The energetics of early platelet responses

Energy consumption during shape change and aggregation with special reference to protein phosphorylation and the polyphosphoinositide cycle

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Among the different platelet responses, secretion requires the greatest amount of metabolic energy. The velocities of dense, α - and acid hydrolase granule secretion vary in parallel with the increase in energy consumption seen in thrombin-stimulated cells. This covariance is preceded by a phase in which energy consumption is increased without the extracellular appearance of secretion markers. By treating the platelets with thrombin and hirudin we have stimulated the platelets for short intervals and succeeded in separating shape change, single platelet disappearance and secretion to a great extent. In this report we show that the early increase in energy consumption reflects the energy requirement of aggregation but not of shape change. The cost of 100% of single platelet disappearance is $2.8 \mu\text{mol}$ of $\text{ATP}_{\text{eq}} \cdot (10^{11} \text{ platelets})^{-1}$. Concurrent analysis of phosphorylation of $M_r 20\,000$ and $47\,000$ proteins and of ^{32}P -labelled phosphatidylinositol metabolites led to the following observations. Firstly, shape change is neither accompanied by an increase in protein phosphorylation nor by changes in the steady state levels of ^{32}P -labelled phosphatidylinositol metabolites. Secondly, when aggregation occurs both proteins are phosphorylated, but the phosphatidylinositol metabolites do not change. Thirdly, when secretion follows, more phosphorylation of the $M_r 47\,000$ protein occurs and initially only phosphatidic acid accumulates. At a later stage of the secretion responses, more protein phosphorylation and phosphatidic acid accumulation become evident, and are now accompanied by alterations in the steady state levels of ^{32}P -labelled (poly)phosphoinositides. Hence, the early increase in energy consumption coincides with protein phosphorylation and, at a later stage, with alterations in (poly)phosphoinositides metabolites. This demonstrates that metabolic energy is directly involved in stimulus-response coupling in aggregating platelets.

The requirement of platelet responses for metabolic energy is illustrated by the acceleration of ATP-regenerating sequences during cell activation and the abolishment of the responses by metabolic inhibitors (McElroy *et al.*, 1971; Chaudry *et al.*, 1973; Fukami *et al.*, 1976; Akkerman & Holmsen, 1981; Mürer *et al.*, 1967; Holmsen *et al.*, 1974, 1982; Akkerman *et al.*, 1979). The sensitivity for

metabolic blockade increases in the order: shape change, aggregation and dense, α - and acid hydrolase granule secretion, suggesting that different responses require different amounts of energy (Akkerman *et al.*, 1979; Holmsen *et al.*, 1982). Indeed, less metabolic ATP is hydrolysed during dense granule secretion than during the combined secretion from α - and acid hydrolase granules (Akkerman *et al.*, 1983; Verhoeven *et al.*, 1984a). Aggregation requires little ATP hydrolysis, but formation and maintenance of aggregates is completely abolished once ATP hydrolysis is prevented (Verhoeven *et al.*, 1984b).

Abbreviations used: PtdIns, phosphatidylinositol; PtdIns(4)P, phosphatidylinositol 4-phosphate; PtdIns(4,5)P₂, phosphatidylinositol 4,5-bisphosphate; PtdA, phosphatidic acid.

More detailed analysis of the energy requirement of secretion by platelets reveals that each type of secretion response shows a quantitative covariance with the extra energy consumed in stimulated platelets, but not with the energy consumption seen before and after execution of these functions (Verhoeven *et al.*, 1984a). Hence, the cells provide extra energy for the various processes that connect receptor occupancy with shape change, aggregation and extrusion of granule contents. Among these are various energy-requiring steps such as polyphosphoinositide metabolism (Lloyd & Mustard, 1974; Vickers *et al.*, 1984), protein phosphorylation (Daniel *et al.*, 1981; Imaoka *et al.*, 1983; Carroll & Gerrard, 1982) and actomyosin-contractile activity (Daniel *et al.*, 1981; Lebowitz & Cooke, 1978). The present report addresses the question as to the importance of metabolic energy in the early processes that precede the extracellular appearance of granule contents. The study makes use of the fact that thrombin-induced secretion requires an intact agonist-receptor complex and that thrombin inactivation by excess of hirudin interferes with the extrusion of granule contents (Detwiler & Feinman, 1973; Holmsen *et al.*, 1981). It will be shown that transient receptor occupancy initiates an increase in energy consumption which is paralleled by alterations in (poly)PtdIns metabolites and protein phosphorylation, but not by extrusion of granule contents. This increment in energy consumption reflects the energy cost of aggregation, whereas the shape-change reaction appears to be independent of the extra energy consumed in stimulated platelets.

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). After centrifugation (200g, 10 min, room temperature) the supernatant, platelet-rich plasma, was incubated with $1\text{ }\mu\text{M}$ -5-hydroxy[side chain-2- ^{14}C]tryptamine ($[^{14}\text{C}]$ serotonin, sp. radioactivity $58\text{ Ci}\cdot\text{mol}^{-1}$; Amersham International) and $1\text{ }\mu\text{M}$ -[2- ^3H]adenine (sp. radioactivity $10\text{ Ci}\cdot\text{mmol}^{-1}$; Amersham International) for 45 min at 37°C to label the contents of the dense granules and the metabolic pool of adenine nucleotides, respectively. In another series of experiments the platelets were labelled with 0.1 mCi of carrier-free $[^{32}\text{P}]$ orthophosphate (New England Nuclear)/ml of platelet-rich plasma (120 min). Platelets were then gel-filtered at room temperature on Sepharose 2B (Pharmacia; column size $2.5\text{ cm} \times 15\text{ cm}$) into Ca^{2+} -free and glucose-free Tyrode's solution (pH 7.25, osmolality 300 mos-

$\text{mol}\cdot\text{kg}^{-1}$) as described by Walsh (1972), except that albumin was replaced by gelatin (Merck, Darmstadt, Germany; Aharony *et al.*, 1982). Platelet numbers were standardized at $(1.5\text{--}2.5) \times 10^{11}\text{ cells}\cdot\text{l}^{-1}$ by dilution in gel-filtration buffer. The platelet suspension was kept in capped polystyrene tubes at room temperature until the start of the experiments. All experiments were completed within 1 h after elution of the platelets from the column.

