Interrelationships Among Platelet Responses: Studies on the Burst in Proton Liberation, Lactate Production, and Oxygen Uptake During Platelet Aggregation and Ca\(^{2+}\) Secretion

By Jan Willem N. Akkerman and Holm Holmsen

The interrelationships between several functional and metabolic platelet responses to physiologic stimuli have been studied in gel-filtered platelets by continuous and simultaneous registration of shape change, aggregation, Ca\(^{2+}\) secretion, oxygen uptake, and proton liberation. At various rates of aerobic and anaerobic energy metabolism, proton liberation quantitatively paralleled the production of lactate. However, immediately following thrombin addition, a lactate-unrelated burst in proton liberation was apparent, which correlated with acid hydrolase secretion but not with dense granule or \(\alpha\)-granule secretion, ATP-hydrolysis, or hydrolysis of membrane constituents. Following platelet stimulation with a low dose of thrombin (0.1 U \(\cdot\) ml\(^{-1}\)), a specific order of cellular responses was recorded: (1) onset of shape change; (2) acceleration of lactate-related proton liberation; (3) onset of aggregation; (4) onset of Ca\(^{2+}\) secretion; and (5) a minor increase in oxygen uptake. Stimulation with a high dose of thrombin (5 U \(\cdot\) ml\(^{-1}\)) initiated (1) onset of shape change; (2) onset of aggregation, Ca\(^{2+}\) secretion, and lactate-unrelated proton liberation; (3) acceleration of mitochondrial respiration; and (4) start of arachidonate oxygenation. Maximal arachidonate oxygenation was found at 5 U \(\cdot\) ml\(^{-1}\) of thrombin and could be inhibited completely without affecting Ca\(^{2+}\) secretion or aggregation. At 0.1 U \(\cdot\) ml\(^{-1}\) of thrombin, arachidonate oxygenation was hardly detectable, but when inhibited, Ca\(^{2+}\) secretion (velocity and extent) and aggregation were greatly impaired and became detectable only after a time delay 2-3-fold that of uninhibited suspensions. The results suggest that glyco(geno)lytic but not oxidative ATP production supports aggregation and Ca\(^{2+}\) secretion. They demonstrate that thrombin-induced aggregation precedes Ca\(^{2+}\) secretion and therefore is independent of products secreted from the dense granules.

**When platelets** are properly stimulated they change shape, secrete the contents of specific granules, become sticky, and form aggregates. These functional responses are accompanied by alterations in the cell's metabolism that lead to increased synthesis of prostaglandins and accelerated ATP production in glycolysis, glyco(gen)olysis, and oxidative phosphorylation. So far, it has been difficult to establish how the various functional and metabolic responses interact. Platelet functions are best studied in platelet-rich plasma or in suspensions of platelets isolated by mild separation procedures, such as gel-filtration. On the other hand, most metabolic assays require concentrated cell suspensions that are usually obtained by washing of the cells with EDTA-containing salt solutions by centrifugation. A comparison between functional and metabolic aspects of platelet behavior is further complicated by poor time-resolution of current methodology that mainly consists of single point determinations of secreted compounds or metabolic end products and therefore insufficiently discriminates between rapid processes, such as aggregation and secretion, that are completed within 2 min.

We report here the use of a device designed for continuous registration of different functional and metabolic parameters in the same suspension of human platelets. Platelet shape change and aggregation are measured with the conventional light transmission technique. The secretion of dense granule constituents, which in human platelets include high amounts of Ca\(^{2+}\) ions, is monitored with a Ca\(^{2+}\) electrode. A microoxygen electrode measures oxygen uptake that takes place both in mitochondrial respiration and in arachidonate oxygenation. A pH electrode is included for monitoring proton liberation, which conflicting reports have contributed to lactic acid formation, hydrolysis of metabolic ATP, and proteolysis of membrane constituents. A special amplifier is used to make the electrode readings sensitive enough for use in a suspension of gel-filtered platelets, which closely resembles platelet-rich plasma in many functional and biochemical aspects. The cuvette therefore enables a direct comparison between three important platelet functions and several metabolic parameters and makes it possible to evaluate in detail how these processes interact.

*In contrast to granular, nonmetabolic ATP.*

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MATERIALS AND METHODS

Platelet Isolation

Freshly drawn venous blood (160-ml portions) was collected from healthy volunteers into citrate (0.1 volume of 130 mM trisodium citrate). The donors claimed not to have taken any medication during the previous 10 days. After centrifugation (200 g, 15 min, 22°C), the supernatant, platelet-rich plasma, was placed on a Sepharose 2B column (Pharmacia, Uppsala, Sweden; column-size 3 cm × 20 cm) equilibrated in Sr2+ and albumin containing Tyrode’s solution. After gel-filtration (at 22°C; completed within 30 min) 1.9 mM MgCl2 and 0.05 mM CaCl2 were added. This provided the extracellular bivalent cations that are essential for aggregation and enabled measurement of extracellular Ca2++ with a Ca2+ electrode in a range where the amplified electrode output is linear with respect to Ca2+ concentration. The gel-filtered platelets were prewarmed at 37°C in capped polystyrene tubes for 15 min before the start of the metabolic and functional studies. The final concentration of platelets ranged between 2.0 and 4.5 × 10^10 cells · ml⁻¹, unless otherwise stated.

