Differential energy requirements for platelet responses

A simultaneous study of aggregation, three secretory processes, arachidonate liberation, phosphatidylinositol breakdown and phosphatidate production

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Previous studies have indicated different energy requirements for some platelet responses; these differences could, however, be due to inadequate methodology and differences in platelet preparation. The present study describes the effect of decreasing ATP availability on seven platelet responses measured in gel-filtered human platelets. The cells, prelabelled with 5-hydroxy[³H]tryptamine, [³H]- or [¹⁴C]adenine, [³²P]P_i or [³H]arachidonate, were incubated with antimycin A and 2-deoxy-D-glucose. Platelet responses induced by thrombin and collagen (secretion only), level of metabolic ATP and the adenylate energy charge (AEC) were determined at various times during incubation. Platelet aggregation was rapidly inhibited after a lag of 5–15 min and with 50% inhibition at AEC = 0.55–0.60. Secretion of 5-hydroxy[¹⁴C]tryptamine and ATP + ADP from dense granules and of fibrinogen and β -thromboglobulin from α -granules were inhibited in parallel, without a lag and with 50% inhibition at AEC = 0.65–0.70. The inhibition of secretion of platelet factor 4 from the α -granules followed another pattern with 50% inhibition at AEC = 0.70–0.80. Breakdown of [³H]-phosphatidylinositol, formation of [³H]- and [³²P]-phosphatidate, liberation of [³H]arachidonate and secretion of acid hydrolases were inhibited in parallel and inhibition was present at the start of incubation with 50% inhibition at AEC = 0.80–0.87. These results suggest that the responses have different energy requirements, increasing in the order: aggregation < dense granule and α -granule secretion < acid hydrolase secretion, phosphatidylinositol breakdown, phosphatidate formation and arachidonate liberation. The powerful inhibition of phosphatidylinositol breakdown by metabolic inhibitors suggests that energy-requiring steps are involved in the activation of phospholipase C.

Stimulated platelets display a series of responses: shape change (Born, 1970), aggregation (Kinlough-Rathbone *et al.*, 1977), secretion of the contents of three storage granules (Holmsen & Day, 1970; Holmsen, 1978; Kaplan *et al.*, 1979) and liberation of arachidonate esterified in phospholipids (Bills *et al.*, 1976; Rittenhouse-Simmons, 1979; Billah *et al.*, 1981). ADP and serotonin (5-hydroxytryptamine) secreted from the dense granules (Holmsen, 1978), as well as prostaglandins and thromboxanes formed by oxygenation of the non-esterified arachidonate (Samuelsson, 1977), are potent platelet-stimulating agents that potentiate the propagation of responses.

Abbreviations used: PtdIns, phosphatidylinositol; PtdA, phosphatidic acid.

The interrelations between these responses and the possibility that they are all triggered by increase in the concentration of cytoplasmic Ca²⁺ have been discussed previously (Charo *et al.*, 1977; Holmsen, 1978; Akkerman & Holmsen, 1981).

Several studies suggest that ATP energy is required for platelet responses. Inhibition of ATP production markedly decreases shape change (Holmsen *et al.*, 1974), aggregation (Mürer *et al.*, 1967; Holmsen *et al.*, 1974; Kattlove, 1974), arachidonate liberation (Rittenhouse-Simmons & Deykin, 1977), dense-granule secretion (Mürer, 1968; Mürer & Holme, 1970; Holmsen *et al.*, 1979) and acid hydrolase secretion (Holmsen *et al.*, 1979), as well as ADP-induced adhesion of platelets to glass and platelet-promoted clot retraction (Mürer *et*

al., 1967). Platelet responses differ, however, in their sensitivity to metabolic inhibitors, and these studies suggested that ATP is required for platelet responses in this order: shape change < primary aggregation < secondary aggregation (equivalent to dense-granule secretion and/or arachidonate liberation) < acid hydrolase secretion. Three responses, dense-granule secretion, α -granule secretion and arachidonate liberation, have not been studied directly with respect to relative energy requirement. Furthermore, the greater ATP requirement for acid hydrolase secretion than for dense-granule secretion may have been due to the adverse effects of washing platelets with EDTA-containing solutions at low temperature (Akkerman *et al.*, 1978). Platelets respond to stimuli with an increased turnover of PtdIns (Lloyd & Mustard, 1974; Lapetina & Cuatrecasas, 1979; Broekman *et al.*, 1980), a process that directly consumes ATP.

In the present work, we have studied the effect of ATP deprivation on aggregation, all three secretory processes, arachidonate liberation, PtdIns breakdown and production of PtdA. The platelets used have been gel-filtered, a method that preserves platelet responsiveness better than washing in the cold with EDTA-containing salt solutions (Akkerman *et al.*, 1978).

