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A novel technique for rapid determination of energy consumption in platelets

Demonstration of different energy consumption associated with three secretory responses

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A novel method has been developed for rapid and quantitative determination of the rate of energy consumption in platelets. In platelets suspended in a cyanide-containing medium, ATP resynthesis is abruptly blocked by addition of 2-deoxyglucose and D-glucono-1,5-lactone. We demonstrate that the subsequent changes in the levels of cytoplasmic ATP and ADP reflect the velocity of energy consumption in the platelets immediately before addition of the inhibitors. Despite the arrest in ATP resynthesis the platelets remain responsive to stimulation by thrombin (5 units · ml^{-1}) which triggers the secretion of the contents of dense, α- and acid hydrolase granules. Unstimulated platelets were found to consume about 3.5 and 0.5 μmol of ATP equivalents · min^{-1} · $(10^{11}$ cells$)^{-1}$ at 37°C and 15°C, respectively; the thrombin-treated platelets consumed respectively 16 and 2 μmol of ATP equivalents · min^{-1} · $(10^{11}$ cells$)^{-1}$ at these temperatures. When the velocity of energy consumption was varied by (*a*) changing the temperature and (*b*) preincubation with glyco(geno)lytic inhibitors, it was found to be linearly related to the initial rate of secretion from the three types of granules. The precise nature of this relationship differed between the three types of secretion responses and indicated an increasing requirement for metabolic energy for secretion from the three types of granules in the order: dense granule < α-granule < acid hydrolase granule. The results obtained with changes in temperature were superimposable on those obtained with the glyco(geno)lytic inhibitors for dense granule secretion and α-granule secretion, suggesting an apparent coupling between energy consumption and the rate of these secretion responses. The rate of secretion of acid hydrolase was always higher when energy consumption was varied by temperature changes than when glyco(geno)lytic inhibitors were used, probably as a result of metabolic changes prior to induction of secretion. On the basis of these experiments, we calculated an incremental energy consumption during complete secretion of dense, α- and acid hydrolase granule contents of 2.5, 4.2 and 6.7 μmol of ATP equivalents · $(10^{11}$ platelets$)^{-1}$, respectively.

The occupation of specific receptors on secretory cells leads to a sequence of events generally denoted as stimulus–secretion coupling (Rubin, 1970*a*). A secretagogue exerts its effect without entering the cell and triggers a process in which a signal is transduced by secondary messengers to the mechanisms that execute exocytosis. Depending on the tissue and species, receptor occupancy might trigger changes in the levels of cyclic nucleotides (Goldberg *et al.*, 1974), an increase in phosphatidylinositol turnover (the PI response; Michell *et al.*, 1976) and changes in membrane permeability that result in an increase in intracellular Ca^{2+} content (Putney, 1979). In combination with a specific Ca^{2+}-binding protein, calmodulin, Ca^{2+} mediates various processes that are probably linked to secretion such as actomyosin contractile activity (Herman & Pollard, 1979), myosin light chain kinase activity and microtubule assembly and disassembly (Welsh *et al.*, 1979).

Along this sequence there are various steps that depend on metabolic energy. ATP is a source for cyclic nucleotide generation, it is consumed in the PI response (Lapetina & Michell, 1973), it is a substrate for $(Na^+ + K^+)$-ATPase which in some cells is closely linked to Ca^{2+} transport (Putney,

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1979) and it is a prerequisite for actomyosin contractile activity (Adelstein *et al.*, 1973). One might therefore expect that inhibition of energy metabolism would lead to inhibition of secretory responses. This has indeed been demonstrated in various secretory cells such as exocrine pancreatic cells (Palade, 1975), adrenal medulla (Rubin, 1969, 1970*a*), ox pituitary glands (McPherson & Schofield, 1974), rat salivary glands (Rossignol *et al.*, 1974) and blood platelets (Mürer, 1968; Holmsen *et al.*, 1979). When energy metabolism is intact, one would expect it to increase during secretory responses and this is especially apparent in platelets, which respond to secretion inducers with an increase in generation of ATP by both glycolysis and oxidative phosphorylation (Akkerman & Holmsen, 1981).

So far, it has been difficult to measure more specifically how energy metabolism and secretory responses are linked, since in platelets these responses are extremely fast (completed within 25–40s after maximal stimulation at 37°C), rendering useless the conventional methods for measuring ATP consumption. In the present work, we describe a novel method which measures energy consumption within 20–25s on the basis of fluctuations in cytoplasmic ATP and ADP after abrupt arrest of ATP regeneration. Concurrent analysis of secretion responses shows that secretion velocity indeed correlates with the rate of energy consumption and that the apparent coupling differs quantitatively between the three different secretory processes in platelets.

Materials and methods

Platelet isolation

Freshly drawn venous blood (40ml portions) was collected from healthy human volunteers into citrate (0.1vol. of 129mM-sodium citrate). After centrifugation (200*g*, 10min, 22°C) the supernatant platelet-rich plasma was incubated with 1μM-5-hydroxy[β-^{14}C]tryptamine ([^{14}C]serotonin, sp. radioactivity 58Ci·mol^{-1}; The Radiochemical Centre, Amersham) and 1μM-[2-^{3}H]adenine (sp. radioactivity 3Ci·mmol^{-1}; The Radiochemical Centre, Amersham) for 45min at 37°C in order to label the serotonin in the dense granules and the metabolic cytoplasmic pool of adenine nucleotides, respectively (in contrast with granular, non-metabolic ATP and ADP; Holmsen *et al.*, 1974). The platelets were then isolated by gel filtration at 22°C on Sepharose 2B (Pharmacia; column size 5cm × 20cm), equilibrated in Ca^{2+}-free Tyrode's solution (pH7.35, osmolality 300mosmol·kg^{-1}; Walsh, 1972) containing 1mM-KCN and no glucose. Under these conditions mitochondrial energy generation and glucose utilization are absent, leaving glycogen as the only source for ATP generation (Akkerman & Gorter, 1980; Akkerman *et al.*, 1978). Since glycogenolysis alone insufficiently maintains ATP homeostasis (Akkerman *et al.*, 1978), 1mM-glucose (final concentration) was added to the platelet suspensions immediately after gel filtration was completed, i.e., within 20–30min after the start of gel filtration. The suspensions were then incubated for 20min (22°C), which restored ATP homeostasis (see the Results section). Control experiments, in which aliquots of the platelet suspension were fixed and centrifuged after this incubation (see below under 'Analysis of functional parameters'), showed that less than 3% of total cell-bound secretable constituents had leaked into the supernatant.

