Comparative studies on the energetics of platelet responses induced by different agonists

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The correlation between energy consumption and platelet responses induced by collagen, A23187 and ADP was investigated and compared with the energetics of thrombin-stimulated platelets established in earlier work. Aggregation, measured as single-platelet disappearance, and secretion correlated quantitatively with the increment but not with the total consumption of energy, suggesting that the former reflects the energy cost of these responses. The cost of complete aggregation was 2–3 μmol of ATP equivalents/ 10^{11} platelets with collagen, ADP and thrombin as the stimulus. The cost of complete dense-granule secretion was 0.5–0.8 μmol of ATP equivalents/ 10^{11} platelets with all agonists tested. The cost of combined secretion of α -granule and acid hydrolase granule contents was 5–7 μmol of ATP equivalents/ 10^{11} platelets with thrombin and collagen. However, in the presence of A23187 much more energy was consumed during aggregation and secretion. Also ADP triggered more energy consumption during secretion than was seen with the other inducers. The effect of inhibitors of aggregation and secretion was investigated in thrombin-stimulated platelets. Raising the cellular cyclic AMP content sharply decreased the increment in energy consumption as well as aggregation and secretion. The cytoskeleton-disrupting agents cytochalasin B and colchicine left the increment in energy consumption intact, but decreased the basal consumption seen in unstimulated platelets. This was accompanied by normal (cytochalasin B) or diminished (colchicine) aggregation and secretion. Apart from the latter exception, all inhibitors decreased secretion and incremental energy consumption in parallel, thereby preserving the energy-versus-secretion relationship established in earlier work. In contrast, aggregation and energy consumption varied independently, suggesting that the coupling with energy consumption is much weaker for this response.

INTRODUCTION

Platelet aggregation and secretion are accompanied with an increase in ATP-regenerating pathways (Akkerman & Holmsen, 1981; Chaudry *et al.*, 1973; Detwiler, 1972; Fukami *et al.*, 1976), whereas pretreatment with metabolic inhibitors abolishes these responses (Akkerman *et al.*, 1979; Holmsen *et al.*, 1982; Kattlove, 1974; Mürer, 1968). These observations have led to the concept that platelet functions require metabolic energy and have stimulated the search for techniques to measure how much energy is involved in a specific response. We have employed the fall in energy content immediately after complete abolition of ATP regeneration as a means to measure energy consumption in small periods of time (Akkerman *et al.*, 1983; Verhoeven *et al.*, 1984, 1985a,b). It was shown that in platelets stimulated with thrombin each secretion response was accompanied by a specific amount of energy consumption and also that aggregation required energy. Part of the energy was used for phosphorylation of M_r -20000 and 47000 proteins and accumulation of phosphorylated inositides, which appear to be crucial steps in the mechanism of stimulus–response coupling in platelets (Verhoeven *et al.*, 1985b).

In the present study we have investigated how close the coupling between a platelet response and the concurrent consumption of energy is. This has been performed by two approaches: first, by comparing agonists that activate platelets via different mechanisms, resulting in

different aggregation and secretion patterns; second, by employing inhibitors that interfere with the mechanisms that execute aggregation and secretion. The results favour the concept that energy consumption is an intrinsic part of each platelet response and is relatively independent of the mechanisms by which an agonist initiates the responses.

MATERIALS AND METHODS

Platelet isolation

Freshly drawn venous blood was collected from healthy human volunteers into citrate (0.1 vol. of 129 mM-sodium citrate); the donors claimed to have taken no medication known to interfere with platelet function during the preceding 10 days. After centrifugation (200 g, 10 min, room temperature) the supernatant, platelet-rich plasma, was incubated with 1 μM -5-hydroxy-[side chain-2- ^{14}C]tryptamine (sp. radioactivity 58 Ci/mol; Amersham International) and 1 μM -[2- ^3H]adenine (sp. radioactivity 10 Ci/mmol; Amersham International) for 45 min at 37 °C, to label the contents of the dense granules and the metabolic pool of adenine nucleotides respectively. Platelets were then isolated by gel filtration on Sepharose 2B (Pharmacia, column size 2.5 cm $\times$ 15 cm) into a Ca^{2+} -free Tyrode's solution (137 mM-NaCl, 2.68 mM-KCl, 0.42 mM- NaH_2PO_4 , 1.7 mM- MgCl_2 , 11.9 mM- NaHCO_3 , pH 7.25, osmolality

Abbreviations used: ATP_{eq} , ATP equivalents; PGE_1 , prostaglandin E_1 ; Na^+, K^+ -ATPase, ($\text{Na}^+ + \text{K}^+$)-dependent ATPase.

