Activation of human platelet phospholipase C by ionophore A23187 is totally dependent upon cyclo-oxygenase products and ADP

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Human platelets exposed to the Ca^{2+} ionophore A23187 form cyclo-oxygenase metabolites from liberated arachidonic acid and secrete dense granule constituents such as ADP. I have shown previously that A23187 causes activation of phospholipase A_2 and some stimulation of phospholipase C. I now report that, in contrast to the case for thrombin, the activation of phospholipase C in response to ionophore is completely dependent upon the formation of cyclo-oxygenase products and the presence of ADP. The addition of A23187 to human platelets induces a transient drop in the amount of phosphatidylinositol 4,5-bisphosphate, a decrease in the amount of phosphatidylinositol, and the formation of diacylglycerol and phosphatidic acid. In addition, lysophosphatidylinositol and free arachidonic acid are produced. The presence of cyclo-oxygenase inhibitors or agents which remove ADP partially impairs these changes. When both types of inhibitor are present, the changes in phosphatidylinositol 4,5-bisphosphate and the formation of diacylglycerol and phosphatidic acid are blocked entirely, whereas formation of lysophosphatidylinositol and free arachidonic acid are relatively unaffected. The prostaglandin H_2 analogue U46619 activates phospholipase C. This stimulation is inhibited partially by competitors for ADP. I conclude that phospholipase C is not activated by Ca^{2+} in the platelet, and suggest that stimulation is totally dependent upon a receptor coupled event.

The role played by phospholipase C-catalysed phosphoinositide hydrolysis in serving as a Ca^{2+} -gating mechanism has been the subject of recent controversy (Michell, 1975, 1982; Hawthorne, 1982). A corollary of the hypothesis that such hydrolysis promotes the movement of Ca^{2+} is that a flux in Ca^{2+} *per se* should not activate phospholipase C in intact cells (Michell, 1975). I have reported that addition of the Ca^{2+} ionophore A23187 to human platelets can stimulate phospholipase C activity, albeit inefficiently in comparison with a potent agonist such as thrombin (Rittenhouse-Simmons, 1981). In keeping with these findings, it has been proposed that platelets contain Ca^{2+} -activatable phospholipase C (Billah & Lapetina, 1982a). The present results indicate

that this is unlikely. Although A23187 can stimulate phospholipase A_2 directly, and thereby permit the release of arachidonic acid and formation of cyclo-oxygenase metabolites which promote secretion and phospholipase C activity, in the absence of such secondary stimuli the activation of phospholipase C does not occur.

Materials and methods

Preparation of platelets

Blood (450 ml) was obtained from normal human donors who had been free from aspirin for at least 10 days. Platelet-rich plasma, free from erythrocytes, was prepared as described (Rittenhouse, 1983) in the presence of $0.5 \mu\text{M}$ -PGE₁ (Upjohn Company, Kalamazoo, MI, U.S.A.), and centrifuged at 3000g for 5 min at room temperature. Platelets were suspended in plasma/ Ca^{2+} - and P_i -free gel-filtration buffer (see below) (1:4, v/v), pH 6.5 (Rittenhouse-Simmons & Deykin, 1976) to a concentration of $6 \times 10^9/\text{ml}$, and were incubated for 60 min at 37°C in the presence of 3.3 units of

Abbreviations used: DG, diacylglycerol; PtdIns, phosphatidylinositol; PtdIns4P, phosphatidylinositol 4-monophosphate; PtdIns(4,5) P_2 , phosphatidylinositol 4,5-bisphosphate; PA, phosphatidic acid; U46619, (15S)-hydroxy-11 α ,9 α -(epoxymethanol)prosta-5,13-dienoic acid; PG, prostaglandin; TXA₂, thromboxane A₂.