Incubation with metabolic inhibitors

After the 20min preincubation, platelet suspensions were subsequently incubated in capped polystyrene tubes at temperatures ranging from 10 to 42°C. In these suspensions ATP regeneration was blocked by adding a mixture of 2-deoxy-D-glucose (Merck), an inhibitor of glycolysis (Holmsen & Robkin, 1980), and D-gluconic acid-1,5-lactone (gluconolactone; Sigma, grade IX), an inhibitor of glycogen phosphorylase (Gold *et al.*, 1971; Tu *et al.*, 1971; Holmsen & Akkerman, 1980), to final concentrations of 30 and 10mM, respectively. Since gluconolactone is rapidly hydrolysed at neutral pH, the dry powder was dissolved in the deoxyglucose solution immediately before addition to the platelet suspension. The subsequent hydrolysis in the cell suspension made the pH fall from 7.35 to 6.95 over a 5min period; it stabilized thereafter. Control experiments run at pH7.35 in a pH titrator gave similar results, indicating that the drop in pH was of no significance for the metabolic and functional parameters investigated in this study. Furthermore, incubation of the platelet suspension with the inhibitors for 5min (37 or 15°C) did not cause further release of the secretion markers than during the 20min incubation with glucose (above).

Analysis of metabolic parameters

At different times, samples were collected for analysis of lactate and of [^{3}H]adenine nucleotides and their metabolites. Platelet suspension (1vol.) was mixed with 2vol. of freshly prepared EDTA/ethanol [0.1M-EDTA/96% (v/v) ethanol, 1:9v/v; pH7.4, 0°C]. After centrifugation (10000*g*, 2min, 4°C) the supernatant was analysed for the radioactivity of ethanol-soluble ATP, ADP, IMP, AMP and hypoxanthine + inosine. These metabolites were separated by high voltage paper electrophoresis (60V·cm^{-1}) in 0.05M-citrate buffer (Holmsen *et al.*, 1974) and the radioactivity was counted according to standard procedures. The prelabelling and ethanol

extractions used are essential for metabolic studies in platelets. Firstly, only one-third of total platelet ATP and ADP is cytoplasmic and participates in metabolism; the remainder is present in the dense granules in a slowly exchangeable, metabolically inert form (Reimers *et al.*, 1977). The cytoplasmic pool is labelled by radioactive adenine and the radioactivity is distributed among the cytoplasmic adenine nucleotides according to their concentrations (Daniel *et al.*, 1979, 1980). Secondly, a substantial portion of cytoplasmic ADP is bound to actin and inaccessible for metabolism; ethanol does not extract the protein-bound ADP, but extracts the remainder of the nucleotides (Daniel *et al.*, 1979, 1980). With the combined ethanol extraction/electrophoresis technique cytoplasmic ATP is determined with a precision of 1.2%. Lactate was assayed as described elsewhere with a precision of 4% (Hohorst, 1965).

Analysis of functional parameters

Since platelets contain at least three types of secretory granules, the secretion response was monitored by measuring the extracellular appearance of markers that were specific for each class of granules. These were: [^{14}C]serotonin for the dense granules (Holmsen *et al.*, 1973), β-thromboglobulin for the α-granules (Kaplan *et al.*, 1978) and *N*-acetyl-β-D-glucosaminidase (EC 3.2.1.30), for the acid hydrolase-containing granules (Dangelmaier & Holmsen, 1980). At 5 min before initiation of secretion, 2.5 μM-imipramine (Geigy, Basel, Switzerland) was added to prevent re-uptake of secreted [^{14}C]serotonin by the platelets (Walsh & Gagnatelli, 1974). Secretion was started with 5 N.I.H. units of bovine α-thrombin · ml^{-1} (La Roche, Basel, Switzerland). At various times thereafter, samples were withdrawn and immediately mixed with 0.15 vol. of 1.035 M-formaldehyde in saline (0°C) in order to stop secretion (Costa & Murphy, 1975). After centrifugation (10000 *g*, 1 min, 4°C) the supernatants were collected and used for analysis of [^{14}C]serotonin (counted according to standard procedures) and β-thromboglobulin (measured with the β-thromboglobulin kit from The Radiochemical Centre, Amersham), after diluting the samples in Ca^{2+}-free Tyrode solution to different extents (depending on the amount of β-thromboglobulin in the samples) followed by a final dilution in 4 vol. of albumin-free Tyrode solution containing 20% (v/v) horse serum. This procedure minimized non-specific binding and allowed accurate kinetic measurements of β-thromboglobulin when all data were expressed relative to maximal secretable amounts (Akkerman *et al.*, 1982). β-*N*-Acetylglucosaminidase was measured according to Troost *et al.* (1976).

Miscellaneous

Platelets were counted in a Thrombo counter (Coulter Electronics, Harpenden, Herts., U.K.). Cell lysis was minimal (2.6 ± 0.7%, mean ± S.D., $n = 8$; based on liberation of lactate dehydrogenase) and was the same whether or not the suspensions were treated with metabolic inhibitors.