Incubation conditions

The gel-filtered platelets were incubated at 37°C and stirred at 900 rev./min . Secretion responses were initiated with different doses of bovine α -thrombin (La Roche, Basel, Switzerland; stock solutions prepared of $1000\text{ NIH units}\cdot\text{ml}^{-1}$ and dialysed for 24 h against 300 vol. of gelatin- and glucose-free Tyrode's solution). The thrombin was rapidly neutralized with an excess of hirudin (Pentapharm, Basel, Switzerland; stock solutions prepared at $1000\text{ AT-units}\cdot\text{ml}^{-1}$, and dialysed as described for thrombin), by either one of two procedures. In the first set of experiments, platelets were stimulated with thrombin ($0.2\text{ unit}\cdot\text{ml}^{-1}$) and at various times thereafter thrombin was neutralized with a 50-fold excess of hirudin. In the second series of experiments, platelets were preincubated with 4 units of hirudin $\cdot\text{ml}^{-1}$ for 1 min, and then thrombin was added at final concentrations ranging between 0.2 and $1.0\text{ unit}\cdot\text{ml}^{-1}$. At different times after thrombin addition, samples were collected for analysis of functional and biochemical responses (see below).

A set of parallel incubations were used for the determination of energy consumption at different stages after stimulation. Platelets were incubated under the same conditions as described above. At the times indicated, ATP regeneration was abruptly blocked by the addition of a mixture of D-gluconic acid-1,5-lactone (gluconolactone; Sigma) and antimycin A (Boehringer Mannheim, Germany) at final concentrations of 10 mM and $15\text{ }\mu\text{M}$, respectively, as described previously (Verhoeven *et al.*, 1984a). At 5, 10 and 15 s thereafter, samples were collected for the analysis of metabolic adenine nucleotides. Throughout the incubation cell lysis was minimal ($2.5 \pm 0.5\%$, $n = 5$) based on the extracellular appearance of lactate dehydrogenase and was the same in the presence and absence of thrombin, hirudin and the metabolic inhibitors.

Assessment of energy consumption

Energy consumption was measured by analysis of the fall in metabolic ATP and ADP immediately following complete arrest of ATP regeneration, as described in detail elsewhere (Akkerman *et al.*,

1983; Verhoeven *et al.*, 1984a). In short, samples of cell suspension were collected in 2 vol. of EDTA/ethanol (10 mM-EDTA in 86% ethanol, pH 7.4, 0°C). After centrifugation (10000g, 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.

On the basis of a metabolic ATP content of $4.5 \mu\text{mol} \cdot (10^{11} \text{ platelets})^{-1}$ and the fact that in normal [^3H]adenine-labelled platelets 80% of the total radioactivity is found in ATP, a 1% change of ^3H radioactivity corresponds to $0.056 \mu\text{mol}$ of nucleotide $\cdot (10^{11} \text{ platelets})^{-1}$. The energy stored in metabolic ATP and ADP was expressed in terms of ATP equivalents ($\text{ATP}_{\text{eq.}}$; Atkinson, 1977) which reflect the energy liberated in the conversion of ATP to ADP. Rates of energy consumption were derived from the decline in the number of $\text{ATP}_{\text{eq.}} \cdot (10^{11} \text{ platelets})^{-1}$ during the initial 15 s after addition of metabolic inhibitors by linear regression analysis, expressed as ($\Delta \text{ATP}_{\text{eq.}} / \Delta t$) and plotted at the halfway point of each 15 s interval. This method accurately determines metabolically active ATP and ADP, since it is neither disturbed by actin-bound ADP, nor by ATP and ADP stored in the dense granules which are unavailable for energy metabolism (Daniel *et al.*, 1979, 1980). The energy consumption data obtained with this technique correlates well with conventional methods for determination of energy consumption (Akkerman *et al.*, 1983; Verhoeven *et al.*, 1984a).

Analysis of functional responses

Platelet shape change was measured in a Payton Dual Channel Aggregometer (Scarborough, Ontario, Canada) by two techniques. Firstly, by monitoring the decrease in light transmission of a stirred (900 rev./min) suspension following addition of thrombin. Secondly, by monitoring the light transmission of the suspension at two different stirring speeds, 900 and 200 rev./min, according to the method of Latimer *et al.* (1977). These authors and others (Holme & Murphy, 1978; Patscheke *et al.*, 1984) have shown that the disk-sphere transformation proportionally decreases the difference in light transmission found at the two stirring speeds, which provides a sensitive means for the quantification of shape change.

Platelet aggregation was measured as the disappearance of single platelets, as described elsewhere (Verhoeven *et al.*, 1984a). Studies were carried out without added fibrinogen in order not to interfere with thrombin-platelet interaction, taking advantage of the fact that gel-filtered platelets contain enough adsorbed fibrinogen for the early stages of aggregation (Kaplan *et al.*, 1981). In short, samples were collected from the aggregating suspension

into 9 vol. of 0.5% (v/v) glutaraldehyde (Fluka, Buchs, Switzerland) in saline (0°C). Then, platelet numbers were determined in a Platelet Analyzer 810 (Baker Instruments, Allentown, PA, U.S.A.) with apertures set between 3.2 and $16 \mu\text{m}^3$. Single platelet disappearance was expressed as a percentage of the platelet number found within these settings in the unstimulated suspension.