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300 mosmol/kg) containing 0.2% (w/v) gelatin (Merck, Darmstadt, Germany) and no glucose unless otherwise stated (Aharony *et al.*, 1982; Walsh, 1972). Platelet numbers were standardized at $(1.5\text{--}2.5) \times 10^{11}$ cells/litre. The platelet suspension was kept in capped polystyrene tubes at room temperature until the start of the experiments. Batches of gel-filtered platelets were incubated at 37 °C for 3 min in silicone-treated glass cuvettes (1.48 cm diameter) and then stirred (900 rev./min). After 1 min, the platelets were stimulated with thrombin, collagen, the bivalent cationophore A23187 or ADP

Stimulation with collagen and A23187

Energy consumption and secretion responses were measured at various times after stimulation with horse collagen (Hormon-Chemie, München, Germany; 1 mg/ml stock solution, dialysed overnight against 1000 vol. of 5 mM-acetic acid in saline in order to remove glucose) or A23187 (Boehringer, Mannheim, Germany; in ethanol), essentially as described previously (Akkerman *et al.*, 1983; Verhoeven *et al.*, 1984). In short, to one set of gel-filtered platelets the stimulus was added, and at the indicated times samples were withdrawn for the analysis of secretion responses. Another set of gel-filtered platelets was incubated under the same conditions, except that a mixture of antimycin A (Boehringer) and D-gluconic acid-1,5-lactone (gluconolactone; Sigma) was added at final concentrations of 15 μM and 10 mM respectively, either simultaneously with the stimulus, or at 15, 30, 45, 60, 75 and 105 s thereafter. At 5, 10 and 15 s after addition of the inhibitor mixture, samples were withdrawn for the analysis of ^3H -labelled adenine nucleotides and derivatives.

Human fibrinogen (Kabi, Stockholm, Sweden; stock solutions prepared at 20 mg/ml, dialysed for 72 h in gelatin- and glucose-free Tyrode's solution and stored at -70°C) was added at 1 min before the stimulus to a final concentration of 0.5 mg/ml. In the experiments with collagen, gelatin was omitted from the platelet suspension, since it may interfere with collagen-platelet interaction. In the experiments with A23187, MgCl_2 was omitted, since A23187-induced platelet responses are inhibited in the presence of extracellular Mg^{2+} or Ca^{2+} (Holmsen & Dangelmaier, 1981).

Stimulation with ADP

Platelets were suspended in a Tyrode's solution containing 1 mM-glucose, 1 mM-KCN, and 0.2% (w/v) albumin (Organon Technika, Oss, The Netherlands) instead of gelatin. The gel-filtered platelets were stimulated with 100 μM -ADP (Boehringer) in the presence of 0.5 mg of fibrinogen/ml (final concns.). At various times thereafter, samples were withdrawn from the main incubation cuvette and incubated further in another cuvette. To the latter, a mixture of 2-deoxy-D-glucose (Merck) and gluconolactone was added at final concentrations of 30 mM and 10 mM respectively, in order to block ATP regeneration completely, as described previously (Akkerman *et al.*, 1983). At 5, 10 and 20 s thereafter, samples were withdrawn for the analysis of ^3H -labelled adenine nucleotides; at 15 s another sample was collected for the analysis of secretion responses, and at 25 s a sample was collected for the analysis of aggregation.

Stimulation with thrombin

Platelets were stimulated with bovine α -thrombin (Roche, Basel, Switzerland; stock solutions prepared at 1000 NIH units/ml and dialysed for 24 h against 300 vol. of protein- and glucose-free Tyrode's solution and stored at -70°C). Abrupt arrest of ATP regeneration was induced by antimycin A/gluconolactone, added simultaneously with thrombin as described previously (Verhoeven *et al.*, 1984). At 5, 10 and 20 s after addition of thrombin plus inhibitor mixture, samples were withdrawn from the suspension for analysis of ^3H -labelled nucleotides, at 15 s for analysis of secretory responses and at 25 s for analysis of aggregation. For the studies with platelet-function inhibitors, the following agents were used (all final concentrations): 20 μM -indomethacin (Merck), 0.28 mM-phosphoenolpyruvate/3 units of pyruvate kinase/ml (both from Boehringer), 100 μM -cytochalasin B (Sigma), 10 mM-colchicine (Sigma), 10 mM- N^6,O^2' -dibutyrylcyclic AMP (Sigma), 5 mM-theophylline (Sigma)/10 μM -PGE₁ (Cayman Chemical, Denver, CO, U.S.A.), 1 mM-ouabain (Sigma) and 50 μM -quercetin (Sigma). PGE₁ was added 1 min before the stimulus; all other drugs were present in the platelet suspension from the start of the incubation. Stock solutions of indomethacin, cytochalasin B, colchicine, PGE₁ and quercetin were prepared in ethanol; the other drugs were prepared in the gel-filtration buffer. The drugs were tested at final concentrations that were maximally effective in suspensions of washed platelets (Dayal *et al.*, 1983; Fox & Phillips, 1981; Harfenist *et al.*, 1981; Haslam, 1964; Kinlough-Rathbone *et al.*, 1977; Okuda & Nemerson, 1971; Ribbi-Jaffe & Apitz-Castro, 1979; Rittenhouse-Simons, 1980). The Na^+, K^+ -ATPase inhibitor quercetin has not been used with platelets previously; the final concentration used here was maximally effective in intact tumour cells (Suollina *et al.*, 1975). In all experiments the final ethanol concentration never exceeded 0.4% (v/v).