Expressions

The amount of metabolic ATP in platelets has recently been established by means of: (*a*) rapid isolation of the cytosolic compartment followed by direct quantification (Akkerman *et al.*, 1980); (*b*) subtraction of secreted ATP from total ATP (Daniel *et al.*, 1980; Holmsen *et al.*, 1969); and (*c*) calculation from the specific radioactivity of actin-bound, ethanol-insoluble ADP (Daniel *et al.*, 1980). The result ranged narrowly between 4.40 (Akkerman *et al.*, 1980) and 4.70 (Daniel *et al.*, 1980) μmol · (10^{11} platelets)$^{-1}$. After prelabelling the cells with radiolabelled adenine under the same conditions, 83–85% of total radioactivity is found in the ATP fraction (Akkerman & Gorter, 1980; Akkerman *et al.*, 1978). Since the ATP and ADP in the dense granules remain unlabelled during a short incubation (Holmsen *et al.*, 1974) it follows that a 1% change in ATP radioactivity corresponds to 0.0532 μmol of metabolic ATP · (10^{11} platelets)$^{-1}$. Based on the measured radioactivity the absolute amounts of ATP and metabolites were calculated and expressed as μmol of ATP equivalents ($ATP_{eq.}$; Atkinson, 1977), which reflects an energy yield of 2 $ATP_{eq.}$ for the conversion of ATP to AMP and of 1 $ATP_{eq.}$ for the conversion of ATP to ADP. Throughout the manuscript energy consumption is defined as the loss of metabolic energy stored in ATP and ADP.

Secretion of granule markers was expressed as a percentage of maximally secretable amounts, the latter being the amount of marker secreted after 5 min incubation with 5 units of thrombin · ml^{-1} (37°C) in the presence of 1 mM-KCN and 1 mM-glucose. The effect of the inhibitors on secretion velocity was measured by comparing inhibited and uninhibited suspensions; here too secretion was expressed as a percentage of maximally secretable amounts found in CN^--treated platelets. The total secretable amounts (no inhibitors present) of [^{14}C]serotonin, β-thromboglobulin and β-*N*-acetylglucosaminidase were respectively 92 ± 5, 95 ± 7 and 57 ± 13% (mean ± S.D., $n = 5$) of the total amounts found in extracts of non-stimulated platelets.

Results

Assessment of energy consumption by abrupt arrest of ATP regeneration

When platelet-rich plasma was incubated with [^{3}H]adenine (45 min, 37°C), about 80% of total ^{3}H

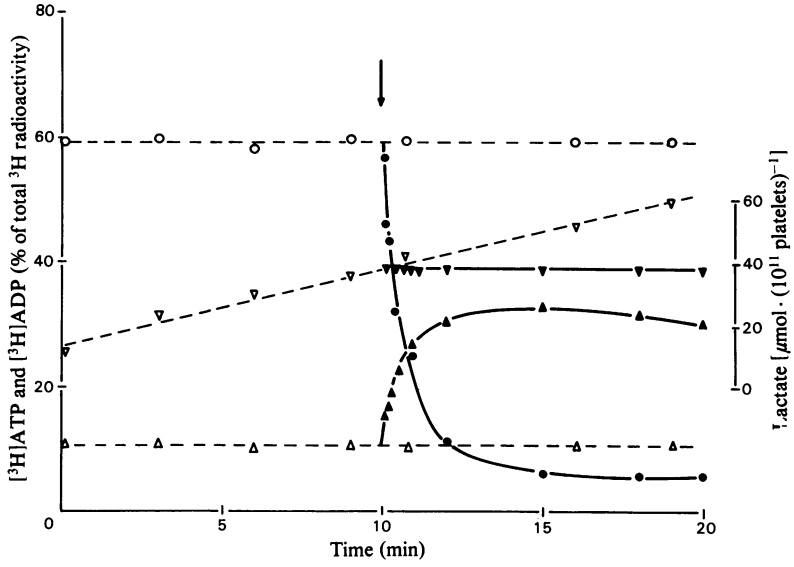

Fig. 1. *Effect of deoxyglucose/gluconolactone on lactate production and [3H]ATP and [3H]ADP of CN⁻-treated platelets*
[3H]Adenine-labelled platelets were gel-filtered in a medium containing 1 mM-CN^- without glucose and subsequently incubated with 1 mM-glucose (37°C). Lactate (▼,▽), expressed as $\mu mol \cdot (10^{11}$ platelets$)^{-1}$, [3H]ATP (○,●) and [3H]ADP (△,▲), both expressed as % of total [3H]radioactivity, were measured in control suspensions (no additional inhibitors, open symbols) as well as after addition of a mixture of deoxyglucose (30 mM) and gluconolactone (10 mM) (arrow; closed symbols).

radioactivity was incorporated into ATP. Subsequent gel-filtration in CN^--containing, glucose-free medium reduced the level of [3H]ATP to about 60% of total 3H radioactivity. This level stabilized upon addition of 1 mM-glucose, which induced a constant production of lactate of $2.8 \mu mol \cdot min^{-1} \cdot (10^{11}$ cells$)^{-1}$. Since mitochrondrial ATP production was blocked (1 mM-CN^-) and glycogenolysis is absent at 1 mM-glucose or more (Akkerman & Gorter, 1980), the production of 1 mol of lactate reflected the regeneration of one mol of ATP from ADP. This rate of ATP production maintained ATP homeostasis for at least 20 min (Fig. 1). Addition of a mixture of deoxyglucose and gluconolactone abolished the production of lactate within 5 s, and induced an abrupt fall in [3H]ATP. Simultaneously, [3H]ADP accumulated until it and [3H]ATP levelled off at 3 and 0.1 times their initial levels, respectively. The remainder of the 3H radioactivity was recovered in AMP, IMP and hypoxanthine/inosine; the sum of 3H-labelled fractions remained constant throughout any experiment.