purified potato apyrase/ml (Valenzuela *et al.*, 1973), and either 3 μ Ci of [5,6,8,9,11,12,14,15- 3 H]-arachidonic acid/ml (72 Ci/mmol) and 20–40 μ Ci of carrier-free [32 P] P_i /ml (Rittenhouse, 1983), or with 1 μ Ci of 5-hydroxy[2- 14 C]tryptamine binoxalate/ml to label the storage granule pool (Rittenhouse-Simmons & Deykin, 1976). Radioisotopes were purchased from New England Nuclear (Boston, MA, U.S.A.). Platelets were then filtered through a 30 ml column (5 cm internal diameter) of Sepharose 2B equilibrated with a Ca^{2+} - and P_i -free gel-filtration buffer, pH 7.3, containing 1 mg of gelatin/ml, 3 munit of apyrase/ml, 0.8 mM- $MgCl_2$, 129 mM- $NaCl$, 10.9 mM-sodium citrate, 8.9 mM- $NaHCO_3$, 5 mM-glucose, 10 mM-Tris/HCl, 2.8 mM-KCl, and 1 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid].

Incubation with inhibitors of cyclo-oxygenase and ADP

Filtered human platelets were incubated, where appropriate, with 0.5 mM-aspirin (Sigma Chemical Co., St. Louis, MO, U.S.A.) at room temperature for 15 min. In some experiments, aspirin was added prior to gel filtration, or indomethacin (1 μ g/ml) (Sigma) was substituted for aspirin. Platelet suspensions (1.5×10^9 /ml) containing 0.25 mM- Ca^{2+} were incubated for up to 3 min at 37°C with 0–20 units of apyrase/ml, and with 4 units of adenosine deaminase/ml (Boehringer-Mannheim, Indianapolis, IN, U.S.A.) before exposure to stimuli. Apyrase converts ADP to AMP, which can be transformed to adenosine in the presence of cellular 5'-nucleotidase. Adenosine deaminase was included as a means of eliminating possible inhibitory effects of adenosine (which can increase levels of cyclic AMP) on platelet activation (Born *et al.*, 1965). In other experiments, 200 μ M-ATP (an antagonist of ADP binding to platelets; Nachman & Ferris, 1974), 200 μ M each of ATP plus ADP, or 5 mM-phosphocreatine plus 40 units of creatine kinase/ml (which converts ADP to ATP) (both from Sigma) were added 15 s prior to the addition of a stimulus. Platelets were incubated for 0–60 s with the Ca^{2+} ionophore A23187 (0.5 μ M), U46619 (8 μ M) or buffer. A23187 and U46619 were provided by Dr. Robert Hosley (Eli Lilly, Indianapolis, IN, U.S.A.) and Dr. John Pike (Upjohn), respectively. Human α thrombin (2 units/ml, kindly provided by Dr. John W. Fenton II, New York State Department of Health) was substituted for A23187 in some studies. Incubations were terminated by the addition of 3.75 vol. of chloroform/methanol/conc. HCl (1:2:0.03, by vol.) for $^3H/^{32}P$ -labelled platelets (HCl was omitted for neutral lipid extractions) or 0.1 vol. of 1 M-formaldehyde/50 mM-EDTA (Costa *et al.*, 1977) for ^{14}C -labelled platelets. Lipid extracts were

mixed with 1 vol. each of 2 M-KCl and chloroform and the resulting upper phases were washed twice with 2 vol. of chloroform. The pooled chloroform phases were concentrated under a flow of N_2 and applied to boric acid- or potassium oxalate/EDTA-impregnated silica plates and chromatographed as described (Rittenhouse, 1983) for resolution of acidic phospholipids. Other extracts were applied to silica G plates for resolution of DG (Rittenhouse-Simmons, 1979). Lipids were located by brief staining with iodine and autoradiography, and quantified by using a cocktail of 1 ml of methanol and 10 ml of Liquescent (National Diagnostics, Somerville, NJ, U.S.A.) by scintillation spectrophotometry in a Tracor BetaTrac 6895 programmed to discriminate between 3H and ^{32}P . Samples containing 5-hydroxy[^{14}C]tryptamine were centrifuged at 3000 g for 10 min at 4°C and the supernatant solutions, containing released 5-hydroxy[^{14}C]tryptamine, were counted.

Studies with varied concentrations of A23187

Incubations of $^3H/^{32}P$ - or ^{14}C -labelled platelets, with and without aspirin and phosphocreatine/creatine kinase, were conducted as above in the presence of buffer or 0.25–5 μ M-A23187 for 30 s. Such studies were performed to assess the effect of increasing concentrations of A23187 on inhibited lipid metabolism and secretion. Leakage of lactate dehydrogenase was monitored as an indicator of platelet damage in response to high concentrations of A23187, and was assayed as described previously (Rittenhouse-Simmons & Deykin, 1977).

