1  $\mu\text{M}$  A23187. No comparable increase in phosphatidylinositol 4-monophosphate or phosphatidylinositol 4,5-diphosphate was observed within this period, as

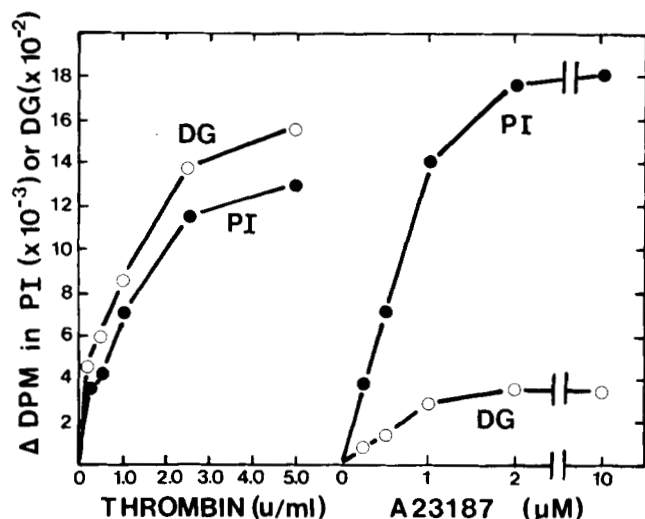


FIG. 1. Change in content of [ $^3\text{H}$ ]arachidonic acid in PI or DG after exposure of platelets to thrombin or A23187. Platelets ( $2 \times 10^9/\text{ml}$ ) containing esterified [ $^3\text{H}$ ]arachidonic acid (40,000 dpm in PI, 100 dpm in DG) were exposed to stimuli for 15 s at 37  $^{\circ}\text{C}$ . Incubations were terminated by the addition of chloroform/methanol. The gain of radiolabel in DG versus the loss in PI was determined.

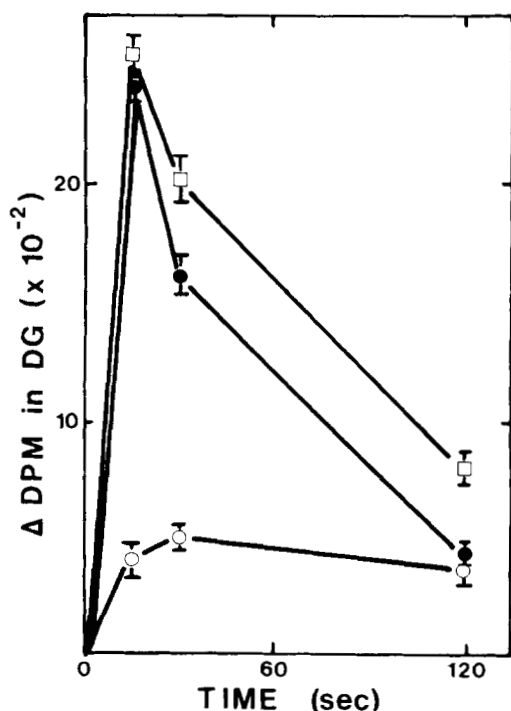


FIG. 2. Effect of separate or simultaneous additions of thrombin and A23187 on the formation of DG in platelets. Washed, labeled platelets (as in Fig. 1) were incubated with 5 units/ml of thrombin (●) or 1  $\mu\text{M}$  A23187 (○) or thrombin and A23187 (□). The range of values for a representative experiment performed in duplicate is indicated.

determined by two-dimensional chromatography on silica-impregnated papers. However, the formation of DG in response to platelet stimulation differed markedly depending upon whether thrombin or ionophore was employed. A23187 was significantly less effective than thrombin in producing DG, although comparable amounts of PI had been hydrolyzed (Fig. 1). The specific activities (disintegrations per min of [ $^3\text{H}$ ]arachidonic acid/ $\mu\text{g}$  of DG) of the DG formed in response to thrombin or A23187 were similar, thus measurement of [ $^3\text{H}$ ]DG was an accurate gauge of the amount of DG present (2). Increasing the concentration of A23187 beyond 1  $\mu\text{M}$  did not enhance significantly the amount of DG formed in platelets.