The changes of [3H]ATP and [3H]ADP following abolition of ATP regeneration reflected a net loss of metabolic energy, probably as a result of continuing energy consumption. For the first 25 s after addition of the inhibitors the changes in [3H]ATP and [3H]ADP were practically linear with time indicating a constant consumption of ATP by energy-requiring processes during this period (Figs. 1 and 2). Moreover, the rates of these linear changes were practically identical after inhibitors were added to successive aliquots of the same platelet suspension (Fig. 2). Since ATP homeostasis was maintained for at least 20 min (Fig. 1) it was possible that the constant linear changes reflected directly ATP consumption before abolition of ATP regeneration. If this were true, the rate of ATP consumption calculated from lactate production before addition of inhibitors should be identical with that derived from the changes in [3H]ATP and [3H]ADP after this addition. This possibility was tested by measuring ATP consumption with both methods using variations in temperature as a means to induce different rates of energy consumption. Fig. 3 illustrates that both methods correlated well over a broad range of energy consumption velocities. Even at a low temperature (10–15°C) they gave similar results despite the fact that it took more time (10 s instead of 5 s) for the inhibitors to block ATP resynthesis and induce a fall in [3H]ATP.

Energy consumption in platelets with and without thrombin at various temperatures

The measurement of ATP consumption by determining the rate of decrease in ATP equivalents after

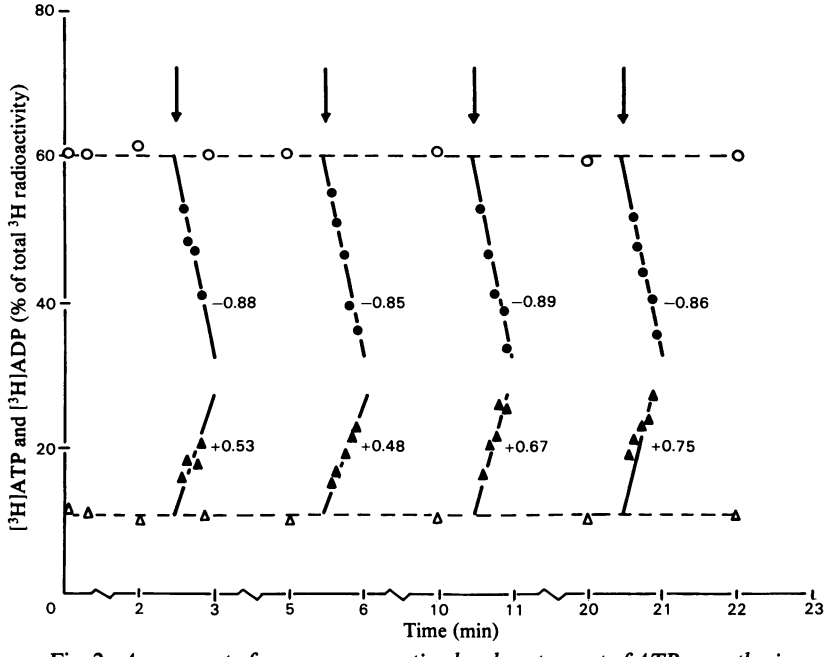

Fig. 2. *Assessment of energy consumption by abrupt arrest of ATP resynthesis*

Changes in [³H]ATP (○,●) and [³H]ADP (△,▲) expressed as % of total [³H]radioactivity after arrest of ATP resynthesis by addition of deoxyglucose/gluconolactone (arrows; closed symbols). Open symbols refer to control suspensions (no deoxyglucose/gluconolactone). The numbers to the right of the solid lines give % change in [³H]ATP and [³H]ADP per s.

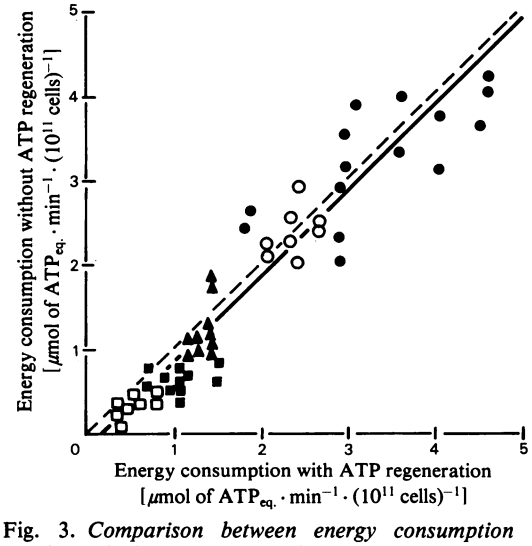

Fig. 3. *Comparison between energy consumption in uninhibited platelets and in platelets with blocked ATP resynthesis at various temperatures*

Energy consumption [expressed as μmol of ATP$_{eq.}$ consumed · min^{-1} · (10^{11} platelets)$^{-1}$] was calculated from lactate production in CN^--treated platelets in which the steady-state levels of ATP and ADP (measured as [³H]ATP and [³H]ADP) remained constant (platelets with ATP regeneration). Deoxyglucose and gluconolactone were then added and the ATP consumption of the same suspension of platelets, now without ATP regeneration, was calculated from the changes in [³H]ATP and [³H]ADP during the first 25 s. The data show the results of incubation studies at 37°C (●), 30°C (○), 22°C (▲), 15°C (■) and 10°C (□). The solid line is that calculated from the experimental points [regression line with coefficient of correlation: $y = 1.02x - 0.18$; $r = 0.92$ ($n = 52$)]. The dotted line indicates a 1:1 relationship.

abrupt abolition of ATP regeneration could be accomplished within 25 s. Such rapidity allows this new technique to be applied to rapid cellular processes such as secretion which, in platelets, is completed within 25–40 s with maximal stimulation. Energy consumption was temperature-dependent both in unstimulated platelets and in platelets stimulated with 5 units of thrombin · ml^{-1}. At 37°C unstimulated platelets consumed about 3.5 μmol of ATP$_{eq}$ · min^{-1} · (10^{11} cells)$^{-1}$. Lowering the temperature to 10°C decreased the ATP consumption by about 85%. Stimulated suspensions at 37°C

consumed 4.5 times more ATP than did unstimulated suspensions and reached 16μmol of $ATP_{eq} \cdot min^{-1} \cdot (10^{11}$ cells$)^{-1}$. Again, lowering the temperature reduced the ATP consumption, but at all temperatures between 10 and 42°C ATP consumption in stimulated cells was 3.5–4.5 times higher than in unstimulated suspensions.