Patient

Platelets from one patient with storage pool deficiency (patient III-4, see reference 11), were used and shown to be devoid of secretable ATP and ADP.

Simultaneous Measurement of Metabolic and Functional Parameters

The platelets were incubated in a multiresponse monitor, which has been described in detail previously. It consists of an incubation chamber with a built-in pH combination electrode, a Ca2+ electrode, a micro Clark oxygen electrode, a lamp-phototransistor pair, and a magnetic stirring device. Except for a 1-mm diameter outlet, the cuvette is sealed off by a plunger and does not contain any gas space, thus allowing O2 measurements and preventing CO2 liberation from the platelet suspension. The outlet, in combination with the plunger, enables sampling of small quantities of cell suspension without interference with the measurements. The electrode outputs are conditioned by a multichannel amplifier. Amplified electrode outputs were linear with platelet count over the range between 0.35 and 4.5 × 10^10 cells · ml⁻¹. The suspensions (final volume 3–6 ml) were incubated at 37°C (unless otherwise stated) and stirred at 900 rpm.

Platelet responses were induced with 5 μM epinephrine (Sigma, St. Louis, Mo.), 10 μM ADP (Boehringer, Mannheim, Germany), or 0.05-10 NIH units · ml⁻¹ of thrombin (Parke Davis, Michigan; dialyzed against the gel-filtration buffer for 24 hr at 4°C and stored at −20°C in 1000 U · ml⁻¹ samples). Epinephrine and ADP-induced aggregations were carried out in the presence of 1 g · l⁻¹ human fibrinogen (grade 1, Kabi, Stockholm), dialyzed for 48 hr at 4°C against the gel-filtration buffer.

In some experiments, total arachidonate oxygenation was inhibited by 30 μM 5,8,11,14 eicosatetraynoic acid (ETYA, Hoffman-La Roche, Nutley, N.J.), whereas cyclooxygenase activity was specifically blocked by preincubation (15 min, 37°C) of the gel-filtered platelets with 1 mM acetylsalicyclic acid (Sigma, St. Louis, Mo.), freshly prepared as a 100 mM stock solution in 0.12 M NaHCO3 (pH 7.2). In other experiments, cellular ATP production was inhibited by preincubating the suspensions for 30 min (37°C) with 30 mM 2-deoxy-D-glucose (Sigma) and/or 8.2 μg · ml⁻¹ antimycin-A (Sigma). The final ethanol concentration introduced with the antimycin or ETYA (0.175% v/v) did not affect the results. Lactate was measured fluorimetrically. For measurement of metabolic ATP and ADP, platelet-rich plasma was incubated with 0.4 μM U-14C adenine (specific activity 286 Ci · mole⁻¹, Code CFA, 436, Amersham, Arlington Heights, Ill.), for 30 min at 37°C prior to gel-filtration. The ethanol-soluble, radioactive adenine nucleotides and their derivatives were extracted from the gel-filtered platelets and quantitated according to standard procedures. Secretion of the contents of α-granules and acid hydrolase-containing vesicles was followed by measurement of extracellular β-thromboglobulin (measured by the β-thromboglobulin kit of the Radiochemical Center, Amersham) and N-acetyl-β-glucosaminidase, respectively. Samples for these measurements were collected from the incubation chamber and centrifuged (10,000 g, 2 min, 22°C) in the presence of 135 mM formaldehyde; the secreted activities were measured in the supernatant.

![Fig. 1. Simultaneous measurement of oxygen uptake (O), extracellular Ca2+ (Ca2+), proton liberation (H+), and light transmission (T) in the same suspension of gel-filtered platelets. Data for O, Ca2+ T, and H (+) are expressed as n-atoms · ml⁻¹. The downward deflection of the O and Ca2+ curves reflect oxygen consumption and an increase in extracellular Ca2+ concentration, respectively. The upward deflection in H+ and light transmission (T, arbitrary units) reflect increase in extracellular H+ and aggregation, respectively. Inducer: 1.0 U · ml⁻¹ of thrombin (arrow).](attachment:fig1.png)
RESULTS
Proton Liberation and O₂ Consumption During Thrombin-Induced Aggregation and Ca²⁺ Secretion

Figure 1 depicts a typical experiment in which normal platelets were stimulated with 1.0 U · ml⁻¹ of thrombin. Before stimulation, the platelets liberated H⁺ ions at a constant rate and showed a steady uptake of oxygen. Following thrombin addition, aggregation and Ca²⁺ secretion became apparent, paralleled by a transient increase in O₂ uptake and a rapid rise in proton liberation.

