The Effects of ATP on Platelets: Evidence Against the Central Role of Released ADP in Primary Aggregation

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The influence of freshly purified ATP on the effects of aggregating agents on human platelets was studied. ATP inhibited aggregation induced by ADP competitively (Ki = 20 μM) and immediately without need for prior incubation. ATP had no effect on primary aggregation induced by adrenaline, thrombin, vasopressin, or 5-hydroxytryptamine (5HT). ATP inhibited the shape change and the consumption of metabolic ATP induced by ADP but did not inhibit these effects when induced by thrombin, vasopressin, or 5HT. ATP counteracted the inhibition by ADP of PGE1-stimulated cyclic AMP production in platelets but did not reduce inhibition by adrenaline. It is concluded that adrenaline, thrombin, 5HT, and vasopressin each can induce primary aggregation of human platelets by a mechanism independent of extracellular ADP.

Since the discovery that adenosine diphosphate (ADP) causes the aggregation of platelets,1 the mechanism of this effect has been intensively studied. This work has been reviewed by Mustard and Packham2 and more recently by Smith and Macfarlane.3 Several observations have led to the suggestion that ADP is an essential mediator of the actions of other aggregating agents including thrombin, collagen, 5-hydroxytryptamine (5HT), and adrenaline. Firstly, human platelets store ADP in a metabolically inert form and can release it when stimulated by these agents.4–6 Secondly, aggregation induced by ADP is inhibited by several of its structural relatives.7–10 In the case of adenosine this inhibition has been


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reported to have competitive kinetics, suggesting an interaction at the receptor level. The fact that some of these analogues also inhibit aggregation induced by other agents suggests that their action may involve ADP. Thirdly, enzyme systems that remove ADP can inhibit aggregation induced by other agents.

This evidence has been taken to imply a central role for ADP in the induction of aggregation, and this has generated a number of hypotheses attempting to explain aggregation in terms of ADP alone (see reference 2).

However, there are several observations which are difficult to reconcile with the hypothesis that ADP is the common mediator of aggregation. Release of ADP has not been detected during the first phase of aggregation induced by adrenaline, thrombin, or vasopressin, although it has been clearly demonstrated during the second phase. The effects of adenosine and 2-chloroadenosine can be accounted for by their ability to stimulate the adenylate cyclase system of platelets. AMP is rapidly degraded to adenosine in plasma; when this breakdown is blocked by cyanide or when the accumulation of adenosine is prevented by adenosine deaminase the effect of AMP is greatly reduced. Nonmammalian thrombocytes and washed human platelets resuspended without fibrinogen are aggregated by thrombin but not by ADP. Lastly, it has been shown that apyrase can inhibit aggregation by exogenous ADP without affecting aggregation by adrenaline.

We report here results of investigations of the effects of ATP on aggregation induced by ADP and other agents that lead us to the conclusion that thrombin, adrenaline, 5-HT, and vasopressin induce aggregation and other specific platelet reactions through mechanisms which are not mediated by extracellular ADP.

**MATERIALS AND METHODS**

*Citrated Platelet-rich Plasma (PRP)*

Blood was drawn from volunteers who had not taken aspirin for 5 days and was anticoagulated with 3.8% trisodium citrate. PRP was prepared by differential centrifugation of the blood at room temperature and was stored in capped plastic tubes at room temperature for up to 1.5 hr before use.

*Platelet Aggregation*

Platelet aggregation was observed photometrically using the apparatus described by Mills and Roberts. Cuvettes of either 8-mm diameter containing 1.0 ml plasma and a plastic-coated flea magnet or 6-mm diameter containing 0.8 ml plasma and an uncoated wire stir bar (Payton Associates, Buffalo, N.Y.) were used.

*Platelet Shape Change*

Platelet shape change was observed in the same apparatus, with the recorder sensitivity doubled. In these experiments the plasma was incubated at 37°C for 30-60 min before use.

*Adenylate Energy Charge*

Adenylate energy charge was determined isotopically. PRP was incubated with 2 μM 14C adenine for 1-2 hr. After incubation of samples (0.2 ml) of this PRP with drugs for the times indicated, 20 μl of 6 M perchloric acid containing 10 mM ATP, ADP, AMP, IMP, adenosine, and adenine and 5 mM hypoxanthine and inosine was added and immediately mixed on a vortex mixer. Samples were cooled to 0°C and centrifuged. One hundred microliters of the supernatant was
neutralized with 2 M K2CO3, cooled, and centrifuged again. For chromatography, 25 µl was applied as a streak on a 1-inch strip of Whatman 3MM paper and developed by descending elution for 22 hr with n-butanol, acetone, glacial acetic acid, NH4OH (specific gravity = 0.880), water (45:15:10:0.5:30 v/v). For electrophoresis, 20 µl was applied as a spot on 3 MM paper moistened with 0.05 M sodium citrate/citric acid buffer, pH 4.05, and electrophoresed on a Shandon L24 HVE apparatus at 3000 V (about 50 V/cm) for 1-1.5 hr. Spots visible under 254-nm light were cut out and counted in toluene containing PPO. 6.5 g/liter, and POPOP, 0.3 g/liter. The adenylate energy charge (AEC) was calculated from the formula AEC = (2ATP + ADP)/2(ATP + ADP + AMP), where ATP = radioactivity in the ATP spot etc.

