Ca^{2+}-independent secretion is dependent on cytoplasmic ATP in human platelets

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The secretion evoked in human platelets by physiological agonists is an energy-requiring process that depends on the metabolic ATP pool. Diacylglycerol, phorbol ester and collagen evoke a secretory response, which is not associated with an increase of cell Ca^{2+} levels, and is attributed to activation of protein kinase C [(1983) Nature 305, 317–319]. The secretion evoked by these agonists decreased along with cytoplasmic ATP depletion in the same way that the thrombin-induced secretion did. The secretory response was restored by raising again the cytoplasmic ATP levels. These results support the idea that the secretory response takes place by the physiological ATP-dependent mechanisms rather than by membrane perturbations in these instances.

ATP level *Release* *Diacylglycerol* *Phorbol ester*

1. INTRODUCTION

Although Ca^{2+} has been regarded as the universal trigger for secretory exocytosis, it has been shown recently that Ca^{2+}-independent secretion can be obtained in platelets when either phorbol ester (TPA) or diacylglycerol (DG), both activators of protein kinase C, are externally added in a lipophilic vehicle [1]. The same kind of response is elicited by collagen, a physiological agent, in aspirin-treated cells. Endogenously generated DG is thought to trigger protein kinase C in this case [2]. DG may be a fusogenic agent [3], TPA causes a rather atypical vacuolization of the platelet [4] and collagen-induced secretion in aspirin-treated cells is always quantitatively small and dependent on stirring, suggesting that collagen fibrils may cause some sort of membrane distortion. For these reasons it seemed worth testing whether the secretion was caused in these instances by the physiological exocytic mechanism and not by another kind of membrane perturbation. The physiological platelet secretory response induced by thrombin is known to decline along with the decrease of the ATP metabolic pool size [5]. In this work the effect of changes of the metabolic ATP pool on the platelet release reaction induced by either TPA, DG or collagen has been studied. All the experiments were performed in quin2-loaded platelets to mimic the previous experimental conditions [1].

2. EXPERIMENTAL

Platelet-rich plasma (PRP) was prepared from freshly drawn blood anti-coagulated with acid-citrate dextrose [6]. The cells were loaded with the fluorescent calcium indicator quin2 (a generous gift of Dr T.J. Rink, Physiological Laboratory, Cambridge, England) by incubating the PRP with 20 μM quin2 acetoxymethyl ester for 30 min at 37°C. The platelets were then centrifuged at 350 × g for 20 min and resuspended in a medium containing 145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$ and 10 mM Na-Hepes, pH 7.4, at a cell density of about 1×10^8 cells/ml. Glucose (10 mM) was used as the metabolic substrate in control experiments. ATP-depleted platelets were prepared by incubation in the same medium containing 200 ng/ml rotenone and 30 mM 2-deoxyglucose (DOG) in-

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stead of glucose. In the experiments in which reversible depletion was attempted, platelets were first depleted by incubation at 1×10^9 cells/ml in medium containing 4 mM DOG and 0.5 mM NaCN for 60 min. Then the cell suspension was diluted with 10 vols of standard medium containing 10 mM glucose and gassed with oxygen to remove the cyanide. Stimulation under stirring was performed in a Payton aggregometer in which changes in absorbance of the cell suspension were followed. 1 mM $CaCl_2$ or 1 mM K_2H_2 EGTA were added as required. Secretion was followed by ATP determination [7] in ethanol extracts of the cell suspension (total ATP) or direct analysis of the incubation medium after sedimentation of the cells by centrifugation at $10000 \times g$ for 1 min (secreted ATP). Collagen (Horn) and thrombin (Calbiochem) were added from concentrated buffered stock solutions to give final concentrations of 10 μg/ml and 0.5 U/ml, respectively. TPA (Sigma) and DG (a generous gift from Dr T.J. Rink) were added from concentrated stock solutions in DMSO to give final concentrations of 20 nM and 60 μg/ml, respectively. The DMSO did not exceed 0.2% (v/v) which by itself had no obvious effect.

3. RESULTS AND DISCUSSION

Fig.1A shows the time course of ATP depletion by incubation with rotenone and DOG. The figure also shows the amount of ATP secreted by thrombin under conditions of maximal stimulation. By subtracting this value from the total ATP the size of the metabolic pool could be estimated. It can be seen that, after 60 min incubation with the inhibitors, the metabolic ATP pool decreased to negligible values while the ATP levels in control cells were scarcely modified. Fig.1B shows the secretory response induced by thrombin, TPA, DG or collagen after different ATP-depletion periods. The $t_{1/2}$ values for depletion of metabolic ATP and inhibition of agonist-induced secretory exocytosis were very similar in all cases (8–10 min). This result suggests that the secretion is supported by the metabolic ATP pool in every case. Fig.2 shows the relation between total cell ATP and agonist-induced ATP secretion, both parameters plotted on a logarithmic scale. The relation was linear in all 4 cases studied. Aggregation also declined along

