Mechanisms Involved in Adenosine Triphosphate–Induced Platelet Aggregation in Whole Blood

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Objective—Effects on platelet aggregation of adenosine triphosphate (ATP) released from damaged cells and from platelets undergoing exocytosis have not been clearly established. In this study we report on the effects of ATP on platelet aggregation in whole blood.

Methods and Results—Aggregation, measured using a platelet-counting technique, occurred in response to ATP and was maximal at 10 to 100 μmol/L. It was abolished by MRS2179, AR-C69931, and creatine phosphate/creatine phosphokinase, implying that conversion to adenosine diphosphate (ADP) is required. ATP did not induce aggregation in platelet-rich plasma, but aggregation did occur when apyrase or hexokinase was added. Aggregation also occurred after addition of leukocytes to platelet-rich plasma (as a source of ecto-ATPase), and this was potentiated on removal of adenosine by adenosine deaminase, indicating that adenosine production modulates the response. Dipyridamole, which inhibits adenosine uptake into erythrocytes, inhibited aggregation induced by ATP in whole blood, and adenosine deaminase reversed this. DN9693 and forskolin synergized with dipyridamole to inhibit ATP-induced aggregation.

Conclusions—ATP induces aggregation in whole blood via conversion of ATP to ADP by ecto-ATPases on leukocytes. This is inhibited by agents that prevent adenosine removal. Reduced aggregation at high concentrations of ATP (>100 μmol/L) may be a consequence of inhibition by ATP of ADP action at ADP receptors.

Materials

Hirudin (recombinant desulphato-hirudin, Revasc) was a gift from Novartis. Mono-Poly resolving medium was supplied by ICN Biomedicals. AR-C69931, a purinergic P2Y12 receptor antagonist, was a gift from Astra Charnwood. Dipyridamole, an adenosine uptake inhibitor, was obtained from Boehringer Ingelheim. ATP, adenosine

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Large quantities of adenosine triphosphate (ATP) and adenosine diphosphate (ADP) are present in erythrocytes, platelets, and other cells and tissues and can leave the cells via physical damage or exocytosis. The amount of ATP released from erythrocytes is approximately 10 times the amount of ADP, and ATP and ADP in platelet secretory granules are present in approximately equimolar concentrations. The released ADP can interact with P2Y1 and P2Y12 (formally known as P2Y1 and P2Y12) receptors on platelets and induce platelet aggregation, which contributes to normal hemostasis and to thrombus formation. However, the effect of the released ATP is unclear. It is known that ATP can interact with P2X1 receptors on platelets, causing a transient Ca2+ mobilization, but this does not result in platelet aggregation, and the importance of P2X1 receptors to overall platelet function is unknown. It is also known that ATP acts as an antagonist of the effects of ADP at P2Y1 and P2Y12 receptors and that high concentrations can inhibit ADP-induced platelet aggregation.

When considering the possible effects of ATP on platelets in vitro and in vivo, the presence of enzymes present on blood cells and endothelial cells and in plasma that metabolize ATP must be taken into account. These include enzymes that convert ATP to ADP and ADP to AMP (NTPDase-1, also known as ATP diphosphohydrolase, CD 39 and EC 3.6.1.5), ATP to AMP and ADP to AMP (5′-monophosphate phosphoanhydrolase/phosphodiesterase, NMPP), and AMP to adenosine (5′-nucleotidase), the latter being an inhibitor of platelet aggregation. Adenosine can be taken up and neutralized by erythrocytes and other blood cells, thus limiting its potential inhibitory action. Thus, the overall effect of ATP could depend on several competing influences.

In this study, we have investigated the effects of ATP on platelet aggregation in whole blood and in platelet-rich plasma (PRP). We used hirudin as the anticoagulant to ensure that the conditions used were as near physiological as possible. We found that ATP induces platelet aggregation in whole blood but not in PRP, and we have investigated the mechanisms that are involved.

