

## Tight coupling of thrombin-induced acid hydrolase secretion and phosphatidate synthesis to receptor occupancy in human platelets

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Human platelets incubated with [ $^{32}\text{P}$ ]P<sub>i</sub> and [ $^3\text{H}$ ]arachidonate were transferred to a P<sub>i</sub>-free Tyrode's solution by gel filtration. The labile phosphoryl groups of ATP and ADP as well as P<sub>i</sub> in the metabolic pool of these platelets had equal specific radioactivity which was identical to that of [ $^{32}\text{P}$ ]phosphatidate formed during treatment of the cells with thrombin for 5 min. Therefore, the  $^{32}\text{P}$  radioactivity of phosphatidate was a true, relative measure for its mass. The thrombin-induced formation of [ $^{32}\text{P}$ ]phosphatidate had the same time course and dose–response relationships as the concurrent secretion of acid hydrolases.  $^{125}\text{I}$ - $\alpha$ -Thrombin bound maximally to the platelets within 13 s and was rapidly dissociated from the cells by hirudin; readdition of excess  $^{125}\text{I}$ - $\alpha$ -thrombin caused rapid rebinding of radioligand. This binding–dissociation–rebinding sequence was paralleled by a concerted start–stop–restart of phosphatidate formation and acid hydrolase secretion. [ $^3\text{H}$ ]Phosphatidylinositol disappearance was initiated upon binding but little affected by thrombin dissociation and rebinding. ATP deprivation caused similar changes in the time courses for [ $^{32}\text{P}$ ]phosphatidate formation and acid hydrolase secretion which were different from those of [ $^3\text{H}$ ]phosphatidylinositol disappearance. The metabolic stress did not alter the magnitude (15%) of the initial decrease in phosphatidylinositol-4,5-bis[ $^{32}\text{P}$ ]phosphate, but did abolish the subsequent increase of phosphatidylinositol-4,5-bis[ $^{32}\text{P}$ ]phosphate in the thrombin-treated platelets. It is concluded that in thrombin-treated platelets (1) phosphatidate synthesis, but not phosphatidylinositol disappearance, is tightly coupled to receptor occupancy and acid hydrolase secretion in platelets, (2) successive phosphorylations to phosphatidylinositol-4,5-bisphosphate is unlikely to be the main mechanism for phosphatidylinositol disappearance, and (3) only a small fraction (15%) of phosphatidylinositol-4,5-bisphosphate is susceptible to hydrolysis.

Stimulation of platelets is associated with rapid accumulation of PtdA and DG and disappearance and synthesis of PtdIns (Lloyd & Mustard, 1974; Lapetina & Cuatrecasas, 1979; Rittenhouse-Simmons, 1979; Walenga *et al.*, 1980; Broekman *et al.*, 1980; Bell & Majerus, 1980; Lapetina *et al.*, 1981; Holmsen *et al.*, 1981). Changes in PtdIns(4)*P* and PtdIns(4,5)*P*<sub>2</sub> have also been demonstrated (Lloyd *et al.*, 1972; Vickers *et al.*, 1982; Billah & Lapetina,

1982*a*; Agranoff *et al.*, 1983). Concurrent with the activation of phosphoinositide metabolism, thrombin initiates a number of cellular responses that are associated with (Feinstein & Hadjian, 1982; Rink *et al.*, 1982) and can be caused by (Holmsen, 1978; Hallam *et al.*, 1981; Knight *et al.*, 1982; Daniel *et al.*, 1982) an increase in the cytoplasmic calcium ion concentration.

Rapid stimulation of phosphoinositide metabolism during receptor-mediated induction of cell responses has been observed in a vast variety of cells, and thought to be involved in mechanisms responsible for mobilization of calcium ions in the cytoplasm (for reviews see Putney, 1981; Michell *et al.*, 1981). For reasons that are not entirely clear, it is generally believed that the agonist–receptor complex controls or causes a diesteratic hydrolysis

Abbreviations used: DG, diacylglycerol; PtdA, phosphatidic acid; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdIns, phosphatidylinositol; PtdIns(4)*P*, phosphatidylinositol 4-phosphate; PtdIns(4,5)*P*<sub>2</sub>, phosphatidylinositol 4,5-bisphosphate; PtdSer, phosphatidylserine.

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of PtdIns or of PtdIns(4,5) $P_2$  (Michell, 1982; Agranoff *et al.*, 1983; Berridge, 1983). Both reactions yield DG which is phosphorylated by DG kinase (Hokin & Hokin, 1963; Pieringer & Kunnes, 1965; Call & Rubert, 1973) to PtdA which has characteristics as a calcium ionophore (Salmon & Honeyman, 1980; Putney *et al.*, 1980; Serhan *et al.*, 1981; Sommermeyer *et al.*, 1983). PtdA is eventually cycled back to PtdIns(4,5) $P_2$ . This cyclic metabolism of the phosphoinositides ('PtdIns cycle', 'PtdIns(4,5) $P_2$  cycle') appears to be the same in all cells investigated.

The cellular responses and metabolic steps in the PtdIns cycle in thrombin-stimulated platelets can be subdivided into those that require sustained occupancy of the thrombin receptor and those that do not (Holmsen *et al.*, 1981). Thus, addition of hirudin, which removes platelet-associated thrombin (Tam *et al.*, 1979) by complex formation (Fenton, 1981), to thrombin-stimulated platelets caused an instantaneous arrest of acid hydrolase secretion, PtdA formation and arachidonate liberation (Holmsen *et al.*, 1981). These observations suggested a general use of hirudin to study the coupling of thrombin receptor occupancy to individual responses and steps in the phosphoinositide metabolism, and indicated specifically that there might exist tight coupling of acid hydrolase secretion and PtdA formation to receptor occupancy. However, hirudin did not cause an abrupt stop of PtdIns disappearance, so that the generally believed coupling of this step to receptor occupancy and PtdIns disappearance was not revealed. Thrombin-induced PtdIns disappearance is strongly inhibited by metabolic blockers, suggesting an involvement of ATP in the mechanism(s) of PtdIns removal (Holmsen *et al.*, 1982). In the present study hirudin interruption of, and metabolic interference in, the thrombin-platelet interaction have been used to characterize further the relationships of receptor occupancy and acid hydrolase secretion to PtdIns and PtdIns(4,5) $P_2$  metabolism.

## Materials and methods

### Preparation of pulse-labelled human platelets

Platelet-rich plasma was prepared as described previously (Holmsen *et al.*, 1972a) and incubated at 37°C for 60 min with 0.2 mCi of [ $^{32}$ P]P $_i$  (carrier-free, New England Nuclear)/ml and 0.01  $\mu$ M-[ $^3$ H]-arachidonic acid (90 Ci/mmol; code TRK, Amersham International). The platelets were then gel-filtered (Lages *et al.*, 1975) into a calcium-free Tyrodes solution containing 5 mM-glucose and 0.2% human albumin (crystallized and lyophilized; Sigma) but no added P $_i$ .