Maximal responses were found at 5 U · ml⁻¹ of thrombin. Here, the acidification velocity rose from 5.0 ± 0.8 μatoms H⁺ formed · min⁻¹ · 10⁻¹¹ cells (n = 12) before stimulation to values 4–8 times higher immediately after stimulation. The proton liberation leveled off about 3 min after stimulation to 11.0 ± 2.9 μatoms H⁺ · min⁻¹ · 10⁻¹¹ cells (n = 12). The slow O₂ uptake in nonstimulated suspensions was abolished by antimycin-A, and thus reflected a mitochondrial respiration of 0.77 ± 0.26 μatoms O₂ · min⁻¹ · 10⁻¹¹ cells (n = 9). Thrombin at 5 U · ml⁻¹ induced a burst in O₂ uptake that lasted for 2–3 min; thereafter, the O₂ uptake stabilized at 0.72 ± 0.46 μatoms O₂ · min⁻¹ · 10⁻¹¹ (n = 11). Maximum secretion was also found at 5 U · ml⁻¹ of thrombin and amounted to 13.4 ± 1.7 μatoms Ca²⁺ · 10⁻¹¹ platelets (n = 14).

Characterization of Proton Liberation

Several possible sources of the increased proton liberation following platelet stimulation were considered, i.e., (1) production of lactate, (2) secretion of granule contents, (3) hydrolysis of metabolic ATP, and (4) proteolysis of membrane components.

Previous work already demonstrated a good correlation between lactate production and proton liberation in nonstimulated platelets. We could extend this observation by varying lactate production with metabolic inhibitors or by varying the amount of extracellular glucose in antimycin-A-treated cells (Fig. 2). Also, in thrombin (5 U · ml⁻¹) treated platelets, both parameters correlated well after aggregation and secretion were well completed (Fig. 2). During aggregation and Ca²⁺ secretion induced by thrombin, however, an extra burst in H⁺ liberation took place that was not accounted for by lactate production (Fig. 3).

The lactate-unrelated burst was then compared with the secretion of the contents of dense granules, which in pig platelets have a pH of 5.7, and with secretion of constituents of α-granules and acid hydrolyase vesicles, the latter having a pH of 5.0 in most types of cells. The results are shown in Table 1. Stimulation of antimycin-A-treated platelets with a low dose of thrombin (0.1 U · ml⁻¹; 37°C) reduced the H⁺ burst by 65% and acid hydrolase secretion by 70% as compared to platelets treated with 5 U · ml⁻¹ of thrombin in the absence of the inhibitor. Ca²⁺ and β-thromboglobulin secretion, however, were little affected. Lowering the temperature from 37°C to 15°C reduced the H⁺ burst and acid hydrolase secretion by about 80%, whereas Ca²⁺ secretion and

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**Fig. 2.** Correlation between proton liberation and lactate production in nonstimulated platelet suspensions (open symbols) and in suspensions in which aggregation-secretion were well completed (at 3 min after thrombin addition and later). The platelet suspensions were incubated with and without 8.3 μg antimycin-A · ml⁻¹ and 0–5 mM glucose in order to induce different rates of lactate formation. Regression line with correlation coefficient: y = 1.01x + 2.01, r = 0.85. Data (n = 33) originate from 7 suspensions.
β-thromboglobulin secretion were distinctly less reduced. The lactate-unrelated H+ burst was completely absent following stimulation of platelets with ADP (10 μM) or epinephrine (5 μM). These agents did, however, increase lactate production, and they induced a quantitatively similar rise in proton liberation. Neither ADP nor epinephrine induced acid hydrolase secretion, although dense granule and α-granule secretion were normal. This suggested that the dense granule and α-granule contents were not the source of the lactate-unrelated H+ burst.

A normal H+ burst in response to 5 U·ml⁻¹ of thrombin was found with platelets from a patient with severe storage pool deficiency. These platelets did not secrete Ca²⁺ but showed normal acid hydrolase secretion (data not shown). The lactate-unrelated H+ burst induced by thrombin therefore appeared to correlate closely with the secretion of the acid hydrolase vesicles.

Finally, in platelets pretreated with 2-deoxy-D-glucose and antimycin-A (30 min, 37°C), addition of thrombin (5 U·ml⁻¹) did not induce any functional responses and there was no H+ liberation either before or after thrombin addition. This result indicates that the proteolysis of membrane constituents that takes place during platelet-thrombin interaction was apparently below our detection limits.

Acceleration of Proton Liberation During Platelet Stimulation

The burst in H+ liberation, which was unrelated to lactate production and took place during thrombin-induced aggregation and secretion, masked the acceleration in the proton liberation that was due to or coincided with lactate formation. This made it difficult to establish precisely when lactate formation accelerated. Since no burst in H+ liberation took place after stimulation with ADP or epinephrine (see above), the increase in lactate-related proton liberation was first studied following stimulation by these agents. Within 5 sec after addition of 10 μM ADP, the rate of proton liberation in resting platelets (Fig. 4, phase I) increased abruptly and remained constant thereafter (phases II and III). Only after about 2 min did dense granule secretion start, as illustrated by a slow liberation of Ca²⁺ ions (phase III). During Ca²⁺ secretion, no change in acidification velocity took place. The early increase in proton liberation

### Table 1. Comparison Between the Burst in H+ Liberation and Granule Secretion

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Secretion After 3 min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H⁺</td>
</tr>
<tr>
<td>5.0 U·ml⁻¹</td>
<td>37°C</td>
</tr>
<tr>
<td>0.1 U·ml⁻¹</td>
<td>37°C</td>
</tr>
<tr>
<td>5.0 U·ml⁻¹</td>
<td>15°C</td>
</tr>
</tbody>
</table>

Data give mean ± SD of 6 experiments and are expressed as percent of values found following addition of 5.0 U·ml⁻¹ of thrombin at 37°C. Controls (no thrombin added) were less than 2%.