Methods
deaminase (ADA), creatine phosphate (CP), creatine phosphokinase (CPK), hexokinase, apyrase, and MRS2179, a purinergic P2Y1 receptor antagonist were from Sigma Chemical Co. The platelet-fixing solution contained 150 mmol/L NaCl, 4.6 mmol/L Na2EDTA, 4.5 mmol/L NaH2PO4, 1.6 mmol/L KH2PO4, and 0.16% wt/vol formaldehyde, pH 7.4. PBS was from Unipath Ltd, and saline (sodium chloride, 0.9% wt/vol) was from Baxter Healthcare Ltd.

Blood Collection
Venous blood was obtained from volunteers who denied taking any medication in the 2 weeks before sampling. It was taken by forearm venepuncture using a polypropylene syringe and 19G needle and immediately dispensed into polystyrene tubes containing hirudin (fc 50 μg/ml) or EDTA (final concentration 4 mmol/L) as anticoagulant. The blood was then kept at 37°C for 30 minutes before experimentation (whole blood investigations) or immediately processed to obtain PRP. The EDTA blood was used for leukocyte preparation.

PRP Preparation
The blood was centrifuged (180g, 10 minutes), and the resulting supernatant (PRP) was removed. The remaining blood was centrifuged (1500g, 10 minutes) to obtain platelet-poor plasma. The platelet count of the PRP was determined using a Sysmex KX-21 hematology analyser, and the PRP was diluted using autologous platelet-poor plasma to a standard platelet count; the final platelet count in each experiment was 300×10^3/L.

Leukocyte Preparation
Polymorphonuclear leukocytes (PMNLs) and mononuclear leukocytes (MNLs) were isolated from EDTA blood using Mono-Poly resolving medium. Briefly, blood was centrifuged (180g, 10 minutes), and the resulting PRP was removed and discarded. The remaining blood was reconstituted to the original volume with saline and then carefully layered (3.5 mL) onto the Mono-Poly resolving medium (3 mL) in polystyrene tubes, which were then centrifuged (200g, 30 minutes). The resulting leukocyte layers (PMNLs and MNLs) were removed and washed twice in PBS. Each cell population was finally resuspended in PBS at a concentration ×10 that was obtained in whole blood, such that when added to PRP, the number of leukocytes was equivalent to the white cell count in the original whole-blood sample. (The mean leukocyte counts for MNLs and PMNLs were 2900 and 4100/μL, respectively.)

Platelet Aggregation
An aliquot of whole blood or PRP was dispensed into a polystyrene LP3 tube (64×11 mm) containing a stirrer bar and any agent under investigation (20 μL) or leukocyte preparation (50 μL). The final volume of whole blood or PRP was always 500 μL. The tube was placed in the stirring well of a Multi-Sample Agitator (University of Nottingham) operating at 1000 rpm at 37°C. After 2 minutes, 20 μL of a solution of ATP was added. Aliquots (15 μL) of the sample were removed and mixed with fixative solution (30 μL) at various time points after the addition of ATP. The platelet count of the fixed samples was determined using an Ultra-Flo 100 whole-blood platelet counter (whole blood) or a Sysmex KX-21 hematology analyser (PRP).19,20 Platelet aggregation was calculated as the percentage loss of single platelets compared with the platelet count of a fixed sample of unstimulated whole blood or appropriately diluted PRP. Data were analyzed using ANOVA (repeated measures) on SPSS 11.0 software. Significance was assigned to P<0.05. Platelet aggregation was confirmed by microscopy and flow cytometry.

Results
The effects of adding various concentrations of ATP to whole blood and to PRP are shown in Figure 1. Addition of ATP to whole blood resulted in platelet aggregation, the extent of which was dependent on the concentration used and was most extensive at concentrations of 10 to 100 μmol/L. Increasing the ATP concentration beyond 100 μmol/L resulted in a reduced aggregation response. In contrast, ATP was unable to induce an aggregation response in PRP. The time course of aggregation induced in whole blood by selected concentrations of ATP is shown in Figure 2.