Assessment of energy consumption

Energy consumption is defined here as the loss of energy stored in metabolic ATP and ADP after abrupt arrest of ATP regeneration. It was measured by two techniques: first, by adding antimycin A/gluconolactone to platelets suspended in albumin-free and glucose-free medium (Verhoeven *et al.*, 1984); second, by adding 2-deoxyglucose/gluconolactone to platelets suspended in CN^- (1 mM)-, and glucose (1 mM)-containing medium (Akkerman *et al.*, 1983). Both techniques are to some extent supplementary, but give similar data for the increase in energy consumption seen in stimulated platelets. For the determination of metabolic ATP and ADP, samples of gel-filtered platelets were collected into 2 vol. of EDTA/ethanol (10 mM-EDTA in 86% ethanol, pH 7.4, 0°C). After centrifugation (10000 g, 2 min, 4°C), the supernatants were analysed for ^3H -labelled ATP, ADP, AMP, IMP and hypoxanthine/inosine after separation by high-voltage paper electrophoresis (Holmsen *et al.*, 1972). As outlined previously (Daniel *et al.*, 1979a, 1980), this technique is disturbed by neither actin-bound ADP nor dense-granule ATP and ADP, since during the short incubation times used in this study there is no significant exchange of adenine nucleotides between the metabolic and the granule pool. On the basis of a metabolic ATP content of 4.5 $\mu\text{mol}/10^{11}$ platelets and the fact that in normal [^3H]adenine-labelled platelets

Fig. 1. Comparison between aggregation and secretion and concurrent energy consumption in collagen-stimulated platelets

[³H]Adenine- and 5-hydroxy[¹⁴C]tryptamine-labelled platelets were stimulated with 8 μ g of collagen/ml (final concn.). At various times samples were collected for analysis of single-platelet disappearance (■) and secretion of 5-hydroxy[¹⁴C]tryptamine (○) and *N*-acetyl- β -D-glucosaminidase (Δ) (a). Also shown are the number of single platelets at 5 s before addition of the inducer (□). Results for secretion of β -thromboglobulin (not shown) were always intermediate between those of 5-hydroxy[¹⁴C]tryptamine and the acid hydrolase. Concurrently, energy consumption, expressed as μ mol of ATP_{eq.}/min per 10¹¹ platelets, was measured (b) in both stimulated (●) and unstimulated (□) suspensions. Data are expressed as means $\pm$ s.d. ($n = 4$). Between 15 and 90 s after stimulation, energy consumption was significantly higher ($P < 0.01$) than in unstimulated cells.

80% of the total radioactivity is found in ATP, 1% of total ³H radioactivity corresponds to 0.056 μ mol of nucleotide/10¹¹ platelets (Akkerman *et al.*, 1983). The energy stored in metabolic ATP and ADP was then expressed in terms of ATP equivalents (ATP_{eq.}; Atkinson, 1977), which reflect the energy liberated in the conversion of ATP into ADP. Rates of energy consumption were derived from the decline in the number

of ATP_{eq.} during the initial 15 or 20 s interval after addition of the inhibitor mixture by linear regression analysis, expressed as (Δ ATP_{eq.}/ Δt) and plotted at the midpoint of the corresponding interval (Verhoeven *et al.*, 1984).}

Analysis of functional responses

Aggregation was measured as single-platelet disappearance, as described elsewhere (Verhoeven *et al.*, 1984). Briefly, samples of cell suspension were collected into 9 vol. of 0.5% (v/v) glutaraldehyde (Fluka, Buchs, Switzerland) in saline (0 °C). The number of platelets was determined in a Platelet Analyzer 810 (Baker Instruments, Allentown, PA, U.S.A.) with apertures set at 3.2 and 16 μ m³. Single-platelet disappearance was expressed as a percentage of the platelet number found within these settings in the unstimulated suspension. The technique was not disturbed by adhesion of platelets to collagen

Fig. 2. Comparison between aggregation and secretion and concurrent energy consumption in A23187-stimulated platelets

Experiments were performed as described for Fig. 1, except that 4.5 μ M-A23187 (final concn.) was used as the stimulus. Symbols are as defined for Fig. 1. Between 0 and 90 s after stimulation, energy consumption was significantly higher ($P < 0.001$) than in unstimulated cells.

fibres, since EDTA, which is known to inhibit aggregation but leaves adhesion unchanged, completely prevented single-platelet disappearance.