Determination of platelet cyclic AMP content

Assays to determine the effect of inhibitors on cyclic AMP levels in gel-filtered platelets were carried out in quadruplicate. Platelets were incubated at 37°C with adenosine deaminase and apyrase, phosphocreatine/creatine kinase, aspirin, aspirin + apyrase, PGE_1 (10 μ M), or buffer, as above, in a final volume of 500 μ l. An equal volume of ice-cold 10% (w/v) trichloroacetic acid was added to terminate the incubation, and 100 μ l of [3H]cyclic AMP (tracer quantity) was added as an internal standard. Precipitated protein was spun down at 2300 g for 10 min at 4°C, and supernatants were applied to 1 ml Bio-Rad AG50W X4 columns which had been washed with 2 ml of 1 M-HCl and 15 ml of water. Trichloroacetic acid was removed with two 1 ml washes of water, and cyclic AMP was eluted with 4 ml of water. Eluates containing cyclic AMP were evaporated by vacuum centrifugation in a SpeedVac Concentrator (Savant Instruments, Inc., New York, NY, U.S.A.) and assayed by using a New England radioimmunoassay kit.

Results

Hydrolysis of phosphoinositides by phospholipase C results in the formation of DG which can be phosphorylated by DG kinase, yielding PA. Table 1 presents the effects of various inhibitors on the stimulated formation of these metabolites, lysoPtdIns (a product of phospholipase A₂ activity), and free arachidonic acid. Secretion of a dense granule marker, 5-hydroxytryptamine, is similarly presented. Stimulus-free controls incubated with the inhibitors did not differ substantially from buffered controls (not shown). The data are shown as the ratio of values in the presence of a stimulus divided by the values for stimulus-free controls under the various incubation conditions. A control value of 2000 c.p.m. and a stimulated value of 30000 c.p.m. for free arachidonic acid plus arachidonic acid metabolites would thus give a ratio of 15. A control value of 5% and a stimulated value of 80% for secreted 5-hydroxytryptamine would give a ratio of 16.

When thrombin was employed as a stimulus, none of the inhibitors had a significant effect on the parameters monitored. The formation of lyso-

PtdIns resulting from phospholipase A₂ activity represented a minor (15%) portion of the total PtdIns hydrolysed in 30 s (not shown). In contrast, some of the effects of the Ca²⁺ ionophore A23187, shown in the middle of Table 1, were markedly reduced by the presence of inhibitors. Aspirin (or indomethacin at 1 µg/ml; results not shown), which blocks cyclo-oxygenase and therefore PGH₂/TXA₂ formation, inhibited the accumulation of [³²P]PA and [³H]DG and secretion, while having no effect on the lysoPtdIns and arachidonic acid plus metabolites produced. Higher concentrations of indomethacin (50–100 µg/ml; results not shown) partially blocked PtdIns degradation and the production of lysoPtdIns and free arachidonic acid, apparently by inhibiting phospholipase A, as previously described (Jesse & Franson, 1979). The presence of agents removing ADP eliminated the stimulated PA, DG, and secretion, while having little effect on the increase in phospholipase A₂ products. Similarly, ATP, which partially inhibits ADP binding to platelets (Nachman & Ferris, 1974), decreased PA accumulation. This effect was counteracted by the simultaneous addition of ADP. Finally, the addition of the PGH₂ analogue

Table 1. *Effect of inhibitors on stimulated increases in lipid metabolites and secretion*