Materials and methods

Preparation of labelled gel-filtered platelets

Platelet-rich plasma, prepared from human blood (9 vol.) collected in 0.11 M-sodium citrate (1 vol.) was incubated with various radioactive compounds: 0.4 μ M-[U- ^{14} C]adenine (code CFA 436; sp. radioactivity 260–300 Ci/mol; Amersham) or [G- ^{3} H]adenine (code NET-063; sp. radioactivity 4500–6000 Ci/mol; New England Nuclear) for 60 min at room temperature, 0.01 μ M-[^{3} H]arachidonic acid (code TRK 508; sp. radioactivity 90–130 Ci/mmol; Amersham) or 0.2 mCi of [^{32} P]P_i/ml (carrier-free; New England Nuclear) for 60 min at 37°C. In some experiments, the platelet-rich plasma was incubated for at least 10 min (37°C) with 5-hydroxy[*side-chain-2- ^{14} C*]tryptamine ([^{14} C]serotonin; code CFA 170; sp. radioactivity 57 Ci/mol; Amersham). After incubation, the platelets were transferred into a Ca²⁺-free Tyrode's solution containing 5 mM-glucose and 0.2% human crystalline serum albumin (Sigma) by gel filtration (Lages *et al.*, 1975). When the platelets had been labelled with [^{32} P]P_i, P_i was excluded from the Tyrode's solution.

Incubation with metabolic inhibitors

The labelled gel-filtered platelets were divided into two portions, which were equilibrated at 37°C for 5 min. NaCl (30 μ l; 0.15 M) and 1.67 μ l of ethanol

was added per ml of gel-filtered platelets to one of the portions ('control'), and 30 μ l of 1 M-2-deoxy-glucose in 0.15 M-NaCl and 1.67 μ l of antimycin A (5 mg/ml) in ethanol was added per ml to the other portion ('test'). Both the control and the test mixtures were incubated at 37°C and samples were removed at various times for determination of the levels of metabolic adenine nucleotides and of platelet responses.

Determinations

Metabolic adenine nucleotides. A portion (0.2 ml) of the incubation mixture was extracted with 0.2 ml of freshly prepared EDTA/ethanol (9 vol. of 95% ethanol plus 1 vol. of 77 mM-EDTA, pH 7.4). The extracts were stored at –20°C. If the platelets had been labelled with [^{14} C]adenine, the ^{14} C-labelled adenine nucleotides in the extracts were separated by high-voltage paper electrophoresis and counted for radioactivity as described previously (Holmsen *et al.*, 1974). For ^{32} P-labelled platelets, the adenine nucleotides in the extracts were separated by two-dimensional paper chromatography, detected by autoradiography and counted for radioactivity as described previously (Holmsen, 1965).

Platelet responses. The concentrations of agonist and times used are given in the Figures. Thrombin was a topical, bovine preparation (Parke Davis) and was stored in portions at concentrations of 100 units/ml at –20°C. Collagen was from tendon and was prepared, stored and used as described previously (Holmsen *et al.*, 1973). Agonist solution (0.1 vol.) was added per 1 vol. of gel-filtered platelets.

Measurements

Aggregation in response to thrombin. This was determined as the initial rate of increase in light transmission in gel-filtered platelets (after shape change) as described previously for platelet-rich plasma (Holmsen *et al.*, 1972a).

Secretion. The portions that had been incubated with agonist for the given time were mixed with 0.1 vol. of 77 mM-EDTA in tubes prechilled in ice. The mixtures were centrifuged for 2 min at 12000 g (room temperature), and extracts for determination of secreted substances were made from the supernatants (TS). In addition, extracts were made from non-centrifuged mixtures of gel-filtered platelets and agonist solvent for determination of total amounts of substances (C), and from supernatants of such mixtures, centrifuged as above (CS). The extent of secretion is expressed as a proportion of total cellular amounts:

$$\text{Secretion (\%)} = \frac{A_{\text{TS}} - A_{\text{CS}}}{A_{\text{C}} - A_{\text{CS}}} \times 100$$

where A represents the amounts of constituent determined in the fractions TS, CS and C.

Determination of secreted constituents

Total amounts of ATP + ADP (dense-granule constituents). EDTA/ethanol extracts (see above) were prepared and their content of ATP + ADP was determined by a firefly luciferase method (Holmsen *et al.*, 1972b).

[¹⁴C]Serotonin (dense-granule constituent). This was regarded as the ¹⁴C radioactivity in 100 μ l portions of supernatants and non-centrifuged controls and determined by scintillation counting (Holmsen *et al.*, 1973).

Platelet factor 4, β -thromboglobulin and fibrinogen (α -granule constituents). To all samples were added hirudin (VEB Arzneimittelwerk, Dresden, Germany) to a concentration ten times (on a per unit base) that of thrombin used and Triton X-100 to a final concentration of 0.1%. Platelet factor 4 and β -thromboglobulin were determined by radioimmunoassay as previously described (Kaplan *et al.*, 1979). Fibrinogen was determined by using the radioimmunoassay for fibrinopeptide A described by Nossel *et al.* (1974). Samples from thrombin-treated platelets were assayed after bentonite extraction of remaining fibrinogen and those from collagen-treated platelets were treated with thrombin to cleave all fibrinopeptide A from the released fibrinogen and then assayed (Kaplan *et al.*, 1981).