In experiments where the phosphorus contents of

the phospholipid fractions were to be determined, the platelets were washed at 4°C with an EDTA-containing salt solution (Holmsen & Robkin, 1980).

### Incubations

Suspensions of pulse-labelled platelets were incubated at 37°C without stirring with various concentrations of thrombin (bovine, Parke-Davis; human  $\alpha$ -thrombin, a generous gift from J. W. Fenton II). Samples were taken at the times indicated for determination of phospholipid radioactivity and secreted constituents. In some experiments hirudin (VEB Arzneimittelwerk, Dresden, Germany) was added as indicated followed by a new addition of excess thrombin. In experiments with ATP deprivation, the platelet-rich plasma was incubated with 0.4  $\mu$ M-[ $^{14}$ C]adenine (Holmsen *et al.*, 1972a) in addition to the other labelled material.

### Extractions

Phospholipids other than the PtdIns polyphosphates were extracted according to Bligh & Dyer (1959). A sample (0.5–3 ml) of the incubation mixture was mixed with 3.6 vol. of chloroform/methanol (1:2, v/v). Water (1.2 vol.) and chloroform (1.2 vol) were added with vigorous shaking and the resulting mixture was centrifuged at approx. 200g (room temperature) to enhance phase separation. The bottom phase was collected, concentrated to dryness under N $_2$  flow, and dissolved in 25  $\mu$ l of chloroform/10 $^8$  platelets (for gel-filtered platelets) or 10 $^9$  platelets (for EDTA-washed platelets); these extracts are referred to as 'concentrated extract'. In experiments where the PtdIns polyphosphates were to be determined, the above extraction fluid was replaced by chloroform/methanol/conc. HCl (20:40:1, by vol.) (Imai *et al.*, 1983); further procedure was as above.

### Secretion

Samples (0.4 ml) of the incubation mixture were mixed with 0.1 ml of 0.633 M-formaldehyde/50 mM-EDTA in ice in order to stop secretion (Costa & Murphy, 1975; Holmsen & Setkowsky-Dangelmaier, 1977). These fixed samples were centrifuged at 12000g at room temperature, and EDTA/ethanol extracts for ATP+ADP determination (Holmsen *et al.*, 1972b) and 0.1% Triton X-100 extracts for acid hydrolase determination (Dangelmaier & Holmsen, 1980) were made from the supernatants. Such extracts were also made from non-centrifuged reaction mixtures for determination of total levels of constituents and from supernatants of mixtures not treated with thrombin for determination of control extracellular levels of constituents.

### Determinations

The concentrated phospholipid extract of gel-filtered platelets (25  $\mu$ l) was chromatographed at room temperature on aluminum sheets coated with silica gel 60 (Merck, cat. no. 5538, without fluorescent indicator) in chloroform/methanol/acetic acid/water (81:20:45:5, by vol.) as described by Hauser & Eichberg (1975). This one-dimensional system gave adequate separation of PtdCho, PtdIns, PtdSer, PtdEtn, PtdA and arachidonate + DG + arachidonate oxygenation products. Another 25  $\mu$ l portion was chromatographed on the same sheets with the upper phase of ethyl acetate/acetic acid/2,2,4-trimethylpentane (iso-octane)/water (9:2:5:10, by vol.) according to Hong & Levine (1976). This system was used for PtdA determination, as this phospholipid specifically separates from the other phospholipids which remain at the application point (Lapetina & Cuatrecasas, 1979).

Separation of PtdIns(4)P, PtdIns(4,5)P<sub>2</sub>, PtdIns and PtdA from each other and other phospholipids was done on the same aluminum sheets as above in chloroform/acetone/methanol/acetic acid/water (40:15:13:12:8, by vol.) according to Jolles *et al.* (1981). The spots were localized by measuring their content of <sup>32</sup>P by overnight radioautography with Chronex 4 X-ray film. For determination of phospholipid radioactivity only, the procedure described previously (Holmsen *et al.*, 1982) was used.

When both the <sup>32</sup>P radioactivity and the P<sub>i</sub> content of PtdA and PtdIns were determined, 45  $\mu$ l portions of the concentrated extract of EDTA-washed platelets were chromatographed, and the spots were localized by radioautography as above. The spots were scraped into thoroughly washed (HCl) digestion tubes, 600  $\mu$ l of 70% (v/v) HClO<sub>4</sub> was added and the mixture was boiled for 15 min. Water (1 ml) was then added; 200  $\mu$ l was removed for determination of <sup>32</sup>P radioactivity and 800  $\mu$ l was mixed with 400  $\mu$ l of 5.6 M-KOH in an ice-bath and the KClO<sub>4</sub> formed was removed by centrifugation. The phosphate content was determined in 1.0 ml of the supernatant by a molybdate/Malachite Green method (Hess & Derr, 1975).

The specific radioactivity of ATP, ADP and P<sub>i</sub> in the metabolic pool of the platelets was determined as described previously (Holmsen *et al.*, 1983).

### Binding experiments

<sup>125</sup>I- $\alpha$ -Thrombin (500000 c.p.m./unit; a generous gift from Dr. G. Tuszynski) was incubated at various concentrations and times with gel-filtered platelets at 37°C. A sample (200  $\mu$ l) of the incubation mixture was layered on top of 50  $\mu$ l of DC 500/DC 200 (4:1, v/v) silicone oil (William F. Nye Inc., New Bedford, MA, U.S.A.) in a micro-

centrifuge tube with an elongated, narrow tip (Sarstedt, cat. no. 72.702) which was centrifuged in a Beckman Microfuge B for 2 min at 12000g. The platelets became pelleted in the elongated tip of the tubes; this tip was cut off above the pellet, but still within the oil layer, and placed in a counting vial. The remainder of the tube (containing the extracellular phase of the incubation medium) was placed in another vial. Both vials were counted in an Intertechnique  $\gamma$ -spectrometer. Non-specific binding of <sup>125</sup>I was determined by using the same protocol, except that hirudin in a concentration 10 times that of <sup>125</sup>I- $\alpha$ -thrombin was added to the platelets before thrombin.

### Calculation of specific radioactivity of <sup>32</sup>P

The pulse-labelling technique with [<sup>32</sup>P]P<sub>i</sub> used in the present study causes equal labelling of the  $\beta$ - and  $\gamma$ -phosphoryl groups of ATP and of the  $\beta$ -phosphoryl group of ADP, while the  $\alpha$ -phosphoryl group of both nucleotides remains unlabelled (Holmsen *et al.*, 1983). Therefore, the specific radioactivity of each labelled phosphoryl group is: c.p.m. in ATP/2  $\times$  content of ATP, and c.p.m. in ADP/content of ADP. The specific radioactivity of PtdIns and PtdA was calculated as c.p.m. of PtdIns/content of PtdIns phosphate and c.p.m. of PtdA/content of PtdA phosphate, respectively.