Energy consumption in thrombin-treated platelets at a decreasing ATP level

Another way of modulating the availability of ATP in platelets was to stimulate the cells with thrombin at various times after arresting ATP resynthesis. Fig. 4 shows that the fall in [^{3}H]ATP induced by the inhibitors accelerated upon thrombin addition, but the lower the ATP level was when thrombin was added, the slower was the subsequent decrease in [^{3}H]ATP. At an initial [^{3}H]ATP level of 65–70% of total radioactivity, the total thrombin-induced energy consumption was about 18μmol of $ATP_{eq.} \cdot min^{-1} \cdot (10^{11}$ cells$)^{-1}$. This value decreased to 5–6μmol of $ATP_{eq.} \cdot min^{-1} \cdot (10^{11}$ cells$)^{-1}$ when the initial [^{3}H]ATP level had decreased to 20% of total radioactivity. At high initial [^{3}H]ATP levels, [^{3}H]ADP accumulated (as shown in Figs. 1 and 2), but, as the initial [^{3}H]ATP level fell, less and less [^{3}H]ADP accumulated and when the initial [^{3}H]ATP level fell below 25–30% of total radioactivity [^{3}H]ADP actually decreased after thrombin addition (results not shown).

Correlation between the initial velocity of secretory responses and the accompanying ATP consumption rates in thrombin-treated platelets

We have shown above that the thrombin-induced ATP consumption in platelets can be varied by (1) changing the temperature and (2) reducing the initial ATP level by preincubation with deoxyglucose/gluconolactone. The secretion of constituents from the dense granules ([^{14}C]serotonin), α-granules (β-thromboglobulin) and acid hydrolase-containing granules (β-*N*-acetylglucosaminidase) was immediately stopped by transferring aliquots of the reaction mixture to ice-cold formaldehyde solution; the resulting mixtures were then centrifuged and the amounts of the secretion markers were determined in the supernatants. Secretion of all markers was approximately linear with time between 0 and 10s after addition of thrombin at 37°C and between 0 and 40s at 15°C. The amounts secreted after 10s at 37°C and 30s at 15°C, the latter divided by 3, were therefore taken as a measure for initial secretion velocity. Secretion was expressed as percentage of the amounts liberated after maximal stimulation (see

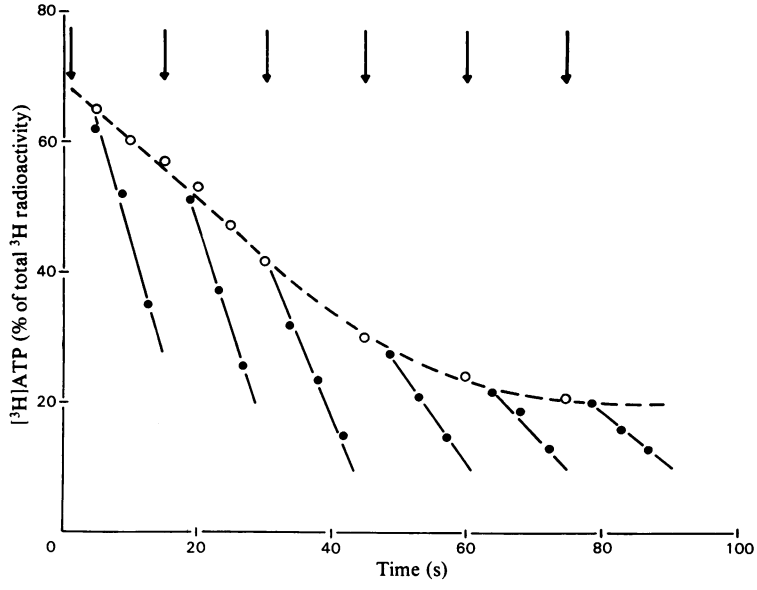

Fig. 4. *Effect of deoxyglucose/gluconolactone and subsequent thrombin stimulation on [^{3}H]ATP levels of CN$^-$ treated platelets*

CN$^-$-treated platelets were incubated with 1mM-glucose (37°C). At time zero, deoxyglucose and gluconolactone were added followed by 5 units of thrombin $\cdot$ ml^{-1} at 1, 15, 30, 45, 60 and 75s later. [^{3}H]ATP and [^{3}H]ADP (% of total [^{3}H]radioactivity) were measured at various times after thrombin addition (arrows, ●) as well as in control (no thrombin added) suspensions (○). For the sake of clarity the [^{3}H]ADP data have been omitted.

Table 1. *Effect of abrupt arrest of ATP regeneration on secretory responses in platelets*

One batch of gel-filtered platelets containing no cyanide was incubated with 1 mM-glucose for 20 min at 22°C and divided into two halves. One half was then incubated with and the other half without 1 mM-KCN for a further 15 min. Samples of both portions were equilibrated for 3 min at 15 or 37°C before the CN^--containing samples were mixed with thrombin, 30 mM-deoxyglucose (dGlc) and 10 mM-gluconolactone (GLAC) while the CN^--free portions were mixed with thrombin only. The final concentration of thrombin was 5 units · ml^{-1} in either mixture. The secretion of granule markers was analysed after 10 s (37°C) and 30 s (15°C) and the data were normalized by dividing the 30 s data by 3. Data were corrected for liberation of markers in the absence of thrombin, which was below 3% of maximal secretable at both temperatures for all markers, with or without the inhibitors. Data are means ± S.D. from five different suspensions and are expressed as % of maximal secretable amounts found in CN^--treated platelets (defined as 'maximal secretable'; see the Materials and methods section). The effect of CN^- is illustrated by comparing maximal secretable amounts with total secretable amounts, previously defined as the amounts secreted after 5 min incubation with 5 units of thrombin · ml^{-1} at 37°C in the absence of inhibitors (Holmsen, 1978).