β -Hexosaminidase and β -glucuronidase. All samples were made 0.1% with respect to Triton X-100 and frozen and thawed once. The enzyme activities in these extracts were determined with 4-methylumbelliferyl β -N-acetylglucosaminide and 4-methylumbelliferyl β -glucuronide as substrate (Dangelmaier & Holmsen, 1980).

Liberation of [³H]arachidonate from platelet phospholipids

Gel-filtered platelets prepared from [³H]arachidonate-labelled platelet-rich plasma were used. Samples (1 ml) of control or test gel-filtered platelets that had been incubated with thrombin for noted times were extracted with chloroform/methanol and processed as described previously (Holmsen *et al.*, 1981).

Formation of [³H]PtdA; breakdown of [³H]PtdIns

Gel-filtered platelets prepared from platelet-rich plasma labelled with [³²P]P_i plus [³H]arachidonate was incubated with thrombin and extracted as described above for arachidonate liberation. However, the amounts of PtdA present in extracts from 1 ml of thrombin-stimulated gel-filtered platelets were usually too small to be detectable with the phosphomolybdc reagent used (Holmsen *et al.*, 1981). The thin-layer chromatograms were therefore kept with X-ray film overnight by which time distinct spots were produced by PtdA.

Expressions

The extent of all responses measured in the control mixtures usually decreased during incubation at 37°C. Therefore, the extent of a response in test mixtures at any given time of incubation was corrected for this decrease in the control:

$$\text{Corrected response (\%)} = \frac{R_t - \text{test}}{R_t - \text{control}} \times 100$$

where R = % measured response (e.g., initial slope of aggregation, percentage secretion, relative change in [³H]arachidonate content of PtdIns, PtdA or arachidonate, ³²P content in PtdA) at time *t*.

The sum of the radioactivities of ³H- or ¹⁴C-labelled ATP, ADP, AMP, IMP and hypoxanthine was constant in a given gel-filtered platelet preparation irrespective of treatment (100 000–150 000 c.p.m./ml of gel-filtered platelets). The radioactivity of each metabolite was therefore expressed as the percentage of total radioactivity. The adenylate energy charge (AEC) was calculated according to the formula:

$$\text{AEC} = \frac{[\text{ATP}] + \frac{1}{2}[\text{ADP}]}{[\text{ATP}] + [\text{ADP}] + [\text{AMP}]} \quad (\text{Atkinson, 1969})$$

in which the concentration of each nucleotide was replaced by the percentage radioactivity (Mills, 1973). We have shown previously that the radioactivities of [¹⁴C]adenine-labelled nucleotides are directly proportional to their concentration in the platelet cytoplasm (Daniel *et al.*, 1980).

Likewise, the sum of the radioactivity in [³H]-arachidonate-labelled fractions was constant (10 000–15 000 c.p.m./ml) in a given gel-filtered platelet preparation irrespective of treatment. The radioactivity of each ³H-containing fraction was expressed as a percentage of this sum. In contrast, the ³²P content of PtdA (and of PtdIns) increased markedly during thrombin treatment and was expressed in c.p.m./ml of gel-filtered platelets.

Results

Differential inhibition of aggregation and dense-granule secretion

The aggregation response to thrombin, which was always monophasic, was measured together with secretion of ATP + ADP and/or pre-absorbed [¹⁴C]-serotonin at various times during incubation of gel-filtered platelets with antimycin A and 2-deoxyglucose. Fig. 1 shows the results from a representative experiment (12 similar experiments were performed, although with various concentrations of thrombin). It is apparent that the aggregation response is unaffected by incubation of gel-filtered platelets with the metabolic inhibitors for 10 min.

Fig. 1. Variations in thrombin-induced aggregation and dense-granule secretion and in [^{14}C]adenine-labelled metabolites during incubation of gel-filtered platelets with antimycin A and 2-deoxyglucose

One portion of gel-filtered platelets prepared from [^{14}C]adenine-labelled platelet-rich plasma was incubated without (control) and another portion incubated with (test) antimycin A and 2-deoxyglucose. At the times indicated portions from control and test mixture were analysed for aggregation (○—○) and secretion of ATP+ADP (●) in response to 0.4 unit of thrombin/ml with stirring (900 rev./min) for 60s and AEC (○—○) immediately before thrombin addition. For further details, see the Materials and methods section.

whereas secretion of ATP + ADP was progressively inhibited within this time period, reaching 48% inhibition at 10 min. After 10 min, the aggregation response decreased rapidly, and was abolished after 25 min of incubation; the secretion response also decreased during this time period, although not so sharply as the aggregation response. The aggregation response is unaffected by a decrease in AEC from 0.93 to 0.68, under the conditions used,

whereas secretion is sensitive to any decrease in AEC from its initial value. Similar results were obtained when dense-granule secretion was monitored with pre-absorbed [^{14}C]serotonin (for similar behaviour of secretion of ATP + ADP and [^{14}C]serotonin during incubation with the metabolic inhibitors, see Figs. 3 and 6). Neither dense-granule secretion nor aggregation were affected by the mere presence of the metabolic inhibitors; that is, there is no inhibition at the start of incubation (zero-time, Fig. 1).