Cyclic AMP Synthesis

Cyclic AMP synthesis was measured isotopically by a modification of the previously described method.16 PRP was incubated with freshly purified 2-3H adenine (0.2-0.5 µM) for 1-2 hr. After incubation of 0.5-ml aliquots with drugs as indicated, the reaction was stopped by the addition of 0.5 ml 1.2 M perchloric acid containing 0.1 µmole cyclic AMP and 1500 cpm 14C cyclic AMP as an internal standard for the subsequent recovery of 3H cyclic AMP. After centrifugation, the supernatant was placed on a 1-ml column of Dowex 50 cation exchange resin in a Pasteur pipette and washed with 1.5 ml H2O and then eluted with a further 2 ml H2O. The eluate was treated with 0.2 ml 10% ZnSO4 and 0.2 ml Ba(OH)2 (equivalent concentration) to precipitate metabolites other than cyclic AMP.26 The precipitation step was repeated, and the resulting supernatant was counted in a 15-ml scintillation cocktail (dioxane containing naphthalene, 85 g/liter, and butyl PBD, 8 g/liter) using an Intertechnique SL30 counter. Efficiency of counting for 14C was about 50% and for 3H about 10%, Crossovers were about 17%, 14C counted in the tritium channel and 1.8% 3H in the carbon channel. After correction for background, crossover, and recovery (20%-40%), the 3H cyclic AMP content was expressed as a percentage of the total intracellular 3H.

Purification of 3H Adenine

Commercially available 3H adenine contains a contaminant that gives rise to falsely high cyclic AMP levels by the above method. It was therefore purified before use as follows: 1.0 mCi in 1 ml H2O was put on a 1-ml column of Dowex 50, washed with 5 ml H2O, and eluted with 5% NH4OH. 0.5-ml fractions were collected, and the peak fraction(s) appearing after about 4.0 ml NH4OH were evaporated to dryness under reduced pressure, redissolved in Tris-buffered saline, and used immediately.

Purification of ATP

One milliliter 50 mM ATP (disodium salt) was placed on a 20 × 0.5-cm column of Dowex 50 and eluted with water at 4°C. Fractions (0.5 ml) were collected and their absorbance determined at 258 nm. The first half of the peak, containing ATP free of ADP, was neutralized with Tris base and assayed at 258 nm assuming E1%1% = 14,700 in 0.01 M HCl. The ATP solution was stored at 0°C for up to 2 hr before use, or at −60°C for up to 24 hr.

Materials

2-3H adenine (specific activity, 18 Ci/mmmole) and U-14C adenine (specific activity, 281 Ci/mole) were obtained from Amersham Searle Ltd., Arlington Heights, Ill. 8-14C adenosine 3':5' cyclic monophosphate (specific activity, 49.2 Ci/mole) was obtained from Schwartz/Mann, Orangeburg, N.Y. Dowex AG50 Wx4 100-200 mesh, H+ form, was from BioRad Laboratories, Richmond, Calif.

Nucleotides, nucleosides and bases, grade I-S lysine vasopressin (100 IU/mg), grade VI arginine vasopressin (370 IU/mg), and 5 hydroxytryptamine creatinine sulfate were obtained from Sigma. Thrombin (bovine) was from Parke Davis, Detroit, Mich. Adrenaline hydrogen tartrate was obtained from Winthrop Laboratories, New York. PGE1 was a gift from Dr. J. E. Pike, Upjohn, Kalamazoo, Mich. RA 233 (2, 6-bis-(diethanolamino)-4-piperidinopyrimido-[5,4d]-pyrimidine was a gift from Dr. J. W. Bell of Boehringer Ingelheim Ltd., Isleworth, Middlesex, England.
RESULTS

The addition of ADP to stirred PRP in an aggregometer causes a change in the shape of the platelets which is associated with a characteristic change in the pattern of light transmission. The rapid random fluctuations caused by swirling of discoid platelets disappear, and a net reduction in the amount of light transmitted occurs. Since these changes may be obscured by the large increase in light transmission associated with aggregation, they can best be seen when aggregation is inhibited, for example by the use of EDTA. Similar changes are induced by other aggregating agents including thrombin and 5HT (Fig. 1), although not by adrenaline. On some occasions vasopressin caused a shape change similar to that seen with ADP in the presence of excess EDTA (Fig. 1), but on others it was not seen unless the EDTA concentration was reduced to

Fig. 1. Effect of ATP on platelet shape change induced by various agents. Samples of citrate PRP (0.8 ml) incubated at 37°C for 30-60 min were placed in the aggregometer. Tracings of light transmission are shown. EDTA was added to a final concentration of 5 mM in a volume of 50 µl, and 30 sec later either Tris-buffered saline (left-hand tracings) or purified ATP (25 µl, final concentration 300 µM, right-hand tracings) were added. Thirty seconds later (at the times indicated by the dashed vertical lines), the aggregating agent was added in less than 5 µl to give the final concentration shown. A reduction in net optical transmission (upward movement of pen) together with loss of rapid oscillations indicates the platelet shape change. Similar results were obtained using platelets from a different donor.