		Secretory response (% of maximal secretable)		
Conditions	Inhibitors	Serotonin	β-Thromboglobulin	*N*-Acetyl-β-D-glucosaminidase
15°C, 10 s	None	11.7 ± 2.2	2.4 ± 0.4	4.2 ± 0.9
	CN^-/dGlc/GLAC	15.6 ± 1.8	3.2 ± 0.5	4.6 ± 0.8
37°C, 10 s	None	78.5 ± 4.4	39.2 ± 5.3	23.6 ± 6.4
	CN^-/dGlc/GLAC	83.7 ± 5.2	47.5 ± 4.6	29.1 ± 4.2
37°C, 5 min	None	103.5 ± 1.2	109.1 ± 7.0	143.2 ± 10.5
	CN^-	(100)	(100)	(100)

the Materials and methods section). Table 1 shows that, surprisingly, the secretion velocities of all markers were slightly but consistently potentiated by the inhibitors. The total amounts secreted after 5 min in the presence of CN^- only were comparable with those found in the absence of inhibitors, with the exception of β-*N*-acetylglucosaminidase which was reduced by about 30%.

The initial rate of the individual secretory responses decreased both with decreasing temperature and with decreasing initial ATP level. In Fig. 5 the velocities of secretion of serotonin, β-thromboglobulin and β-*N*-acetylglucosaminidase are compared with the concomitant energy consumption; both the total and incremental ATP consumption are plotted in Figs 5(*a*) and 5(*b*), respectively, the latter being derived from the difference between the energy consumption in thrombin-treated and in unstimulated cells. The velocity of secretion of serotonin was almost linearly related to the corresponding total and incremental ATP consumption (left hand panels, Figs. 5*a* and 5*b*, respectively). Most importantly, the velocity-versus-energy-consumption relationship was the same for the temperature experiments as for the experiments with decreasing ATP levels. The results with secretion of β-thromboglobulin were generally similar (middle panels, Figs. 5*a* and 5*b*). However, the slope of the curve of secretion velocity versus ATP consumption for α-granule secretion was distinctly smaller than that for dense granule secretion (compare left and middle panels, Figs. 5*a* and 5*b*). For example, at a total ATP consumption rate of 12.5 μmol of $ATP_{eq.}$ · min^{-1} · $(10^{11}$ cells$)^{-1}$ the velocity of α-granule secretion is 39% of total secretable in contrast to 76% for dense granule secretion (per 10 s).

For the secretion of β-*N*-acetylglucosaminidase, the velocity also increased with increasing ATP consumption, but not as linearly as for the two other secretory processes. The velocity at a given consumption rate was distinctly higher in the temperature experiments than in the experiments with lowering of the ATP level (Figs 5*a* and 5*b*, right hand panels). Also, the slope of the curves of velocity versus ATP consumption, irrespective of experimental approach, was smaller for secretion of β-*N*-acetylglucosaminidase than for the secretion of the two other markers at ATP consumption rates above 10 μmol of $ATP_{eq.}$ · min^{-1} · $(10^{11}$ cells$)^{-1}$. Below this rate, the velocities of secretion of β-*N*-acetylglucosaminidase and of β-thromboglobulin were the same at any ATP consumption rate in the temperature experiments, while they were distinctly lower in the experiments with decreasing ATP level.

Apparent energy requirement for the individual secretory responses

The apparent energy consumption associated with the individual secretory responses could be expressed as an 'energy quotient', i.e., the amount of ATP consumed per unit of secretory response. Such energy quotients were calculated from our results obtained at 37°C (Fig. 5) and are shown in Table 2.

With 5 units of thrombin · ml^{-1} secretion of the three markers measured is complete within 25 s and