Secretion from dense granules, α -granules and acid-hydrolase-containing granules was monitored by the extracellular appearance of 5-hydroxy[14 C]tryptamine, which is > 99% accumulated in the dense granules, β -thromboglobulin and *N*-acetyl- β -D-glucosaminidase (EC 3.2.1.30) respectively, in accordance with previous studies (Akkerman *et al.*, 1983; Verhoeven *et al.*, 1984). Samples of cell suspension were collected into 0.15 vol. of 1.035 M-formaldehyde in saline (0 °C) and centrifuged (10000 g, 1 min, 4 °C). The supernatants were analysed for 5-hydroxy[14 C]tryptamine (counted for radioactivity by standard procedures), β -thromboglobulin (by using the radioimmunoassay kit from Amersham International) and enzymic activity of the acid hydrolase (Troost *et al.*, 1976). All incubations were performed in the presence of 3 μ M-imipramine (Geigy, Basel, Switzerland) to prevent re-uptake of secreted 5-hydroxytryptamine by the platelets (Walsh & Gagnatelli, 1974). Secretion was expressed as a percentage of the maximal secretable amount, the latter being the amount secreted by the gel-filtered platelets after 5 min incubation with 5 units of thrombin/ml in the absence of inhibitors (Akkerman *et al.*, 1983).

Miscellaneous

In all experiments, cell lysis (based on lactate dehydrogenase activity) was less than 2.5% and not affected by the metabolic and functional inhibitors. All data were expressed as means $\pm$ s.d.; statistical significances were determined by Student's *t* test.

RESULTS

Energetics of collagen- and A23187-induced platelet responses

As illustrated in Fig. 1, collagen (8 μ g/ml) induced a decrease in the number of single platelets, which started after about 10 s and was complete after about 1.5 min. Secretion from dense granules, α -granules (not shown) and acid-hydrolase-containing granules also started after a 10 s delay and reached an extent of 50, 45 and 40% of maximal secretable amounts respectively. Concurrent energy consumption increased in parallel with these responses. During the first 15 s the rate of energy consumption was hardly affected, but thereafter it increased to a maximum of about 12 μ mol of ATP_{eq.}/min per 10¹¹ platelets, which was 6 μ mol/min per 10¹¹ platelets higher than before collagen addition. Thereafter energy consumption gradually decreased again, and

Fig. 3. Comparison between the increment in energy consumption and extent of aggregation (a) and secretion (b and c) induced by collagen and A23187

The amount of energy, expressed as μ mol of ATP_{eq.}/10¹¹ platelets, that was consumed from the moment of stimulation with different doses of collagen (open symbols) or A23187 (closed symbols) was calculated as $\int_0^t (\Delta \text{ATP}_{\text{eq.}}/\Delta t)_{\text{total}} dt$ from $(\Delta \text{ATP}_{\text{eq.}}/\Delta t)$

measured over each 15 s interval. The incremental energy consumption was obtained by subtracting $\int_0^t (\Delta \text{ATP}_{\text{eq.}}/\Delta t)_{\text{resting}} dt$ measured simultaneously in an unstimulated suspension, and plotted versus the corresponding extents of single-platelet disappearance (a) and 5-hydroxy[14 C]tryptamine (b) and acid hydrolase (c) secretion. Shown are the data for suspensions stimulated with 50 (○), 12.5 (□), 8 (△) and 4 (▽) μ g of collagen/ml, and 9 μ M- (●) and 4.5 μ M- (■) A23187 (all final concns.). Similar results were obtained for β -thromboglobulin secretion (not shown). The correlation between incremental energy consumption and secretion was tested by linear regression analysis; when the extent of secretion was less than 5% of the maximal secretable amount, the data were excluded. The results were: for 5-hydroxy[14 C]tryptamine secretion, $y = -14.6 + 17.7x$ ($r = 0.921$, $n = 15$, $P < 0.001$) and $y = -21.8 + 8.3x$ ($r = 0.933$, $n = 8$, $P < 0.001$) for collagen- and A23187-stimulated platelets respectively. For acid hydrolase secretion, the respective values were: $y = -21.3 + 14.1x$ ($r = 0.943$, $n = 15$, $P < 0.001$) and $y = -8.5 + 3.1x$ ($r = 0.955$, $n = 7$, $P < 0.001$). The broken lines represent the energy-versus-aggregation and energy-versus-secretion relationships that were previously established for thrombin-stimulated platelets (Verhoeven *et al.*, 1984).

Fig. 4. Comparison between aggregation and secretion and concurrent energy consumption in ADP-stimulated platelets

[³H]Adenine- and 5-hydroxy[¹⁴C]tryptamine-labelled platelets were stimulated with 100 μM-ADP (final concn.), and samples were collected at the indicated times for the analysis of functional responses (a) and concurrent energy consumption (b). In (a), the values for single-platelet disappearance (■) and secretion of 5-hydroxy[¹⁴C]tryptamine (○) and β-thromboglobulin (▲) are shown. The number of single platelets present in the suspension at 5 s before stimulation is also shown (□). (b) Concurrently, energy consumption, expressed as μmol of ATP_{eq.}/min per 10¹¹ platelets, was measured in both stimulated (●) and unstimulated (□) suspensions. Data are expressed as means ± S.D. (*n* = 3). During the first 1 min and between 4 and 7 min after stimulation, energy consumption was significantly higher (*P* < 0.05) than in unstimulated cells.

normalized after about 2 min, when the responses were completed.

