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Quantification of energy consumption in platelets during thrombin-induced aggregation and secretion

Tight coupling between platelet responses and the increment in energy consumption

Adrie J. M. VERHOEVEN, Marlène E. MOMMERSTEEG and Jan Willem N. AKKERMAN
Department of Haematology, University Hospital Utrecht, P.O. Box 16250, 3500 CG Utrecht, The Netherlands

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The involvement of metabolic energy in platelet responses was investigated by measuring the energy consumption during aggregation and secretion from dense, α- and acid-hydrolase-containing granules. Gel-filtered human platelets were stimulated with different amounts of thrombin (0.05–5.0 units·ml^{-1}). At various stages during aggregation and secretion the energy consumption was measured from the changes in metabolic ATP and ADP following abrupt arrest of ATP resynthesis. Stimulation with 5 units of thrombin·ml^{-1} increased the energy consumption from 6.2 ± 0.9 to 17.8 ± 0.4 μmol of $ATP_{eq.}$·min^{-1}·$(10^{11}$ platelets$)^{-1}$ during the first 15 s. It decreased thereafter and returned to values found in resting cells after about 30 s. With 0.05 unit of thrombin·ml^{-1}, the energy consumption accelerated more slowly and took at least 3 min before it normalized. A strong positive correlation was found between the velocities of the three secretion responses and the concurrent energy consumption (*a*) at different stages of the responses induced by a given dose of thrombin, and (*b*) at different secretion velocities initiated by different amounts of thrombin. When, at different stages of the responses, the extent of secretion was compared with the amount of energy that had been consumed, a strong linear correlation was found with the increment in energy consumption but not with the total energy consumption. This correlation was independent of the concentration of thrombin and indicated that complete secretion from dense, α- and acid-hydrolase-containing granules was paralleled by an increment of 4.0, 6.5 and 6.7 μmol of $ATP_{eq.}$·$(10^{11}$ platelets$)^{-1}$, respectively. An energy cost of 0.7 μmol of $ATP_{eq.}$·$(10^{11}$ platelets$)^{-1}$ was calculated for separate dense-granule secretion, whereas the combined α- and acid-hydrolase granule secretion required 5.3 μmol of $ATP_{eq.}$·$(10^{11}$ platelets$)^{-1}$. There was no correlation between energy consumption and optical aggregation. In contrast, the rate of single platelet disappearance, which is a measure for the early formation of small aggregates, correlated closely with the rate of energy consumption. Compared with secretion, however, the energy requirement of single platelet disappearance was minor, since 2 mM-EDTA completely prevented this response but decreased the energy consumption only slightly. An increase of 0.5–1.0 μmol of $ATP_{eq.}$·$(10^{11}$ platelets$)^{-1}$ was seen before single platelet disappearance and the three secretion responses were initiated, indicating an increase in energy consuming processes that preceded these responses. The current studies indicate that, throughout the responses, secretion is tightly coupled to the increment in energy consumption. The association with energy metabolism is much weaker for aggregation.

The activity of secretory cells is regulated by a limited number of second messengers that link receptor occupancy to the mechanisms that execute exocytosis in a sequence known as stimulus–response coupling. Along this sequence, there are several steps that depend on metabolic energy, e.g.

the phosphatidylinositol response (Billah & Michell, 1979; Jones *et al.*, 1979; Lapetina & Cuatrecasas, 1979) and actomyosin contractile activity (Lebowitz & Cooke, 1978; Daniel *et al.*, 1981), which suggests that metabolic energy is required for secretion responses. Recent observations in many types of secretory cells are in line with this concept and demonstrate that secretion is accompanied with a transient fall in ATP concentration and adenylate energy charge (Mills, 1973; Akkerman & Holmsen, 1981; Borregaard & Herlin, 1982), and an increase in ATP resynthesizing processes (Rossignol *et al.*, 1974; Malaisse *et al.*, 1977; Akkerman & Holmsen, 1981), apparently in response to an increased demand for metabolic energy.

Recently, more insight in the role of metabolic energy in secretion responses has been gained by the development of a quantitative method for the assessment of energy consumption in platelets, which, when properly stimulated, aggregate and secrete the contents of at least three types of granules (Akkerman *et al.*, 1983*a*). In this technique the rate of energy consumption is derived from the changes in metabolic ATP and ADP immediately following abrupt arrest of ATP resynthesis. Analysis of the first 10s after stimulation with a fixed, maximal dose of thrombin showed that during this initial phase secretion was accompanied by consumption of a specific amount of metabolic energy. At a given secretion velocity this amount differed between secretion from dense, α- and acid-hydrolase-containing granules. It remained unclear, however, what was the relation between secretion and energy at later stages and whether all energy consumption was involved, or just the increment seen in stimulated cells. The present study is addressed to these questions and demonstrates that the energetics of secretion are independent of the dose of thrombin with which these functions are initiated. In addition, it shows that, compared with that of secretion, the energy requirement of aggregation is minor.

Materials and methods

Platelet isolation

Freshly drawn venous blood was collected from healthy human volunteers into citrate (0.1 vol. of 129mM-sodium citrate). After centrifugation (200**g**, 10min, room temperature) the supernatant, platelet-rich plasma, was incubated with 1 μM-5-hydroxy[*side-chain*-2-^{14}C]tryptamine ([^{14}C]serotonin, sp. radioactivity 58Ci·mol^{-1}; Amersham International) and 1 μM-[2-^{3}H]adenine (sp. radioactivity 10Ci·mmol^{-1}; Amersham International) for 45min at 37°C to label the contents of the dense granules and the metabolic pool of adenine nucleotides, respectively. Platelets were then transferred into Ca^{2+}-free Tyrode's solution (pH7.25, osmolality 300mosM·kg^{-1}) by gel filtration at room temperature on Sepharose 2B (Pharmacia; column size 5cm × 15cm) as described by Walsh (1972), except that albumin and glucose were omitted from the medium. Platelet numbers were standardized at $(1.5–2.5) \times 10^{11}$ cells·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 1h after elution of the platelets from the column.