H⁺: the extra H⁺ formation after correcting for lactate-related H⁺ liberation measured between 3 and 10 min after thrombin addition (see Fig. 3);

suggested that lactate production accelerated during the beginning of aggregation and well before dense granule secretion started. Similar conclusions could be drawn from experiments with low concentrations of thrombin. Following addition of 0.1 U·ml⁻¹ of thrombin (Fig. 5) proton liberation became increasingly rapid and then levelled off again. Superimposed on the proton liberation of resting cells, this pattern was quite similar to that of Ca²⁺ secretion except that the alterations in proton liberation preceded the changes in the rate of Ca²⁺ secretion. The kinetics of Ca²⁺ secretion have been shown to follow a mathematical model composed of three phases: a very slow exponential phase immediately after thrombin addition, then a rapid, exponential phase in which most of the Ca²⁺ is secreted, and finally a slow, linear phase (details shown in Table 4). By analogy, we have subdivided the changes in proton liberation relative to resting cells (Fig. 5, phase I) that took place by thrombin addition into a slow phase (phase II), a rapid phase (phase III), and finally a slow linear phase (phase IV). The onset and duration of the various phases varied among different platelet suspensions. Phase II started between 35 and 45 sec after thrombin addition, phase III after 90-100 sec, and phase IV after 130-150 sec (range for 5 different suspensions). The beginning of
phase II was always later than the onset of shape change (after 20–25 sec), while phase III started always after the onset of detectable aggregation (after 50–60 sec) and Ca\(^{2+}\) secretion (after 65–75 sec). As shown before, the acidification in phases I and IV correlated well with the formation of lactate, whereas the burst in proton liberation during phase III correlated with the secretion of acid hydrolase vesicles. The extent and duration of phase II was too small for accurate comparison between H\(^+\) and lactate production. By analogy with Ca secretion, phase II acidification may reflect an early phase in acid hydrolase secretion. It might also be caused by an increased formation of lactate as demonstrated with ADP and epinephrine (Fig. 5). The fact that phase II acidification was approximately linear and fell well within the range of acidification rates of phase IV (differences were less than 10% in 5 different suspensions) makes us favor the latter possibility.

**O\(_2\) Uptake by Mitochondrial Respiration and Arachidonate Oxygenation**

O\(_2\) uptake increased rapidly following thrombin addition, until it stabilized after 2–3 min (Fig. 1). The burst in O\(_2\) uptake amounted to \(2.57 \pm 0.63\) (\(n = 5\)) and \(4.15 \pm 0.59\) (\(n = 8\)) \(\mu\)atoms O\(_2\) \(\cdot 10^{-11}\) cells at 1.0 and 5.0 U \(\cdot\) ml\(^{-1}\) of thrombin, respectively. The burst was abolished by a mixture of antimycin-A and ETYA; addition of these inhibitors separately and in the presence and absence of acetylsalicylic acid (ASA) showed that 21% of the burst in O\(_2\) uptake was related to mitochondrial respiration (antimycin-A-sensitive), while 55% could be attributed to arachidonate metabolism via cyclooxygenase (ASA-sensitive) and 23% to lipooxygenase oxygenation of arachidonate (difference between ETYA-sensitive and ASA-sensitive O\(_2\) uptake with 5 U \(\cdot\) ml\(^{-1}\) of thrombin, mean of four experiments).

**Response Times of Metabolic and Functional Platelet Responses**

The response time was defined as the time between addition of the agonist to the platelet suspension and the first deflection of the recorder tracings. Where necessary, the number of channels that was monitored simultaneously was reduced to shorten the interval between the signals. The response times greatly depended on the strength of the stimulus. A low thrombin concentration (0.1 U \(\cdot\) ml\(^{-1}\)) induced shape change after about 25 sec, an acceleration in proton liberation after about 40 sec, an increase in light transmission after about 55 sec, and a small Ca\(^{2+}\) secretion (k, phase) after about 70 sec. At this thrombin concentration, the burst in O\(_2\) uptake was not detectable (Fig. 5). At higher thrombin concentrations, the differences in response times became smaller and the burst in O\(_2\) uptake became more apparent (Fig. 1). The acceleration in mitochondrial O\(_2\) uptake (ETYA present) started about 6 sec later than aggregation and secretion, which started simultaneously at 1 U \(\cdot\) ml\(^{-1}\) of thrombin or more. Arachidonate oxygenation (antimycin-A present) also began after aggregation and secretion were initiated and started about 6 sec later than mitochondrial respiration (Fig. 6). Upon addition of ADP or epinephrine, no burst in oxygen uptake could be detected. Again, proton liberation (lactate-related) started early, whereas Ca\(^{2+}\) secretion became apparent after the beginning of aggregation (Table 2).