The bivalent cationophore A23187 (4.5 μM) induced an immediate disappearance of single platelets, which resulted in a complete aggregation after 1.5 min (Fig. 2). There was a delay of about 15 s before the start of secretion of 5-hydroxy[¹⁴C]tryptamine, β-thromboglobulin (not shown) and *N*-acetyl-β-D-glucosaminidase, which reached 60, 50 and 30% of maximal secretable amounts after 2 min respectively. The increase in energy

consumption was immediate and amounted to 7 μmol of ATP_{eq.}/min per 10¹¹ platelets in the first 15 s interval, where only aggregation occurred. The initiation of secretion did not further increase the rate of energy consumption, but was accompanied with a gradual decline to control values. At 2 min after stimulation, when the responses were complete, energy consumption had normalized.

A more detailed comparison between aggregation and concurrent energy consumption could be made by stimulating the cells to different extents with different amounts of collagen or A23187. At various stages after stimulation, the extent of aggregation correlated with the increment in energy consumption (Fig. 3a), but not with the total amount of energy consumed in the same period (results not shown). The curvilinear relationship for collagen-induced aggregation was close to the relationship found previously in thrombin-stimulated cells (Verhoeven *et al.*, 1984), whereas A23187-induced aggregation was accompanied by much more energy consumption.

A comparison between secretion and the increment in energy consumption revealed linear relationships (Fig. 3b), as found previously in thrombin-stimulated cells (Verhoeven *et al.*, 1984). Before secretion of 5-hydroxy[¹⁴C]tryptamine, β-thromboglobulin (results not shown) and acid hydrolase became apparent, energy consumption was already higher than in unstimulated cells. This threshold energy amounted to about 1 and 2.5 μmol of ATP_{eq.}/10¹¹ platelets with collagen and A23187 respectively. As with aggregation, the relationships for secretion in collagen-stimulated cells were in the same range as in thrombin-stimulated cells, whereas ionophore-induced secretion was accompanied by a much higher energy consumption.

Energetics of ADP-induced platelet responses

Fig. 4 shows a comparison between platelet function and concurrent energy consumption after stimulation with ADP. Immediately after addition of ADP, aggregation started and was more than 90% complete within 1.5 min. At the end of the aggregation response, secretion of dense-granule and α-granule contents started, which reached about 40% of maximal secretable amounts. These responses were accompanied by a biphasic change in energy consumption. There was a rapid increase of about 2.5 μmol of ATP_{eq.}/min per 10¹¹ platelets in the first 15 s, which subsequently levelled off to control values. Thereafter a second increase followed, which was slower but lasted longer than the first peak of energy consumption. The first increase in energy consumption coincided with aggregation, whereas the second peak was in the period in which most of the secretion took place. When these functions were completed, energy consumption stabilized in the range found in unstimulated cells.

Energy costs of separate responses

When the increase in energy consumption was compared with the responses induced by collagen, A23187 and ADP, the following data could be calculated (Table 1). First, the energy cost of aggregation (measured as single-platelet disappearance) was 2–3 μmol of ATP_{eq.}/10¹¹ platelets after stimulation with collagen, thrombin and ADP, but almost twice as high with A23187. Second, the increase in energy consumption that accompanied dense-granule secretion ranged narrowly

Table 1. Energy costs for separate responses induced by different agonists

The data, expressed as μmol of $\text{ATP}_{\text{eq.}}/10^{11}$ platelets, were calculated from periods where energy consumption could be related to a single response. The energy consumed during 1% response (energy quotient; Akkerman *et al.*, 1983) was then multiplied by 100, assuming a constant energy requirement during the complete response. Hence, data on α -granule plus acid hydrolase secretion were derived from periods where aggregation and dense-granule secretion were completed. The cost of dense-granule secretion could then be calculated by correcting for simultaneous α -granule and acid hydrolase secretion. Similarly, the cost of aggregation could be calculated for collagen- and A23187-activated platelets, where aggregation and secretion started simultaneously. The data for ADP- and thrombin-induced aggregation were derived from periods where no secretion occurred. (The data for thrombin-stimulated platelets were taken from Verhoeven *et al.*, 1984, 1985b.)

Agonist	Energy cost of 100%			
	Single-platelet disappearance	Dense-granule secretion	α -Granule secretion	α -Granule plus acid hydrolase secretion
Collagen	2.0	0.8	—	6.9
Thrombin	2.8	0.7	—	5.3
A23187	4.9	0.6	—	32.3
ADP	2.8	0.5	11.2	—

Table 2. Effect of inhibitors on energy consumption and thrombin-induced responses

[^3H]Adenine- and 5-hydroxy[^{14}C]tryptamine-labelled platelets were preincubated with the inhibitors as indicated in the Materials and methods section. Energy consumption was measured in unstimulated platelets, and during the initial 20 s after stimulation with 5 units of thrombin/ml. The increment in energy consumption was derived from the total energy consumption by subtracting the energy consumption in unstimulated suspensions ($6.5 \pm 1.0 \mu\text{mol}$ of $\text{ATP}_{\text{eq.}}/\text{min}$ per 10^{11} platelets). Secretion and single-platelet disappearance were measured at 15 and 25 s after stimulation respectively. The data obtained in the presence of inhibitors were expressed as a percentage of the corresponding data from a simultaneously run control in the absence of inhibitors, and expressed as means $\pm$ S.D. ($n = 4$): *significant ($P < 0.05$) difference from control.