Throughout this paper, secretion responses were defined as the extrusion of granule contents. Secretion from dense, α - and acid-hydrolase-containing granules was determined by monitoring the extracellular appearance of markers that were specific for each type of granule (Akkerman *et al.*, 1983). Samples of cell suspension were collected in 0.15 vol. of 1.035 M-formaldehyde in saline (0°C) and centrifuged (10000g, 1 min, 4°C). [^{14}C]Serotonin (counted for radioactivity according to standard procedures) was used as a marker for the dense granules, β -thromboglobulin (measured with the radioimmunoassay kit from Amersham International) was a marker for the α -granules and *N*-acetyl- β -D-glucosaminidase (EC 3.2.1.30; measured spectrophotometrically according to Troost *et al.*, 1976) was a marker for the acid-hydrolase-containing granules. All secretion experiments were performed in the presence of $3 \mu\text{M}$ -imipramine (Geigy, Basel, Switzerland) to prevent reuptake of secreted serotonin by the platelets (Walsh & Gagnatelli, 1974). Secretion data were expressed as the percentages of maximal secretable amounts, the latter being the amount of marker secreted by the gel-filtered platelets after 5 min incubation with 5 units of thrombin $\cdot \text{ml}^{-1}$.

Analysis of biochemical responses

For the determination of (poly)phosphoinositides, 0.5 ml samples of ^{32}P -labelled gel-filtered platelets were mixed with 2 ml of chloroform/methanol/13 M-HCl (100:50:1 by vol.) at 0°C. The phospholipids were extracted and subsequently separated by high-performance t.l.c. as described by Jolles *et al.* (1981). Labelled lipids were visualized by overnight radioautography on Kodak Royal X-Omat film. The spots were scraped from the plates and counted for radioactivity according to standard procedures. The radioactivity of each spot was expressed as a percentage of that in the unstimulated platelet suspension; these were 2900 ± 700 , 2300 ± 700 , 1900 ± 500 and 250 ± 150 c.p.m./ 10^8 platelets for $\text{PtdIns}(4,5)\text{P}_2$, $\text{PtdIns}(4)\text{P}$, PtdIns and PtdA , respectively (means $\pm$ s.d. for 15 different platelet suspensions).

For the determination of protein phosphorylation, samples of ^{32}P -labelled platelets were collected into 5 vol. of 3-fold concentrated Laemmli sample buffer (Laemmli, 1970) and incubated at 100°C for 5 min. Aliquots were then separated by

electrophoresis through 11% sodium dodecyl sulphate/polyacrylamide gels according to Laemmli (1970). Proteins were stained with Coomassie Brilliant Blue; the distribution of radioactivity was determined by radioautography of the dried gels on Kodak Royal X-Omat film. For the determination of ^{32}P radioactivity in M_r 47000 and 20000 proteins the specific areas were cut out from the dried gels and heated for 2 h at 80°C in 30% H_2O_2 , the radioactivity was determined by liquid-scintillation counting (Lapetina & Siegel, 1983). The ^{32}P content of each protein was expressed as a percentage of that in unstimulated platelets. These were 200 ± 100 and 400 ± 100 c.p.m./ 10^8 platelets for the M_r 20000 and 47000 proteins, respectively (data derived from five different platelet suspensions).

Statistical treatment

Data are expressed as means $\pm$ s.d.; statistical significances were determined by Student's *t*-test unless otherwise stated, and were considered not significant at $P > 0.05$.

Results

Energy consumption during the initiation of secretion

Stimulation of platelets with thrombin (5 units $\cdot$ ml $^{-1}$) triggered rapid secretion of dense, α - and acid hydrolase granule contents, which after 15 s amounted to 95, 45 and 30% of maximal secretable amounts, respectively. With lower doses of thrombin, secretion during the first 15 s after stimulation was proportionally slower and reached 7, 4 and 2% of the respective maximal secretion responses with 0.05 unit $\cdot$ ml $^{-1}$. Concomitantly with secretion, thrombin induced shape change and aggregation, and a dose-dependent increase in energy consumption. As illustrated in Fig. 1, the velocities of the three secretion responses were related linearly to the increase in energy consumption. This covariance, however, held only above a certain threshold, below which energy consumption was higher than in unstimulated platelets but not accompanied by the extracellular appearance of granule markers. This threshold amounted to $3.1 \pm 0.9 \mu\text{mol}$ of $\text{ATP}_{\text{eq.}} \cdot \text{min}^{-1} \cdot (10^{11} \text{ platelets})^{-1}$.

A better separation between the early stages of platelet activation and the final extrusion of granule contents could be obtained by transient stimulation of the platelets followed by abrupt dissociation of the agonist-receptor complex. This was achieved by stimulating the cells with thrombin (0.2 unit $\cdot$ ml $^{-1}$) followed by neutralization of thrombin with excess of hirudin. As illustrated in Table 1, hirudin alone (10 units $\cdot$ ml $^{-1}$) neither affected energy consumption nor triggered any functional response, whereas under the same

Fig. 1. Relationship between thrombin-induced secretion responses and the concurrent consumption of metabolic energy

[^3H]Adenine- and [^{14}C]serotonin-labelled platelets were stimulated with thrombin at final concentrations ranging between 0.05 and 5 units $\cdot$ ml $^{-1}$; antimycin A and gluconolactone were added simultaneously with thrombin. At 15 s thereafter, secretion of [^{14}C]serotonin (Δ), β -thromboglobulin (Δ), and *N*-acetyl- β -D-glucosaminidase ($\square$) was measured. Energy consumption during the 0–15 s interval after stimulation, expressed as μmol of $\text{ATP}_{\text{eq.}} \cdot \text{min}^{-1} \cdot (10^{11} \text{ platelets})^{-1}$, was determined in the same suspensions. The energy consumption data were corrected for the energy consumption in unstimulated suspensions [$7.0 \pm 0.8 \mu\text{mol}$ of $\text{ATP}_{\text{eq.}} \cdot \text{min}^{-1} \cdot (10^{11} \text{ platelets})^{-1}$]. Data originate from an experiment with the platelets from one donor, and are representative of four similar experiments.