Incubation conditions

One set of samples of gel-filtered platelets, prewarmed at 37°C for 3min in silicone-treated glass cuvettes (1.48cm diameter) was stirred (900rev./min). After 1min, the platelets were stimulated with different concentrations of bovine α-thrombin (La Roche, Basel, Switzerland; stock solutions prepared at 1000 NIH units·ml^{-1} and dialysed for 24h against 300vol. of albumin- and glucose-free Tyrode's solution). These suspensions were used for analysis of the aggregation and secretion responses.

Under the same conditions another set of samples of gel-filtered platelets was incubated with the same amount of thrombin and used for the determination of energy consumption at different times after stimulation. Control experiments showed that the platelet functions were not affected by the presence of the metabolic inhibitors during the 15s intervals in which energy consumption and platelet responses were compared. Secretion data at 15s after addition of thrombin (0.3unit·ml^{-1}) were $39.5 \pm 3.8\%$ and $39.1 \pm 5.2\%$ for [^{14}C]serotonin ($P > 0.1$; $n = 8$), $18.5 \pm 2.1\%$ and $20.5 \pm 2.6\%$ for β-thromboglobulin ($P > 0.1$; $n = 6$) and $15.0 \pm 1.0\%$ and $15.6 \pm 2.0\%$ for *N*-acetyl-β-D-glucosaminidase ($P > 0.1$; $n = 8$) in the absence and presence of metabolic inhibitors, respectively. It is therefore justified to compare the metabolic studies performed in one sample with the functional studies performed in the other sample.

Assessment of energy consumption

The rate of energy consumption during a 15s interval was derived from the changes in metabolic ATP and ADP between 0 and 15s after abrupt arrest of ATP resynthesizing sequences by using a modification of a previously reported procedure (Akkerman *et al.*, 1983*a*). Gel-filtered platelets were incubated in a glucose-free medium in the absence of metabolic inhibitors. Since platelets lack detectable amounts of glucose (Karpatkin, 1967; Solomon & Gaut, 1970) this treatment

abolished ATP resynthesis in the glycolytic pathway. In contrast, glycogenolysis and mitochondrial respiration were left intact and these pathways together provided sufficient metabolic energy to maintain homeostasis, as reflected by the maintenance of a high adenylate energy charge (0.901 ± 0.013; $n = 14$) during a 20 min incubation. Abrupt arrest of ATP resynthesis was achieved by addition of a mixture of D-gluconic acid-1,5-lactone (gluconolactone, grade IX; Sigma) and antimycin A (Boehringer Mannheim) to final concentrations of 10 mM and $8.25\,mg \cdot l^{-1}$, respectively. Gluconolactone is an effective blocker of glycogenolysis in platelets (Akkerman *et al.*, 1983*a*), whereas antimycin A rapidly abolishes mitochondrial ATP resynthesis, provided that the medium is free of albumin (Slater, 1973; Holmsen & Robkin, 1980). Determinations of lactate production (Gutman & Wahlefeld, 1974) and oxygen consumption (Akkerman *et al.*, 1979*b*) confirmed that energy generation came to a complete standstill within 5 s after addition of these inhibitors.

To determine the rate of energy consumption at various 15 s intervals after stimulation, the inhibitors were added to separate incubation tubes either simultaneously with thrombin, or at 15, 30, 45, 60 and 75 s thereafter. At 5, 10 and 15 s after addition of the inhibitors, samples were collected from the incubation mixture in 2 vol. of freshly prepared 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 ^{3}H-labelled ATP, ADP, IMP, AMP and hypoxanthine/inosine. These metabolites were separated by high-voltage paper electrophoresis ($60\,V \cdot cm^{-1}$) in 50 mM-citrate buffer (Holmsen *et al.*, 1972) and the radioactivity was counted according to standard procedures. As outlined earlier (Daniel *et al.*, 1979, 1980), this technique accurately determines metabolically active ATP and ADP and is neither disturbed by actin-bound ADP nor by ATP and ADP stored in the dense granules, which are unavailable for energy metabolism.

Analysis of functional parameters

Aggregation was measured by two techniques. First, by monitoring the change in light transmission in a Payton Dual Channel Aggregometer (Scarborough, Ontario, Canada). Secondly, by measuring the disappearance of single platelets with a modified version of the method of Frojmovic *et al.* (1983). For the latter technique, samples were collected from the aggregating suspension in 9 vol. of 0.5% glutaraldehyde (Fluka, Buchs, Switzerland) in phosphate-buffered saline (15 mM-sodium phosphate, pH 7.25, at 0°C, filtered through a $0.22\,\mu m$ Millipore filter); $150\,\mu l$ was then further diluted in 10 ml of phosphate-buffered saline and immediately thereafter analysed in a Platelet Analyzer 810 (Baker Instruments, Allentown, PA, U.S.A.) with apertures set between 3.2 and $16\,\mu m^3$. In an unstimulated suspension $92.5 \pm 1.0\%$ (mean $\pm$ S.D. for nine donors) of total platelets fell within these settings. Control studies were performed to evaluate how effective this procedure stopped aggregation and prevented disaggregation. The same results were obtained when the glutaraldehyde solution contained an additional $4\,\mu M$-prostaglandin E_1 (Upjohn, Kalamazoo, MI, U.S.A.) and 10 mM-theophylline (Sigma) which are known to inactivate the platelets by raising the cell's cyclic AMP content (Mills, 1974). Furthermore, the presence of 2 mM-Gly-Pro-Arg-Pro (UCB, Brussels, Belgium), which inhibits aggregation by interfering with fibrinogen binding (Plow & Marguerie, 1982), did not change the results. Aggregates collected in 9 vol. of glutaraldehyde remained stable for at least 5 h, indicating that disaggregation was minimal. This procedure therefore offers an accurate means to evaluate the disappearance of single platelets in an aggregating suspension.

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*a*). Samples of cell suspension were collected at different times after thrombin addition in 0.15 vol. of 1.035 M-formaldehyde in saline (0°C) in order to stop secretion (Costa & Murphy, 1975; Akkerman *et al.*, 1982). [^{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 vesicles. All studies were carried out in the presence of $3\,\mu M$-imipramine (Geigy, Basel, Switzerland) to prevent reuptake of secreted [^{14}C]-serotonin by the platelets (Walsh & Gagnatelli, 1974).