## Results

### Specific radioactivity of <sup>32</sup>P in PtdIns, PtdA, ATP, ADP and P<sub>i</sub>

The labelled phosphoryl groups of ATP ( $\beta, \gamma$ ) and of ADP ( $\beta$ ), as well as of P<sub>i</sub> in the metabolic pool of platelets labelled as in the present study have the same specific radioactivity and are hence in complete equilibrium (Holmsen *et al.*, 1983). The ATP-derived phosphorus in PtdA formed 0.5, 2 and 5 min after thrombin addition to the labelled washed platelets had also the same specific radioactivity as the labelled groups in ATP, ADP and P<sub>i</sub> (Table 1). Therefore, the [<sup>32</sup>P]phosphate atoms in PtdA, ATP, ADP and P<sub>i</sub> are in complete equilibrium in the pulse-labelled platelets used, and the total radioactivity of [<sup>32</sup>P]PtdA represents a direct, relative measure of the PtdA mass. In contrast, since the <sup>32</sup>P/<sup>3</sup>H ratio of PtdA increases 6–10-fold following thrombin stimulation (Holmsen *et al.*, 1981) the changes in the <sup>3</sup>H radioactivity of PtdA do not reflect the corresponding changes in the PtdA mass. The specific radioactivity of [<sup>32</sup>P]-PtdIns was markedly lower than that of PtdA (Table 1), showing that the diester-bound phosphate in PtdIns does not equilibrate within 5 min of thrombin treatment with the phosphate in PtdA, ATP ( $\beta, \gamma$ ), ADP ( $\beta$ ) and P<sub>i</sub> in the metabolic pool of the platelets.

Table 1. *Specific radioactivity of [<sup>32</sup>P]PtdA, [<sup>32</sup>P]PtdIns, [<sup>32</sup>P]ATP, [<sup>32</sup>P]ADP and [<sup>32</sup>P]P<sub>i</sub> in platelets pulse-labelled with [<sup>32</sup>P]P<sub>i</sub>*

Platelet-rich plasma was incubated with [<sup>32</sup>P]P<sub>i</sub> and the cells isolated by washing with EDTA-containing salt solution as described in the Materials and methods section. The dense granule pool of ATP, ADP and P<sub>i</sub> was removed by thrombin-induced secretion as described previously (Holmsen *et al.*, 1983) in some aliquots of the platelet suspension, and the amounts and radioactivity of ATP, ADP and P<sub>i</sub> were determined in the thrombin-treated platelets (metabolic pool). Other aliquots were incubated with 0.2 unit of thrombin/ml for the times given and the amounts and radioactivity of PtdA and PtdIns were determined in portions of 3 ml. Values for radioactivity, mass and specific radioactivity (columns 3, 4 and 5) are from a typical single experiment ( $11.8 \times 10^8$  cells/ml). Values for normalized specific radioactivity (column 6) are the means ( $\pm$ S.D.) from four experiments. In this column the specific radioactivity of ATP ( $\gamma$  or  $\beta$  phosphoryl group) in each individual experiment was set equal to 100 and the specific radioactivities of the other substances were calculated relative to that of ATP. Abbreviation used: n.d., not detectable.

Substance	Incubation with thrombin (min)	Radioactivity (c.p.m./ml)	Mass (nmol/ml)	Specific radioactivity (c.p.m./nmol)	Normalized specific radioactivity
ATP	–	226800	22.0†	5150*	100
ADP	–	49800	10.2†	4880*	98.1 $\pm$ 6.3
P <sub>i</sub>	–	359100	68.9	5210	104.6 $\pm$ 11.6
PtdA	No thrombin added	145	n.d.	–	–
	0.5	4000	0.73	5530	–
	2.0	6700	1.23	5470	101.9 $\pm$ 8.7
	5.0	2300	0.57	4030	97.1 $\pm$ 9.8
PtdIns	No thrombin added	660	4.89	140	2.7 $\pm$ 1.7
	5.0	1800	2.67	670	12.3 $\pm$ 6.8

\* Per  $\gamma$ - or  $\beta$ -phosphoryl group (see under 'Calculations' in the Materials and methods section).

† The ATP/ADP ratio in the cytoplasmic compartment of resting platelets is 7–10 (Daniel *et al.*, 1980) but decreases considerably by treatment of the cells with thrombin, as done here.

In these experiments the maximal PtdA radioactivities reached after thrombin treatment were 4–7% of the radioactivity of one labelled phosphate group of ATP of the metabolic pool (results not shown). The size of this pool is  $3.2 \mu\text{mol}/10^{11}$  cells (Holmsen & Robkin, 1980); the maximal levels of PtdA found in these experiments are thus 128–243 nmol/ $10^{11}$  cells, which is slightly lower than those reported by Broekman *et al.* (1980).

#### *Dose-response relationship for thrombin-induced [<sup>32</sup>P]PtdA formation, secretion and binding of thrombin*

The amounts of [<sup>32</sup>P]PtdA formed 60s after addition of thrombin increased rapidly with increasing thrombin concentration to about 1 unit/ml and thereafter levelled off (Fig. 1a). The variations in the rate of secretion of  $\beta$ -hexosaminidase and  $\beta$ -glucuronidase with the thrombin concentration were very similar to those of [<sup>32</sup>P]-PtdA (Fig. 1a). The decrease in [<sup>3</sup>H]PtdIns, which could not be measured with great accuracy, also followed the same pattern as [<sup>32</sup>P]PtdA (results not shown). In contrast, secretion of ATP + ADP (Fig. 1a), or of preadsorbed [<sup>14</sup>C]serotonin (results not shown), reached maximal values at 0.2 unit/ml and did not change with higher thrombin concentrations.

Pure  $\alpha$ -thrombin binds rapidly and specifically to washed platelets with dose-binding character-

istics suggestive of low- and high-affinity binding sites (Martin *et al.*, 1976; Shuman *et al.*, 1976; White *et al.*, 1981). The only thrombin-induced platelet response that has been measured concomitantly with thrombin binding is dense granule secretion, which is saturated when only a small fraction of the thrombin-binding sites is occupied. <sup>125</sup>I- $\alpha$ -Thrombin also showed specific binding characteristics with gel-filtered platelets (Fig. 1b) that were similar to those previously found for platelets washed in the cold with EDTA-containing solutions. Moreover, dense granule secretion, as measured by secretion of ATP + ADP, was saturated when about 20% of maximal binding was achieved. In contrast,  $\beta$ -hexosaminidase secretion increased with the binding throughout the 0–3 unit/ml thrombin range studied, but the secretion and binding curves were not superimposable (Fig. 1b). Thus, there is a distinctly better correspondence of agonist binding to acid hydrolase secretion than to dense granule secretion in the range of thrombin concentration where PtdA formation is saturated (Fig. 1a).