Additions	Unstimulated platelets	Thrombin (5 units/ml)-stimulated platelets		
	Energy consumption	Energy consumption	Single-platelet disappearance	5-Hydroxy[^{14}C]tryptamine secretion
None	100	100	100	100
Indomethacin	105 ± 17	91 ± 12	103 ± 11	99 ± 2
Phosphoenolpyruvate/ pyruvate kinase	97 ± 11	93 ± 10	94 ± 10	97 ± 3
Dibutyl cyclic AMP	110 ± 11	$46 \pm 13^*$	0*	$14 \pm 1^*$
Theophylline + PGE_1	$81 \pm 11^*$	$34 \pm 14^*$	$3 \pm 3^*$	$12 \pm 1^*$
Cytochalasin B	$45 \pm 8^*$	112 ± 17	85 ± 21	106 ± 7
Colchicine	$75 \pm 8^*$	101 ± 17	$37 \pm 20^*$	$67 \pm 4^*$
Ouabain	98 ± 16	103 ± 13	97 ± 14	95 ± 6
Quercetin	88 ± 15	100 ± 17	$43 \pm 21^*$	93 ± 8

between 0.5 and 0.8 μmol of $\text{ATP}_{\text{eq.}}/10^{11}$ platelets and appeared independent of the type of agonist. Third, the energy cost of combined secretion from α -granules and acid-hydrolase-containing granules, which in our experiments almost overlapped, was similar in collagen- and thrombin-treated platelets (5–7 μmol of $\text{ATP}_{\text{eq.}}/10^{11}$ platelets). In contrast, this value was about 5-fold higher with A23187, whereas with ADP this value was twice as high for separate α -granule secretion.

Effects of inhibitors on the energetics of thrombin-induced responses

In the absence of inhibitors the platelets consumed $6.5 \pm 1.0 \mu\text{mol}$ of $\text{ATP}_{\text{eq.}}/\text{min}$ per 10^{11} platelets (mean $\pm$ S.D., $n = 6$). During the first 20 s after stimulation

with 5 units of thrombin/ml the consumption increased by 8.4 ± 1.9 to $14.9 \pm 1.5 \mu\text{mol}/\text{min}$ per 10^{11} platelets. Under these conditions single-platelet disappearance (measured at 25 s after stimulation) and 5-hydroxy-[^{14}C]tryptamine secretion (measured after 15 s) were $66 \pm 12\%$ of the initial number of single platelets and $84 \pm 4\%$ of the maximal secretable amount respectively. A low dose of thrombin (0.1 unit/ml) increased the energy consumption by $4.0 \pm 0.6 \mu\text{mol}/\text{min}$ per 10^{11} platelets, which was now accompanied with $50 \pm 18\%$ single-platelet disappearance and $12 \pm 9\%$ 5-hydroxy-[^{14}C]tryptamine secretion. Previous studies have shown that thrombin-induced single-platelet disappearance varies with energy consumption according to a curvilinear pattern, whereas the secretion-versus-energy relationship

Fig. 5. Effect of functional inhibitors on the energy-versus-response relationships in thrombin-stimulated platelets

[³H]Adenine- and 5-hydroxy[¹⁴C]tryptamine-labelled platelets were stimulated with 0.1 (+ and open symbols) or 5 (× and closed symbols) units of thrombin/ml. For the determination of energy consumption during the first 20 s after stimulation, a mixture of antimycin A and gluconolactone was added simultaneously with thrombin, and samples were collected at 5, 10 and 20 s. Single-platelet disappearance was determined at 25 s and 5-hydroxy[¹⁴C]tryptamine secretion was determined at 15 s. Similar studies were performed with platelet suspensions that were preincubated for 4 min at 37 °C with 10 mM-dibutyl cyclic AMP (Δ, ▲), 5 mM-theophylline/10 μM-PGE₁ (▽, ▼; PGE₁ added 3 min after theophylline), 10 mM-colchicine (□, ■), 100 μM-cytochalasin B (+, ×) or 50 μM-quercetin (◇, ◆). Incremental energy consumption, expressed as μmol of ATP_{eq.}/min per 10¹¹ platelets, was calculated from (ΔATP_{eq.}/Δt)_{total} by subtracting (ΔATP_{eq.}/Δt)_{resting}, measured simultaneously in an unstimulated suspension, and plotted versus the corresponding values of single-platelet disappearance (a) and 5-hydroxy[¹⁴C]tryptamine secretion (b). The data for control platelets are expressed as means ± S.D. for six different platelet suspensions; the other data are means ± S.D. (n = 4).

is linear above a threshold of about 3 μmol of ATP_{eq.}/min per 10¹¹ platelets (Verhoeven *et al.*, 1984). We studied the effect of several functional inhibitors on these characteristics by first comparing their effect on energy consumption and responses induced by 5 units of thrombin/ml. When these parameters were affected by the inhibitors, the studies were repeated with 0.1 unit of thrombin/ml in order to clarify whether their effect was primarily on the slope or on the intercept of the energy-versus-response relationship.