**Energy Consumption During Aggregation and Ca\(^{2+}\) Secretion**

The variations in H\(^+\) liberation suggested that lactate production increased rapidly at the end of the shape change phase and remained constant thereafter for at least 20 min. This enabled calculation of the rate of energy consumption in glycolysis and glycogenolysis during the aggregation–secretion responses. Together with the measurement of mitochondrial respiration and determination of metabolic adenylate levels with isotopic tracer techniques in the same platelet suspension, the rate of ATP consumption could be calculated (Table 3). The data show that in resting platelets, glycolytic energy production supplied 65% of the total energy yield, while the remainder was furnished by mitochondrial respiration. Upon stimulation with 5 U \(\cdot\) ml\(^{-1}\) of thrombin, lactate production increased twofold, leading to about two times more ATP production (depending on the contribution of glycogen catabolism, which has a higher ATP yield per mole of lactate than glycolysis). Oxidative phosphorylation accelerated later. This acceleration, however, was hyperbolic, and the average over the first 30 sec (Table 3) therefore underestimates its contribution in
these functions. This was found also when contribution of mitochondrial energy production and catabolism. At lower thrombin concentrations, the generating sequences, about 40% of the total energy also show that despite the acceleration in ATP- and secretion. Arachidonate is made aggregation of thromboxane A2, which is a strong inducer of
Induced Aggregation and Ca2+ Secretion
ADP or epinephrine were the aggregating agents.
Role of Arachidonate Oxygenation in Thrombin-Induced Aggregation and Ca2+ Secretion
Oxygenation of arachidonate precedes the formation of thromboxane A2, which is a strong inducer of aggregation and secretion. Arachidonate is made available from phospholipids during the platelet-thrombin interaction. We quantitated arachidonate oxygenation, aggregation, and Ca2+ secretion in response in thrombin by measuring the burst in O2 uptake following thrombin addition to antimony-A-treated platelets. The burst was maximal at 5 U · ml⁻¹ of thrombin and declined rapidly at lower concentrations of thrombin. At 1 U · ml⁻¹ of thrombin, the burst in oxygen uptake was reduced to 24%, but this hardly affected the aggregation velocity (78% of maximum) or Ca2+ secretion (100% of maximum). When arachidonate oxygenation was inhibited by ETYA, the burst in oxygen consumption was absent. This did not affect aggregation and Ca2+ secretion at thrombin concentrations of about 1.0 U · ml⁻¹ or higher. At lower thrombin levels, however, ETYA retarded aggregation
Table 2. Response Times of Energy-Generating Sequences and Platelet Functions

<table>
<thead>
<tr>
<th>Inducer</th>
<th>Glycolysis</th>
<th>Oxidative Phosphorylation</th>
<th>Shape Change</th>
<th>Aggregation</th>
<th>Ca²⁺ Secretion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thrombin (U · ml⁻¹)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>41</td>
<td>—</td>
<td>24</td>
<td>54</td>
<td>70</td>
</tr>
<tr>
<td>1.0 -</td>
<td>8</td>
<td>21</td>
<td>4</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td>2.5 -</td>
<td>8</td>
<td>14</td>
<td>2</td>
<td>9</td>
<td>8</td>
</tr>
<tr>
<td>5.0 -</td>
<td>8</td>
<td>12</td>
<td>2</td>
<td>9</td>
<td>8</td>
</tr>
<tr>
<td>ADP, 10 µM</td>
<td>5</td>
<td>—</td>
<td>5</td>
<td>12</td>
<td>139</td>
</tr>
<tr>
<td>Epinephrine, 5 µM</td>
<td>5</td>
<td>—</td>
<td>—</td>
<td>5</td>
<td>154</td>
</tr>
</tbody>
</table>

The data present the time (sec) between addition of the aggregation agent and the first deflection of electrode outputs and light transmission measurement from the values found in nonstimulated suspensions. The onset of shape change is defined as the beginning of a decrease in light transmission. Data originate from the same suspension of gel-filtered platelets and are representative for 5 different suspensions. It should be noted that the response times of all electrodes is less than 1 sec.¹ The geometry of the incubation cuvette and the large volumes (3–5 ml) of platelet suspensions used in these studies greatly determine the time between addition of the stimulus and the onset of the various responses.

The first second of the increased respiration. The data also show that despite the acceleration in ATP-generating sequences, about 40% of the total energy consumption during the first 30 sec came from ATP catabolism. At lower thrombin concentrations, the contribution of mitochondrial energy production and ATP catabolism to total energy supply diminished, thus leaving glyco(geno)lysis as the only energy source supporting these functions. This was found also when ADP or epinephrine were the aggregating agents.