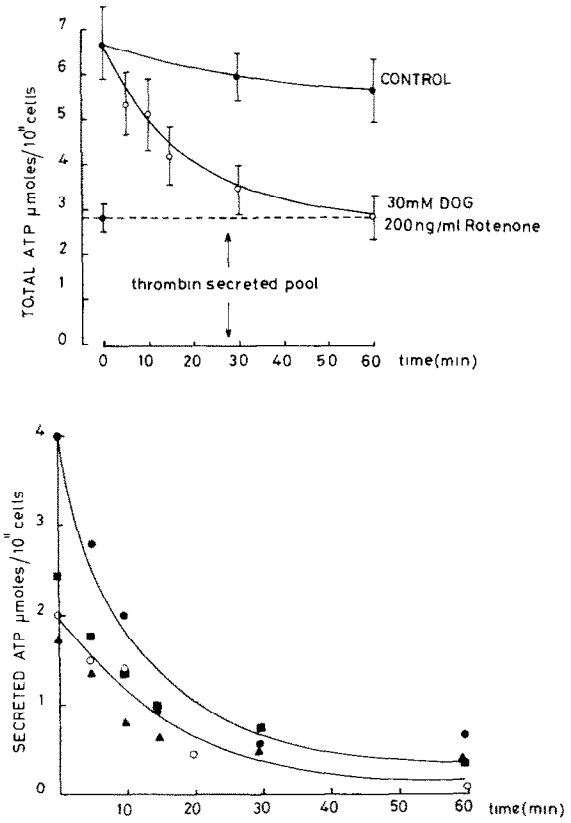

Fig.1. (A) Time course of the changes in ATP content of platelets resuspended in control medium plus 10 mM glucose (●) or the same plus 30 mM 2-DOG + 200 ng/ml rotenone (○). Incubation was carried out at 37°C without stirring. The broken line represents the dense granule-secretable pool taken as the thrombin-induced ATP secretion in control cells at the beginning of the experiment (●). Each value is the mean ± SD of 5 different experiments. (B) Time course decay of ATP secretion in platelets preincubated with 30 mM 2-DOG plus 200 ng/ml rotenone for different periods. Platelets were taken from the preincubation pool and aggregation and secretion assayed in the same medium under stirring. Agonist concentrations: 0.5 U/ml thrombin (●), 20 nM TPA (○), 10 μg/ml collagen (▲) and 60 μg/ml DG (■). Stimulations were done in 1 mM $(Ca^{2+})_o$.

with cytoplasmic ATP levels (not shown). It has been shown that some platelet proteins are phosphorylated preceding aggregatory and secretory response. These proteins have been identified as a 40 kDa protein, a substrate for protein kinase C, and a 20 kDa protein, the myosin light