conditions thrombin (0.2 unit $\cdot$ ml $^{-1}$) initiated almost complete shape change, single platelet disappearance and dense granule secretion within 60 s. These responses were accompanied by an increase in energy consumption of about $8 \mu\text{mol}$ of $\text{ATP}_{\text{eq.}} \cdot \text{min}^{-1} \cdot (10^{11} \text{ platelets})^{-1}$ between 5 and 20 s after stimulation. Preincubation of thrombin with excess of hirudin completely abolished these responses. When hirudin was added at 2 s after thrombin no dense granule secretion (Table 1) or α - and acid hydrolase granule secretion (results not shown) could be detected in the first 1 min following stimulation. Even after 5 min no secretion markers could be detected extracellularly. The increase in energy consumption during the first 20 s was reduced to about $3.4 \mu\text{mol}$ of $\text{ATP}_{\text{eq.}} \cdot \text{min}^{-1} \cdot (10^{11} \text{ platelets})^{-1}$, which is similar to the increase in energy consumption that preceded secretion, as shown in Fig. 1 ($P > 0.05$). Single platelet disappearance was only reduced by

Table 1. *Energy consumption during transient stimulation of platelets*

[³H]Adenine- and [¹⁴C]serotonin-labelled platelets were incubated with and without thrombin (0.2 unit·ml⁻¹) and hirudin (10 unit·ml⁻¹). When both thrombin and hirudin were present these agents were added after a 10 min preincubation (designated as 'complex'), or with a 2 s and 5 s interval in between (thrombin added before hirudin). The energy consumption, expressed as $\mu\text{mol of ATP}_{\text{eq}} \cdot \text{min}^{-1} \cdot (10^{11} \text{ platelets})^{-1}$, was measured between 5 and 20 s after thrombin addition. Shape change was expressed as percentage of the decrease in light transmission induced by a stirring-speed transition from 900 to 200 rev./min. Single platelet disappearance (%) and [¹⁴C]serotonin secretion (%) were measured at 60 s after thrombin addition. Data are means $\pm$ S.D. ($n = 5$) and considered significantly different from unstimulated platelets at * $P < 0.05$.

	Addition . . .	None	Hirudin	Thrombin	Thrombin + hirudin		
					Complex	$\Delta t = 2\text{ s}$	$\Delta t = 5\text{ s}$
Energy consumption during the first 20s							
Total		6.6 ± 0.8	6.6 ± 0.5	$14.7 \pm 1.2^*$	6.9 ± 0.6	$10.0 \pm 0.5^*$	$12.3 \pm 1.5^*$
Incremental		—	0 ± 0.9	$8.1 \pm 1.4^*$	0.3 ± 1.0	$3.4 \pm 0.9^*$	$5.7 \pm 1.7^*$
Functional responses after 60s							
Shape change		0	0	$79 \pm 10^*$	0	$85 \pm 8^*$	$79 \pm 7^*$
Single platelet disappearance		0	0	$86 \pm 3^*$	0	$49 \pm 5^*$	$85 \pm 5^*$
[^{14}C]Serotonin secretion		2 ± 1	2 ± 1	$85 \pm 3^*$	2 ± 1	2 ± 1	$63 \pm 6^*$

about 40%, whereas shape change was not affected. When the period between thrombin and hirudin addition was extended to 5 s, about 60% dense granule secretion took place, whereas energy consumption was increased to about $6 \mu\text{mol of ATP}_{\text{eq}} \cdot \text{min}^{-1} \cdot (10^{11} \text{ platelets})^{-1}$. Hence, a 2 s period of stimulation with thrombin was critical for increasing the rate of energy consumption without triggering the three secretion responses.

Fig. 2(a) shows the increase in energy consumption during the first 60 s after stimulation with thrombin (0.2 unit·ml⁻¹). The addition of hirudin at 2 s after thrombin not only reduced the energy consumption during the first 20 s, but also normalized it after 30 s, whereas without hirudin the increase in energy consumption lasted twice as long. Hirudin did not affect the shape change response (Fig. 2b). In contrast, single platelet disappearance was slightly retarded during the first 10 s and thereafter ceased completely at about 60% of the uninhibited suspension. Hence, the successive addition of thrombin and hirudin revealed a period with increased energy generation which was accompanied by a normal shape change and impaired single platelet disappearance, whereas even after prolonged incubation the extrusion of granule markers was completely absent. This made it possible to compare the energetics of shape change and single platelet disappearance with the two early energy-dependent steps in stimulus-response coupling, the phosphorylation of proteins and the metabolism of (poly)phosphoinositides.

Early energy-requiring steps in stimulus-response coupling

Fig. 3 illustrates the effect of hirudin on

thrombin (0.2 unit·ml⁻¹)-induced changes in the ³²P radioactivity of PtdIns(4,5)P₂, PtdIns(4)P, PtdIns and PtdA. In the absence of hirudin, thrombin induced an extensive increase in [³²P]PtdIns(4,5)P₂ and [³²P]PtdA, a moderate increase in [³²P]PtdIns(4)P and a small decrease in [³²P]PtdIns during the first 60 s. The changes in [³²P]PtdIns(4)P and [³²P]PtdA occurred almost instantaneously, whereas the increase in [³²P]PtdIns(4,5)P₂ and the concomitant decrease in [³²P]PtdIns were delayed by about 10 s. All these changes were affected by hirudin but to different extents. When thrombin was neutralized after 2 s, the increase in [³²P]PtdIns(4,5)P₂ continued unaltered for about 30 s after which it rapidly decreased. The increase in [³²P]PtdIns(4)P was completely prevented, whereas the fall in [³²P]PtdIns seen in the absence of hirudin changed into a transient accumulation in its presence. Addition of hirudin left the increase in [³²P]PtdA unaltered during the first 10 s; thereafter, [³²P]PtdA gradually declined to control values. Thus, the increase in energy consumption found during the first 20 s after transient stimulation with thrombin is accompanied by accumulation of [³²P]PtdIns(4,5)P₂, [³²P]PtdIns and to a lesser extent of [³²P]PtdA. The accumulation of these phospholipids may therefore be responsible for part of the increase in energy consumption seen in this period.

