PLATELET AGGREGATION AND Ca^{2+} SECRETION ARE INDEPENDENT OF SIMULTANEOUS ATP PRODUCTION

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1. Introduction

It is generally believed that platelet aggregation and secretion depend on the simultaneous production of energy [1]. Immediately after induction of these processes, both lactate production and mitochondrial oxygen consumption increase [2,3]. Consequently, more ATP is generated and since the level of ATP involved in metabolism (metabolic-ATP) changes only slightly, it has been assumed that ATP is consumed in processes related to platelet function [1,4]. Inhibition of energy production lowers the metabolic-ATP level gradually with a sequential disappearance of the platelets' ability to perform secretion, aggregation and shape change. Specific threshold levels of metabolic-ATP were therefore postulated for each platelet function with shape change, aggregation and secretion requiring increasing amounts of metabolic-ATP [5]. Although the energy balance in non-stimulated platelets clearly affects subsequent platelet behaviour, it is difficult to assess the role of metabolic energy during platelet function. It is, for example, unclear whether the increase in energy production during platelet function directly supports these functions or merely reflects a disturbance in control mechanisms that regulate energy metabolism. This paper demonstrates that aggregation and Ca^{2+} secretion depend mainly on the energy state of the platelet at the moment of stimulation with only minor involvement of energy production during these platelet functions.

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2. Materials and methods

Freshly drawn venous blood (160 ml portions) was collected from healthy volunteers into citrate (1.0 vol. 130 mM trisodium citrate). After centrifugation (200 × *g*, 15 min room temp.), the supernatant, platelet-rich plasma, was incubated with 0.4 μM [U-^{14}C]adenine (spec. act. 286 mCi.nmol^{-1}, Amersham, Arlington Heights, IL, code CFA, 436) for 30 min at 37°C to label the metabolic adenine nucleotides [6]. The adenine nucleotides localized in the dense granules and released during secretion are not labelled in this short incubation period [7]. The platelets were then isolated by gel filtration on a Sepharose 2B column (20 × 3 cm, 22°C) [8] equilibrated with a Sr^{2+} and albumin-containing, Ca^{2+}-free Tyrode's solution [9] without glucose. In some experiments 8.2 μg.ml^{-1} antimycin A (Sigma, St Louis, MO) was included in the buffer. After gel filtration (completed within 20–30 min) 1.9 mM $MgCl_2$, 0.05 mM $CaCl_2$ and 1 mM glucose were added. This provided the bivalent cations which are essential to aggregation and enabled measurement of Ca^{2+} secretion with a Ca^{2+} electrode in a range where electrode output in linear with respect to Ca^{2+} concentration [10]. Platelets do not lose their functional ability during gel filtration in glucose-free media provided that sufficient glucose is added some few minutes prior to induction of function. The addition of 1 mM glucose restores energy production completely [11] and on the other hand, this concentration was sufficiently low to enable rapid suppression of glucose utilization by adding an excess of the competitive inhibitor 2-deoxyglucose [12].

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Platelet aggregation and secretion was measured in a 3 ml cuvette (at 37°C) equipped with a device for continuous measurement of light transmission (aggregation) and extracellular Ca^{2+} content (secretion), using a Ca^{2+} electrode (type F 2112, Radiometer, Copenhagen 10) with a pH combination electrode (type S-900C, Sensorex, Irvine, CA) as a reference. Response time of the Ca^{2+} electrode was <1 s; interference of pH changes was <5%. The electrode output was conditioned by a specially constructed amplifier system (Instech Laboratories, Philadelphia, PA) equipped with a digital voltmeter over a ± 1999 mV range. A multiplexer pen lift controller permitted recording of light transmission and Ca^{2+} electrode output on a single channel (Hewlett-Packard 7123 A) 1 V full scale recorder with 8 s intervals. The suspensions were stirred at 900 rev./min [13]. Samples were collected before and during induction of platelet function with thrombin for measurement of ^{14}C-radioactivity in ethanol-soluble adenine nucleotides and lactate in extracts of 1 vol. platelet suspension and 2 vol. 7.7 mM EDTA in 96% ethanol kept at 0°C [6].

3. Results and discussion

In human platelets metabolic ATP is the only known intermediate for energy transduction from sites of generation to sites of utilization [1]. Its level can be monitored accurately by measuring [^{14}C]ATP in extracts of [^{14}C]adenine-labelled platelets [6].