Expressions

Throughout this paper energy consumption is defined as the loss of energy stored in metabolic ATP and ADP following metabolic arrest. On the basis of a metabolic ATP content of $4.5\,\mu mol \cdot (10^{11}\ platelets)^{-1}$ and the fact that in normal [^{3}H]-adenine-labelled platelets 80% of the total radioactivity is found in ATP, a 1% change in ^{3}H radioactivity corresponds to $0.056\,\mu mol$ of $ATP \cdot (10^{11}\ platelets)^{-1}$ (Akkerman *et al.*, 1983*a*). The energy potential, defined as the energy stored in metabolic

ATP and ADP, was expressed in terms of ATP equivalents ($ATP_{eq.}$; 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 energy potential during the initial 15s after addition of antimycin A/glucono-lactone by linear regression analysis, expressed as ($\Delta ATP_{eq.}/\Delta t$) and plotted at the halfway point of each 15s interval. The adenylate energy charge [(ATP + ½ADP)/(ATP + ADP + AMP)] was calculated according to Atkinson (1977).

Secretion of granule markers was expressed as a percentage 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 $\cdot ml^{-1}$ in the absence of metabolic inhibitors. Coefficients of variation for the secretion measurements at 15s after stimulation with 5 units of thrombin $\cdot ml^{-1}$ were 4, 10 and 8% for [^{14}C]serotonin, β-thromboglobulin, and N-acetyl-β-D-glucosaminidase, respectively ($n = 10$). The rate of secretion at different stages of the responses was determined by measuring the increase in extracellular marker during a 15s interval. The data were expressed as percentage secretion per 15s and plotted at the halfway point of each interval.

Throughout incubation, cell lysis was minimal ($3.8 \pm 0.8\%$; $n = 5$) based on the liberation of lactate dehydrogenase, and was the same in the presence and absence of thrombin and the metabolic inhibitors. Data are expressed as means ± S.D. Statistical significances were determined by Student's t-test.

Results

Energy consumption in resting platelets and during thrombin-induced secretion.

Platelets that were incubated in a glucose-free medium produced 0.7 ± 0.5 ($n = 4$) μmol of lactate $\cdot min^{-1} \cdot (10^{11}$ platelets$)^{-1}$ and consumed 1.0 ± 0.4 ($n = 4$) μmol of $O_2 \cdot min^{-1} \cdot (10^{11}$ platelets$)^{-1}$. Assuming an energy yield of 1.5 $ATP_{eq.}$ for lactate formation (mol/mol) and 6 $ATP_{eq.}$ for O_2 uptake (mol/mol) these data are equivalent to an energy production of about 7.0 μmol of $ATP_{eq.} \cdot min^{-1} \cdot (10^{11}$ platelets$)^{-1}$. In spite of this energy generation, [^{3}H]ATP was slowly converted to [^{3}H]-hypoxanthine/inosine at a rate of 0.25 ± 0.05 ($n = 4$) μmol of $ATP_{eq.} \cdot min^{-1} \cdot (10^{11}$ platelets$)^{-1}$. From these data a total energy consumption of 7.3 ± 2.5 μmol of $ATP_{eq.} min^{-1} \cdot (10^{11}$ platelets$)^{-1}$ can be calculated. From the fall in [^{3}H]ATP and ADP following abrupt inhibition of ATP resynthesis a consumption of 6.2 ± 0.9 μmol of $ATP_{eq.} \cdot min^{-1} \cdot (10^{11}$ platelets$)^{-1}$ (25 different donors) was calculated. These figures did not differ significantly ($P > 0.1$).

Stimulation with thrombin (5 units $\cdot ml^{-1}$) induced a rapid acceleration of energy consumption to a rate of 17.8 ± 0.4 μmol of $ATP_{eq.} \cdot min^{-1} \cdot (10^{11}$ platelets$)^{-1}$ (five different donors) during the first 15s after stimulation. Subsequently, the

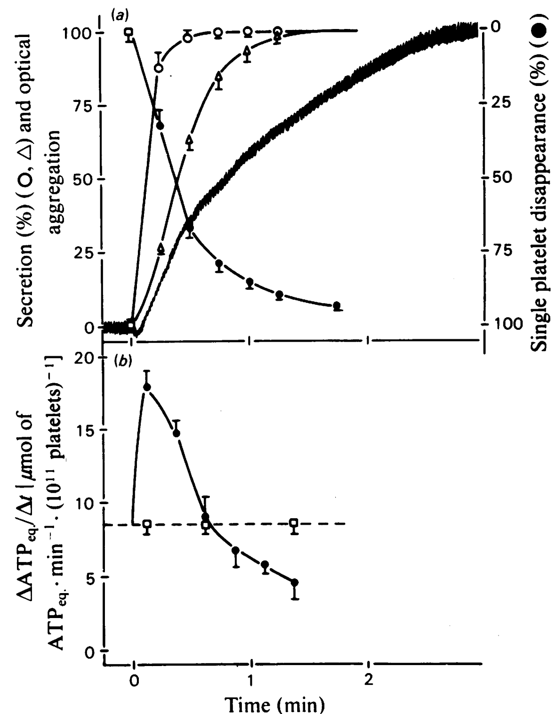

Fig. 1. *Comparison between aggregation and secretion and the concurrent consumption of metabolic energy*
Platelets were stimulated with thrombin (5.0 units $\cdot ml^{-1}$ final concentration) and optical aggregation was monitored. At various times samples were collected for analysis of single platelet disappearance (●) and of secretion (expressed as percentage of maximal secretable amount of marker) of [^{14}C]serotonin (○) and N-acetyl-β-D-glucosaminidase (△) (a). Also shown are the values for secretion and single platelet disappearance at 5s before addition of thrombin (□). Data on secretion of β-thromboglobulin were always intermediate between those of [^{14}C]serotonin and the acid hydrolase; these data were omitted for the sake of clarity. Concurrently energy consumption, expressed as μmol of $ATP_{eq.} \cdot min^{-1} \cdot (10^{11}$ platelets$)^{-1}$, was measured (b) both in thrombin-stimulated (●——●) and unstimulated (□--□) suspensions. Data are expressed as means ± S.D. ($n = 4$). Between 0 and 30s after stimulation energy consumption was at significantly higher rates ($P < 0.0001$), and at 45s and thereafter at significantly lower rates ($P < 0.01$) than in resting cells.

consumption of energy slowed down and reached the values of resting platelets between 30 and 45s (Fig. 1*a*). Concurrent analysis of platelet responses showed that most of the secretion took place between 0 and 45s after the addition of thrombin, that is in the period of increased energy consumption (Fig. 1*b*). Within this period the single platelet disappearance was 75% complete. In contrast, most of the optical aggregation occurred after the energy consumption had normalized (Fig. 1*b*). Secretion velocities and rates of single platelet disappearance were maximal in the period where energy consumption was also maximal, suggesting that the rates of these responses were linked to the rate of energy consumption.