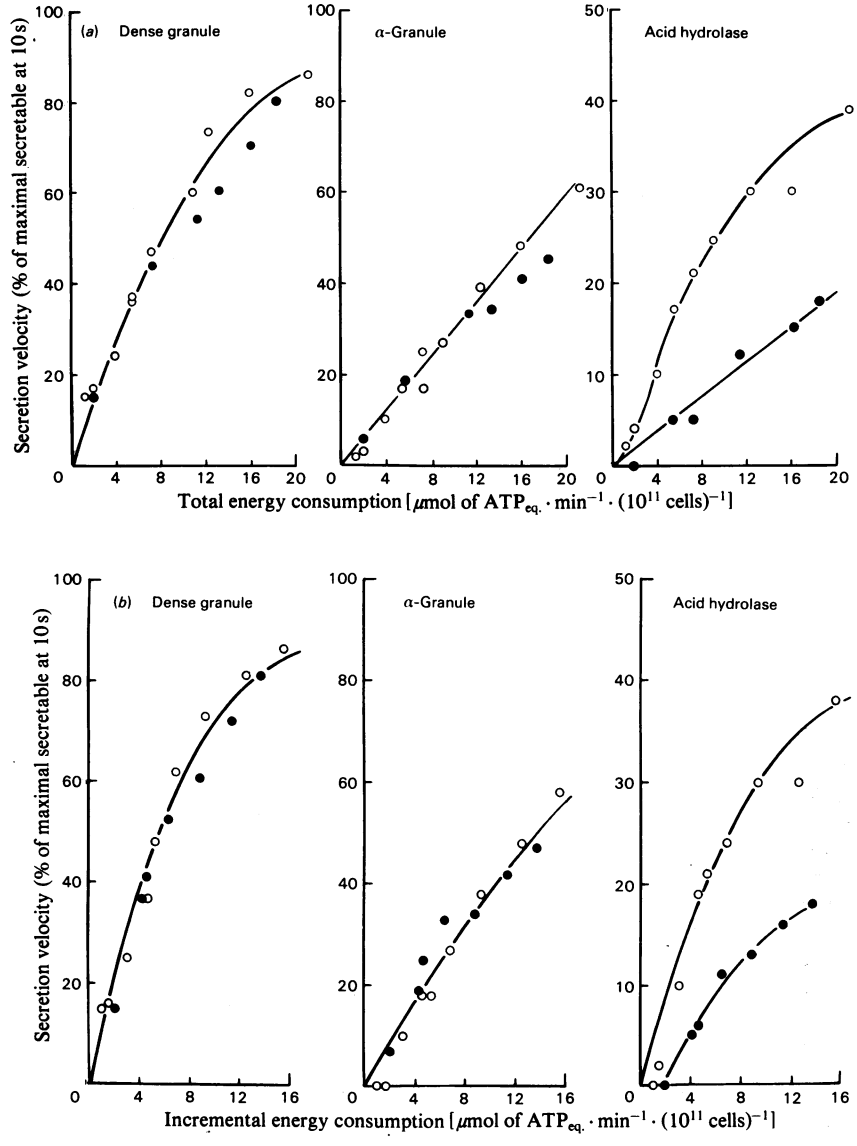

Fig. 5. *Comparison between secretion velocity and simultaneous energy consumption*
Energy consumption [expressed as μmol of $ATP_{eq.} \cdot min^{-1} \cdot (10^{11}$ cells)$^{-1}$] at various temperatures (open circles) and at various initial ATP levels (closed circles) versus the corresponding initial secretion velocity (expressed as % of maximal secretable at 10s) of serotonin (dense granule), β-thromboglobulin (α-granule) and *N*-acetyl-β-D-glucosaminidase (acid hydrolase). (*a*) Total energy consumption in thrombin-treated platelets; (*b*) incremental energy consumption (difference between thrombin-treated and unstimulated suspensions).

each parameter is secreted almost linearly with time. Since the increased rate of ATP consumption triggered by thrombin also is almost constant during this period of time (see Fig. 4), it is justified to calculate how much ATP would be consumed for complete secretion by multiplying the energy quotients by 100 (Table 2). Thus, platelets consume 3.2, 5.4 and 8.6 μmol of $ATP_{eq.}$ per 10^{11} cells during complete dense granule, α-granule and acid hydrolase secretion, respectively.

Discussion

We demonstrate here that ATP consumption in platelets can be determined as the rate of the

Table 2. *Energy quotients for secretory processes in platelets*

The numbers are calculated from the results obtained at 37°C by dividing the total and incremental energy consumption (nmole of ATP · s^{-1}) by the individual secretion velocities (% of total · s^{-1}). The unit for the energy quotient becomes nmol of ATP consumed by 10^{11} platelets for 1% of total secretion. The data for acid hydrolase secretion are based on the values found in the experiments with varying temperatures.

Secretory response	Energy quotient (nmol · 10^{11} cells · % secretion^{-1})		Energy consumed for complete secretion [μmol of $ATP_{eq.}$ · (10^{11} cells)$^{-1}$]	
	Total	Incremental	Total	Incremental
Dense granule secretion	32	25	3.2	2.5
α-Granule secretion	54	42	5.4	4.2
Acid hydrolase secretion	86	67	8.6	6.7

decrease in the cytoplasmic concentration of ATP equivalents after abrupt abolition of ATP regeneration. This new technique, with which ATP consumption is determined within 15–20 s, makes use of the following facts. Firstly, in human platelets metabolic ATP and ADP are the only known intermediates in the transduction of metabolic energy from sites of generation to sites of utilization (there is no phosphocreatine, and exchange with UTP, GTP etc. is slow; see Meltzer & Guschwan 1972; Rivard *et al.*, 1975). Secondly, metabolic ATP and ADP can be precisely measured with isotopic tracer techniques which do not label the granule pools (Daniel *et al.*, 1980). Thirdly, platelets have a complete Pasteur effect which permits ATP homeostasis to be maintained when oxidative resynthesis of ATP is abolished (Akkerman & Gorter, 1980). Fourthly, the minimal concentrations of glucose required for ATP homeostasis in CN^--treated platelets (Akkerman & Gorter, 1980; Akkerman *et al.*, 1978), as well as the optimal concentrations of deoxyglucose and gluconolactone for rapidly blocking glycolysis and glycogenlysis, have been established (Holmsen & Robkin, 1980; Holmsen & Akkerman, 1980).

Addition of the deoxyglucose/gluconolactone mixture to the CN^--containing platelet suspensions induced an abrupt abolition of glyco(geno)lytic lactate production within 5 s, i.e., ATP production was abolished. During the first 30 s after this abolition the changes in [^{3}H]ATP and [^{3}H]ADP were approximately linear and the rate of energy consumption calculated from these changes corresponded well with the velocity of energy consumption calculated from lactate measurements prior to abolition of ATP production. These results show that abrupt arrest of ATP resynthesis does not immediately affect the velocity of energy-consuming processes, so that the changes in ATP and ADP following sudden metabolic arrest are an accurate and true reflection of cellular energy consumption. They also indicate that the phosphorylation of 2-deoxyglucose hardly interferes with these rapid measurements.

With this method, we demonstrate that resting, gel-filtered platelets incubated at 37°C consume about 3.5 μmol of $ATP_{eq.} \cdot min^{-1} \cdot (10^{11}$ cells$)^{-1}$. With a metabolic ATP content of 4.5 μmol · (10^{11} cells$)^{-1}$ this implies that the metabolic ATP pool turns over once every 77 s. By lowering the temperature this rate declines and reaches 0.03 μmol of $ATP_{eq.} \cdot min^{-1} \cdot (10^{11}$ cells$)^{-1}$ at 10°C, which is equivalent to an ATP turnover of 2½h. When the platelets were treated with thrombin there is a 3.5–4.5 fold increase in ATP consumption, which is almost independent of the temperature. At 37°C thrombin causes an increase from 3.5 to 16 μmol of $ATP_{eq.} \cdot min^{-1} \cdot (10^{11}$ cells$)^{-1}$, which is in the range found in uninhibited gel-filtered platelets on the basis of lactate production and mitochondrial O_2 uptake (Akkerman & Holmsen, 1981), but much higher than in EDTA-washed cells (Holmsen & Robkin, 1980). Secretion is generally accompanied by an increase in the production of ATP (Rossignol *et al.*, 1974; Akkerman & Holmsen, 1981; Malaisse *et al.*, 1977) and has led to the current belief that secretory responses require an intact ATP resynthesizing apparatus. Our present data show that ATP resynthesis is not essential for initiation of secretory responses but they do stress the need for ATP.