In other studies, DG production was compared for the dose of thrombin (5 units/ml) yielding the maximum amount of DG and hydrolysis of PI and the dose of A23187 (1  $\mu\text{M}$ ) inducing a comparable amount of hydrolysis of PI in  $2 \times 10^9$  platelets (see Fig. 2). The presence of A23187 in cell suspensions exposed to thrombin did not reduce the amount of measurable DG or alter significantly the kinetics of formation of DG. Platelets stimulated by ionophore alone produced much less DG after 5 s than after 15 s (Fig. 3). It is improbable, therefore, that DG is metabolized more rapidly in platelets stimulated by A23187. These observations are consistent with findings reported by Lapetina and Cuatrecasas (6) and by Broekman *et al.* (11), who have observed (and we confirm) that A23187 is a much less efficient stimulus than thrombin for the generation of phosphatidic acid. If phosphatidic acid is derived from DG, the limited formation of this phosphoglyceride could be explained by the restricted availability of precursor. Further, whereas the presence of indomethacin (50  $\mu\text{g}/\text{ml}$ ), which inhibits DG lipase (4), led to an accumulation of DG produced in response to thrombin, comparable accu-

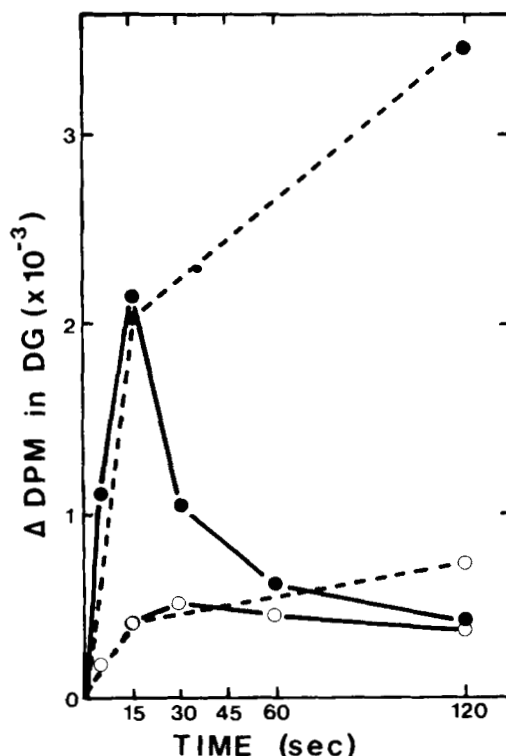


FIG. 3. Effect of indomethacin on the formation and accumulation of DG in platelets exposed to thrombin or A23187. Washed platelets (as in Fig. 1) were exposed to 5 units/ml of thrombin (●) or 1  $\mu\text{M}$  A23187 (○) in the presence (---) or absence (—) of 50  $\mu\text{g}/\text{ml}$  of indomethacin.

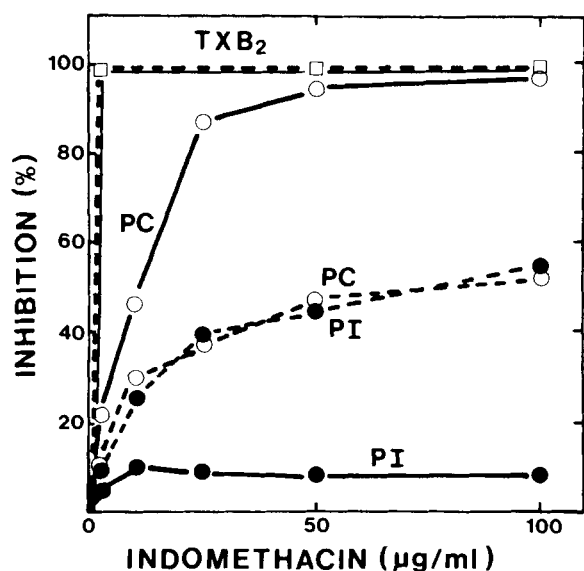


FIG. 4. Inhibition by indomethacin of thromboxane  $B_2$  production and [ $^3H$ ]arachidonic acid loss from PC and PI in platelets exposed to thrombin or A23187. Washed, labeled platelets (see Fig. 1) were exposed for 2 min to  $1 \mu M$  A23187 (---) or 5 units/ml of thrombin (—) in the presence of varied concentrations of indomethacin. The inhibition of change in content of [ $^3H$ ]arachidonic acid in thromboxane  $B_2$  (TXB<sub>2</sub>) (□), PC (○), and PI (●) was determined.