#### *Reversibility of thrombin-induced [<sup>32</sup>P]PtdA formation, acid hydrolase secretion and binding of <sup>125</sup>I- $\alpha$ -thrombin*

Fig. 2(a) shows that the hirudin-induced arrest of PtdA formation and of the secretion of both  $\beta$ -hexosaminidase and  $\beta$ -glucuronidase are both

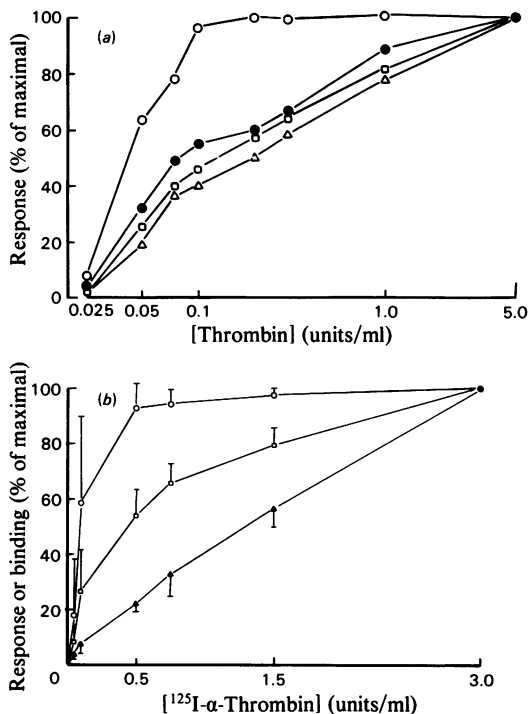


Fig. 1. Dose-response relationships for thrombin-induced secretion and  $[^{32}\text{P}]\text{PtdA}$  formation (a) and effect of thrombin concentration on binding of  $^{125}\text{I}\text{-}\alpha\text{-thrombin}$  to platelets and secretion (b)

(a) Gel-filtered,  $[^{32}\text{P}]\text{P}_i$ -labelled platelets ( $2.4 \times 10^8$  cells/ml) were incubated with bovine thrombin in the concentrations shown for 60 s in the presence of 2 mM-EGTA, and secreted ATP+ADP ( $\circ$ ),  $\beta$ -hexosaminidase ( $\square$ ), and  $\beta$ -glucuronidase ( $\triangle$ ) besides  $[^{32}\text{P}]\text{PtdA}$  ( $\bullet$ ) were determined. The values has been normalized by setting the values at 5 units/ml equal to 100. The actual values at this concentration were: ATP + ADP, 55.6% of total;  $\beta$ -hexosaminidase, 46.2% of total;  $\beta$ -glucuronidase, 18.2% of total;  $[^{32}\text{P}]\text{PtdA}$ , 3653 c.p.m./ml. (b) Gel-filtered platelets were incubated for 60 s with  $^{125}\text{I}\text{-}\alpha\text{-thrombin}$  in the concentrations shown and analysed for specific binding of thrombin ( $\blacktriangle$ ), secreted ATP+ADP ( $\circ$ ) and secreted  $\beta$ -hexosaminidase ( $\square$ ). The numbers are averages ( $\pm$ S.D.) of results obtained with four different preparations, and the values obtained with 3 units/ml have been arbitrarily set equal to 100 (maximal binding). Further increase in the concentration of  $^{125}\text{I}\text{-}\alpha\text{-thrombin}$  above 3 units/ml gave a linear relationship of binding versus thrombin concentration up to 30 units/ml; acid hydrolase secretion increased by only 10% throughout this 3–30 units/ml thrombin range.

bin treatment gave a very reproducible 'start-stop-restart' pattern of PtdA formation (Figs. 2a and 2b, left panels; representative of 16 experiments). In contrast, the corresponding changes in the level of PtdIns did not give a reproducible pattern.  $[^3\text{H}]\text{PtdIns}$  always decreased after the initial addition of thrombin, while the subsequent addition of hirudin produced a slightly lesser decrease (no stop), no change or even an acceleration of the decrease as illustrated in Fig. 2(b) (right panel); the post-hirudin addition of thrombin produced either no, or only a small, further decrease in  $[^3\text{H}]\text{PtdIns}$  (Fig. 2b, right panel).

$^{125}\text{I}\text{-Thrombin}$  binds maximally within seconds to platelets washed in the cold with EDTA-containing solutions, and excess hirudin completely dissociates the bound thrombin from the cells provided it is added less than 1 min after thrombin (Tam *et al.*, 1979). With gel-filtered platelets we found maximal binding of  $^{125}\text{I}\text{-}\alpha\text{-thrombin}$  within the 13 s that lapsed between mixing the radioligand with the sample and the time that maximal centrifugation speed was obtained; there was no further changes in the bound  $^{125}\text{I}\text{-}\alpha\text{-thrombin}$  throughout the 6-min observation period (Fig. 2c, upper panel). When a 10-fold excess of hirudin was added 30 s after  $^{125}\text{I}\text{-}\alpha\text{-thrombin}$  (i.e. conditions similar to those in Fig. 2a), all specifically bound  $^{125}\text{I}\text{-}\alpha\text{-thrombin}$  was lost from the platelets within 13 s (results not shown). With a 3-fold excess of hirudin (i.e. conditions similar to those in Fig. 2b), the loss of cell-bound  $^{125}\text{I}\text{-}\alpha\text{-thrombin}$  was slower, and 10% of the originally bound thrombin was still associated with cells 60 s after addition of hirudin; re-addition of  $^{125}\text{I}\text{-}\alpha\text{-thrombin}$  caused rapid re-binding; this specifically bound thrombin slowly dissociated from the cells (Fig. 2c, left panel). Secretion of  $\beta$ -hexosaminidase was measured concurrently with the binding-dissociation-rebinding of  $^{125}\text{I}\text{-}\alpha\text{-thrombin}$ , and it started, stopped and restarted (Fig. 2c, right panel) exactly as with non-radioactive thrombin (Fig. 2a).

Thus, treatment of thrombin-activated platelets with hirudin does not perturbate their ability to bind thrombin, synthesize PtdA or secrete acid hydrolases. Most importantly, the above findings strongly suggest a tight coupling of receptor occupancy, PtdA formation and acid hydrolase secretion.  $[^3\text{H}]\text{PtdIns}$  disappearance does not appear to be tightly coupled to these processes.