Although the need for ATP thus appears evident, this does not automatically imply that velocity or extent of secretory responses are determined by the availability of ATP. In platelets, however, several observations do favour such a coupling. Firstly, platelet stimulation with ADP is accompanied by a rapid and transient fall in the adenylate energy charge (Mills, 1973). Secondly, several platelet responses are gradually inhibited when the availability of ATP is reduced, both in an irreversible (with metabolic inhibitors; Holmsen *et al.*, 1974, 1979. 1982) and reversible manner (during starvation–

refeeding; Akkerman & Gorter, 1980). Thirdly, such responses are more closely correlated with the adenylate energy charge than with ATP levels, the former being a better reflection of the cell's capacity to provide ATP (Holmsen & Robkin, 1977). Fourthly, once ATP regeneration is blocked, such responses do correlate with the ATP level which is then the only remaining source for ATP (Akkerman *et al.*, 1979).

These observations suggest that the various reactions that are involved in stimulus–response coupling are coupled to the cell's energy metabolism and require different amounts of energy, depending on the response they trigger. Our strongest evidence for an apparent coupling between platelet responses and ATP consumption is the finding that the proportionality between secretion velocity and concomitant ATP consumption remained identical irrespective of how the velocity was varied, by altering the temperature or by changing the initial ATP level (Fig. 5). The finding that the velocity of secretory responses was directly proportional to the energy consumption also argues strongly for coupling. However the slope of the curve for the velocity versus ATP consumption was steeper for dense granule secretion than for α-granule secretion, and the latter was in turn steeper than for acid hydrolase granule secretion, indicating that the energy requirement increases in the order of dense granule, α-granule and acid hydrolase secretion. At 37°C, this difference in associated ATP consumption could be expressed quantitatively by comparing the energy quotients, which shows a 1.7 times greater coefficient for α-granule secretion than for dense granule secretion. An even higher coefficient is found for acid hydrolase secretion.

Both the variations in temperature and the variations in initial ATP level affect subsequent studies on energy consumption by changing factors in the expression of the free energy of hydrolysis of ATP ($\Delta G = \Delta G^0 + RT \ln [ADP][P_i]/[ATP]$). It is difficult to quantify these effects from the present study, since ATP, ADP and P_i participate in many reactions, such as the phosphorylation of fructose-6-phosphate, some of which occur in different cell compartments. The variation in the absolute temperature is minor (about 10%) but its contribution to ΔG is uncertain. Readjustment of our data for different values of ΔG therefore awaits further identification of these effects.

With 5 units of thrombin $\cdot$ ml^{-1} at 37°C the rates for ATP consumption and secretion are fairly constant. Our results show that complete dense-, α- and acid hydrolase granule secretion are accompanied by a total consumption of 3.2, 5.4 and 8.6 μmol of $ATP_{eq.} \cdot (10^{11}$ cells$)^{-1}$, respectively (Table 2). Since platelets contain 4.5 μmol of metabolic ATP and 0.5 μmol of metabolic ADP per 10^{11} cells (Daniel *et al.*, 1979, 1980), they contain an energy potential of 9.5 μmol of $ATP_{eq.}$ once ATP regeneration is blocked. One would therefore expect that secretion is normal in platelets with blocked ATP and ADP levels. The present paper shows that the velocity of all three secretion responses is normal despite the arrest in ATP regeneration (they are even slightly increased; Table 1). The extent of dense granule secretion (Akkerman *et al.*, 1979) and α-granule secretion (J. W. N. Akkerman, unpublished work) is also unaffected. Only the extent of acid hydrolase granule secretion is diminished, which is mainly due to the CN^- treatment (Table 1). Acid hydrolase secretion is also inhibited by antimycin A (Holmsen *et al.*, 1979). These results suggest that mitochondrial energy support may play a role in the later stages of acid hydrolase granule secretion.

The slight stimulation of the secretory responses observed when thrombin was added together with the metabolic inhibitors (Table 1) could be caused by a greater availability for ATP immediately after stimulation of the cells. Within seconds after addition of 5 units of thrombin $\cdot$ ml^{-1}, a considerable part of ATP (more than 10%) is consumed in phosphorylation of fructose 6-phosphate (J. W. N. Akkerman & H. Holmsen, unpublished work). This process is abolished by 2-deoxyglucose and gluconolactone, thus leaving more ATP available in the first seconds after stimulation than in uninhibited platelets.

The velocity of acid hydrolase secretion increased with increasing velocity of energy consumption. However, in sharp contrast to the other two secretory responses, the relationship of acid hydrolase secretion versus ATP consumption was dependent on the method used for altering the secretion velocity. Thus, with variation of the ATP level less secretion occurred at a given rate of ATP consumption than when the rate was altered by changing the temperature. The ATP level was altered by use of metabolic inhibitors, so that the platelets had experienced metabolic blockade before thrombin was added. It is therefore possible that acid hydrolase secretion, in contrast to the other secretory responses, depends on energy-dependent processes that take place in the resting platelet.

In conclusion, the present study shows that the three secretory responses have different apparent energy requirements, and that these are maximally 3.2, 5.4 and 8.6 μmol of $ATP_{eq.} \cdot (10^{11}$ cells$)^{-1}$ for complete dense granule secretion, α-granule secretion and acid hydrolase secretion, respectively. It is possible that the basal ATP consumption occurring in resting platelets continues unaltered after platelet stimulation, and this is supported by the better correspondence of the curve points for the two approaches in Fig. 5 when the basic energy consumption is subtracted. This reduces the energy

consumption that occurs during completion of the three secretory processes to the incremental values, 2.5, 4.2 and 6.7 μmol of $ATP_{eq.} \cdot (10^{11}$ platelets$)^{-1}$.

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