To investigate the possibility that the aggregatory effect of ATP in whole blood is mediated by conversion of ATP to ADP, we determined the effects of the ADP antagonists MRS2179 (a P2Y1 antagonist, 100 μmol/L)21,22 and AR-C69931 (a P2Y12 antagonist, 1 μmol/L)23–25 (Figure 3). We also studied the effect of removing ADP from the system using CP/CPK (300 μmol/L CP and 22U/mL CPK).26 The extensive aggregation response induced in whole blood by ATP was virtually abolished in the presence of either ADP antagonist or CP/CPK.

From the experiments described above, it would seem that the aggregatory effects of ATP in whole blood are mediated by conversion of ATP to ADP and that this does not occur in PRP. Consequently, we carried out experiments in PRP in which we sought to bring about the conversion of ATP to ADP. We investigated the effects of ATP in PRP to which either apyrase (an enzyme with adenosine 5′ triphosphatase activity and adenosine 5′ diphosphatase activity) or hexokinase (an enzyme that converts ATP to ADP but has no

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**Figure 1.** Extent of platelet aggregation induced by addition of a range of concentrations of ATP to whole blood (WB) and PRP. Aggregation was measured 4 minutes after the addition of ATP to the blood. Mean±SEM, n=11 (WB) and n=6 (PRP).

![Image](http://atvb.ahajournals.org/)

**Figure 2.** Platelet aggregation induced by selected concentrations of ATP added to whole blood. Mean±SEM, n=11.

![Image](http://atvb.ahajournals.org/)

**Figure 3.** Platelet aggregation induced by selected concentrations of ATP added to whole blood. Mean±SEM, n=11.
additional phosphatase activity) had been added. The results are shown in Figure 4. As before, the addition of ATP to PRP did not produce an aggregation response. However, in the presence of apyrase or hexokinase, ATP brought about a marked aggregation response.

Subsequent experiments were designed to investigate the role of leukocytes and also the influence of any adenosine that may be produced from the ATP. To examine the contribution of leukocytes, we carried out experiments in which PMNLs and MNLs were isolated from whole blood and then added back to autologous PRP (at a concentration equivalent to that in whole blood) before stimulation with ATP. The results are shown in Figure 5. As previously, ATP produced an aggregation response in whole blood but not in PRP. However, on addition of leukocytes to PRP, a small degree of aggregation was observed that was slightly more extensive in the presence of PMNLs compared with MNLs. Similar results were obtained in response to 10 μmol/L ATP (results not shown). The results of control experiments showed that neither leukocyte preparation caused platelet aggregation in the absence of ATP (results not shown). To examine the contribution of adenosine in this system, the experiment was also performed in the presence of ADA, which converts adenosine to inosine. Again similar results were obtained in response to 10 μmol/L ATP (results not shown).

To additionally investigate the contribution of adenosine, we studied the effects of dipyridamole, an agent that inhibits the uptake of adenosine into red cells, on ATP-induced aggregation in whole blood. We also looked at the effects of DN9693 (a cAMP PDE inhibitor) and forskolin (a stimulator of adenylyl cyclase), low concentrations of which are known to synergize with adenosine to raise platelet cAMP. The results are shown in Figure 6. Dipyridamole (10 μmol/L) limited the extent of aggregation observed in response to ATP, and this inhibitory effect of dipyridamole was significantly enhanced by DN9693 and forskolin. This inhibition of aggregation by dipyridamole used alone and in combination with DN9693 or forskolin was completely prevented in the presence of ADA.

Discussion

In considering the factors that might determine the overall effect of ATP on platelet aggregation, we thought that some of the following might be relevant: possible promotion of aggregation via conversion to ADP by ecto-ATPases on blood cells (platelets or leukocytes), competitive inhibition by ATP of the effects of ADP at ADP receptors on the platelet surface, and inhibition of aggregation via conversion of ATP to adenosine and consequent elevation of platelet cAMP.

Our first results confirmed earlier preliminary findings (that so far have only been reported in abstract form) and demonstrated that ATP induces platelet aggregation in whole blood but not in PRP. The aggregation was dependent on the concentration of ATP that was used and was maximal at concentrations between 10 and 100 μmol/L. Less aggregation
was obtained when lower or higher concentrations of ATP were used.