#### *Effect of metabolic inhibitors on the time course of $[^{32}\text{P}]\text{PtdA}$ formation, $[^3\text{H}]\text{PtdIns}$ disappearance and acid hydrolase secretion*

Incubation of gel-filtered platelets with antimycin A and 2-deoxyglucose causes a parallel inhibition of thrombin-induced  $[^{32}\text{P}]\text{PtdA}$  formation,  $[^3\text{H}]\text{PtdIns}$  disappearance and acid hydrolase

reversible; they both start again when thrombin is added in concentrations exceeding that of hirudin. Thus, both PtdA formation and acid hydrolase secretion can be started, stopped and restarted in parallel. This sequential thrombin-hirudin-throm-

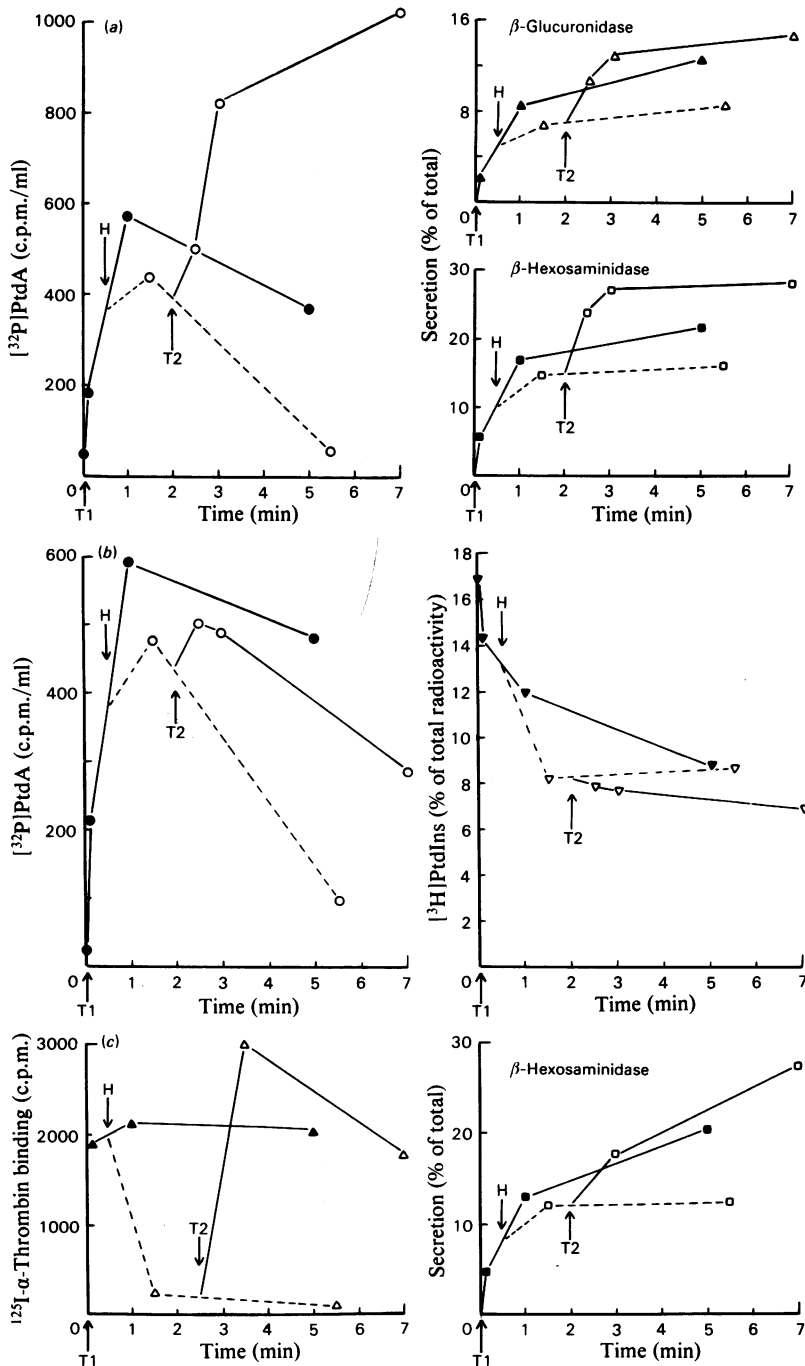


Fig. 2. Reversibility of the arrest of acid hydrolase secretion, arrest of  $[^{32}\text{P}]\text{PtdA}$  formation and loss of bound  $^{125}\text{I}$ - $\alpha$ -thrombin caused by hirudin

(a) Gel-filtered,  $[^{32}\text{P}]\text{PtdA}$ -labelled platelets ( $1.8 \times 10^8$  cells/ml) were incubated with 0.2 units of bovine thrombin/ml (T1) and samples were removed at the time indicated for determination of  $[^{32}\text{P}]\text{PtdA}$ , secreted  $\beta$ -hexosaminidase and secreted  $\beta$ -glucuronidase (filled symbols, solid lines). At 30s after start of incubation, an aliquot of the platelet/thrombin mixture was mixed with 5 units of hirudin/ml (H) and incubated further; samples from this platelet/thrombin/hirudin mixture were collected and analysed (open symbols, broken lines). At 120s after start of incubation (i.e. 90s after addition of hirudin) and aliquot of the platelet/thrombin/hirudin mixture was mixed with 6 units of thrombin/ml (T2) and incubated further; samples from the latter mixture was collected and analysed (open symbols, solid lines). The transfer of thrombin-treated, and hence potentially sticky, platelets in these experiments