Differential inhibition of dense-granule secretion and acid hydrolase secretion

We found previously that acid hydrolase secretion occurs more slowly than dense-granule secretion when EDTA-washed platelets are treated with thrombin (Holmsen *et al.*, 1979). In the gel-filtered platelets used here this was clearly the case for higher (≥ 0.1 unit/ml) but not at lower (0.05 unit/ml) concentrations of thrombin (Fig. 2). At the lower concentration the secretion of ATP + ADP and β -hexosaminidase had the same time course. However, the secretion of another acid hydrolase, β -glucuronidase, was markedly slower than that of β -hexosaminidase at the low thrombin concentration; at higher concentrations its secretion was also slower, although not as markedly so (Fig. 2).

There seems to be an inverse correlation between the velocity of appearance of a platelet response and the relative degree of inhibition by metabolic blockade: shape change is faster than aggregation and is inhibited less by metabolic inhibitors (Holmsen *et al.*, 1974); aggregation occurs before dense-granule secretion and is inhibited less (Fig. 1); dense-granule secretion in EDTA-washed platelets is faster than acid hydrolase secretion and inhibited less (Holmsen *et al.*, 1979). In the gel-filtered platelets acid hydrolase secretion was inhibited much more than dense-granule secretion during incubation with the inhibitors, and acid hydrolase secretion was inhibited at the very start of incubation (Fig. 3). Greater inhibition of secretion of β -glucuronidase than of β -hexosaminidase is consistent with the hypothesis that there is an inverse relationship between response velocity and degree of inhibition.

Inhibition of α -granule secretion by incubation with antimycin A and 2-deoxyglucose: comparison with dense-granule secretion and acid hydrolase secretion

Effect of time of incubation gel-filtered platelets with inhibitors. Fig. 4 demonstrates that incubation of gel-filtered platelets with the metabolic inhibitors caused a progressive inhibition of the thrombin-induced secretion of platelet factor 4, β -thromboglobulin and fibrinogen (monitored as fibrinopeptide

Fig. 2. Time course of secretion of ATP + ADP and two acid hydrolases with various concentrations of thrombin. Gel-filtered platelets were incubated with 0.05 (a), 0.1 (b) and 0.5 (c) unit of thrombin/ml. Samples were taken at the times indicated for determination of ATP + ADP (●), β -hexosaminidase (Δ) and β -glucuronidase (∇). The results are expressed as percentages of maximal secretion, which is the amount of each constituent secreted after incubation with 5 units of thrombin/ml for 5 min.

A). The degree of inhibition of the secretion of β -thromboglobulin and fibrinogen was the same at all times of incubation, and not statistically different from that of dense-granule secretion. During the first 5 min of incubation the inhibition of secretion of platelet factor 4 was not different from the inhibition of secretion of β -thromboglobulin and fibrinogen, ATP + ADP or [^{14} C]serotonin, but was less than the inhibition of acid hydrolase secretion. Between 10 and 25 min of incubation the secretion of platelet factor 4 was significantly lower ($P < 0.05$; $n = 19$) than that of ATP + ADP and not different from inhibition of acid hydrolase secretion.

The same effect of time of incubating gel-filtered platelets with the inhibitors on the secretory responses was obtained with collagen as inducer (Fig. 5). Inhibition of secretion of pre-absorbed [^{14} C]-serotonin and of ATP + ADP was practically identical with that of secretion of β -thromboglobulin and fibrinogen. As with thrombin, the secretion of platelet factor 4 by collagen was markedly more inhibited in the later period of incubation (after about 5 min). The secretion of β -hexosaminidase was inhibited at zero-time and more than that of the secretion of ATP + ADP, [^{14} C]serotonin, β -thromboglobulin and fibrinogen all throughout the incubation; its inhibition was statistically not different from that of platelet factor 4 secretion at the later stages of incubation.

Effect of agonist concentration after a constant time of incubation with the inhibitors. The secretory responses of control platelets (after 15 min of incubation with inhibitor solvents) to increasing

concentrations of thrombin are shown in Fig. 6(a). The dose-response relationships for the secretion of ATP + ADP and of β -thromboglobulin and platelet factor 4 were practically identical, and distinctly different from that of β -hexosaminidase secretion. The latter increased $2\frac{1}{2}$ times by increasing the thrombin concentration from 0.1 to 1.0 unit/ml, whereas dense-granule and α -granule secretion were maximal at 0.1 unit/ml. The secretion of fibrinogen followed an intermediate pattern, being less than β -hexosaminidase and identical with ATP + ADP and the other α -granule markers at high concentrations of thrombin. This is likely to be a function of measuring fibrinogen as fibrinopeptide A, with less complete cleavage at low thrombin concentrations.