Platelets (1.5×10^9 /ml), labelled with [³H]arachidonic acid and ³²P, or 5-hydroxy[¹⁴C]tryptamine, were incubated with buffer, thrombin (2 units/ml), A23187 (0.5 µM), or U46619 (8 µM) in the presence or absence of inhibitors. Incubations were terminated with chloroform/methanol (³H/³²P-labelled cells) or formaldehyde/EDTA (¹⁴C-labelled cells) after 30 s. Lipids were resolved and quantified. 5-Hydroxy[¹⁴C]tryptamine-labelled cells were sedimented and the ¹⁴C which was present in the supernatant was determined as a function of the total ¹⁴C that had been incorporated. Results are expressed as the values for stimulated cells divided by the values for stimulus-free controls, and represent the means ± S.E.M. for three (or * two) experiments performed in duplicate. A value of 1.0 indicates no change from stimulus-free controls. Further abbreviations used: AA, arachidonic acid plus oxygenated metabolites; PC/CK, phosphocreatine/creatine kinase; N.D., not determined. Data are presented as ratios in order to allow the comparison of results from nine separate experiments which differed in basal incorporation of ³²P and ³H into complex platelet lipid. Over this range of experiments, resting levels varied as follows: [³²P]PA, 200–1000 c.p.m.; [³²P]lysoPtdIns, 80–310 c.p.m.; [³H]arachidonic acid, 600–2000 c.p.m.; [³H]DG, 150–360 c.p.m. Basal secretion varied from 2 to 5%.

Stimulus	Response (+ stimulus/– stimulus) of:				
	PA	lysoPtdIns	AA	DG	Secreted 5-Hydroxytryptamine
Thrombin	39 ± 5	1.8 ± 0.2	15 ± 2	6.1 ± 0.9	16 ± 3
+ aspirin	38 ± 4	1.9 ± 0.3	16 ± 3	6.3 ± 0.8	17 ± 3
+ aspirin + apyrase	35 ± 3	1.7 ± 0.1	14 ± 2	5.9 ± 0.8	15 ± 2
+ aspirin + PC/CK	37 ± 4	1.7 ± 0.2	15 ± 3	6.0 ± 0.7	16 ± 2
A23187	8.1 ± 0.9	5.8 ± 0.2	21 ± 2	3.1 ± 0.4	8.6 ± 0.7
+ aspirin	2.3 ± 0.4	5.6 ± 0.3	20 ± 1	2.4 ± 0.3	3.2 ± 0.2
+ aspirin + apyrase	1.0 ± 0.1	5.4 ± 0.2	18 ± 2	1.1 ± 0.1	1.2 ± 0.1
+ aspirin + PC/CK	1.1 ± 0.1	5.6 ± 0.3	19 ± 3	0.9 ± 0.1	1.3 ± 0.2
+ ATP*	5.8 ± 0.3	5.7 ± 0.5	19 ± 2	N.D.	N.D.
+ ATP + ADP*	7.7 ± 0.6	6.2 ± 0.6	24 ± 2	N.D.	N.D.
+ aspirin + U46619*	21 ± 3	6.1 ± 0.7	22 ± 4	4.3 ± 0.2	N.D.
U46619	20 ± 2	1.1 ± 0.2	1.8 ± 0.2	4.1 ± 0.3	7.2 ± 0.8
+ aspirin	20 ± 3	1.2 ± 0.2	1.7 ± 0.3	3.9 ± 0.4	7.3 ± 0.5
+ aspirin + apyrase	13 ± 2	1.0 ± 0.1	1.5 ± 0.2	2.8 ± 0.3	3.9 ± 0.4
+ aspirin + PC/CK	15 ± 2	1.0 ± 0.2	1.6 ± 0.3	2.9 ± 0.1	4.2 ± 0.5

U46619 overcame the inhibitory effects of aspirin on A23187-induced changes. In further contrast to the case for thrombin, A23187-associated formation of lysoPtdIns accounted for 65% of the loss of PtdIns in the absence of inhibitors and 90% in the presence of inhibitors (results not shown). U46619 (bottom of Table 1) was observed to stimulate PA and DG production, and secretion of 5-hydroxytryptamine, in the absence of substantial lysoPtdIns or arachidonic acid accumulation. Aspirin had no effect on the changes induced. However, agents which eliminated ADP partially inhibited lipid changes and secretion. Higher concentrations of apyrase or phosphocreatine/creatine kinase did not lead to further inhibition. These inhibitors were found to be equally effective whether added 3 min before or simultaneously with the addition of stimulus (not shown).