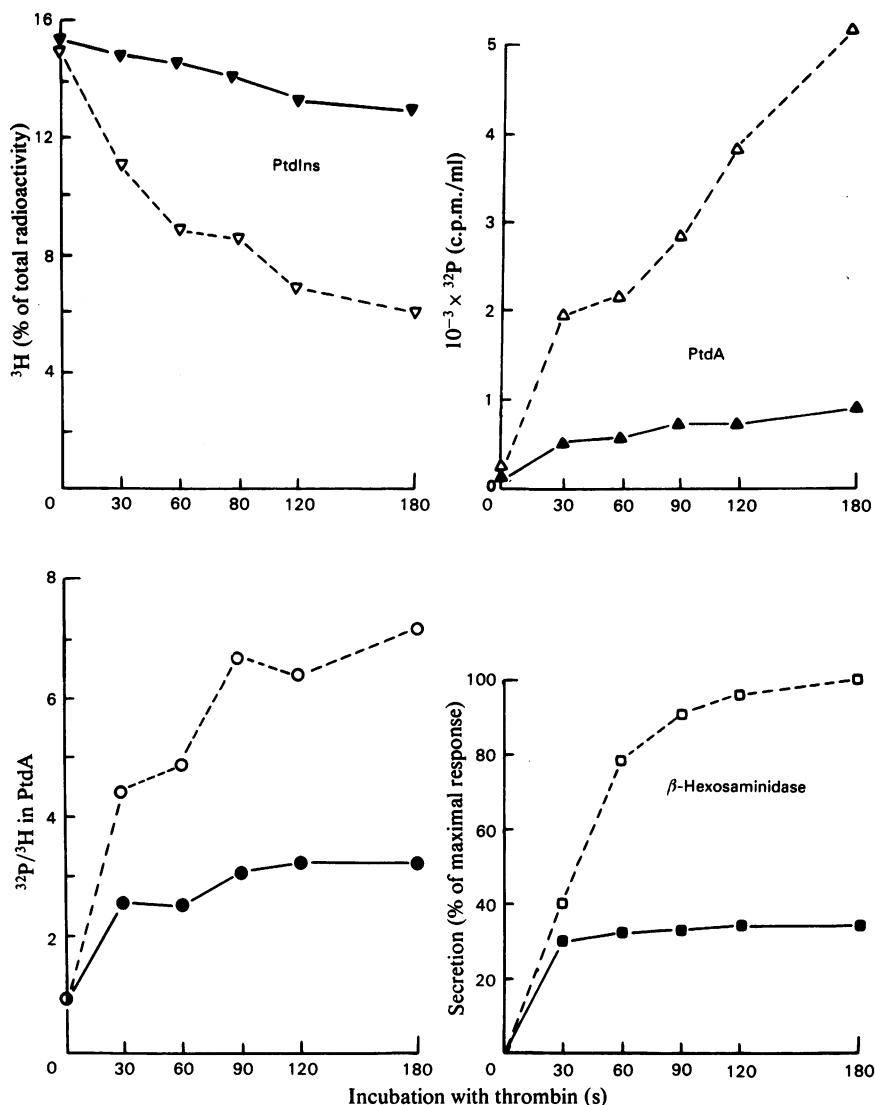


Fig. 3. Effect of metabolic blockers on the time course of secretion and phospholipid metabolism. Gel-filtered, [<sup>3</sup>H]arachidonate- and [<sup>32</sup>P]P<sub>i</sub>-labelled platelets ( $1.8 \times 10^8$  cells/ml) were preincubated with (—) or without (---) 4  $\mu$ g of antimycin A/ml and 30 mM-2-deoxyglucose for 15 min. The platelets were then incubated with 1 unit of bovine thrombin/ml and samples were removed at the times indicated for determination of the parameters given in the individual panels. Note that the time refers to incubation with thrombin. After 3 min incubation with thrombin of platelets that had not been preincubated with the inhibitors, 38% of the total content of  $\beta$ -hexosaminidase had been secreted.

was quantitative; the amount of lipid-extractable <sup>3</sup>H per ml was the same in all three subdivisions of a given platelet suspension that had been prelabelled with [<sup>3</sup>H]arachidonate. (b) The experiment was performed essentially as described above for (a), except that the platelets ( $3.4 \times 10^8$  cells/ml) had also been pulse-labelled with [<sup>3</sup>H]arachidonate, and that human  $\alpha$ -thrombin was used as follows. The first thrombin addition (T1) was 0.5 unit/ml and the second addition (T2) was 2 units/ml; hirudin (H) was 1.5 unit/ml. Parameters measured are given in each panel. [The more elevated PtdA accumulation upon the second thrombin addition in (a) than in (b) was reproducible. It may have resulted from a more effective stop of PtdA accumulation at the lower initial (T1) on thrombin concentrations in A (H/T1) = 25; T1 = 0.2 unit/ml than in B (H/T1) = 4; T1 = 0.5 unit/ml). Thus, the PtdA accumulation achieved at hirudin addition was relatively less in (a) than in (b), and a restart would cause more PtdA accumulation in (a) than in (b)]. (c) The experimental design was as in (b) above, except that non-labelled platelets ( $3.1 \times 10^8$  cells/ml) and <sup>125</sup>I-thrombin was used. Parameters measured are shown in each panel.

secretion (Holmsen *et al.*, 1982). These results were based on single time point determinations, i.e. the extent of response 3 min after addition of thrombin. In the present work the time courses of individual responses were determined after 15 min incubation of the platelets with and without the inhibitors (Fig. 3). After incubation without antimycin A and 2-deoxyglucose, thrombin induced an initially rapid disappearance of [ $^3\text{H}$ ]PtdIns which became gradually slower; after incubation with the inhibitors the disappearance was very slow and linear (Fig. 3, upper left panel). A different effect of the preincubation with the inhibitors was revealed for the time courses of [ $^{32}\text{P}$ ]PtdA formation (Fig. 3, upper right panel), increase in the  $^{32}\text{P}/^3\text{H}$  ratio of PtdA (Fig. 3, lower left panel) and acid hydrolase secretion (Fig. 3, lower right panel). All three parameters increased most within the first 30 s of the thrombin-platelet interaction and changed little thereafter.

Thus, reduction of ATP availability has effects on [ $^3\text{H}$ ]PtdIns disappearance that are different from those on acid hydrolase secretion and PtdA production; PtdIns disappearance is relatively more inhibited than the two latter processes in the early period of the platelet-thrombin interaction.

#### *Metabolism of polyphosphoinositides during the thrombin-platelet interaction*

A rapid (maximal at 15–20 s) and transient decrease in [ $^{32}\text{P}$ ]PtdIns(4,5) $P_2$  has been noted in platelets prelabelled with [ $^{32}\text{P}$ ]P $_i$  upon stimulation with ADP (Vickers *et al.*, 1982), platelet-activating factor (Mauco *et al.*, 1983; Billah & Lapetina, 1983) and thrombin (Billah & Lapetina, 1982c; Agranoff *et al.*, 1983). However, these studies were done with platelets washed with EDTA- or EGTA-containing salt solutions or at low pH, methods that are known to decrease markedly platelet responsivity relative to gel filtration (Akkerman *et al.*, 1978). With gel-filtered platelets, Perret *et al.* (1983) found no significant decrease in the mass of the phosphoinositides immediately after thrombin addition, whereas the mass of both PtdIns(4) $P$  and PtdIns(4,5) $P_2$  increased 30 s after thrombin, and in parallel with acid hydrolase secretion. As shown in Fig. 4, our gel-filtered platelets also responded to thrombin with a rapid, transient decrease in [ $^{32}\text{P}$ ]PtdIns(4,5) $P_2$ , but the subsequent increase was very modest compared with that reported by Agranoff *et al.* (1983) and Perret *et al.* (1983). There was no significant changes in [ $^{32}\text{P}$ ]PtdIns(4) $P$  (results not shown). The marked increase in PtdA over the 90 s incubation period was the same whether the cells were extracted with acid (Fig. 4) or neutral (Figs. 1–3 and Table 1) chloroform/methanol.

We demonstrated previously that incubation of

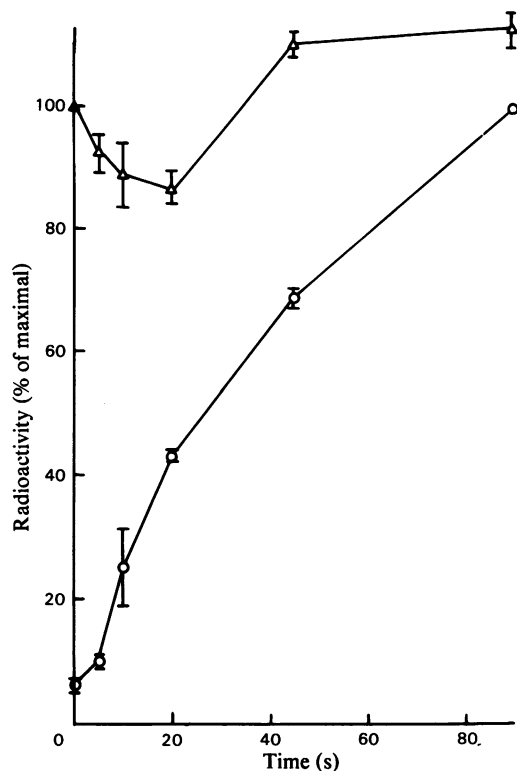


Fig. 4. Metabolism of [ $^{32}\text{P}$ ]PtdIns(4,5) $P_2$  and [ $^{32}\text{P}$ ]PtdA during the platelet-thrombin interaction