The effect of antimycin A and 2-deoxyglucose on the responses at the same thrombin concentrations is shown in Fig. 6(b). Inhibition was greatest at the lowest concentration of thrombin, but increase in the thrombin concentration gradually overcame the inhibition. Secretion of ATP + ADP and of β -thromboglobulin and fibrinogen showed similar dependence of inhibition on thrombin concentration and inhibition was abolished at 0.5–1.0 unit of thrombin/ml. In contrast, neutralization of the inhibition of platelet factor 4 secretion required higher thrombin concentrations, and inhibition was not abolished within the range of thrombin concentrations used. Neutralization of inhibition of β -hexosaminidase secretion was similar to that for secretion of ATP + ADP, β -thromboglobulin and fibrinogen by increase from 0.05 to 0.1 unit of

Fig. 3. *Progressive inhibition of dense-granule secretion and acid hydrolase secretion during incubation of gel-filtered platelets with antimycin A and 2-deoxyglucose*

Gel-filtered platelets prepared from platelet-rich plasma labelled with [³H]adenine and [¹⁴C]serotonin was incubated as described in the legend to Fig. 1. Samples were taken at the times indicated for determination of secretion of ATP+ADP (●), [¹⁴C]serotonin (○—○), β-hexosaminidase (Δ) and β-glucuronidase (□) in response to 0.5 unit of thrombin/ml for 30s and of AEC (○----○) immediately before thrombin addition.

thrombin/ml, but further increase in the thrombin concentration to 1 unit/ml had no effect on the inhibition of β-hexosaminidase secretion, which remained constant at 50% of control.

Similar results were obtained with collagen in the 1–10 μg/ml range (results not shown).

Thrombin-induced changes in the radioactivity of PtdIns, PtdA and arachidonate

When platelets prelabelled with [³H]arachidonate and [³²P]P_i were treated with thrombin for 2 min, the

Fig. 4. *Progressive inhibition of thrombin-induced secretory responses during incubation of gel-filtered platelets with antimycin A and 2-deoxyglucose*

The experiment was performed as described in the legend to Fig. 3. Values are means of five experiments; s.d. for each point is not shown (for clarity) and did not exceed 15% of the mean value. ○, AEC. Secretion markers are: ●, ATP+ADP; Δ, β-hexosaminidase; ▼, platelet factor 4; ▲, fibrinogen (fibrinopeptide A); □, β-thromboglobulin.

level of [³H]PtdIns fell, whereas that of [³H]PtdA, [³²P]PtdA and [³H]arachidonate (+ oxygenation products) increased. Incubation of the same platelets for 22.5 min with antimycin A + 2-deoxyglucose caused a marked decrease in these changes. Both the thrombin-induced increase in [³²P]PtdA in control platelets and the decrease in this increase by antimycin A + 2-deoxyglucose were greater than the corresponding changes in [³H]PtdA. There were no changes in ³H and ³²P radioactivity of these substances in platelets incubated with antimycin A + 2-deoxyglucose for up to 45 min and subsequently incubated with 0.1 volume of 0.15 M-NaCl (vehicle for thrombin) for 2 min (results not shown).

Fig. 5. Progressive inhibition of collagen-induced secretory responses during incubation of gel-filtered platelets with antimycin A and 2-deoxyglucose

Gel-filtered platelets were prepared from platelet-rich plasma labelled with [^3H]adenine and [^{14}C]serotonin and used in the same type of experiment as described in Fig. 3, except that the platelets were stimulated by $50\mu\text{g}$ of collagen/ml for 3 min with stirring (900 rev./min) to induce secretion. The symbols are defined in the legend to Fig. 4, except for the inclusion of results of [^{14}C]serotonin secretion (■). The data are means of two experiments.

The effects of the time of incubating the platelets with antimycin A + 2-deoxyglucose on the thrombin-induced phospholipid changes are illustrated in Fig. 7. Since the platelets were incubated with [^3H]arachidonate and [^{32}P]P $_i$ and not [^{14}C]adenine as in the experiments above, the AEC could not be assessed. Instead, the combined ^{32}P radioactivity of the ethanol-soluble nucleoside triphosphates was used to follow energy deprivation during incubation. The decrease in ^{32}P -labelled nucleoside triphosphates as incubation with antimycin A + 2-deoxyglucose proceeded (Fig. 7) was similar to that of [^{14}C]ATP in the experiments above where [^{14}C]adenine labelling was used. The thrombin-induced decrease in [PtdIns] and formation of PtdA was inhibited by the mere

presence of antimycin A + 2-deoxyglucose since these responses were reduced by 40–50% at the beginning of incubation with the inhibitors (zero-time, Fig. 7). The responses decreased further as incubation proceeded, and became 10–18% of control values after 30 min of incubation. The time course of the reduction in the decrease of [^3H]PtdIns concentration and of the decrease in the formation of [^3H]PtdA or [^{32}P]PtdA were indistinguishable, and followed a distinctly different pattern from aggregation (Figs. 1 and 7), dense-granule secretion (Figs. 1, 3 and 4) and α -granule secretion (Fig. 4), but were similar to that of acid hydrolase secretion (Figs. 3 and 4).