Although it is known that there are P2X$_1$ receptors on the platelet surface that interact with ATP, there is little evidence that this interaction plays a significant role in platelet aggregation. The fact that ATP did not cause aggregation in PRP seemed to confirm this. Also, the lack of aggregation in PRP in response to ATP showed that the aggregation was not merely a consequence of ADP present in the ATP preparation. Consequently, we believed we needed to look elsewhere for an explanation of the effects of ATP that we had observed.

To test the possibility that the aggregation in whole blood might be brought about by ADP generated from the added ATP, we investigated the effects of 2 agents, MRS2179 and AR-C69931, on ATP-induced platelet aggregation. ADP-induced aggregation is mediated by ADP interacting with P2Y$_1$ and P2Y$_{12}$ receptors. MRS2179 and AR-C69931 act as antagonists at the P2Y$_1$ receptors and the P2Y$_{12}$ receptors, respectively, and it is well-known that blocking ADP interaction with either receptor inhibits ADP-induced platelet aggregation. We found that both these agents markedly inhibited ATP-induced aggregation in whole blood. Additional confirmation of a role for ADP in the response came from an experiment in which we added CP/CPK to remove any ADP that might be generated from the ATP. This also abolished the aggregation.

Because ATP was unable to induce aggregation in PRP, we considered that platelets themselves may have insufficient ATPase activity to generate enough ADP for aggregation to occur. This is despite the fact that platelets themselves are known to have an ecto-ATPase. Consequently, we looked to see whether addition to PRP of an enzyme that converts ATP to ADP would be sufficient for ATP to induce an aggregation response and thus reproduce the results obtained in whole blood. This proved to be the case with both apyrase (which has combined ATPase and ADPase activity) and hexokinase (an ATPase) able to bring about an aggregation response to ATP.

Having identified an involvement of ADP in ATP-induced aggregation in whole blood and a possible involvement of an ATPase, we went on to try to identify the source of the ATPase. Leukocytes were obvious candidates for which the presence of ecto-nucleotidase activity has been described. We performed experiments in which purified PMNLs and MNLs were added to autologous PRP. The number of leukocytes added was always equivalent to the number in the whole blood from which they were derived. Introduction of either type of leukocyte was sufficient for ATP to induce a small, reversible aggregation response in the PRP. This was slightly more extensive in the case of PMNLs compared with MNLs, but the aggregation induced by the ATP proved to be nothing like as extensive as that obtained in whole blood itself. We thus looked for an explanation for this.

In whole blood and PRP, ATP and ADP are metabolized to adenosine, which is an inhibitor of platelet aggregation. It stimulates adenylate cyclase and increases the intracellular level of cAMP, which in turn inhibits calcium mobilization and other signal transduction pathways. We thought it possible that differences in response to ATP in whole blood and in PRP containing leukocytes might relate to the relative availability of generated adenosine in the 2 systems. Erythrocytes rapidly take up and remove adenosine and thereby might make it unavailable to act as an inhibitor of platelet aggregation. To investigate the role of adenosine, we used adenosine deaminase, an enzyme that rapidly deamidates any adenosine that is generated. Whereas ADA had no effect on ATP-induced aggregation in whole blood, it was found to markedly potentiate the aggregation induced by ATP in PRP that contained leukocytes as a source of ATPase.

It would seem that ATP-induced aggregation in PRP containing leukocytes and ADA is a consequence of ATP conversion to ADP via ecto-ATPases on the leukocytes. The presence of ADA removes any adenosine that is produced by additional breakdown of ATP and ADP, thus making it unavailable to reduce the degree of aggregation that occurs via an effect on platelet cAMP. It follows that ATP-induced platelet aggregation in whole blood could also be via ATP conversion to ADP, with the erythrocytes providing the means of removing any adenosine that is produced through rapid uptake of the latter.

To explore this possibility further, we decided to study the effects of dipyridamole, a drug that inhibits uptake of adenosine into erythrocytes. We found that dipyridamole limited the extent of aggregation obtained after addition of ATP to whole blood and that ADA reversed this inhibition.
results that are entirely in accord with the mechanism proposed.