Platelet stimulation with 0.2 unit of thrombin·ml⁻¹ initiated rapid phosphorylation of two proteins with M_r values of 47 000 and 20 000 (Fig. 4). The phosphorylation was maximal after 10 s (M_r 20 000 protein) to 30 s (M_r 47 000 protein). Thrombin neutralization with hirudin did not

Fig. 2. *Rapid neutralization of thrombin by hirudin*

[^3H]Adenine-labelled gel-filtered platelets were stimulated with thrombin ($0.2 \text{ unit} \cdot \text{ml}^{-1}$). To some of the suspensions, hirudin ($10 \text{ units} \cdot \text{ml}^{-1}$; indicated by the arrow) was added at 2 s after thrombin (closed symbols), whereas the remainder were incubated without hirudin (open symbols). In (a), the effect of hirudin on the thrombin-induced increase in energy consumption is shown. At 5, 20, 35 and 50 s after thrombin, antimycin A and gluconolactone were added for the determination of energy consumption, expressed as $\mu\text{mol of ATP}_{\text{eq}} \cdot \text{min}^{-1} \cdot (10^{11} \text{ platelets})^{-1}$. Also shown is the energy consumption in unstimulated cells ($\square$). Data are expressed as means $\pm$ S.D. ($n = 4$). In the thrombin-stimulated platelets energy was consumed at significantly higher rates than in unstimulated cells between 5 and 50 s in the absence and between 5 and 20 s in the presence of hirudin ($P < 0.01$). In (b), the effect of hirudin on thrombin-induced shape change (Δ , $\blacktriangle$) and single platelet disappearance ($\circ$, $\bullet$) is shown. The $\square$ symbols reflect single platelet disappearance in the unstimulated suspension. Platelet shape change was continuously monitored in an aggregometer, and quantified at various times by expressing the change in light transmission as a percentage of the maximal decrease in light transmission induced by a stirring speed transition of 900 to 200 rev./min, measured in the same suspension. Note that shape change was studied in the absence of a chelator of divalent cations; in the absence of hirudin aggregation became apparent at 15 s after thrombin addition, making further analysis of shape change impossible. Differences between single platelet disappearance with and without hirudin were significant at 10 s and thereafter ($P < 0.05$).

affect the rapid phosphorylation of both proteins during the first 10 s. Thereafter, the further increase in ^{32}P -labelled $M_r 47000$ protein was prevented, whereas the ^{32}P radioactivity in the $M_r 20000$ protein even declined. Thus, protein phosphorylation occurred despite the prevention of secretion and at least partly coincided with the increase in energy consumption.

Energy consumption during early platelet responses

A further separation of these early processes was achieved by an alternate method for transient stimulation of platelets, in which the cells were first preincubated with hirudin ($4 \text{ units} \cdot \text{ml}^{-1}$) and then stimulated with thrombin. A low dose of thrombin ($0.2 \text{ unit} \cdot \text{ml}^{-1}$) added to hirudin-treated platelets did not significantly change the rate of energy consumption ($P > 0.05$, $n = 7$), but did induce the shape change response. In contrast, aggregation and secretion were completely prevented and no alterations in (poly)phosphoinositides and protein phosphorylation could be detect-

ed at 7, 10, and 20 s (results not shown) or 30 s (Table 2) following thrombin addition. When the thrombin concentration was raised to $0.4 \text{ unit} \cdot \text{ml}^{-1}$, a slight but significant ($P < 0.01$, $n = 7$) increase in energy consumption was seen, which was accompanied by a further increase in shape change. A slight degree of single platelet disappearance became apparent but secretion remained absent. Under those conditions a 1.5-fold increase in protein phosphorylation occurred, while the (poly)phosphoinositides were not changed at 30 s after stimulation. When the thrombin concentration was further raised to $1.0 \text{ unit} \cdot \text{ml}^{-1}$, energy consumption increased further, and was now accompanied by about 50% single platelet disappearance and a slight secretion ($< 10\%$). The phosphorylation of the $M_r 47000$ protein increased further, while the $M_r 20000$ protein phosphorylation did not change. At this stage, a 1.6-fold increase in [^{32}P]PtdA became apparent. The (poly)phosphoinositides, however, remained unchanged. For comparison, Table 2 also lists the data for thrombin-treated platelets in the absence

Fig. 3. Effect of hirudin on thrombin-induced alterations in the ^{32}P -labelled polyphosphoinositides and ^{32}P PtdA. ^{32}P -labelled gel-filtered platelets were incubated either with thrombin ($0.2 \text{ unit} \cdot \text{ml}^{-1}$) alone (O), or with hirudin ($10 \text{ units} \cdot \text{ml}^{-1}$) added at 2s after thrombin (arrow; ●). At the times indicated, samples were collected for the analysis of ^{32}P -labelled polyphosphoinositides and PtdA. Data are expressed as a percentage of ^{32}P present in each metabolite in an unstimulated suspension, and are means $\pm$ S.D. from five to ten experiments. Hirudin alone had no effect (results not shown). Differences between incubations with and without hirudin were significant ($P < 0.05$) only at 60s for ^{32}P PtdIns(4,5) P_2 and ^{32}P PtdIns(4) P , at 30s and thereafter for ^{32}P PtdA, and at 10s and thereafter for ^{32}P PtdIns. The broken lines represent the unstimulated suspension.

Fig. 4. Effect of hirudin on thrombin-induced phosphorylation of proteins.

Experiments were performed as described in the legend to Fig. 3, except that samples were analysed for ^{32}P radioactivity of the M_r 47 000 (a) and 20 000 (b) proteins. Data are means $\pm$ S.D. from five experiments. For both proteins differences between incubations with and without hirudin were significant ($P < 0.05$) at the 30s point and thereafter. The broken lines represent the unstimulated suspension.

accompanied by complete shape change and single platelet disappearance, and a considerable degree of secretion, alterations in ^{32}P (poly)phosphoinositides and protein phosphorylation.