Table 3. Energy Status During Thrombin (5 U · ml⁻¹) Induced Aggregation and Ca²⁺ Secretion

<table>
<thead>
<tr>
<th></th>
<th>Nonstimulated Platelets</th>
<th>0-30 sec After Thrombin Addition</th>
<th>30-180 sec After Thrombin Addition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxygen consumption</td>
<td>0.77 ± 0.26 (2.3)</td>
<td>1.57 ± 0.37 (4.7)</td>
<td>0.72 ± 0.46 (2.2)</td>
</tr>
<tr>
<td>Lactate production</td>
<td>4.22 ± 1.42 (4.2)</td>
<td>(8.5)</td>
<td>8.52 ± 2.34 (8.5)</td>
</tr>
<tr>
<td>Total energy yield</td>
<td>(6.5)</td>
<td>(13.2)</td>
<td>(10.7)</td>
</tr>
<tr>
<td>Fall in metabolic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATP–ADP</td>
<td>(0)</td>
<td>8.9 ± 1.4 (8.9)</td>
<td>(0)</td>
</tr>
<tr>
<td>Energy consumption</td>
<td>(6.5)</td>
<td>(22.1)</td>
<td>(10.7)</td>
</tr>
<tr>
<td>Extra energy consumption</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>During platelet responses</td>
<td></td>
<td>(15.6)</td>
<td>(4.2)</td>
</tr>
</tbody>
</table>

Data are expressed as µatoms (O2 consumption) or µmole (all other parameters) · min⁻¹ · 10⁻¹¹ platelets. Values between parentheses give ATP equivalents (ATP eq) reflecting an energy yield of 2 ATP eq for the conversion of ATP to AMP and 1 ATP eq for the conversion of ATP to ADP.¹ Energy production was calculated from lactate formation in glycolysis (1 mole of lactate formed equals 1 mole of ATP eq produced) and oxidative phosphorylation (1 at 0 consumed equals 3 mole of ATP eq produced). Glycogenolysis (1 mole of lactate formed equals 1.5 mole of ATP eq produced) was neglected, since before stimulation glycogenolysis is absent,²⁶ whereas in stimulated platelets its maximal flux is about 1.8 µmole lactate formed · min⁻¹ · 10⁻¹¹ cells.²⁷ The data on total energy yield after stimulation might therefore underestimate the actual energy yield by not more than 6%. Energy consumption was taken as the sum of the production and fall in ¹⁴C-ATP + ADP. The latter was recalculated into absolute amounts by taking into account that ¹⁴C-adenine incubation of platelets labels the adenine nucleotides homogeneously³⁵ and that the 80% total of ¹⁴C radioactivity normally found in the ATP fraction equals the metabolic ATP level, that is 3.5 µmole · 10⁻¹¹ platelets.²⁶ The extra energy consumption during platelet function was calculated under the assumption that the energy consumption in processes of nonstimulated suspensions did not change after platelet activation. The small amount of metabolic ATP that is catabolized between 30 sec and 3 min after thrombin addition has been neglected.
and reduced the amounts of Ca\textsuperscript{2+} that were secreted (Fig. 7). The lower the thrombin concentration, the more effective was the inhibition, leading to an almost 50% reduction at 0.1 U ml\textsuperscript{-1} of thrombin. At this concentration, arachidonate oxygenation was hardly detectable. Moreover, when ETYA was present, the response time of Ca\textsuperscript{2+} secretion increased 2-3-fold; the secretion velocity (k\textsubscript{2} phase) was also greatly impaired in the presence of ETYA (Table 4, Fig. 8). The effect of ETYA was enhanced by lowering the platelet count at constant ETYA concentration (data not shown).

**DISCUSSION**

Platelets respond to minor chemical and physical stimuli with pronounced changes in energy metabolism and functional behavior. Accurate comparisons between functions and metabolism can therefore only be made by measuring these parameters simultaneously in the same platelet suspension. We have used electrodes to monitor the extracellular concentrations of H\textsuperscript{+}, Ca\textsuperscript{2+}, and O\textsubscript{2}, which, together with an optical device for measuring shape change and aggregation, enable such comparisons in gel-filtered platelets that resemble platelet-rich plasma in most functional properties. The cuvette also allowed subsampling from the cell suspension during these studies so that any detectable parameter, either intra- or extracellular, could be measured at given times.

A previous observation\textsuperscript{6} that platelets acidify their medium during storage because of lactate formation suggested the use of a pH electrode for continuous monitoring of lactate production. We demonstrate here that the proton liberation equaled lactate production in a mole-to-mole base in nonstimulated gel-filtered platelet suspensions. Thus, the proton liberation was either directly caused by the production of