For additional confirmation of an inhibitory role for adenosine under circumstances where it is not removed from the system (e.g., in PRP or in whole blood containing dipyridamole), we carried out 2 additional experiments. It is known that the effects of adenosine as an inhibitor of platelet aggregation can be amplified by forskolin27 and also by cAMP phosphodiesterase inhibitors such as DN 9693.27 The former synergizes with adenosine in stimulating cAMP production and the latter maintains cAMP by blocking its breakdown. These agents were found to potentiate the inhibitory effects of dipyridamole on ATP-induced aggregation in whole blood, and, again, this was totally reversed by introducing ADA as a means of removing adenosine from the system.

The results of these investigations suggest that ATP-induced platelet aggregation in whole blood occurs mainly via conversion to ADP by ecto-ATPases on blood cells and that the cells involved are both PMNLs and MNLS. The ADP induces aggregation via interaction with P2Y1 and P2Y12 ADP receptors. Reduced aggregation at very high concentrations of ATP may be a consequence of antagonism by ADP of ADP interaction with P2Y1 or P2Y12 receptors.10,11 It is unlikely that this is an artifact of the high concentrations used, because addition of high concentrations of ADP to whole blood or PRP did not result in reduced platelet aggregation (data not shown). There seems to be little inhibition of this aggregation by adenosine generated from the ATP, probably because the adenosine is rapidly removed by the erythrocytes in the blood. Blockade of adenosine uptake by dipyridamole reduces the extent of the aggregation response, and this effect of dipyridamole can be enhanced by other agents that promote the inhibitory effect of adenosine on platelet aggregation. In PRP, ATP does not induce aggregation, because in the absence of blood leukocytes, insufficient ADP is generated to cause an aggregation response, and in the absence of erythrocytes, adenosine generated from the ATP remains available to inhibit platelet aggregation.

It should be noted that all of the experiments that were performed here were conducted in whole blood or PRP that contained hirudin as the anticoagulant. Use of hirudin (unlike citrate, which is more commonly used in investigations of platelet function) maintains plasma divalent cations at normal physiological levels.36 In some experiments, we used citrate in place of hirudin and found that the extent of aggregation induced by ATP in whole blood was less extensive (results not shown). It is therefore possible that normal physiological levels of divalent cations are required for optimum conversion of ATP to ADP to occur.

In considering the relevance of these findings in whole blood and in PRP in vitro to the situation in vivo, we must note that in vivo platelets will also be subject to the influence of endothelial cells on the luminal surface of vessel walls. Endothelial cells are a major source of ecto-ATPase and 5′-nucleotidase, enzymes that collectively dephosphorylate ATP, ADP, and AMP with production of additional adenosine.14,37 These activities will clearly add to the enzyme activities present on blood leukocytes and in plasma. Endothelial cells are also able to synthesize and release prostacyclin, which, like adenosine, inhibits platelet aggregation via stimulation of adenylate cyclase. Also, endothelial cells are able to produce nitric oxide, another agent that inhibits platelet aggregation, in this case through stimulation of guanylate cyclase. Presumably, this powerful protective armory makes it unlikely that platelets will be activated by ATP/ADP under normal conditions, where blood flows over intact endothelium. However, this may not be the case at sites where the endothelium has been breached and where initiation of a hemostatic plug is required or where a platelet thrombus develops. It is already known that ADP emerging from damaged cells and tissues or from previously activated platelets contributes to normal hemostasis and to thrombosis. For example, patients with platelets with defective P2Y12 receptors exhibit bleeding problems,38 and agents that inhibit ADP-induced platelet aggregation (agents such as clopidogrel which blocks the effects of ADP at P2Y12 receptors on platelets) have been shown to be effective antithrombotic agents.39 The role of ATP released in parallel with the ADP has been less certain, despite the fact that very large amounts of ATP can be released. For example, in erythrocytes, the concentration of ATP approaches 1 mmol/L and is 10 times higher than the ADP that is present. The findings described herein suggest that the overall effect of ATP released into whole blood is to add to the proaggregatory effects of released ADP and that ATP should be considered as an agent making an additional contribution to normal hemostasis and to thrombosis.

References


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