Velocity of secretion and rate of concurrent energy consumption

As shown in Fig. 2, the velocity of the three secretion responses depended on the dose of thrombin used for stimulation. A high dose of thrombin (5units·ml^{-1}) triggered the secretion of 90% of the maximal secretable amount of [^{14}C]-serotonin within the first 15s with a rapid fall in secretion velocity immediately thereafter (Fig. 2, left-hand panel). A lower dose of thrombin (0.2–0.1unit·ml^{-1}) triggered slower secretion of [^{14}C]-serotonin, with an optimum after about 15s, whereas a very low dose of thrombin (0.05unit·ml^{-1}) triggered very slow [^{14}C]serotonin secretion with an optimum after about 1min. Similar patterns were found for the secretion of β-thromboglobulin (Fig. 2, middle panel) and *N*-acetyl-β-D-glucosaminidase (Fig. 2, right-hand panel) although these secretion patterns were distinctly slower than those of [^{14}C]serotonin.

The energetic measurements yielded strikingly similar patterns (Fig. 3). A high dose of thrombin (5units·ml^{-1}) induced an immediate increase in energy consumption to a rate of 17.8 ± 0.4 ($n = 5$) μmol of $ATP_{eq.}\cdot min^{-1}\cdot(10^{11}$ platelets$)^{-1}$. This corresponded to an increment of about 11.5μmol of $ATP_{eq.}\cdot min^{-1}\cdot(10^{11}$ platelets$)^{-1}$ compared with resting platelets. About 15s later this increment was reduced to about 4μmol of $ATP_{eq.}\cdot min^{-1}\cdot(10^{11}$ platelets$)^{-1}$ and after 30s the energy consumption had normalized. Lower doses of thrombin (0.2–0.1unit·ml^{-1}) induced a weaker activation of energy consumption with a maximum after about 30s, whereas a very low dose of thrombin (0.05unit·ml^{-1}) triggered only a slight activation of the energy consumption without an apparent maximum between 0 and 1.5min after thrombin addition. At thrombin concentrations between 0.05 and 0.2unit·ml^{-1} the energy consumption stabilized at rates found in resting platelets; at higher doses of thrombin (0.5–5.0 units·ml^{-1}) lower values were obtained than in unstimulated cells (Figs. 1 and 3).

Extent of secretion and extent of energy consumption at various intervals after stimulation by thrombin

The relation between secretion and energy consumption at various stages after stimulation

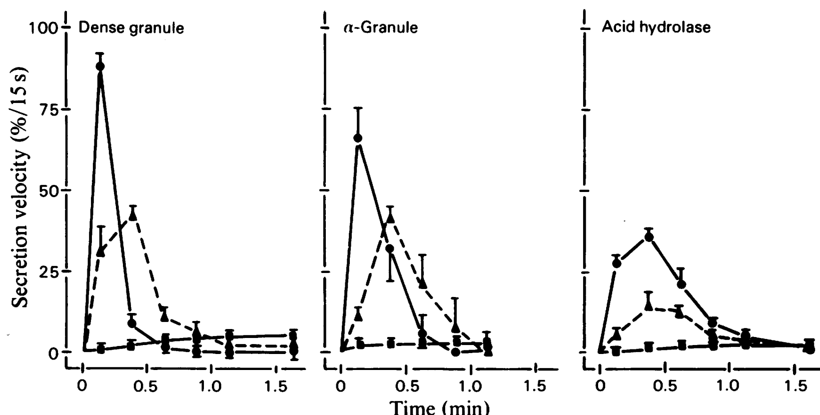

Fig. 2. *Secretion velocity at different stages after stimulation with different concentrations of thrombin*
Platelets were stimulated with thrombin at a final concentration of 0.05 (■—■), 0.2 (▲--▲) and 5.0 (●—●) units·ml^{-1}. Every 15s samples were collected for analysis of secretion of [^{14}C]serotonin (dense granule), β-thromboglobulin (α-granule) and *N*-acetyl-β-D-glucosaminidase (acid hydrolase). The data were averaged over a 15s interval and considered to reflect the secretion velocity at the midpoint of each interval. In the same experiment thrombin at 0.1 and 0.5unit·ml^{-1} was used; the results were intermediate between those obtained with 0.05 and 0.2 and with 0.2 and 5.0units·ml^{-1}, respectively. For the sake of clarity these data have been omitted. Data are expressed as means±S.D. ($n = 5$).

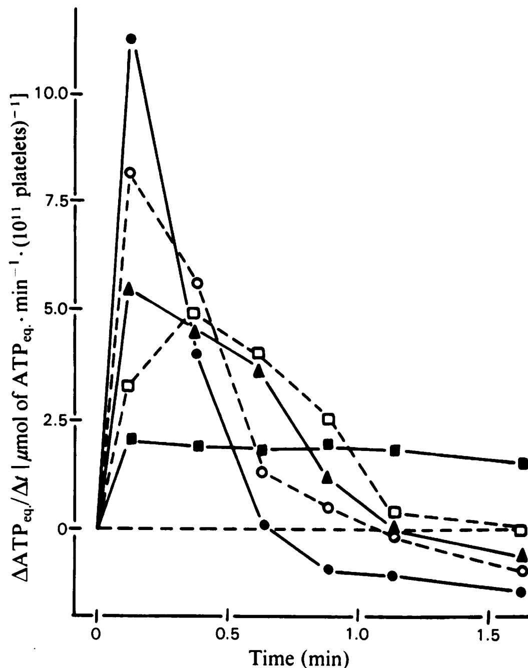

Fig. 3. *Rate of energy consumption at different stages after stimulation with different concentrations of thrombin*
Platelets were stimulated with thrombin at a final concentration of 0.05 (■—■), 0.1 (□--□), 0.2 (▲—▲), 0.5 (○--○) and 5.0 (●—●) units·ml^{-1}. Energy consumption was determined as $(\Delta ATP_{eq.}/\Delta t)$ within the first 15 s after induction of metabolic blockade and plotted at the midpoint of each interval. The rate of energy consumption, expressed as μmol of $ATP_{eq.}\cdot min^{-1}\cdot(10^{11}$ platelets$)^{-1}$, was corrected for $(\Delta ATP_{eq.}/\Delta t)_{resting}$ measured simultaneously in an unstimulated suspension. Data originate from an experiment with the platelets from one donor and are representative of five similar experiments.