**Table 4. Influence of ETYA on the Velocity of Ca\textsuperscript{2+} Secretion (k\textsubscript{2} Phase)**

<table>
<thead>
<tr>
<th>Thrombin Concentration</th>
<th>k\textsubscript{2} (sec\textsuperscript{-1})</th>
<th>Control</th>
<th>ETYA</th>
<th>n</th>
<th>t</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 U ml\textsuperscript{-1}</td>
<td>0.0145 ± 0.0042</td>
<td>0.0062 ± 0.0031</td>
<td>10</td>
<td>0.0009</td>
<td></td>
</tr>
<tr>
<td>0.2 U ml\textsuperscript{-1}</td>
<td>0.0283 ± 0.0116</td>
<td>0.0188 ± 0.0101</td>
<td>7</td>
<td>0.0741</td>
<td></td>
</tr>
<tr>
<td>0.5-5.0 U ml\textsuperscript{-1}</td>
<td>0.0264 ± 0.0112</td>
<td>0.0246 ± 0.0119</td>
<td>8</td>
<td>0.0890</td>
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The kinetics of Ca\textsuperscript{2+} secretion followed the model proposed by Detwiler and Feinman\textsuperscript{22} and consisted of two exponential phases (with velocity constants k\textsubscript{1} and k\textsubscript{2}, respectively) and a slow linear phase (with velocity constant k\textsubscript{3}), which was only apparent after stimulating the cells with low thrombin concentrations (≤0.1 U ml\textsuperscript{-1}). We found values for t\textsubscript{i} (the time between thrombin addition to the inflection point of the Ca\textsuperscript{2+} curve) of 60-90 sec at 0.1 U ml\textsuperscript{-1} and 20-30 sec at 1.0 U ml\textsuperscript{-1} of thrombin, which are about twice the values reported by Detwiler and Feinman\textsuperscript{22} based on murexide binding studies. Our values for k\textsubscript{2} (0.023 ± 0.005 sec\textsuperscript{-1} mean ± SD, n = 14) and for k\textsubscript{3} (8-16 nats Ca\textsuperscript{2+} liberated · sec\textsuperscript{-1} · 10\textsuperscript{-11} platelets; dependent on the thrombin concentration) were in the same range as those reported by these authors. Data are mean ± SD with number of determinations, n. The t value has been calculated with a t test for paired observations. Platelet concentration 2.5 · 10\textsuperscript{8} cells · ml\textsuperscript{-1}. Data originate from 7 platelet suspensions.
lactate or was linked to systems tightly coupled to it. Similarly, production of lactate and liberation of protons were equal in stimulated platelets after aggregation and secretion had been terminated. However, immediately following thrombin addition, a burst in H' liberation took place that did not correlate with the production of lactate. This burst was apparently not caused by extrusion of the contents of dense granules, which are known to have a pH of 5.7,17 since the burst was absent following ADP and epinephrine-induced Ca2+ secretion. On the other hand, such a burst did occur during interaction of thrombin and storage pool deficient platelets, i.e., in the absence of dense granule secretion. The lactate-unrelated H' burst was also not associated with α-granule secretion, since it was absent following ADP and epinephrine-induced β-thromboglobulin secretion. Furthermore, hydrolysis of metabolic ATP does not contribute significantly to overall acidification, since we confirmed other studies that showed that a 70% conversion of ATP into hypoxanthine induced by H2O2 was not accompanied by any change in the acidification rate.4 In addition, membrane proteolysis during platelet–thrombin interaction7 apparently was not detectable with our H' measurements. However, under all conditions studied, acid hydrolase secretion (measured as β-N-acetylglucosaminidase liberation) and the lactate-unrelated H' burst varied in parallel. Since acid hydrolase-containing vesicles in most cells have an acid internal pH,18 it is possible that the extrusion of the contents of these vesicles causes the lactate-unrelated H' production.

Our data show that nonstimulated gel-filtered platelets consume about 0.7 μatoms of O2 by mitochondrial respiration and produce about 4 μmole lactate/min/10¹¹ cells, which is equivalent to the production of about 2 and 4 μmole ATP eq* · min⁻¹ · 10¹¹ cells, respectively. 

Thrombin (0.1 U · ml⁻¹) caused, after a lag of about 40 sec, an increase in proton formation that remained at a high rate for another 30–40 sec (Fig. 5, phase II). This increased rate was the same as the rate found after the lactate-unrelated H' burst; aggregation as well as secretion were terminated. After this termination, H' formation closely correlated with lactate production, and although direct evidence cannot be obtained, it seems reasonable to assume that the same correlation holds during the initial phase (phase II) of increased proton formation. Acceleration of H' formation is also found shortly after addition of ADP or epinephrine, indicating that glyco(geno)lytic energy production accelerates before the initiation of aggregation and Ca²⁺ secretion. Only when high thrombin concentrations are used, and when aggregation and Ca²⁺ secretion approach half-maximal levels, does mitochondrial respiration accelerate. The mitochondrial O2 uptake is exponential and it is therefore difficult to quantitate the ATP yield accurately in the initial phase of accelerated O2 uptake. About 0.75 μatoms O2 · 10⁻¹¹ cells were consumed over the first 30 sec, which equals the production of 2.25 μmole ATP eq · 10⁻¹¹ cells. Under these conditions, the metabolic ATP level falls, liberating about 4.5 μmole ATP eq · 10⁻¹¹ cells over the first 30 sec, thereby contributing to energy liberation during aggregation and secretion. The burst in mitochondrial energy supply is only apparent at high (> about 1 U/ml) thrombin concentrations at which aggregation and Ca²⁺ secretion are rapid and acid hydrolase secretion is maximal. The rapidity of these processes and/or the extent of acid hydrolase secretion, therefore, appear predominant factors for this second acceleration in energy-producing mechanisms. This agrees with recent observations that acid hydrolase secretion is more dependent on mitochondrial energy support than dense granule secretion, which could mean that maximal extent of the former process required ATP more rapidly than glycolysis can produce it.23 Activation of