The liberation of [^3H]arachidonate (+oxygenated products) was inhibited at zero-time and further inhibited by incubation of platelets with antimycin A + 2-deoxyglucose; the pattern of inhibition was superimposable on that for β -hexosaminidase secretion and PtdA formation (Fig. 8).

Discussion

We have shown previously that inhibition of platelet responses is independent of variations in the level of cytoplasmic ATP, i.e., the pool labelled by [^{14}C]adenine and [^{32}P]P $_i$, when the AEC is constant, but correlates well with the level of AEC (Holmsen & Robkin, 1977). In the present study 50% inhibition was obtained for aggregation at AEC = 0.55–0.60, for dense-granule and α -granule secretion at AEC = 0.65–0.70 and for acid hydrolase secretion, PtdIns breakdown, PtdA accumulation and arachidonate liberation at AEC = 0.80–0.87. The AEC level is a measure for the energy balance in the cell; in platelets, an AEC of 0.92–0.95 in ethanolic extracts (Daniel *et al.*, 1979) as used here indicates that synthesis balances consumption of ATP, whereas reduction of AEC from this level indicates that more ATP is consumed than is produced and thus the availability of metabolic energy is decreased. The AEC values corresponding to 50% inhibition of the three classes of responses thus indicates that acid hydrolase secretion, PtdIns breakdown, PtdA accumulation and arachidonate liberation are more dependent on ATP availability than dense-granule and α -granule secretion, which in turn are more dependent than aggregation. These three classes of processes are also distinguished by their time course of inhibition. Thus, the inhibition of aggregation begins after a lag of 5–15 min under the conditions used, and then rapidly becomes complete. Dense-granule secretion and α -granule secretion are not inhibited when tested immediately after addition of antimycin A and 2-deoxyglucose, but inhibition is progressive with incubation with the inhibitors. Acid hydrolase secretion, PtdIns breakdown, PtdA accumulation (both of [^3H] and

Fig. 6. Effect of incubation of gel-filtered platelets with antimycin A and 2-deoxyglucose on the dose-response relationships of thrombin-induced secretory responses. One portion of gel-filtered platelets, prepared from [^{14}C]adenine-labelled platelet-rich plasma, was incubated without (a) and another with (b) antimycin A

[^{32}P]-PtdA) and arachidonate liberation are markedly inhibited immediately after addition of the inhibitors and progressively inhibited as incubation proceeds.

Fig. 7. Progressive inhibition of thrombin-induced breakdown of [^3H]PtdIns and accumulation of [^3H]- and [^{32}P]-PtdA during incubation of gel-filtered platelets with antimycin A and 2-deoxyglucose

The experiment was performed as described in the legend to Fig. 3, except that the gel-filtered platelets were prepared from platelet-rich plasma labelled with [^{32}P]P_i and [^3H]arachidonate, and that the cellular responses were tested in an aggregometer (see the Materials and methods section). The initial rate of aggregation (●) and total nucleoside [^{32}P]-triphosphates (○) are shown together with the breakdown of [^3H]PtdIns (▽) and accumulation of [^3H]PtdA (▲) and [^{32}P]PtdA (■) determined at the times indicated by incubation of portions of control and test gel-filtered platelets with 0.5 unit of thrombin/ml for 2 min. The experiment is representative of four identical experiments.

and 2-deoxyglucose for 15 min. Portions of both mixtures were then incubated for 30 s with the concentrations of thrombin given on the abscissa, and the amounts of secreted markers were determined. The symbols are defined in Fig. 4 and the data (means of two experiments) are expressed in (a) as the percentages of the secretion obtained at 1 unit/ml for 30 s. AEC in the antimycin A-plus-2-deoxyglucose-containing mixture was 0.74 after 15 min of incubation.

Fig. 8. Progressive inhibition of secretory responses, arachidonate liberation and phosphatidate accumulation during incubation of gel-filtered platelets with antimycin A and 2-deoxyglucose

The experiment was performed as described in the legend to Fig. 3 and with the exceptions noted in the legend to Fig. 7. Symbols: ○, nucleoside [^{32} P]triphosphates; ●, secretion of ATP+ADP; ▼, liberation of arachidonate; △, secretion of β -hexosaminidase; ■, accumulation of [^{32} P]PtdA. Portions of control and test mixture were tested with 0.5 unit of thrombin/ml for 2 min. The experiment is representative of three identical experiments.