with different doses of thrombin was analysed from the data in Figs 2 and 3 by comparing the extent of secretion with the amount of energy that was consumed from the moment of stimulation, expressed as:

$$\int_0^t (\Delta ATP_{eq.}/\Delta t)_{total}\, dt$$

Fig. 4 illustrates the lack of correlation between extent of secretion and extent of energy consumption in platelets stimulated with different concentrations of thrombin. At a given amount of energy consumption, the extent of [^{14}C]serotonin secretion was low or high, depending on the concentration of thrombin (Fig. 4*a*, left-hand panel). Similar results were obtained for the secretion of β-thromboglobulin and *N*-acetyl-β-D-glucosaminidase (Fig. 4*a*, middle and right-hand panels, respectively). A much better fit between secretion and energy consumption was obtained when the energy data were corrected for the amount of basal energy consumption:

$$\int_0^t (\Delta ATP_{eq.}/\Delta t)_{resting}\, dt$$

(Fig. 4*b*). This increment in energy consumption closely correlated with the extent of secretion at the different stages of the secretion responses, independent of the thrombin concentration. A given increment in energy consumption always corresponded to a constant amount of [^{14}C]serotonin, β-thromboglobulin and acid hydrolase secretion. From Fig. 4(*b*) it follows that secretion of these three markers was completed at an incremental energy consumption of 4.0, 6.5 and 6.7 μmol of $ATP_{eq.}\cdot min^{-1}\cdot(10^{11}$ platelets$)^{-1}$, respectively. Fig. 4(*b*) illustrates an increase in energy consumption of about 1 μmol of $ATP_{eq.}\cdot(10^{11}$ platelets$)^{-1}$ which is not accompanied by the extracellular appearance of granule markers, suggesting an investment of energy in processes that precede secretion.

Aggregation and energy consumption

Apart from secretion, thrombin triggers aggregation, which is known to depend on energy (Akkerman & Holmsen, 1981; Holmsen *et al.*, 1982). It is possible, therefore, that part of the energy consumed during secretion is reserved for the aggregation process. However, we found no correlation between optical aggregation and energy consumption, since most of the aggregation occurred after energy consumption had normalized (Fig. 1*b*). Furthermore, varying the thrombin concentration between 0.1 and 5.0 units·ml^{-1} changed the energy consumption without altering the optical aggregation curves. In contrast, different rates of single platelet disappearance were obtained at different doses of thrombin (Fig. 5*a*). Analysis by 15 s intervals revealed that single platelet disappearance and energy consumption varied in parallel (results not shown). A comparison between the extent of total energy consumption and extent of single platelet disappearance analogous to the plots for secretion (Fig. 4*a*) did not reveal a constant relationship. However, when only the increment in energy consumption was taken into account a curved relationship was obtained (Fig. 5*b*), suggesting that single platelet disappearance is indeed coupled to the increment in concurrent energy consumption.

In order to discriminate between the energy requirements of single platelet disappearance and secretion, experiments were performed in the

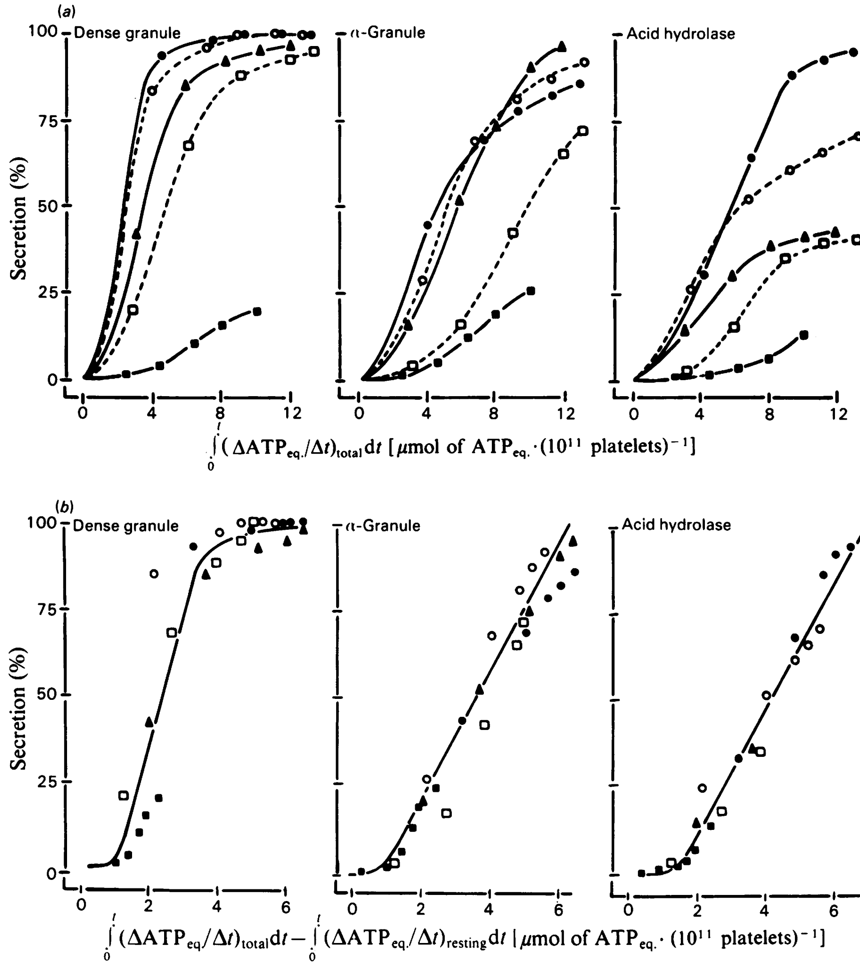

Fig. 4. *Comparison between energy consumption and extent of secretion*

The amount of energy, expressed as μmol of $ATP_{eq.}$·(10^{11} platelets)$^{-1}$, that is consumed from the moment of stimulation was calculated as:

$$\int_0^t (\Delta ATP_{eq.}/\Delta t)_{total}\, dt$$

from ($\Delta ATP_{eq.}/\Delta t$) measured over each 15 s interval and plotted versus the corresponding extent of secretion of [^{14}C]-serotonin (dense granule), β-thromboglobulin (α-granule) and *N*-acetyl-β-D-glucosaminidase (acid hydrolase). The symbols are as shown in Fig. 3 for 0.05 (■—■), 0.1 (□--□), 0.2 (▲—▲), 0.5 (○---○) and 5.0 (●—●) units of thrombin·ml^{-1}. Data are derived from Figs. 2 and 3. (*a*) Total energy consumption in thrombin-treated platelets; (*b*) incremental energy consumption, which is obtained by subtracting:

$$\int_0^t (\Delta ATP_{eq.}/\Delta t)_{resting}\, dt$$

from the data on total energy consumption (difference between thrombin-stimulated and unstimulated platelets). The correlation between the increment in energy consumption and secretion was tested by linear regression analysis. When extent of secretion was less than 10% or more than 90% of maximal releasable amounts the data were excluded. Results were: for [^{14}C]serotonin, $y = -20.83 + 29.69x$ ($r = 0.702$, $n = 9$, $P < 0.025$); for β-thromboglobulin, $y = -14.03 + 17.57x$ ($r = 0.9603$, $n = 19$, $p < 0.0001$); and for *N*-acetyl-β-D-glucosaminidase, $y = -25.88 + 18.90x$ ($r = 0.9638$, $n = 13$, $P < 0.0001$).

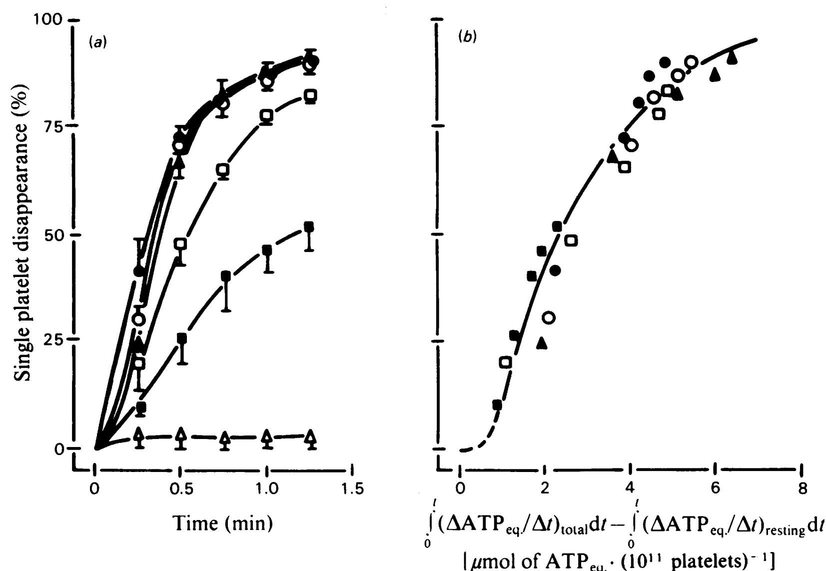

Fig. 5. *Comparison between energy consumption and single platelet disappearance*
(*a*) Platelets were incubated without (△) and with thrombin at final concentrations of 0.05 (■), 0.1 (□), 0.2 (▲), 0.5 (○) and 5.0 (●) units·ml⁻¹. Samples were collected every 15 s for analysis of single platelet disappearance. Data are expressed as means ± S.D. ($n = 5$). (*b*) Extent of single platelet disappearance plotted versus the increment in energy consumption:

$$\int_0^t (\Delta ATP_{eq.}/\Delta t)_{total}\,dt - \int_0^t (\Delta ATP_{eq.}/\Delta t)_{resting}\,dt$$

expressed as μmol of $ATP_{eq.}\cdot(10^{11}\ \text{platelets})^{-1}$. Symbols are defined in (*a*).

presence of 2mM-EDTA, which completely prevented optical aggregation and single platelet disappearance (results not shown). Fig. 6 illustrates the relation between secretion of [^{14}C]serotonin and energy consumption at various stages after stimulation with 0.1 unit of thrombin·ml⁻¹. Compared with control suspensions, EDTA-treated platelets showed a faster [^{14}C]serotonin secretion (Fig. 6*a*) and a slower total energy consumption (Fig. 6*b*). However, the interpretation of these data was complicated by the fact that EDTA significantly reduced the energy consumption in unstimulated platelets from 6.8 ± 1.0 to 5.2 ± 1.0 μmol of $ATP_{eq.}\cdot min^{-1}\cdot(10^{11}\ \text{platelets})^{-1}$ ($n = 7$, $P < 0.025$) (Fig. 6*b*, broken lines). When only the increment in energy consumption was taken into account, the energy data overlapped almost completely. Hence, at a given incremental energy consumption [^{14}C]serotonin secretion was faster in the presence of EDTA than in its absence, as shown in the insert of Fig. 6(*a*), where the cumulative approach is illustrated. Most of the effect of EDTA appears to result in a shift of the secretion-versus-energy relationship to lower energy points, indicating that EDTA predominantly affects the energy-consuming processes that precede secretion.