Gel-filtered, [ $^{32}\text{P}$ ]P $_i$ -labelled platelets were incubated with 2.0 units of thrombin/ml. The reactions were stopped at the times indicated by addition of acid chloroform/methanol as described in the materials and methods section and the radioactivities of [ $^{32}\text{P}$ ]PtdIns(4,5) $P_2$  ( $\Delta$ ) and [ $^{32}\text{P}$ ]PtdA ( $\circ$ ) were determined. The values are means ( $\pm$  S.D.) of results with three different platelet suspensions and are expressed as a percentage of the initial radioactivity of PtdIns(4,5) $P_2$  (4000–5000 c.p.m./ $10^8$  platelets) and the final (after incubation with thrombin for 90 s) radioactivity of PtdA (4500–5800 c.p.m./ $10^8$  platelets). No changes took place when the cells were incubated without thrombin.

platelets with antimycin A and 2-deoxyglucose caused progressive inhibition of the thrombin-induced fall in the level of [ $^3\text{H}$ ]PtdIns (Holmsen *et al.*, 1982). Here we show that the rapid initial fall in the [ $^{32}\text{P}$ ]PtdIns(4,5) $P_2$  level caused by thrombin was unaffected by incubation of the platelets with the metabolic inhibitors for at least 25 min; this incubation caused a gradual inhibition and finally abolition of the thrombin-induced PtdA production (Fig. 5a). However, the time course of the thrombin-induced changes in the [ $^{32}\text{P}$ ]PtdIns(4,5) $P_2$  level was altered by preincubation of



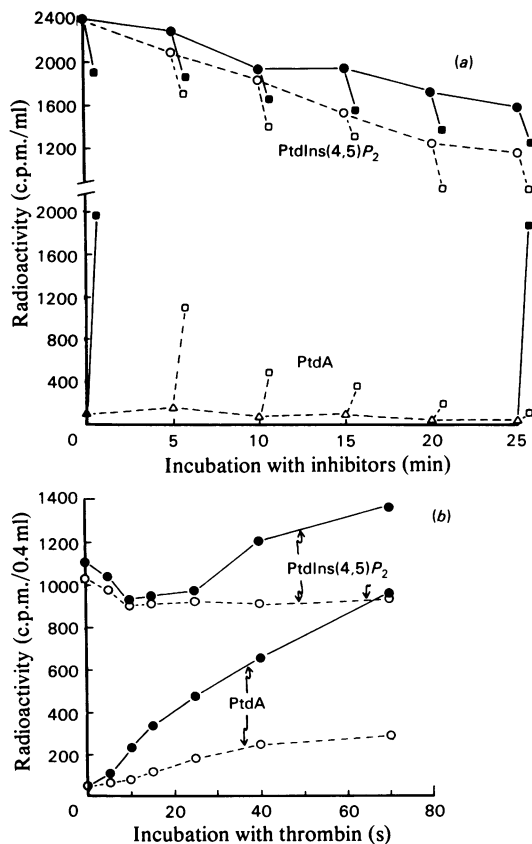


Fig. 5. Effect of time of incubation with metabolic inhibitors on thrombin-induced changes in  $[^{32}\text{P}]\text{PtdIns}(4,5)\text{P}_2$  and  $[^{32}\text{P}]\text{PtdA}$  (a) and effect of preincubation with metabolic inhibitors on the time course of thrombin-induced changes in  $[^{32}\text{P}]\text{PtdIns}(4,5)\text{P}_2$  and  $[^{32}\text{P}]\text{PtdA}$  (b)

(a) Gel-filtered,  $[^{32}\text{P}]\text{P}$ -labelled platelets ( $3.9 \times 10^8$  cells/ml) were incubated at  $37^\circ\text{C}$  with (broken lines, open symbols) and without (solid lines, filled symbols)  $30\text{mM}$ -2-deoxy-D-glucose and  $8.4\mu\text{g}$  of antimycin A/ml. Pairs of samples (0.4ml) were taken out at the times shown. One sample in each pair was extracted directly ( $\Delta$   $\circ$   $\bullet$ ), while the other was incubated further with 1 unit of thrombin/ml for 15s and then extracted ( $\square$   $\blacksquare$ ). (b) Gel-filtered,  $[^{32}\text{P}]\text{P}_i$ -labelled platelets ( $1.9 \times 10^8$  cells/ml) were incubated at  $37^\circ\text{C}$  for 15min with (broken lines, open symbols) and without (solid lines, filled symbols)  $30\text{mM}$ -2-deoxy-D-glucose and  $8.4\mu\text{g}$  of antimycin A/ml. Thrombin (1 unit/ml) was then added and samples extracted at the times shown.

the cells with the inhibitors for 15min. The preincubation had no effect on the initial, rapid decrease while it totally abolished the subsequent increase in  $[^{32}\text{P}]\text{PtdIns}(4,5)\text{P}_2$  (Fig. 5b). This latter effect of ATP deprivation was expected since formation of  $\text{PtdIns}(4,5)\text{P}_2$  from  $\text{PtdIns}$  and  $\text{PtdIns}(4)\text{P}$  consumes ATP directly.

### Discussion

It is widely held that the receptor-controlled step in the  $\text{PtdIns}$  cycle is coupled to breakdown of  $\text{PtdIns}$  (Putney, 1981; Lapetina, 1983) or  $\text{PtdIns}(4,5)\text{P}_2$  (Michell, 1982; Berridge, 1983). The evidence for such coupling is circumstantial and based on temporal and dose-response correlations of agonist binding and response to phospholipid metabolism. These correlations are necessary for, but do not prove, coupling.

The present work demonstrates that under the labelling conditions used, the content of  $^{32}\text{P}$  in  $\text{PtdA}$  is an adequate, relative measure for the mass of this phospholipid. Using this  $^{32}\text{P}$  technique we have established that the time course (Holmsen *et al.*, 1981) and the dependence on the thrombin concentration of  $\text{PtdA}$  formation (the present work) and acid hydrolase secretion are practically identical. These observations, as well as the dependence on the thrombin concentration for binding of  $^{125}\text{I}$ - $\alpha$ -thrombin, are suggestive of coupling between receptor occupancy,  $\text{PtdA}$  formation and secretion. However, although these correlations are necessary, they do not prove coupling. These correlations with binding are only true for the 0–3.0 unit/ml range of thrombin. Considerable amounts of  $^{125}\text{I}$ - $\alpha$ -thrombin became bound by an increase in the  $^{125}\text{I}$ - $\alpha$ -thrombin concentration from 3 to 30 unit/ml without any change in  $\text{PtdA}$  accumulation or acid hydrolase secretion. Thus, there may exist a large reserve of thrombin-binding sites on the platelets' surface that are not coupled to the reactions studied.