The pattern of inhibition of secretion of platelet factor 4 is distinctly different from those of the two other α -granule constituents, β -thromboglobulin and fibrinogen. α -Granules are particularly heterogeneous in appearance (White, 1971) and it is possible that the various markers are not localized in the same subpopulation of α -granules and that the different subpopulations of α -granules have different energy requirements for secretion of their contents. Alternatively, the markers may be present in the same organelle population, but the actual liberation of platelet factor 4 from the organelle matrix to the medium may be (1) more directly dependent on metabolic energy or (2) indirectly dependent on metabolic energy through acid hydrolase secretion. The latter possibility is based on the finding that platelet factor 4 is stored in the α -granules as a complex with a large proteoglycan from which it is set free during secretion (Barber *et al.*, 1972). The radioimmunoassay used here utilizes pure platelet

factor 4 as antigen and uses antibody directed against pure platelet factor 4 (Kaplan *et al.*, 1979). Secretion of an intact complex with less immunological activity towards the platelet factor 4 antibody used and from which immunologically active platelet factor 4 is cleaved by hydrolases made available during acid hydrolase secretion would be in accordance with our results.

β -Hexosaminidase and β -glucuronidase have been shown to have different secretion characteristics (Holmsen & Day, 1970; Holmsen *et al.*, 1975). The results of the present study substantiate this difference and suggest further that a fraction of β -hexosaminidase, but not β -glucuronidase, may be contained in the dense granules.

The finding that treatment of platelets with metabolic inhibitors causes a marked inhibition of the thrombin-induced breakdown of PtdIns is, to our knowledge, the first demonstration of the dependence on ATP availability for agonist-induced PtdIns breakdown in any cell. The classical reaction sequence (Michell, 1975; Putney, 1981) involves an initial activation of phospholipase C, which subsequently hydrolyses PtdIns to diacylglycerol and inositol 1:2-cyclic phosphate. The mechanism of phospholipase C activation is largely unknown, but thought to be coupled to receptor occupancy (Putney, 1981). Our studies indicate that there must be energy-requiring steps in this activation mechanism. The demonstration that platelet phospholipase C is activated by Ca^{2+} (Mauco *et al.*, 1979) suggests that the energy requirement could be connected with Ca^{2+} mobilization.

Tight coupling of receptor occupancy to phospholipase C activation has not yet been demonstrated (Putney, 1981), whereas such coupling to the synthesis of PtdA for thrombin-receptor occupancy has been demonstrated in platelets (Holmsen *et al.*, 1981). Thrombin-induced PtdA accumulation was markedly inhibited by antimycin A and 2-deoxyglucose, which is to be expected, since PtdA synthesis consumes ATP directly. It is therefore possible that the inhibition of breakdown of PtdIns is a result of the inhibition of PtdA synthesis. One may envisage an equilibrium between PtdIns and diacylglycerol in the resting cell, which becomes directed to PtdIns hydrolysis owing to activation of diacylglycerol kinase by the association of thrombin with its receptor. PtdA has been shown to possess ionophoretic properties (Tyson *et al.*, 1976; Salmon & Honeyman, 1980; Putney, 1981) and its accumulation when thrombin is interacting with its receptor may thus lead to liberation of Ca^{2+} , which may stimulate phospholipase C, which in turn provides more diacylglycerol. The gross accumulation of PtdA may therefore be a result of a major positive feedback system. With such a mechanism it is to be expected that the PtdA formation/PtdIns

breakdown steps will be severely inhibited when the availability of ATP for PtdA formation becomes reduced. The parallel inhibition of PtdA formation and PtdIns breakdown supports this mechanism.

Thrombin-induced arachidonate liberation from the pool(s) of phospholipids that can be labelled by [^3H]arachidonate has previously been shown to be markedly inhibited by ATP deprivation (Rittenhouse-Simmons & Deykin, 1977). We have extended this observation by demonstrating that the time course of inhibition is superimposable on that for inhibition of acid hydrolase secretion, PtdIns breakdown and PtdA synthesis. Such parallel inhibition would be expected if these four processes had a common ATP-requiring step. The suggestions that arachidonate originates from PtdIns via diacylglycerol (Bell *et al.*, 1979) would be in accordance with the inhibition patterns presented here.