Although thrombin ($0.2 \text{ unit} \cdot \text{ml}^{-1}$) added to hirudin-treated platelets induced shape change (Table 2), this response was only 50% complete. This is illustrated in Fig. 5, in which shape change has been measured by monitoring the light transmission (i) following thrombin addition at $900 \text{ rev} \cdot \text{min}^{-1}$; and (ii) following a decrease of the stirring speed to $200 \text{ rev} \cdot \text{min}^{-1}$. The successive treatment with hirudin and thrombin ($0.2 \text{ unit} \cdot \text{ml}^{-1}$) induced only about 50% shape change (Fig. 5a), which was further completed by a second dose of thrombin (Figs. 5b and 5c). A further increase of the thrombin concentration, however, induced shape change and aggregation (Fig. 5d). The presence of metabolic inhibitors used for measuring concurrent energy consumption slightly increased the light transmission (Fig. 5e). This did not prevent the shape change response (Fig. 5f). Interestingly, in the presence of metabolic inhibitors the cells recovered their disc shape after 5 min of further incubation, an effect that was not observed in the absence of metabolic blockade (Figs. 5f and 5a). The finding that about 50% of the shape change response proceeds without a concomitant increase in energy consumption indicates that this response does not take part in the energy

of hirudin, which illustrate a further increase in energy consumption to about $7 \mu\text{mol}$ of $\text{ATP}_{\text{eq}} \cdot \text{min}^{-1} \cdot (10^{11} \text{ platelets})^{-1}$. This increase was

Table 2. *Energy consumption in early platelet responses*

Gel-filtered platelets were incubated for 1 min with or without hirudin. Then the platelets were stimulated with different doses of thrombin. At 30 s after stimulation samples were collected for the analysis of functional and biochemical responses. Shape change was recorded continuously; the extent of shape change was determined as described in Table 1. Single platelet disappearance was expressed as a percentage of the platelet number in unstimulated suspensions; secretion was expressed as a percentage of maximal secretable amounts and the polyphosphoinositides and protein phosphorylation were expressed as percentages of ^{32}P present in unstimulated suspensions. Energy consumption was determined during the initial 15 s interval. Incremental energy consumption, expressed as $\mu\text{mol of ATP}_{\text{eq}} \cdot \text{min}^{-1} \cdot (10^{11} \text{ platelets})^{-1}$, was derived from total values after subtraction of $(\Delta\text{ATP}_{\text{eq}}/\Delta t)$ in unstimulated suspensions $[7.8 \pm 0.5 \mu\text{mol of ATP}_{\text{eq}} \cdot \text{min}^{-1} \cdot (10^{11} \text{ platelets})^{-1}]$. Data are means $\pm$ s.d. from 5–15 experiments, and were considered significantly different from unstimulated platelets at $*P < 0.05$.

	Hirudin (units·ml ⁻¹) ...	4	4	4	0
	Thrombin (unit·ml ⁻¹) ...	0.2	0.4	1.0	0.2
Energy consumption					
Total		8.1 $\pm$ 0.6	9.1 $\pm$ 0.2*	11.5 $\pm$ 0.4*	15.2 $\pm$ 0.9*
Incremental		0.3 $\pm$ 0.8	1.3 $\pm$ 0.5*	3.7 $\pm$ 0.6*	7.1 $\pm$ 1.0*
Functional responses					
Shape change		48 $\pm$ 5*	89 $\pm$ 2*	70 $\pm$ 9*	70 $\pm$ 9*
Single platelet disappearance		3 $\pm$ 2	8 $\pm$ 3*	52 $\pm$ 14*	77 $\pm$ 3*
Secretion of:					
[¹⁴ C]Serotonin		0.9 $\pm$ 0.5	0.6 $\pm$ 0.9	9 $\pm$ 4*	97 $\pm$ 4*
β -Thromboglobulin		0.2 $\pm$ 0.2	0.7 $\pm$ 0.3	9 $\pm$ 3*	72 $\pm$ 6*
Acid hydrolase		0.3 $\pm$ 0.3	0.3 $\pm$ 0.3	3 $\pm$ 1*	35 $\pm$ 3*
Polyphosphoinositides					
PtdIns(4,5)P ₂		99 $\pm$ 9	100 $\pm$ 4	97 $\pm$ 7	126 $\pm$ 13*
PtdIns(4)P		102 $\pm$ 3	100 $\pm$ 11	102 $\pm$ 7	118 $\pm$ 10*
PtdIns		100 $\pm$ 3	101 $\pm$ 3	98 $\pm$ 2	87 $\pm$ 6*
PtdA		102 $\pm$ 7	109 $\pm$ 7	161 $\pm$ 9*	427 $\pm$ 62*
Protein phosphorylation					
M _r 47000		99 $\pm$ 6	142 $\pm$ 8*	266 $\pm$ 15*	320 $\pm$ 55*
M _r 20000		96 $\pm$ 5	157 $\pm$ 35*	159 $\pm$ 35*	288 $\pm$ 48*

consumption that precedes the extrusion of granule constituents.

Energy requirement of secretion by a second stimulation with thrombin

The results discussed so far showed that transient stimulation with thrombin increased the energy consumption during a short time in which the platelets changed their shape and aggregated, and in which the M_r20000 and 47000 proteins were phosphorylated and (poly)phosphoinositide metabolism was activated. This raised the possibility that this investment of energy led to a 'cheaper' execution of secretion triggered by a second stimulation with thrombin. Platelets were stimulated with thrombin (0.2 unit·ml⁻¹) and 2 s later the thrombin was neutralized with hirudin (5 units·ml⁻¹). In accordance with the data in Table 1, the cells had fully changed their shape, and were aggregated by 77 $\pm$ 12% ($n = 6$) after 60 s, whereas [¹⁴C]serotonin secretion was negligible (3 $\pm$ 2%, $n = 12$). A second stimulation with thrombin at final concentrations ranging between 4.8 and 10 units·ml⁻¹ induced a dose-dependent secretion of [¹⁴C]serotonin and a concurrent increase in energy consumption. In Fig. 6 the secretion-versus-

energy relationship during the initial 15 s after addition of the second dose of thrombin is shown and compared with the relationship obtained in the same platelet suspension after a first challenge with thrombin. It is clear that the velocities of secretion and concurrent energy consumption varied in parallel. However, the increment in energy consumption that preceded secretion was significantly greater in platelets that had first been treated with thrombin and hirudin ($P < 0.005$). Thus, a first increase in energy consumption during aggregation, protein phosphorylation and (poly)phosphoinositide formation did not lead to less energy consumption during subsequent secretion. Instead, relatively more energy was consumed at a given secretion velocity when platelets were pre-activated by the combined treatment with thrombin and hirudin.