Platelet activation in response to numerous agonists is inhibited by cyclic AMP (Salzman & Weissberger, 1972; Rittenhouse-Simmons & Deykin, 1978); therefore I assayed the platelet content of cyclic AMP after platelets had been exposed to the various inhibitors employed. As a test of platelet adenylate cyclase responsiveness (and the assay), we included an incubation with PGE₁, a known stimulus for this activity (Salzman & Neri, 1969). Within the range of error, no elevation in platelet cyclic AMP occurred from 49.0 ± 2.2 pmol/ 10^9 platelets, except where PGE₁ had been added (yielding 110 ± 5 pmol/ 10^9 platelets).

Fig. 1 presents the effects of increasing concentrations of A23187 on the inhibitions by aspirin + phosphocreatine/creatine kinase of secretion and lipid metabolism. Formation of lysoPtdIns, the monitor for phospholipase A₂ activity, was relatively uninhibited over the range of A23187 concentrations studied. Accumulation of PA and DG (monitoring phospholipase C activity) continued to be totally inhibited, even at $5 \mu\text{M}$ -A23187. In contrast, the inhibitory effects of aspirin and phosphocreatine/creatine kinase upon secretion were overcome with increasing concentrations of A23187, approaching control levels above $1 \mu\text{M}$. A23187 at these concentrations did not cause platelet damage, as gauged by release of cytoplasmic lactate dehydrogenase, which remained around 3.1% of total cellular activity.

The changes in labelled phosphoinositides that occurred in response to A23187 as a percentage of stimulus-free levels are shown in Fig. 2. The broken lines show the inhibitory effects of aspirin plus apyrase on those changes. A rapid drop in both ³H- and ³²P-labelled PtdIns(4,5)P₂ occurred, and to the same degree. The decrease (about 10%) was consistent and significant, and was prevented by the presence of aspirin and apyrase. The

Fig. 1. Inhibitory effects of aspirin plus phosphocreatine/creatine kinase as a function of varied A23187 concentration

³H/³²P- or ¹⁴C-labelled platelets were incubated as in Table 1, with and without aspirin plus phosphocreatine/creatine kinase, followed by a 30 s incubation with buffer or different amounts of A23187. Inhibition of formation of [³²P]PA, [³H]DG, and [³²P]lysoPtdIns, and secretion of 5-hydroxy[¹⁴C]-tryptamine were determined at each concentration of A23187. Results are the mean of two experiments $\pm$ S.E.M.

subsequent increase in labelled PtdIns(4,5)P₂ with time was also inhibited. PtdIns4P did not show a detectable early change, but showed a progressive increase in labelling. Such changes were reduced by the inhibitors. PtdIns (monitored by ³H) also decreased, as reported earlier (Rittenhouse-Simmons, 1981), but this effect was much less susceptible to inhibition by aspirin plus apyrase than was that for PtdIns(4,5)P₂.

In parallel with these events, PA and lysoPtdIns accumulated in response to A23187 (Fig. 3). No formation of either lyso-PtdIns(4,5)P₂ or lysoPA was detectable. PA was labelled with both ³H and ³²P, whereas lysoPtdIns showed an increase only in ³²P. The increase in PA was blocked totally by the combined inhibitors. In contrast, the formation of lysoPtdIns was much less affected, as noted in Table 1.

The PGH₂ analogue, U46619, also induced a decrease in labelled PtdIns, a rapid decrease followed by an increase in PtdIns(4,5)P₂, and an accumulation of PA (Fig. 4). After 60 s, losses in [³H]PtdIns could be accounted for by gains in [³H]-PA, PtdIns(4,5)P₂, and [³H]PtdIns4P.

Fig. 2. Inhibitory effects of aspirin and apyrase on changes in phosphoinositides induced by ionophore A23187

Human platelets, as in Table 1, were incubated for various periods with A23187 in the presence (broken lines) and absence (solid lines) of apyrase and aspirin. The percentage changes in ^3H and ^{32}P of PtdIns4P (Δ) and PtdIns(4,5)P₂ ($\bullet$) are shown in the upper part of the Figure. The percentage changes in ^3H of PtdIns are shown in the lower part of the Figure. Changes were calculated by comparison with agonist-free controls. All results are presented as the mean $\pm$ S.E.M. of three experiments performed in duplicate. Control values for a representative experiment were: PtdIns, 2000 c.p.m. of ^{32}P and 35000 c.p.m. of ^3H ; PtdIns4P, 16500 c.p.m. of ^{32}P and 4600 c.p.m. of ^3H ; PtdIns(4,5)P₂, 28400 c.p.m. of ^{32}P and 2950 c.p.m. of ^3H .