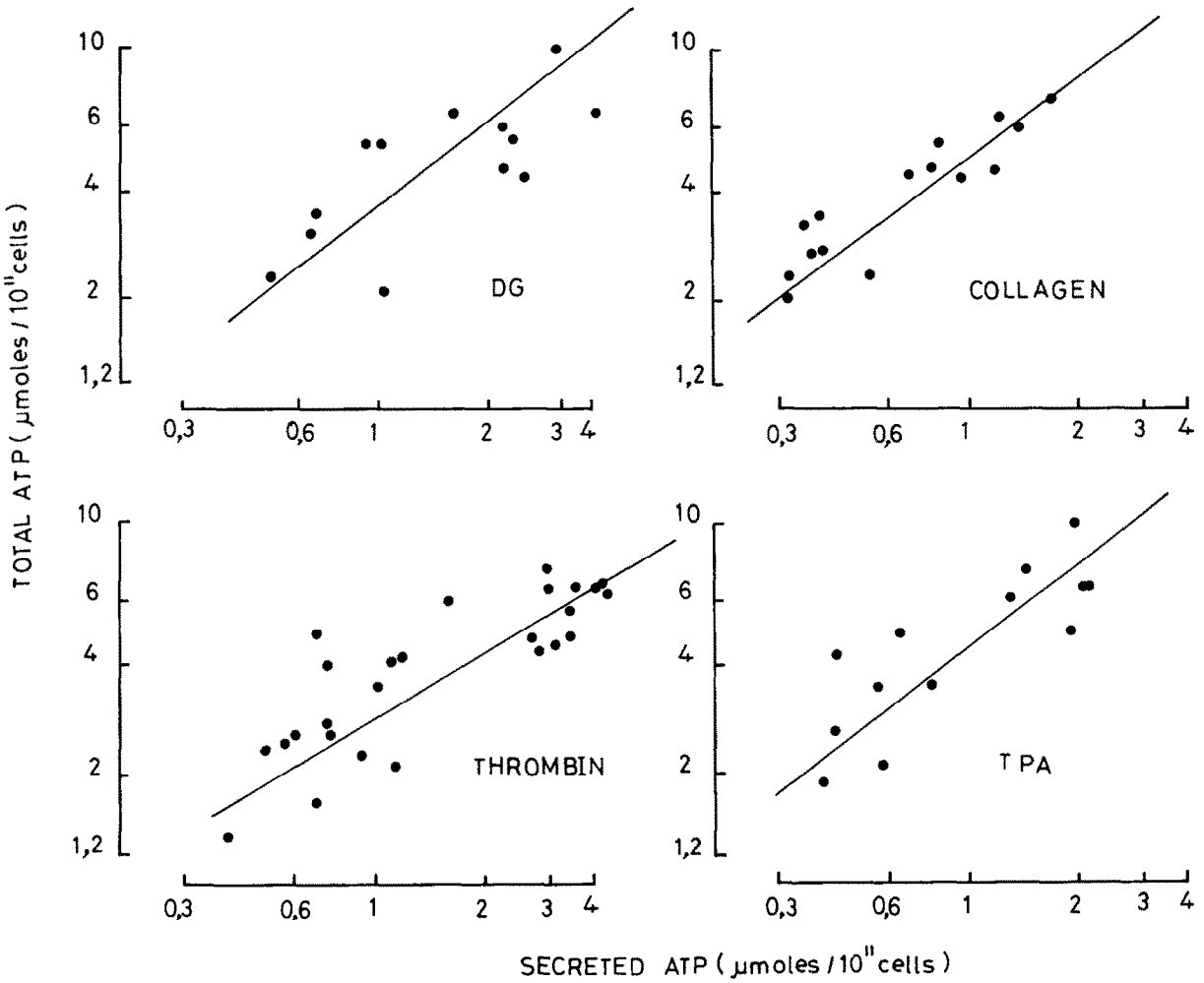

Fig.2. Logarithmic plot of the total ATP contents in platelets at different depletion stages vs dense granule-secreted ATP induced by DG, collagen, thrombin or TPA. Experimental procedure was as described in the legend to fig.1.

chain [8,9]. Since these proteins are involved in platelet secretion and shape change, respectively, one can imagine that these cellular processes will be very sensitive to changes in the availability of ATP such as those expected during depletion of the cytoplasmic ATP pool.

The reversibility of the loss of cellular responses due to ATP depletion was checked by studying the recovery of secretion and aggregation by incubation of ATP-depleted cells in a medium appropriate to raise the cytoplasmic ATP levels again. In these experiments a different procedure was used to deplete the cells, in order to remove the inhibitors and start refeeding without centrifugation (see section 2), since centrifugation of ATP-depleted platelets always led to cell lysis. Fig.3 shows the results of these experiments. From top to bottom the values of the total ATP, agonist-induced ATP secretion and the aggregation traces are presented. The left-hand panels show the responses obtained before any treatment. Incubation with NaCN and DOG during 30 min decreased the total ATP pool to 48% of the initial value and reduced both the secretory and aggregatory responses (central panels). Removal of cyanide and dilution of DOG with fresh glucose (see section 2) restored the cytoplasmic ATP level to 63% of the initial value after 60 min incubation

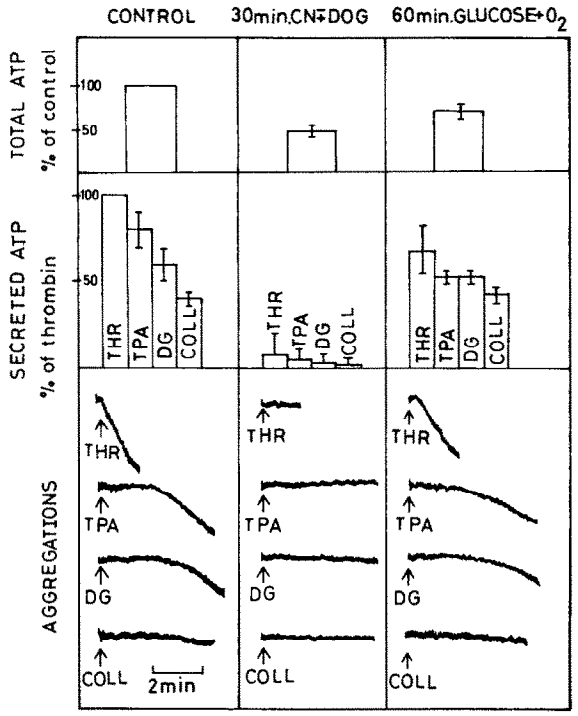

Fig.3. Reversion of inhibition of aggregation and secretory response in ATP-depleted cells by restoration of the ATP level (panels): (left) control cells before treatment, (central) effects of 30 min depletory treatment in 0.5 mM NaCN plus 4 mM 2-DOG for 30 min, (right) ATP-depleted cells were diluted and incubated for an additional period of 1 h in 10 mM glucose (see section 2). All incubations were done at 37°C without stirring. Stimulations were performed with continuous stirring and in the presence of 1 mM $(Ca^{2+})_o$. Agonist concentrations were the same as in fig.1. Each value is the mean ± SD of 3 different experiments.

at 37°C. This relatively small increase of the total ATP content was enough to largely recover the secretory and aggregatory response to all 4 agonists tested (right-hand panels).

These results support the view that the secretory response induced by TPA, DG or collagen, which takes place without changes of the cytoplasmic Ca^{2+} levels [1], occurs via the physiological ATP-dependent mechanism and not by other kinds of membrane perturbations.

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