We have used two other approaches than the 'classical' time, dose and binding relationship to test the existence of coupling.

First, when thrombin binds,  $\text{PtdA}$  formation,  $[^3\text{H}]\text{PtdIns}$  disappearance and acid hydrolase secretion start, but only  $\text{PtdA}$  formation and secretion are arrested when bound thrombin is removed with hirudin; rebinding of thrombin restarts  $\text{PtdA}$  formation and secretion, but has little effect on  $[^3\text{H}]\text{PtdIns}$  metabolism.

Secondly, by reducing the ATP availability,  $\text{PtdA}$  formation and acid hydrolase secretion are inhibited in a similar manner, which is different (less in the early phase of stimulation) from inhibition of  $[^3\text{H}]\text{PtdIns}$  disappearance.

These observations strongly suggest that it is  $\text{PtdA}$  accumulation, and not  $[^3\text{H}]\text{PtdIns}$  disappearance, that is a necessary, receptor-controlled step in the processing of the thrombin signal for execution of acid hydrolase secretion in human platelets. However, other receptor-controlled steps may exist, that occur even earlier in the signal processing sequence than  $\text{PtdA}$  accumulation, and which are necessary for thrombin-induced acid hydrolase secretion in platelets.

The rapid metabolism of PtdIns(4,5) $P_2$  may be such a mandatory receptor-controlled step. The level of [ $^{32}$ P]PtdIns(4,5) $P_2$  decreased immediately after thrombin addition in our gel-filtered platelets, and we have further demonstrated here that this decrease is independent on metabolic energy. This is in sharp contrast to the thrombin-induced decrease in the [ $^3$ H]PtdIns level, which is almost abolished by ATP deprivation. This abolition is to be expected if PtdIns is removed by successive phosphorylations by ATP to replenish the PtdIns(4,5) $P_2$  initially hydrolysed as has been suggested (Agranoff *et al.*, 1983; Berridge, 1983). Our finding that ATP deprivation abolishes the subsequent rise in the [ $^{32}$ P]PtdIns(4,5) $P_2$  level further suggests that this rise is due to ATP-dependent resynthesis of the polyphosphoinositide. Thus, the three effects of ATP deprivation in platelets: (a) no change in the initial breakdown of PtdIns(4,5) $P_2$ , (b) inhibition of PtdIns disappearance and (c) abolition of PtdIns(4,5) $P_2$  resynthesis, are in full accordance with an initial, diesteratic hydrolysis of PtdIns(4,5) $P_2$  followed immediately by its resynthesis from PtdIns (Agranoff *et al.*, 1983; Berridge, 1983). The initial decrease and subsequent increase in the steady-state level of [ $^{32}$ P]PtdIns(4,5) $P_2$  following thrombin addition to the platelets in our study conform to this mechanism.

However, some of our observations contradict a pivotal role of PtdIns(4,5) $P_2$  breakdown in the thrombin-induced acid hydrolase secretion in platelets. First, the ATP deprivation experiments suggest that only a small (15%) fraction of the entire [ $^{32}$ P]PtdIns(4,5) $P_2$  pool is susceptible to thrombin-controlled hydrolysis. Thus, if the entire pool were susceptible one would expect a much greater initial decrease in the level of [ $^{32}$ P]-PtdIns(4,5) $P_2$  when its resynthesis is abolished than when it takes place. This was not the case. Thrombin caused the same 15% decrease in ATP-deprived platelets as in cells with intact energy metabolism. Whether this is due to compartmentalization of PtdIns(4,5) $P_2$  or whether the degree of the thrombin-controlled diesteratic hydrolysis is under metabolic control, after all, can not be distinguished. Second, we have recently demonstrated (A. Opstvedt Nilsen & H. Holmsen, unpublished work) that micromolar concentrations of certain antidepressants abolish the initial decrease in [ $^{32}$ P]PtdIns(4,5) $P_2$  caused by thrombin without affecting acid hydrolase secretion and dense granule secretion. Polyphosphoinositide metabolism may be tightly coupled to occupancy of certain receptors and certain cell responses in many cell types, including platelets. Nevertheless, it does not appear to be important for the signal

processing during thrombin-induced acid hydrolase secretion in platelets.

The abrupt stop in the increase of PtdA by removal of thrombin could be due to inhibition of its synthesis or acceleration of its removal. PtdA can be removed by several pathways, most of which are not activated by thrombin removal. (1) Conversion to PtdIns, as measured by accumulation of [ $^{32}$ P]PtdIns, was not altered by thrombin removal (Holmsen *et al.*, 1981). (2) Removal by PtdA-specific phospholipase  $A_2$  (Billah *et al.*, 1981) is not likely since no [ $^{32}$ P]lysoPtdA accumulated upon removal of thrombin (H. Holmsen & C. A. Dangelmaier, unpublished work). (3) Incorporation of PtdA into phospholipids other than PtdIns did not occur, since their  $^{32}$ P content did not increase by removal of thrombin (results not shown). (4) Dephosphorylation by PtdA phosphatase (Call & Williams, 1973) might be activated by thrombin removal. If this proves not to be the case, the possibility exists that occupancy of the thrombin receptor controls PtdA synthesis. The simplest model would be a direct control of the membrane-bound DG kinase (Call & Rubert, 1973) by the membrane-associated thrombin-receptor complex in a way that the kinase is active when the receptor is occupied and inactive when the receptor is unoccupied. Alternatively, the kinase could be continuously active while a receptor-controlled availability of DG determines PtdA production. One could also envision combinations of these models.

Our results show that there is no apparent correlation of receptor occupancy and secretion with the amount of PtdA in the platelets at any given time. The correlation is strictly with the rate of formation of PtdA, which can be explained by at least two plausible mechanisms. (1) PtdA is only active in the stimulus-response coupling (e.g., as an ionophore) at the locus in the membrane where it is synthesized; once it is released from this locus, it becomes inactive. (2) PtdA itself is inactive while its formation reflects the presence of the active substance, e.g., the substrate, DG.

Experiment with the divalent cationophore A23187 (Holmsen, 1975) and voltage access (Knight *et al.*, 1982) suggest that, while maximal dense granule secretion can be accomplished by a rise in cytoplasmic  $Ca^{2+}$ , acid hydrolase reaction requires additional factors. Since A23187 stimulates platelets without formation of DG or PtdA (Billah & Lapetina, 1982b; Imai & Nozawa, 1982), the results presented here suggest that PtdA formation (not the concentration of PtdA) or DG could be the additional factors needed to support maximal acid hydrolase secretion. If so, PtdA formation or the presence of DG would serve other functions than mobilization of cytoplasmic  $Ca^{2+}$ .

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