Addition of 5 NIH-U.ml^{-1} of thrombin to platelets induced aggregation and rapid secretion of Ca^{2+} (fig.1). Probably as a result of a sudden increase in energy consumption the [^{14}C]ATP level fell. Then, as lactate production and mitochondrial respiration rose (data not shown), a new equilibrium was established by an increased ATP production in glycolysis and oxidative phosphorylation. When the platelet suspension contained antimycin A, which blocks mitochondrial ATP production, thrombin caused a greater fall in the [^{14}C]ATP level indicating that glycolysis alone could not keep up with the increased demand for ATP (fig.2A). Although energy generation thus clearly was suboptimal, aggregation and Ca^{2+} secretion were not affected (fig.2A). An abrupt block in glycolytic energy production could be induced by a mixture

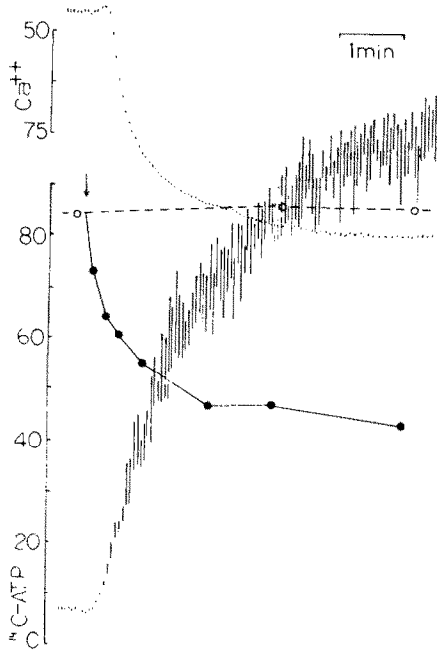

Fig.1. Platelet aggregation, Ca^{2+} secretion and [^{14}C]ATP radioactivity during platelet–thrombin interaction. ^{14}C-labelled, gel-filtered platelets were stirred at 37°C in the cuvette described in section 2. Thrombin (5 NIH-U.ml^{-1}) was added at the arrow and the changes in the extracellular calcium concentration (upper panel, μM), [^{14}C]ATP (closed circles, % of total ^{14}C-radioactivity) and light transmission (arbitrary units, transmission increases upwards with the degree of platelet aggregation) are shown. The open circles show the % ATP radioactivity in the same platelet suspension stirred without thrombin.