Discussion

In a previous report (Akkerman *et al.*, 1983*a*) a method was introduced for the determination of energy consumption based on rapid blockade of anaerobic ATP resynthesis in CN^--pretreated cells. Drawbacks of this procedure were the artificial inhibition of acid hydrolase secretion by 30% and the use of 2-deoxy-D-glucose, which is phosphorylated before it inhibits the glucose-phosphate isomerase reaction and therefore may act as an energy sink. The modification presented here circumvents these problems and still fulfills the criteria for this type of energetic measurements, namely a good correlation with the data from uninhibited suspensions and the maintenance of a high adenylate energy charge until ATP resynthesis is arrested, which is a prerequisite for normal platelet functions (Holmsen & Robkin, 1977; Holmsen *et al.*, 1982; Akkerman *et al.*, 1983*b*).

With the present procedure an energy consump-

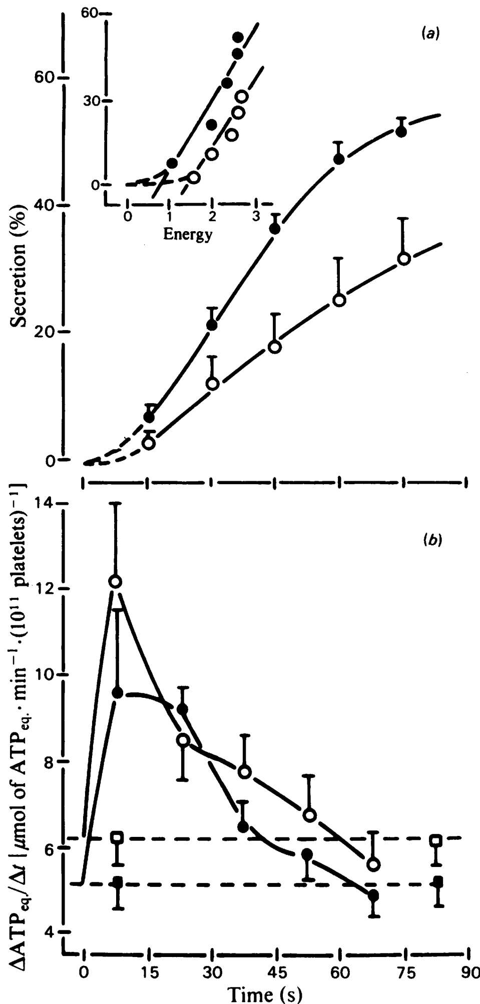

Fig. 6. *Relation between secretion and energy consumption with and without concurrent aggregation*

Platelets were stimulated with thrombin (0.1 unit·ml^{-1}) in the presence (closed symbols) or absence (open symbols) of EDTA (2mM final concentration), added 4min before the inducer. Secretion of [^{14}C]serotonin (*a*) and rates of energy consumption ($\Delta ATP_{eq.}/\Delta t$), expressed as µmol of $ATP_{eq.}\cdot min^{-1}\cdot(10^{11}$ platelets$)^{-1}$, (*b*) were measured simultaneously. In the presence of EDTA, optical aggregation and single platelet disappearance were completely inhibited. Energy consumption was also determined in unstimulated platelets (□, ■). Both secretion data and energy data were expressed as means ± S.D. ($n = 3$). In the insert, extent of secretion was plotted versus the increment of energy consumption:

$$\int_0^t (\Delta ATP_{eq.}/\Delta t)_{total}\,dt - \int_0^t (\Delta ATP_{eq.}/\Delta t)_{resting}\,dt$$

expressed as µmol of $ATP_{eq.}\cdot(10^{11}$ platelets$)^{-1}$. Regression lines were: $y = -26.5 + 28.1x$ ($r = 0.939$, $P < 0.025$) in the presence of EDTA and $y = -32.5 + 22.5x$ ($r = 0.954$, $P < 0.025$) in its absence.

tion rate of about 6µmol of $ATP_{eq.}\cdot min^{-1}\cdot(10^{11}$ platelets$)^{-1}$ is found in resting platelets, which is above the 3.5µmol of $ATP_{eq.}\cdot min^{-1}\cdot(10^{11}$ platelets$)^{-1}$ found previously (Akkerman *et al.*, 1983*a*), but is close to the 6.5µmol of $ATP_{eq.}\cdot min^{-1}\cdot(10^{11}$ platelets$)^{-1}$ that has been calculated from lactate formation and oxygen uptake in uninhibited platelet suspensions (Akkerman & Holmsen, 1981). In thrombin (5units·ml^{-1})-treated platelets both approaches lead to similar results. During the first 15s after stimulation the previous and the present techniques reveal an energy consumption rate of 16 and 17µmol of $ATP_{eq.}\cdot min^{-1}\cdot(10^{11}$ platelets$)^{-1}$ respectively. Since both methods are to some extent supplementary they may help to differentiate between aerobic and anaerobic energy metabolism.

Our observations demonstrate two conditions in which single platelet disappearance and the secretion from dense, α- and acid-hydrolase-containing granules vary in parallel with energy consumption. First, in a fixed time interval following stimulation with increasing amounts of thrombin. Secondly, at different stages of the responses triggered by a constant dose of thrombin. Thus, the relationship between these responses and energy consumption is independent of the strength of stimulation and the actual velocities of these processes. In an earlier publication (Akkerman *et al.*, 1983*a*), the energetics of secretion were studied at different temperatures and at different levels of metabolic ATP. Both conditions are potentially harmful for energetic studies since they change factors in the expression of the free energy change of ATP hydrolysis:

$$\Delta G = \Delta G^0 + RT\ln([P_i][ADP]/[ATP])$$

Although these drawbacks are not features of the present approach, very similar results are obtained. Hence, alterations in temperature, ATP levels, thrombin concentrations and secretion velocities all lead to the same relationship between responses and energy consumption, which strengthens the concept that these parameters are closely mutually dependent.

The present data demonstrate that the relation between secretion and energy holds during the entire secretion responses. This justifies an earlier extrapolation from the initial part of the secretion responses to 100% secretion, which led to the preliminary values of 2.5, 5.2 and 6.7µmol of $ATP_{eq.}\cdot(10^{11}$ platelets$)^{-1}$ for the incremental energy consumption during complete dense, α- and acid-hydrolase granule secretion, respectively (Akkerman *et al.*, 1983*a*). This is close to the respective values of 4.0, 6.5 and 6.7µmol of $ATP_{eq.}\cdot(10^{11}$ platelets$)^{-1}$ obtained with the present approach.