Discussion

The major conclusion that can be drawn from the data presented in this report is that a Ca^{2+} flux, in itself, does not activate phospholipase C in human platelets. Two lines of evidence support this conclusion.

1. Platelets exposed to Ca^{2+} ionophore release arachidonic acid, form $\text{PGH}_2/\text{TXA}_2$ via cyclo-oxygenase, and secrete dense-granule constituents, which include 5-hydroxytryptamine and ADP. ADP (Lloyd *et al.*, 1973) and the cyclo-oxygenase

Fig. 3. Effect of aspirin and apyrase on formation of PA and lysoPtdIns in response to A23187

Labelled platelets were incubated as in Fig. 2 in the presence (broken lines) and absence (solid lines) of inhibitors. The increases in ^{32}P for PA ($\bullet$) and lysoPtdIns (Δ) are presented. Results shown are those for one of three representative experiments performed in duplicate. It should be stressed that the specific activity of PA (resulting from direct phosphorylation of DG) is much greater than that of lysoPtdIns and PtdIns.

metabolites of arachidonic acid (Siess *et al.*, 1983) stimulate the formation of DG and PA. Further, as demonstrated here (Table 1 and Fig. 4), the PGH_2 analogue U46619 promotes the hydrolysis of PtdIns(4,5)P₂ and PtdIns (without yielding lysoPtdIns), and the accumulation of DG and PA. Incubation of platelets with ionophore can induce such changes in PtdIns(4,5)P₂, DG and PA. However, these alterations are prevented by inhibitors which block cyclo-oxygenase (aspirin) and remove or compete with ADP (apyrase, phosphocreatine/creatine kinase, ATP), as indicated by the data in Table 1 and Figs. 2 and 3. Inhibition by aspirin is more than overcome by the addition of U46619, implying that aspirin is doing no more in this context than inhibiting cyclo-oxygenase. The inhibitors used here do not increase cyclic AMP concentrations, nor do they impair thrombin-induced lipid metabolism (Table 1), which would also be blocked by cyclic AMP (Rittenhouse-Simmons & Deykin, 1978).

2. Phospholipase A₂ activity (gauged by lysoPtdIns) accounts for much of the PtdIns hydro-

Fig. 4. Inhibitory effects of apyrase upon U46619-induced changes in acidic phospholipids

Platelets were incubated as in Fig. 2, in the presence (broken lines) or absence (solid lines) of apyrase. Results for PA (○), PtdIns4P (▲) and PtdIns-(4,5)P₂ (●) show the ³²P content of these lipids. Results for PtdIns (Δ) show the ³H content. Data are given for an experiment representative of three in duplicate.

lysed in response to ionophore, and virtually all of the loss in PtdIns in the presence of both ionophore and inhibitors. It is also responsible for the arachidonic acid released from PtdIns and phosphatidylcholine (Rittenhouse-Simmons, 1981; Rittenhouse-Simmons & Deykin, 1978). Formation of lysoPtdIns and liberation of arachidonic acid are not blocked by the inhibitors used to eliminate the actions of PGH₂/TXA₂ and ADP (Table 1, Figs. 1 and 3). This would indicate that sufficient Ca²⁺ is made available by the addition of A23187 in the presence of inhibitors to allow phospholipase A₂ to be active. Studies with purified platelet phospholipase A₂ (Jesse & Franson, 1979) and phospholipase C (Hofmann & Majerus, 1982) have revealed that phospholipase A₂ is absolutely dependent upon Ca²⁺, and requires approximately two orders of magnitude more Ca²⁺ than does

phospholipase C for half-maximal activity. Therefore, it would seem likely that enough Ca²⁺ is present in all incubations with A23187 to fulfil the lesser requirements of phospholipase C, and that some other factor is essential for the activation of this enzyme. Increasing concentrations of ionophore, although sufficient to overcome the inhibition of secretion caused by aspirin and phosphocreatine/creatine kinase, do not restore the formation of PA and DG, i.e. phospholipase C activity (Fig. 1).