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References

- Akkerman, J. W. N. & Holmsen, H. (1981) *Blood* **57**, 956–966
- Akkerman, J. W. N., Doucet-de Bruine, M. H. M., Gorter, G., DeGraaf, S., Holme, S., Lips, J. P. M., Numeuer, A., Over, J., Starkenburg, A. E., Trieschnigg, A. M. C. van der Veen, Vlooswijk, H. A. A., Wester, J. & Sixma, J. J. (1978) *Hemostasis Thromb.* **39**, 146–157
- Atkinson, D. E. (1969) *Annu. Rev. Microbiol.* **23**, 47–68
- Barber, A. G., Käzer-Glanzmann, R., Jakabova, M. & Luscher, E. F. (1972) *Biochim. Biophys. Acta* **286**, 316–325
- Bell, R. L., Kennerly, D. A., Stanford, N., Majerus, P. W., Costrini, N. V. & Bradshaw, R. A. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 3238–3242
- Billah, M. M., Lapetina, E. G. & Cuatrecasas, P. (1981) *J. Biol. Chem.* **256**, 5399–5403
- Bills, T. K., Smith, J. B. & Silver, N. J. (1976) *Biochim. Biophys. Acta* **424**, 303–314
- Born, G. V. R. (1970) *J. Physiol. (London)* **209**, 487–511
- Broekman, M. J., Ward, J. W. & Marcus, A. J. (1980) *J. Clin. Invest.* **66**, 275–283
- Call, F. L. & Rubert, M. (1973) *J. Lipid Res.* **14**, 466–474
- Charo, I. F., Feinman, R. D. & Detwiler, T. C. (1977) *J. Clin. Invest.* **60**, 866–873
- Dangelmaier, C. A. & Holmsen, H. (1980) *Anal. Biochem.* **104**, 182–191
- Daniel, J. L., Robkin, L., Molish, I. R. & Holmsen, H. (1979) *J. Biol. Chem.* **254**, 7870–7873
- Daniel, J. L., Molish, I. R. & Holmsen, H. (1980) *Biochim. Biophys. Acta* **632**, 444–453
- Holmsen, H. (1965) *Scand. J. Clin. Lab. Invest.* **17**, 537–547
- Holmsen, H. (1978) *Adv. Pharmacol. Ther.* **4**, 97–109
- Holmsen, H. & Day, H. J. (1970) *J. Lab. Clin. Med.* **75**, 840–855
- Holmsen, H. & Robkin, L. (1977) *J. Biol. Chem.* **252**, 1752–1757
- Holmsen, H., Day, H. J. & Setkowsky, C. A. (1972a) *Biochem. J.* **129**, 67–82
- Holmsen, H., Storm, E. & Day, H. J. (1972b) *Anal. Biochem.* **46**, 489–501
- Holmsen, H., Ostvold, A.-C. & Day, H. J. (1973) *Biochem. Pharmacol.* **22**, 2599–2608
- Holmsen, H., Setkowsky, C. A. & Day, H. J. (1974) *Biochem. J.* **144**, 385–396
- Holmsen, H., Setkowsky, C. A., Lages, B., Day, H. J., Weiss, H. J. & Scrutton, M. C. (1975) *Blood* **46**, 131–142
- Holmsen, H., Robkin, L. & Day, H. J. (1979) *Biochem. J.* **182**, 413–419
- Holmsen, H., Dangelmaier, C. A. & Holmsen, H.-K. (1981) *J. Biol. Chem.* **256**, 9393–9396
- Kaplan, K. L., Broekman, M. J., Chernoff, A., Lesznik, G. R. & Drillings, M. (1979) *Blood* **53**, 604–618
- Kaplan, K. L., Drillings, M. & Lesznik, G. (1981) *J. Clin. Invest.* **67**, 1561–1568
- Kattlove, H. (1974) *Am. J. Physiol.* **226**, 325–329
- Kinlough-Rathbone, R. L., Packham, M. A., Reiners, J. P., Cazenave, J. P. G. & Mustard, J. F. (1977) *J. Lab. Clin. Med.* **90**, 707–719
- Lages, B., Scrutton, M. C. & Holmsen, H. (1975) *J. Lab. Clin. Med.* **85**, 811–825
- Lapetina, E. G. & Cuatrecasas, P. (1979) *Biochim. Biophys. Acta* **573**, 394–402
- Lloyd, J. V. & Mustard, J. F. (1974) *Br. J. Haematol.* **26**, 243–253
- Mauco, G., Chap, H. & Douste-Blazy, L. (1979) *FEBS Lett.* **100**, 367–370
- Michell, R. H. (1975) *Biochim. Biophys. Acta* **415**, 81–147
- Mills, D. C. B. (1973) *Nature (London) New Biol.* **243**, 220–222
- Mürer, E. H. (1968) *Biochim. Biophys. Acta* **162**, 320–326
- Mürer, E. H. & Holme, R. (1970) *Biochim. Biophys. Acta* **222**, 197–205
- Mürer, E. H., Hellem, A. J. & Rozenberg, M. C. (1967) *Scand. J. Clin. Lab. Invest.* **19**, 280–282
- Nossel, H. L., Yudelman, J., Canfield, R. E., Butler, V. P., Spanondis, K., Wilbur, G. D. & Quereshi, G. D. (1974) *J. Clin. Invest.* **54**, 43–56
- Putney, J. R. (1981) *Life Sci.* **29**, 1183–1194
- Rittenhouse-Simmons, S. (1979) *J. Clin. Invest.* **63**, 582–587
- Rittenhouse-Simmons, S. & Deykin, D. (1977) *J. Clin. Invest.* **60**, 495–498
- Salmon, D. M. & Honeyman, T. W. (1980) *Nature (London)* **284**, 344–345
- Samuelsson, B. (1977) in *Prostaglandins in Hematology* (Silver, M. J., Smith, J. B. & Koscic, J. J., eds.), pp. 123–139, Spectrum Publishing, New York
- Tyson, C. A., Van de Zande, H. & Green, D. E. (1976) *J. Biol. Chem.* **251**, 1326–1331
- White, J. G. (1971) in *The Circulating Platelet* (Johnson, S. A., ed.), pp. 45–121, Academic Press, New York