A major uncertainty in the study of the

energetics of platelet responses is the role of basal energy-consuming processes occurring in unstimulated platelets. It is possible that after stimulation the basal energy consumption continues at the same rate as before stimulation (Akkerman & Holmsen, 1981; Akkerman *et al.*, 1983*a*). Alternatively, there are indications that under conditions of poor energy supply the platelet directs all available energy to secretion responses, probably at the expense of energy-requiring processes occurring in resting platelets (Akkerman *et al.*, 1979*a*). The correlation between extent of secretion and total energy consumption was poor and much affected by the concentration of thrombin (Fig. 4*a*). In contrast, the correlation with the incremental energy consumption was very good and independent of the thrombin concentration (Fig. 4*b*). Similar findings were obtained for single platelet disappearance (Fig. 5). We therefore tend to conclude that the incremental energy consumption is the crucial factor for single platelet disappearance and the secretion responses. However, the possibility that a minor part of the basal consumption is directed to secretion cannot be ruled out. Interestingly, after complete secretion induced by a high dose of thrombin ($>0{\cdot}2$ unit$\cdot$ml^{-1}) energy consumption returns to values below those of resting cells (Fig. 3). If this reduction in basal energy consumption occurs immediately after platelet stimulation our incremental energy consumption data should include this part of the basal energy consumption.

So far, it is impossible to estimate the energy requirement of each process separately. Figs. 4(*b*) and 5 illustrate that at an incremental energy consumption of about 4μmol of $ATP_{eq.}\cdot(10^{11}$ platelets$)^{-1}$ dense granule secretion is completed. Hence, the energy consumption above this level is entirely related to single platelet disappearance and secretion from α- and lysosomal granules. Since the energy requirement of aggregation is probably minor, one can estimate that 1% secretion from α- and lysosomal granules is accompanied by an incremental energy consumption of about 53nmol of $ATP_{eq.}\cdot(10^{11}$ platelets$)^{-1}$. Extrapolation to 100% leads to an energy requirement of 5.3μmol of $ATP_{eq.}\cdot(10^{11}$ platelets$)^{-1}$ for complete secretion of α- and lysosomal granule contents. If one assumes that the energy requirement is the same in periods in which dense granule secretion takes place, an estimated 0.7μmol of $ATP_{eq.}\cdot(10^{11}$ platelets$)^{-1}$ can be obtained as the energy requirement for 100% dense granule secretion. Other studies (Mürer, 1968; Kinlough-Rathbone *et al.*, 1970; Holmsen *et al.*, 1982) have shown an increase in sensitivity towards metabolic blockade in the order of dense, α- and lysosomal granule secretion, suggesting an increase in energy demand of the respective secretion responses. Our data agree with this suggestion. An important point is, however, that it takes much more time for complete acid hydrolase secretion than for dense granule secretion, with α-granule secretion somewhere in between. Sensitivity towards metabolic blockade may therefore be a reflection of the capability to complete a response with the limited energy sources that are available, in which secretion from dense granules stands a better chance than acid hydrolase secretion.

Kinlough-Rathbone *et al.* (1970), Kattlove (1974), Holmsen *et al.* (1972, 1982) and Akkerman *et al.* (1979*a*) have shown that optical aggregation is sensitive to metabolic blockade. In the present study, optical aggregation did not correlate with the increase in energy consumption and was relatively insensitive to variations in thrombin concentrations which greatly affected energy consumption. Single platelet disappearance, however, showed a strong correlation with the increment in energy consumption (Fig. 5*b*). The relation was non-linear and suggested that more energy was needed at the end of the response than at the beginning. However, aggregation is difficult to quantify. The technique used here for measuring single platelet disappearance insufficiently discriminates between free particles and di-, tri- and tetramers. In addition, our experimental conditions require the absence of exogenous fibrinogen, which is a cofactor for aggregation. Kaplan *et al.* (1981) have shown that endogenous fibrinogen and β-thromboglobulin are secreted in parallel and that only 10% secretion is sufficient for more than 40% of optical aggregation. Since single platelet disappearance precedes optical aggregation, α-granule secretion is probably only rate-limiting in the very early start of this response. Its energy requirement, however, is probably minor, since inhibition of this response hardly affects the incremental energy consumption (Fig. 6*b*). But secretion of [^{14}C]serotonin is faster in the presence of EDTA than in control suspensions, suggesting that part of the energy directed to aggregation is made available for secretion. This is primarily reflected by a reduction in the energy consumption that precedes secretion [from about 1 to 0.5μmol of $ATP_{eq.}\cdot(10^{11}$ platelets$)^{-1}$] whereas above that value the dependence on energy is similar to that in the control suspension (Fig. 6*a*, insert). The finding that energy consumption had already increased before secretion and aggregation were apparent indicates the initiation of some energy requiring processes that are probably crucial for those responses. Likely candidates are shape change, the formation of phosphatidic acid from the phosphatidylinositides and the phosphorylation of an unidentified 40kDa protein (Lapetina & Siegel, 1983)

and the myosin light chain (Daniel *et al.*, 1981). EDTA does not prevent the platelets from changing shape, whereas the phosphorylation of both proteins (Lyons & Shaw, 1980) and the formation of phosphatidic acid (Lloyd *et al.*, 1973) are only slightly affected by chelation of bivalent cations.

In conclusion, the present paper demonstrates a tight coupling between thrombin-induced secretion responses and the concurrent consumption of metabolic energy. The degree of coupling is independent of the concentration of thrombin and holds true during the entire responses but differs between secretion from dense and α- or lysosomal granules. In addition, not the total energy consumption, but the increment in energy consumption found after stimulation, appears to be crucial for these platelet responses. Other secretory cells are also sensitive to metabolic inhibitors. Adrenal medulla cells (Rubin, 1970), mast cells (Foreman *et al.*, 1977), exocrine pancreatic cells (Jamieson & Palade, 1971), pituitary glands (McPherson & Schofield, 1974) and salivary glands (Rossignol *et al.*, 1974) all respond to metabolic blockade with decreased extrusion of secretion products. It is therefore possible that the coupling mechanism demonstrated here for platelets bears relevance for other secretory cells as well.

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