The presence of indomethacin, an inhibitor of endoperoxide-thromboxane A₂ formation, affected neither energy consumption nor aggregation and secretion induced by 5 units of thrombin/ml (Table 2). A similar pattern was found in the presence of the ADP scavenger phosphoenolpyruvate/pyruvate kinase. Dibutyl cyclic AMP and a mixture of theophylline and prostaglandin E₁ (PGE₁), which are known to raise the cell's cyclic AMP content, completely prevented aggregation, whereas dense-granule secretion was decreased by more than 85%. This was accompanied by a 60–70% decrease in the increment of energy consumption. A complicating factor was that theophylline + PGE₁ slightly decreased the energy consumption in unstimulated cells, an effect not observed with dibutyl cyclic AMP. The effect of the cytoskeleton-disrupting agents colchicine and cytochalasin B was more complex. Colchicine inhibited energy consumption in resting cells by about 25% and cytochalasin by even more than 50%. The increase in energy consumption, however, was normal. With cytochalasin B this was accompanied by normal aggregation and secretion, but colchicine induced a moderate (secretion) to severe (aggregation) inhibition of platelet function. The

Na⁺,K⁺-ATPase inhibitors ouabain and quercetin affected neither energy consumption nor secretion. However, aggregation was inhibited by quercetin, but was normal with ouabain.

The inhibitors that showed a marked effect on energetic or functional parameters induced by the high dose of thrombin were also studied in suspensions stimulated with 0.1 unit of thrombin/ml. Essentially the same effects were found. With dibutyl cyclic AMP and theophylline + PGE₁, aggregation and secretion were completely abolished, as was the increase in energy consumption. With cytochalasin B, the increase in energy consumption and aggregation and secretion were in the normal range. With colchicine the three parameters were decreased in parallel, whereas with quercetin energy consumption and secretion were normal but aggregation was impaired. The effect of these agents at the high and the low dose of thrombin is illustrated in Fig. 5. Single-platelet disappearance and the increment in energy consumption varied independently. In contrast, the secretion-versus-energy relationship was maintained despite strong inhibition by some of the agents. An exception was colchicine, which inhibited secretion more strongly than incremental energy consumption.

DISCUSSION

The results of this study illustrate that in general the energetic properties of aggregation and secretion established previously in thrombin-stimulated platelets also hold with other agonists. Aggregation and secretion are accompanied by an increase in concurrent energy consumption, and a close correlation is found when only

the increment in energy consumption is considered. A closer study reveals a number of remarkable similarities as well as a few notable differences. The increment in energy consumption that accompanies aggregation by thrombin where it fully overlaps with secretion of dense-granule, α -granule and acid-hydrolase-granule contents is similar to the increment found in collagen- and ADP-stimulated cells, where aggregation precedes a weak and incomplete secretion response. This increment of 2–3 μmol of $\text{ATP}_{\text{eq.}}/10^{11}$ platelets appears to reflect the metabolic price for aggregation. Even more striking is the similarity between the energy costs for complete dense-granule secretion initiated by thrombin (Verhoeven *et al.*, 1984), collagen, A23187 and ADP, which range between 0.5 and 0.8 μmol of $\text{ATP}_{\text{eq.}}/10^{11}$ cells. It is obvious that the mechanisms by which these agonists initiated platelet function vary considerably. Compared with thrombin, collagen adds an adhesion phase which partially requires energy (Mant, 1980; Capobianca *et al.*, 1981) and triggers slower responses. A23187 by-passes several receptor-mediated energy-dependent steps such as the (poly)phosphatidylinositol response (Rittenhouse, 1982), whereas ADP is known for its weak effects on platelets, requiring close cell–cell contact for the induction of secretion responses (Smith *et al.*, 1977). These data may therefore indicate that most of the energy consumed during aggregation and dense-granule secretion is used in a step in stimulus–response coupling that these agonists have in common and which is relatively close to the mechanisms that execute these responses. To some extent the same reasoning holds for the combined secretion from α -granules and acid hydrolase granules, for which the metabolic price varies between 5 (with thrombin) and 7 (with collagen) μmol of $\text{ATP}_{\text{eq.}}/10^{11}$ cells. It should be noted that this number reflects the increment in energy consumption accompanying complete secretion, which is seldom found when collagen is the agonist. The actual energy consumption during collagen-induced secretion is proportionally lower.