Discussion

Among the different platelet responses, secretion shows the greatest need for metabolic energy. In thrombin-stimulated platelets the velocities of dense, α - and acid hydrolase granule secretion vary in parallel with the rate of energy consumption and

Fig. 5. Thrombin-induced shape change in platelet suspensions, preincubated with excess of hirudin

Gel-filtered platelets were incubated in the presence of hirudin (4 units·ml⁻¹) (h). At 2.5 min, the platelets were stimulated with 0.2 ($t_{0.2}$; a, b, f) 0.4 ($t_{0.4}$; c) and 1.0 ($t_{1.0}$; d) unit of thrombin·ml⁻¹ (all final concentrations). In (b), a second dose of thrombin (0.4 unit·ml⁻¹) was added at 30 s after the initial dose. The antimycin A/gluconolactone mixture (i) was added either alone (e) or simultaneously with thrombin (f). Aggregation tracings were recorded with alternate stirring at 900 and 200 rev./min (closed and open bars, respectively). Data originate from an experiment with the platelets from one donor and are representative of five similar experiments.

correlate particularly well with the increment in energy consumption seen only in stimulated platelets. This correlation, however, holds only above a certain threshold. Below this threshold, energy consumption is increased but is not accompanied by extrusion of granule contents. This threshold appears when rates of secretion and energy consumption are compared (Fig. 1), as well as when the extent of both processes is correlated (Verhoeven *et al.*, 1984a), and amounts to $0.8 \mu\text{mol of ATP}_{\text{eq}} \cdot (10^{11} \text{ platelets})^{-1}$. It is possible that the early increase in energy consumption reflects the energy cost of processes that take part in stimulus-secretion coupling, but precede the extrusion step. Alternatively, it may reflect the energy cost of early functional and biochemical responses that are independent of secretion. We found that a good separation between these events was obtained when platelet-thrombin interaction was interrupted by adding hirudin 2 s after thrombin. This procedure abolished secretion and left the early increase in energy consumption intact. Under those conditions hirudin is known to neutralize thrombin completely within 10 s (Tam *et al.*, 1979),

Fig. 6. Relationship between energy consumption and dense granule secretion induced by various doses of thrombin with and without prior transient stimulation

[³H]Adenine- and [¹⁴C]serotonin-labelled gel-filtered platelets were stimulated with thrombin (0.2 unit·ml⁻¹). At 2 s thereafter, thrombin was neutralized by the addition of hirudin (5 units·ml⁻¹). At 60 s the platelets were stimulated with a second dose of thrombin (final concentrations ranging between 4.8 and 10 units·ml⁻¹). Secretion of [¹⁴C]serotonin at 15 s after the second dose (expressed as a percentage of maximal secretable) and concurrent energy consumption during the first 15 s interval [expressed as $\mu\text{mol of ATP}_{\text{eq}} \cdot \text{min}^{-1} \cdot (10^{11} \text{ platelets})^{-1}$], were determined (●). The control suspensions consisted of platelets which were incubated for 60 s with hirudin (5 units·ml⁻¹) without prior activation; then different doses of thrombin were added to final concentrations of 4.8–10 units·ml⁻¹ (○). Energy consumption was corrected for ($\Delta\text{ATP}_{\text{eq}}/\Delta t$) measured simultaneously in the unstimulated suspension [$7.4 \pm 0.9 \mu\text{mol of ATP}_{\text{eq}} \cdot \text{min}^{-1} \cdot (10^{11} \text{ platelets})^{-1}$, $n = 8$]. Data are means $\pm$ S.D. for two to four incubations. Regression lines were $y = -51.92 + 12.73x$ ($r = 0.863$, $n = 17$; $P < 0.01$) and $y = -8.50 + 10.14x$ ($r = 0.868$, $n = 20$; $P < 0.01$) with and without prior stimulation with thrombin (0.2 unit·ml⁻¹), respectively. The slopes of both lines differed not significantly ($P > 0.25$), but the difference between both intercepts was highly significant ($P < 0.005$), as determined by a *F*-distribution test (Snedecor & Cochran, 1967).

and since hirudin, either alone or as part of a hirudin-thrombin complex, does not affect the platelet (Detwiler & Feinman, 1973; Hoffman & Markwardt, 1984; and Table 1), platelet stimulation by thrombin can be interrupted. The result was that some responses changed immediately after hirudin was added, while others first proceeded normally and became affected well after complete neutralization of thrombin. A notable exception was shape change, which proceeded normally despite hirudin addition. An example of

the category with a tight coupling to receptor occupancy was single platelet disappearance, which was decreased within 8 s after hirudin addition. Furthermore, PtdIns changed immediately and accumulated in the presence of hirudin but not in normal thrombin-treated platelets. Examples of the category with a delayed response to thrombin neutralization were the accumulation of PtdIns(4,5) P_2 and PtdA, as well as the phosphorylation of the M_r 20000 and 47000 proteins. These components were unaffected at 8 s after hirudin addition but thereafter significantly diverged from the untreated cells. Other studies have shown that an intact agonist-receptor complex is required for secretion and PtdA formation (Holmsen *et al.*, 1981, 1984), but not for PtdIns catabolism (Holmsen *et al.*, 1984). The latter finding contrasts with our results. In fact, our studies demonstrate the need for an intact agonist-receptor complex for all physiological and biochemical responses studied except shape change, although the coupling efficiency differs between the different responses. It is possible, however, that, during the short stimulation with thrombin, arachidonate metabolism is activated leading to endoperoxide and thromboxane A_2 formation. These intermediates would activate the platelet relatively independent from the action of thrombin. Hence, a definite answer regarding the coupling between PtdIns metabolism and thrombin-platelet interaction awaits the characterization of the kinetics of prostanoid synthesis during short-term activation by thrombin.