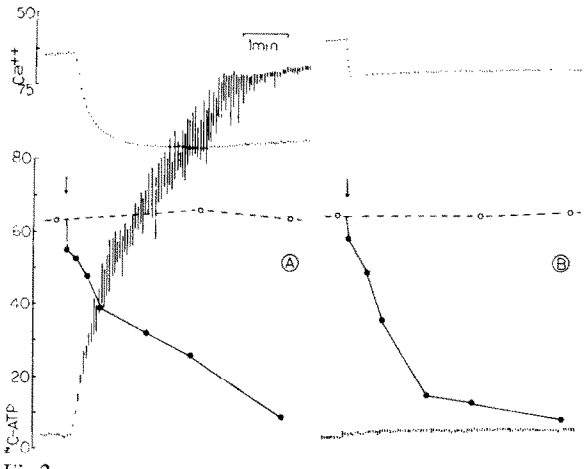

Fig.2

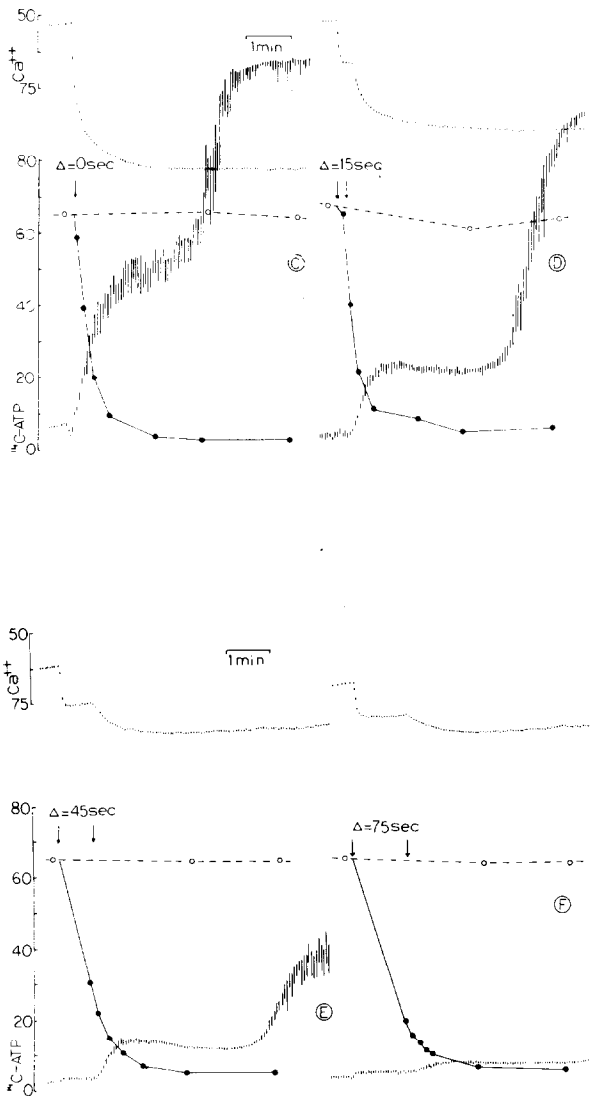

Fig.2. Platelet aggregation, Ca^{2+} secretion and [^{14}C]ATP radioactivity in antimycin A-treated platelets upon addition of 2-deoxyglucose + glucono-lactone and thrombin. ^{14}C-labelled platelets were gel filtered, incubated (for 20 min, 37°C) in the presence of 8.2 $\mu g.ml^{-1}$ antimycin A and finally stirred in the cuvette at 37°C. Shown are the responses, addition (arrow) of 5 $U.ml^{-1}$ of thrombin (A), a mixture of 30 mM 2-deoxyglucose and 10 mM glucono-δ-lactone (B); simultaneously added thrombin and 2-deoxyglucose-glucono-δ-lactone (C); and after an interval between addition of 2-deoxyglucose-glucono-δ-lactone (left arrow) and thrombin (right arrow) of 15 s (D); 45 s (E) and 75 s (F). Platelet concentration was 3.2 × 10^8 cells.ml^{-1}. Further details are described in fig.1.

of 2-deoxyglucose and glucono-δ-lactone* to antimycin A-treated platelets (fig.2B). This mixture inhibited lactate production within 5 s and led to a rapid decrease in [^{14}C]ATP in antimycin A-treated platelets (fig.2B). When 5 $U.ml^{-1}$ of thrombin was included in this mixture an even more rapid fall in [^{14}C]ATP was observed. Ca^{2+} secretion and immediate aggregation, however, occurred normally within the first 2 min after thrombin addition. Further aggregation diminished and was then followed by a secondary increase in light transmission (fig.2C). When the time interval between addition of the 2-deoxyglucose + gluconolactone mixture and of thrombin was increased, Ca^{2+} secretion and aggregation gradually decreased and were completely blocked with a 75 s time interval (fig.2D–F).

The lag time between addition of thrombin and appearance of aggregation and Ca^{2+} secretion was increased by lowering the thrombin concentration, and the inhibition by the metabolic blockers was more prominent. By adding inhibitors and varying amounts of thrombin simultaneously, these functions disappeared at 0.5 $U.ml^{-1}$ of thrombin or lower; with a 20 s interval between addition of inhibitors and thrombin abolishment of the platelet response was already present at 1.0 $U.ml^{-1}$ of thrombin.

Because of the rapid hydrolysis of gluconolactone in solution, pH fell from 7.2 to 6.8 during each of the experiments recorded in fig.2. Control studies carried out at a starting pH of 6.8 showed that the results were not affected by this change in pH. The instability of gluconolactone also prevented removal of contaminating Ca^{2+} (fig.2B). Addition of each metabolic inhibitor alone neither affected the metabolic ATP level nor platelet function.

The second phase in the change in light transmission (fig.2C–E) was not affected by prior addition of 100 $\mu g.ml^{-1}$ apyrase (Sigma, St Louis), which rapidly removed ADP secreted from the platelets and prevents subsequent ADP–platelet interaction. The thrombin inhibitor hirudin (5 $U.ml^{-1}$, Pentapharm, Basel), when added 10 s after thrombin, completely abolished this

* 2-deoxy-D-glucose is a competitive inhibitor of glucose transport and phosphorylation [14,15]. The phosphorylation product, 2-deoxyglucose-6-phosphate, competitively blocks glucose phosphate isomerase activity [15]. Glucono-δ-lactone inhibits glycogen phosphorylase and prevents glycogen degradation [16,17]

'second wave'. During this 'second wave' both energy production and [^{14}C]ATP catabolism were absent. This phenomenon may therefore reflect an energy-independent agglutination process as induced by bovine factor VIII [18], ristocetin [19] or polylysine [20] rather than aggregation [1]. Its sensitivity to hirudin indicates that it is thrombin-mediated and therefore might consist in trapping of platelets by fibrin formed by action of thrombin on fibrinogen secreted from the platelets [21].

These results indicate that Ca^{2+} secretion and aggregation are independent of simultaneous energy production. However, at the moment of stimulation of a sufficiently high metabolic ATP level, i.e., energy potential, is essential for optimal platelet behaviour since these functions gradually disappear when stimulation takes place at a lower ATP level. The parallel inhibition of Ca^{2+} secretion and aggregation contradicts our proposed differences in energy requirement of these processes [5], although the use of a potent stimulator such as thrombin might well mask more subtle differences in energy requirement as revealed with weak inducers such as ADP. The present data, however, indicate that it is not the degree of metabolic inhibition but the time between blocking of energy metabolism and stimulation of the platelets that is the crucial factor.