Notable differences from the energetics of thrombin-induced responses are the energy consumption during A23187-induced responses and that during the later stages of α -granule secretion induced by ADP. A23187 increases the permeability of membranes for bivalent cations (Reed & Lardy, 1972) and is likely to disturb Ca^{2+} - and Mg^{2+} -dependent ATPases, thereby affecting energy consumption. One possibility is that the ionophore disturbs the Ca^{2+} gradient across the mitochondrial inner membrane, thereby affecting proton translocation and the mitochondrial proton-translocating ATPase, resulting in an increase in ATP hydrolysis. Control studies with oligomycin A (3 μM final concn.), which disturbs this step by interfering with the utilization of the proton gradient, showed a $34 \pm 8\%$ ($n = 4$) decrease in ATP hydrolysis during the first 30 s after A23187 addition, suggesting that this mechanism may indeed be responsible for part of the unexpected increase in ATP consumption induced by the ionophore. ADP, however, is a much more physiological platelet activator, and the general opinion that here dense-granule and α -granule secretion are not accompanied by acid hydrolase secretion (Holmsen *et al.*, 1977) makes it an ideal tool with which to separate the energy costs of these secretion responses. However, much more energy is consumed during ADP-induced α -granule secretion than in similar conditions with thrombin, where α -granule secretion is accompanied by acid hydrolase

secretion. Therefore one has to postulate an extra energy-consuming process. Preliminary studies show that, contrary to current belief, ADP-induced dense-granule and α -granule secretion is accompanied by a slight acid hydrolase secretion (about 10%). But even when this extra secretion is taken into account, more energy is consumed than expected.

An alternative means to evaluate how tight aggregation and secretion are coupled to the increment in energy consumption is the use of functional inhibitors that leave energy-producing sequences unaltered. Indomethacin, an inhibitor of formation of prostaglandin endoperoxides and thromboxane A_2 , and the ADP scavenger system phosphoenolpyruvate/pyruvate kinase affected neither the energy consumption nor aggregation and dense-granule secretion in platelets stimulated with a high dose of thrombin (5 units/ml). Under these conditions platelet function is known to be independent of prostanoid synthesis or released ADP (Kinlough-Rathbone *et al.*, 1977), but the fact that the energy requirement remained unchanged may indicate that none of these so-called positive-feedback loops in the mechanism of platelet activation include steps that require considerable amounts of energy. At a low dose of thrombin (0.1 unit/ml), both inhibitors slightly decreased the functions as well as energy consumption, again demonstrating close coupling between these factors. In favour of close coupling were also the effects of dibutyryl cyclic AMP and theophylline + PGE_1 , which are known to raise the cell's cyclic AMP content, and which inhibited the increment in energy consumption as well as aggregation and secretion. An unexpected observation was that PGE_1 decreased the basal energy consumption by about 20%. This fact is puzzling, since protein kinases are activated by cyclic AMP and an increase in energy consumption can therefore be expected (Salama & Haslam, 1984). The cytoskeleton-disrupting agents also decreased the basal energy consumption, by 25% for colchicine and by about 50% for cytochalasin B. The latter compound prevents actin-treadmilling (Fox & Phillips, 1981), and the resulting decrease in basal energy consumption suggests that this process accounts for about 50% of total energy consumption in resting platelets. A slightly lower contribution (30–40%) was calculated by Daniel *et al.* (1979b) from the nucleotide-exchange rate in F-actin. Despite the decrease in basal energy consumption, both cytoskeleton-disrupting agents left the increment seen during aggregation and secretion intact. One would therefore expect normal functions. This was indeed found with cytochalasin B, but colchicine greatly decreased aggregation and secretion. So far this is the first condition where the coupling between platelet secretion and the increment in energy consumption is disturbed. The Na^+, K^+ -ATPase inhibitors ouabain and quercetin did not change the energetic data. In rabbit reticulocytes the Na^+, K^+ -ATPase is known to account for 23% of total energy consumption (Siems *et al.*, 1984). On the basis of cell volume, however, total energy consumption in these cells is only 25% of that in unstimulated platelets. If the Na^+, K^+ -ATPase was as active in platelets as it is in reticulocytes, inhibition of this process would lead to a decrease of basal energy consumption by only 0.4 μmol of $\text{ATP}_{\text{eq.}}/\text{min}$ per 10^{11} platelets; this would not have been detected in the present study. The only effect of the Na^+, K^+ -ATPase inhibitors was the inhibition of aggregation by quercetin.

These findings and similar studies with a low dose of thrombin indicate that all functional inhibitors except colchicine leave the relationship between the increment in energy consumption and dense-granule secretion intact. Preliminary studies on acid hydrolase secretion lead to the same conclusion. This emphasizes the strong degree of coupling between secretion and energy consumption. No such coupling is found with aggregation, which may be explained by assuming that the requirement for energy differs at the different stages of this response. Aggregation depends on exposure of fibrinogen-binding sites and on collisions between cells (Peerschke *et al.*, 1980; Harfenist *et al.*, 1981). The energetics of each of these processes must be assessed before the role of metabolic energy in aggregation can be understood.

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