When the various responses were compared with the increment in energy consumption seen upon stimulation, differences in energy requirement became apparent. Successive treatment with thrombin and hirudin ($\Delta t = 2$ s) revealed that the increment in energy consumption seen between 5 and 20 s after thrombin stimulation (in Table 1 and Fig. 2a, expressed per min) is about $0.8 \mu\text{mol of ATP}_{\text{eq.}} \cdot (10^{11} \text{ platelets})^{-1}$. In this interval the increase in shape change was 75% and in single platelet disappearance 29% (Fig. 2b). Thus, the increment in energy consumption accompanying 1% response was for shape change 0.021 (same units) and for single platelet disappearance 0.028 (same units). Since both processes occurred simultaneously it remained unclear how much energy was consumed in the separate responses. However, when the hirudin treatment preceded the stimulation with thrombin a considerable shape change occurred without an increase in energy consumption (Table 2, Fig. 5). We therefore tend to conclude that the initial increase in energy consumption is fully related to single platelet disappearance. The cost of a full response is therefore estimated at an increment of $2.8 \mu\text{mol of ATP}_{\text{eq.}}$

$(10^{11} \text{ platelets})^{-1}$. A similar calculation based on the data in Table 2 reveals an energy cost for aggregation of 3.2 (same units), but here aggregation has been quantified slightly later than energy consumption (30 s versus 5–20 s, respectively). These data accord with earlier findings (Verhoeven *et al.*, 1984b) that showed that metabolic inhibition abolished optical aggregation, of which single platelet disappearance reflects the early stage. Its energy cost is considerable lower than that of the combined secretion responses [$6 \mu\text{mol of ATP}_{\text{eq.}} \cdot (10^{11} \text{ platelets})^{-1}$ (Verhoeven *et al.* 1984a)] as suggested in studies where energy generation was only partly abolished (Holmsen *et al.*, 1982). On the other hand, it is much above the estimated cost of a separate dense granule secretion response [$0.7 \mu\text{mol} \cdot (10^{11} \text{ platelets})^{-1}$ (Verhoeven *et al.*, 1984a)].

A deeper insight in the mutual relationship between functional and biochemical responses was gained by pretreating the platelets with hirudin and stimulating the cells by subsequent addition of increasing doses of thrombin (Table 2). This stepwise increase in the degree of stimulation initiated a sequence of events that led to the following conclusions. Firstly, shape change is not dependent on an increase in protein phosphorylation and alterations in the steady state levels of ^{32}P -labelled PtdIns metabolites. This is in sharp contrast with other studies in which a direct connection has been proposed between shape change and phosphorylation of the M_r 20000 protein (Daniel *et al.*, 1984), the M_r 47000 protein (Lapetina & Siegel, 1983) and early changes in PtdIns metabolites (Lapetina & Siegel, 1983; Lloyd *et al.*, 1973). Those conclusions were mainly based on similarities in time courses, thereby assuming a causal relationship. Our current findings suggest that these processes are not closely linked to shape change. A notable difference is our use of thrombin as a stimulator and the absence of EDTA, while others used ADP (Daniel *et al.*, 1984) or platelet activating factor (Lapetina & Siegel, 1983) in the presence of EDTA or EGTA. Secondly, the initial part of aggregation (measured as single platelet disappearance) is not accompanied by detectable changes in PtdIns metabolites, but does coincide with a slight (but not significant) increase in PtdA and a considerable increase in protein phosphorylation. Thirdly, when more aggregation occurs (and a slight secretion becomes apparent), PtdA accumulates further whereas the other PtdIns metabolites measured do not change. This accords with recent findings by Vickers *et al.* (1984) who showed that a low thrombin concentration does not stimulate the polyphosphoinositide cycle, whereas PtdA accumulates as a result of synthesis *de novo*. Alternati-

vely, PtdA accumulation may result from a slight increase in the turnover of the polyphosphoinositide cycle at relatively stable steady state concentrations of the polyphosphoinositides. Finally, alterations in polyphosphoinositides become apparent only when these processes are accompanied with a considerable degree of secretion, suggesting that a major part of the activation of the polyphosphoinositide cycle is a step in stimulus-secretion coupling. The role of PtdA formation is less certain, since it occurs in a period in which aggregation and secretion overlap. Since it accumulates in parallel with protein phosphorylation and single platelet disappearance, we tend to conclude that PtdA accumulation is an early step in the initiation of the aggregation response. Lapetina & Cuatrecasas (1979) concluded that in thrombin-stimulated platelets the increased labelling of PtdA is related to secretion and not to aggregation. In contrast, Lloyd *et al.* (1973) found PtdA labelling in ADP-stimulated platelets to be related to optical aggregation, but these authors did not measure single platelet disappearance.

After a first stimulation with thrombin followed by neutralization with hirudin, normal dense granule secretion could be initiated by a second dose of thrombin (Fig. 6). This implies that a normal secretion response could be induced in cells that had undergone transient alterations in PtdIns metabolites and contained elevated levels of phosphorylated proteins. The energetics of this response were partly unchanged as indicated by the parallel lines in the secretion-versus-energy plots for normal and pretreated platelets. This relationship, however, had significantly shifted to higher energy data. Thus, in contrast with the expectation that a preinvestment of energy would make a subsequent secretion response cheaper in terms of energy requirement, an increase in energy cost was evident. However, instead of the amount of phosphorylated product, the velocity of phosphorylation-dephosphorylation is probably the decisive factor in the utilization of energy by these processes. A better correlation with the energetics of secretion may be found when the turnover of these compounds can be assessed. Furthermore, the amount of energy involved in protein phosphorylation and PtdIns metabolism is probably minor, and other processes such as actomyosin contractile activity may consume most of the energy made available during secretion.

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