mulation did not occur when A23187 was the stimulus (Fig. 3). Apparently we could not induce a significant build-up of DG by blocking DG lipase because little DG was being formed in the first place.

We found (not shown) that indomethacin is without effect on the activity of PI-specific phospholipase C in platelet sonicates or their supernatants, in keeping with our observation that indomethacin does not impair the initial formation of DG (Fig. 3). In contrast, high doses of indomethacin have been reported to be inhibitory for purified platelet phospholipase A<sub>2</sub> (12, 13). As shown in Fig. 4, when A23187 is the stimulus, inhibitions of loss of [ $^3H$ ]arachidonic acid from PC and PI are comparable. In contrast, when thrombin is the stimulus, inhibition of such loss from PC is even more pronounced, whereas inhibition of loss from PI is minimal. In all cases, the most marked effects are expressed at concentrations of indomethacin well in excess of those required to block cyclooxygenase, as monitored by measurement of thromboxane  $B_2$  (Fig. 4).

#### DISCUSSION

Ionophore A23187 appears to be an inefficient stimulus for the generation of DG in human platelets. Consequently, we infer that a mechanism involving more than a general flux in calcium controls the activation of PI-specific phospholipase C. Other investigators have reported that ionophore is relatively ineffective in promoting the formation of phosphatidic acid (6, 11). This finding would be understandable were less substrate available to DG kinase.

However, A23187 does promote the hydrolysis of substantial amounts of PI, as noted previously (7). Thus, some hydrolytic enzyme(s) other than PI-specific phospholipase C must be involved in the metabolism of PI in platelets exposed to this ionophore.

Another distinction is made in our experiments comparing the effects of thrombin and A23187. High concentrations of indomethacin, higher than those sufficient to inhibit platelet cyclooxygenase, interfere with the hydrolysis of PI induced by A23187, but not by thrombin. Further, indomethacin inhibits the stimulated metabolism of PC in either circumstance. Indomethacin is known to inhibit both DG lipase (4) and phospholipase A<sub>2</sub> isolated from human platelets (12, 13), but has no such effect on PI-specific phospholipase C. Our studies indicate that indomethacin inhibits neither the formation of DG in platelets, nor PI-specific phospholipase C activity in platelet sonicates or sonicate supernatants. We suggest, therefore, that the bulk of PI hydrolyzed in platelets stimulated by thrombin is acted upon by PI-specific phospholipase C. However, the enzyme responsible for metabolizing both PC in thrombin-activated platelets and PC and PI in A23187-activated platelets is probably a phospholipase A. Indomethacin is somewhat less effective in inhibiting the hydrolysis of PC induced by ionophore than that initiated by thrombin. This phenomenon may reflect a counteracting effect of higher concentrations of  $Ca^{2+}$  mobilized by ionophore. In support of this explanation, we note that  $Ca^{2+}$  has been reported to antagonize the inhibition of phospholipase A<sub>2</sub> by nonsteroidal anti-inflammatory agents (13).

In conclusion, we propose that although PI-specific phospholipase C is dependent upon calcium, it is not activated efficiently by calcium alone. A physiological receptor and/or a sequence of events unique to a physiological stimulus appear to be required for the full expression of this enzyme in human platelets. In contrast, a general flux in platelet calcium pools associated with the presence of divalent cationophore seems to provide sufficient stimulus for a phospholipase A which acts not only upon PC, but on PI as well. It is clear from these studies that the mechanism by which the activity of PI-specific phospholipase C is controlled in platelets merits closer scrutiny.

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