*ATP eq = ATP equivalent, as defined in Table 3.
energy-generating sequences in stimulated platelets is thought to occur in response to the lowering of the ATP level and adenylate energy charge that accompanies stimulation (by thrombin) and which may result from a transient imbalance between energy production and consumption. The present data show that activation of mitochondrial energy production might well be the result of these changes but that glyco(geno)lysis is activated at a much earlier stage. The small transient decrease in energy charge that occurs as early as 5 sec after ADP addition and has disappeared almost completely 2 min later is therefore a more likely cause for glycolytic flux activation. Also, most platelet responses are probably triggered by a rise in cytosolic Ca\(^{2+}\) content, which would activate glycogen catabolism. An increase in glucose-1-phosphate, one of the first intermediates in glycogenolysis, is indeed seen within 3 sec after thrombin stimulation. Activation of aerobic and anerobic energy generation apparently occurs separately and independently, which implies a complicated control mechanism that is still largely obscure. Our data on mitochondrial O\(_2\) uptake are intermediate between those of Fukami et al. (twice as low) and those of Muenzer et al. (2-3 times as high), which in terms of ATP formation has a considerable impact on calculations of energy fluxes. Again, the earlier studies were restricted to EDTA-washed platelets and to measurement of one response at a time. Especially when analyzing H\(^+\) formation in platelet suspensions, simultaneous measurement of secreted parameters and energy-producing pathways proved to be essential for further characterization of the H\(^+\) production that appeared difficult with other techniques.

The maximal burst in O\(_2\) uptake introduced by thrombin in our gel-filtered platelets (2-4 \(\mu\)atoms \(\cdot\) 10\(^{11}\) cells) is the same range as found with EDTA-washed platelets and after collagen stimulation of “bag” washed platelets. Preincubation with ETYA and antimycin-A completely prevented the burst, whereas use of these inhibitors separately showed that about 20% was related to increased mitochondrial respiration and the remaining 80% was involved in arachidonate oxygenation. This distribution agrees with earlier findings by Pickett and Cohen. About 69% of the arachidonate-related oxygen consumption was inhibited by ASA, suggesting the remaining 31% was involved in lipoxygenase activity, but the actual amounts in an uninhibited system might be substantially lower since inhibition of cyclooxygenase is known to stimulate arachidonate oxidation via lipoxygenase. Our data show that above 2.5 U \(\cdot\) ml\(^{-1}\) of thrombin, aggregation and Ca\(^{2+}\)secretion are not inhibited by ETYA, therefore confirming earlier observations that arachidonate oxidation is not essential for aggregation and secretion induced by high amounts of thrombin. However, at low concentrations of thrombin (\(> 2.5\) U \(\cdot\) ml\(^{-1}\)) ETYA retarded shape change, aggregation velocity, and the amount of secreted Ca\(^{2+}\). At 0.1 U \(\cdot\) ml\(^{-1}\) of thrombin, ETYA increased the time lag preceding Ca\(^{2+}\) secretion by 2-3 fold and reduced considerably the velocity by which most of the Ca\(^{2+}\) was secreted (k\(_2\) phase). Although k\(_2\) has been claimed to be independent of thrombin concentration and platelet count, our results clearly show that it is sensitive to inhibition of arachidonate oxygenation, suggesting that it is subject to control by prostaglandins and/or thromboxanes. The effect of ETYA was enhanced by lowering the platelet count. This may have been caused by the increase in the ETYA/platelet ratio, since ETYA is hydrophobic and probably is preferentially bound to the platelets. These effects were only observed at very low thrombin concentrations (0.1 U \(\cdot\) ml\(^{-1}\)) at which the arachidonate-related \(\mathrm{O}_2\) burst became unmeasurable. Thus, in gel-filtered platelets, only a minute fraction of the potential arachidonate oxygenation is of importance for progression platelet responses induced by thrombin.

Our results demonstrate a specific order of events in platelets activated by high concentrations of thrombin (> 0.1 U \(\cdot\) ml\(^{-1}\)): the onset of detectable aggregation always preceded the onset of Ca\(^{2+}\) secretion, whereas the latter always preceded the onset of arachidonate oxygenation, as measured by the \(\mathrm{O}_2\) electrode. Although all electrodes have response times of less than 1 sec, their equilibration times are between 5 and 10 sec and slightly different for the various electrodes (for details see Akkerman et al.). This implies that the onset of the various responses can be measured accurately, but that the duration is subject to differences in electrode characteristics. These inaccuracies are relatively small as compared with the optical measurement of platelet aggregation, which is relatively insensitive to small aggregates. This implies that the onset of aggregation is probably earlier than our data indicate and might coincide with the increase in proton formation. The onset of aggregation well before changes in light transmission makes the time interval between the start of aggregation and dense granule secretion greater than apparent from our trainings. Thus, a considerable part of early thrombin-induced aggregation must be independent of products secreted from the dense granules, such as ADP, which is a potent trigger of aggregation and secretion. The same time sequence is found with weak inducers, e.g.,
ADP and epinephrine, which shows a much longer time lag between aggregation and secretion. Although arachidonate oxygenation leads to formation of potent aggregation-secretion inducers such as thromboxane A₂, the part of the oxygenation that can be measured by the O₂ electrode starts too late to be effective in triggering platelet functions induced by high amounts of thrombin.

ACKNOWLEDGMENT

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REFERENCES

22. Detwiler TC: 
Interrelationships among platelet responses: studies on the burst in proton liberation, lactate production, and oxygen uptake during platelet aggregation and Ca2+ secretion

JW Akkerman and H Holmsen