The role of metabolic ATP was further evaluated

Table 1
Role of energy production during thrombin-induced Ca^{2+} secretion and aggregation

Conditions	Energy status (ATP equiv.: μmol.min^{-1}.10^{-11} cells)			Ca^{2+} secretion		Aggregation (arbitrary units)	
	Production	Fall in ATP + ADP	Consumption	k_2 (s^{-1})	Amount (μ at 10^{-11} cells)	Initial velocity (min^{-1})	Maximum first phase
+ Thrombin − 2DG + GLAC	10.2 ± 1.2	8.9 ± 1.5	19	0.022 ± 0.005	14.48 ± 2.67	13.2 0.9	18.5 ± 2.0
+ Thrombin + 2DG + GLAC	0	16.3 ± 3.6	16	0.022 ± 0.004	14.88 ± 3.18	13.9 ± 1.4	9.9 ± 0.7
− Thrombin + 2DG + GLAC	0	4.7 ± 0.5	5	0	0	0	0

Energy production and steady-state levels of [^{14}C]ATP and [^{14}C]ADP were measured during thrombin (5 U.ml^{-1})-induced Ca^{2+} secretion and aggregation in the presence of antimycin A and with and without simultaneous addition of 2-deoxyglucose + glucono-δ-lactone (2DG + GLAC). Since antimycin A was present, ATP production could be calculated from lactate formation which was measured at 4 min intervals over a 30 min period after thrombin addition. Since lactate production remained linear over this period, values were extrapolated to $t = 0$ (thrombin addition) and compared with the levels of [^{14}C]ATP and [^{14}C]ADP measured at 5 s intervals between 5 s and 30 s after thrombin addition. ^{14}C-Radioactivity data were converted into absolute amounts on the basis that the 80% of total ^{14}C-radioactivity found in ATP in normal platelets equals the amount of metabolic ATP, that is 3.5 μmol.10^{-11} platelets [22,23]. Energy data are expressed in ATP equivalents [24] reflecting an energy yield of 2 equiv. for conversion of ATP to AMP and of 1 equiv. for conversion of ATP to ADP. Energy consumption was taken as the sum of production and fall in [^{14}C]ATP + ADP

The kinetics of Ca^{2+} secretion measured with the Ca^{2+} electrode followed the model proposed [25] consisting of 3 phases: an initial lag phase (k_1-phase), followed by an exponential phase in which most of the Ca^{2+} is secreted and finally a slow linear k_3-phase. The rate constant of the second phase (k_2) was measured with a 15 s interval between addition of 2DG + GLAC and thrombin (fig.2D) to avoid interference of Ca^{2+} contaminated 2DG + GLAC (fig.2B). k_2 data at longer time intervals were within the same range (0.023 ± 0.005). The amount of Ca^{2+} secreted (with simultaneous additions of 2DG + GLAC and thrombin) was corrected for the Ca^{2+} contained in 2DG + GLAC

Aggregation velocity was calculated over the first 30 s after thrombin addition. Since the absorbance of a platelet suspension is linear with cell concentration only at low platelet counts (<1 × 10^8.ml^{-1}) data were gathered from suspensions with similar cell concentrations

Data reflect mean ± SD from 4–7 experiments. Platelet concentrations were 3.2–3.5 × 10^8 cells.ml^{-1}

by calculating the energy made available by glycolysis and the fall in [^{14}C]ATP + ADP during the first 30 s of interaction between thrombin in antimycin A-treated platelets. In the presence of 2-deoxyglucose + gluconolactone almost the same amount of energy was liberated as in the absence of these inhibitors (16 compared to 19 μmol ATP equiv. . min^{-1}.10^{-11} cells, table 1). If the energy consumption in non-stimulated platelets (~5 μmol ATP equiv. . min^{-1}.10^{-11} cells) is temporarily lowered, as occurs during starvation and for aggregation at normal velocity [11], enough energy may be made available for secretion of normal amounts of Ca^{2+} and for aggregation of normal velocity. At a lower [^{14}C]ATP level thrombin induced less energy liberation leading to less Ca^{2+} secretion and slower aggregation (table 1). The fact that the different amounts of Ca^{2+} were secreted at the same relative velocity (constancy of k_2, [25]), suggests that the extent of secretion solely depended on the energy state of the platelet at the moment of platelet–thrombin interaction. When secretion was induced at a lower metabolic ATP level, less Ca^{2+} was secreted. The relation between the amount of Ca^{2+} secreted and the concomitant consumption of metabolic ATP (fig.2 D–F) was linear and the regression line passed through the origin, that is, there was no ATP consumption in the absence of Ca^{2+} secretion. This suggests that platelets deprived of normal energy production direct all available energy to Ca^{2+} secretion, probably at the expense of the many energy consuming processes that are occurring in the resting cell.

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