Another important conclusion is that at lower concentrations of A23187 secretion is dependent upon the formation of cyclo-oxygenase products and the presence of ADP (Fig. 1). Higher concentrations of ionophore over-ride this requirement. It is possible that secretion in response to low concentrations of ionophore is dependent upon phospholipase C activity. Thus, cyclo-oxygenase products and ADP may be required only to stimulate phospholipase C, which then initiates a chain of events leading to secretion. Thrombin, which is not dependent upon cyclo-oxygenase and ADP for phospholipase C activation, can also stimulate secretion in the absence of prostaglandin production and ADP. Kaibuchi *et al.* (1982) have suggested that protein kinase C, which can be activated by DG, plays a role at least as important as that of Ca²⁺ in promoting secretion. DG lowers the requirement of protein kinase C for Ca²⁺, and thus, by implication, the need for DG is decreased when higher quantities of Ca²⁺ are available. It may be for this reason that higher quantities of ionophore can facilitate secretion in the absence of DG formation.

Although the PGH₂ analogue U46619 is capable of activating phospholipase C, this does not necessarily imply that PGH₂ is the active species in stimulated platelets. TXA₂ is known to be a major product in platelets, and it is possible that the receptor involved in stimulating phospholipase C can be occupied by either PGH₂ or TXA₂. Dissection of the relative roles of PGH₂ and TXA₂ awaits further work with specific inhibitors. As noted previously (Rittenhouse & Allen, 1982), U46619 is apparently an ineffective stimulus for phospholipase A₂ or DG lipase, since increases in lysoPtdIns and arachidonic acid were minor in response to this stimulus. Thus, we have demonstrated both stimulation of secretion when release of arachidonic acid is minimal and inhibition of secretion when release of arachidonic acid is substantial (Table 1). Apparently, secretion and phospholipase A₂ activity can be dissociated. The reason for the poor performance of U46619 as a phospholipase A₂ stimulus has yet to be defined.

Human platelets contain phospholipase C active with respect to all phosphoinositides (Rittenhouse,

Fig. 5. Proposed route by which Ca^{2+} ionophore (A23187) causes the activation of phospholipase C and secretion of ADP. Aspirin inhibits cyclo-oxygenase. Apyrase and phosphocreatine/creatine kinase (PC/CK) convert ADP to non-stimulatory products. ATP competes with ADP for binding sites. Specific sub-cellular localisations are not defined here.

1983), and losses in platelet $\text{PtdIns}(4,5)\text{P}_2$ have been reported in response to thrombin (Billah & Lapetina, 1982b; Agranoff *et al.*, 1983) and ADP (Vickers *et al.*, 1982). It is thus reasonable to propose that the early loss in $\text{PtdIns}(4,5)\text{P}_2$ reported here could be due to the action of phospholipase C, followed by replacement via phosphorylation of PtdIns . Although there is insufficient evidence as yet to prove it, the hypothesis of Michell *et al.* (1981) that PtdIns is channelled to phospholipase C via $\text{PtdIns}(4,5)\text{P}_2$ may hold true here. If this is the case, we are observing only a small fraction of the actual initial loss in $\text{PtdIns}(4,5)\text{P}_2$, so rapidly might this species be replaced.

The release of arachidonic acid which follows the ionophore-induced activation of phospholipase A_2 leads to a cascade of effects, as shown in Fig. 5. Arachidonic acid is transformed by cyclo-oxygenase to $\text{PGH}_2/\text{TXA}_2$, which stimulates phospholipase C and secretion of ADP. ADP, either directly or as a potentiator of $\text{PGH}_2/\text{TXA}_2$ effects, induces further activation of phospholipase C and more secretion. We conclude on the basis of the preceding evidence that a general flux in Ca^{2+} , although adequate to stimulate phospholipase A_2 , is insufficient to activate phospholipase C in human platelets in the absence of physiological stimuli such as $\text{PGH}_2/\text{TXA}_2$ or ADP. In view of these findings, it might be well to re-

examine phospholipase C activation in other tissues for which A23187 has been reported to be a stimulus.

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