Fig. 2. Effect of ATP on platelet aggregation induced by various agents. Samples of citrate PRP (0.8 ml) were warmed to 37°C for 4 min before being placed in the aggregometer. Aggregating agents were added at the time indicated by the vertical dashed lines, in 5 µl or less, to give the final concentrations shown. In the tracings on the right, ATP (final concentration, 250 µM) was added in 20 µl, 15 sec before the aggregating agents. An increase in light transmission (downward movement of the pen) indicates aggregation. Similar results were obtained using platelets from three different donors.
2.5 mM. Addition of purified ATP under these conditions neither caused a shape change by itself, nor did it inhibit the change caused by thrombin or vasopressin, and had little effect on the shape change induced by 5HT. However, the effect of ADP was abolished (Fig. 1), although the subsequent addition of either vasopressin, thrombin, or 5HT still resulted in a shape change. The concentration ratio of ATP to ADP used in this experiment to achieve complete inhibition was necessarily very high; increasing the concentration of ADP to 5 μM resulted in a small shape change. This is consistent with a competitive mechanism for the action of ATP (see below).

ADP, vasopressin, thrombin, 5HT, or adrenaline, when added in low concentrations to citrate PRP, cause reversible (single-phase) aggregation (Fig. 2).
Fig. 4. Kinetics of inhibition of ADP-induced platelet aggregation by different inhibitors. Samples of citrate PRP (1.0 ml) were prewarmed to 37°C for 4 min before the addition of ADP (10 μl). Inhibitors (20 μl) were added 30 sec before ADP. Aggregation was measured as the maximum rate of increase of light transmission at three different concentrations of ADP. Results are plotted as rate of aggregation (arbitrary units) against the concentration of ADP (log scale, μM). Concentrations of each inhibitor are shown beside the appropriate lines, and the uppermost line of each set represents results obtained in the absence of an inhibitor. The different inhibitors were tested on different plasma samples. Similar results were obtained on three to five occasions for each inhibitor on platelets from different donors.

The addition of purified ATP, which did not cause aggregation by itself, inhibited the action of ADP added shortly afterwards, while aggregation induced by vasopressin, thrombin, and 5HT was unaffected. A slight enhancement of adrenaline-induced aggregation was seen. At slightly higher concentrations of vasopressin and thrombin, a second phase of aggregation occurs; this second phase was inhibited by ATP, although the first phase remained unaffected. This suggests that the second phase of aggregation by these agents is caused by released ADP, while the first phase is not.

The experiments described above demonstrate the specificity of the antagonism between ATP and ADP. The characteristics of this antagonism were
examined in aggregation experiments in which times of preincubation and concentration were varied. In these experiments, the rate of aggregation proved to be easier to measure than the shape change, and ATP was compared with several other known inhibitors of aggregation. Figure 3 shows that increasing the time of preincubation of platelets with RA233, an inhibitor of platelet cyclic AMP phosphodiesterase,\textsuperscript{16} caused a considerable increase in the degree of inhibition of aggregation. The same was true, in varying degree, for prostaglandin E\textsubscript{1} (PGE\textsubscript{1}), adenosine, AMP, and 2-chloroadenosine. In contrast, ATP caused inhibition which was independent of the time of preincubation.

The results of experiments in which ADP and inhibitor concentrations were varied are shown in Fig. 4. The data are presented as log dose-response plots, using only concentrations of ADP of 5 \( \mu M \) and above to show the extent to which increasing concentrations of ADP can overcome the effect of the inhibitor. Inhibition by ATP was almost completely overcome by increasing the concentration of ADP, as would be expected for a competitive inhibitor. In contrast, inhibition by RA233 was noncompetitive, and that by the other inhibitors was partially competitive. The data for ATP are replotted according to Dixon\textsuperscript{29} in Fig. 5, which gives a value for the inhibitor constant (Ki) for ATP of 20 \( \mu M \).

Metabolic ATP Consumption Induced by Aggregating Agents

ADP and 5HT cause a small but rapid decrease in the metabolically active ATP content of platelets associated with the induction of shape change\textsuperscript{25}; this can be conveniently expressed as a fall in the adenylate energy charge (AEC) and is the earliest biochemical effect of these agents so far detected. A similar fall in AEC is caused by both lysine- and arginine-vasopressin (Fig. 6) and with thrombin. The effect of the simultaneous addition of purified ATP together with these aggregating agents is shown in Fig. 7, which demonstrates that ATP inhibits the action of ADP but causes no significant inhibition of the effects of
Fig. 6. Depression of adenylate energy charge (AEC) by vasopressin and ADP. Duplicate samples of $^{14}$C adenine-labeled citrate PRP (0.2 ml) were incubated with ADP or vasopressin (10 µl) for 15 sec before the addition of perchloric acid. The AEC was determined by paper chromatographic separation of the extracted nucleotides (see Materials and Methods). Each circle represents one determination.

vasopressin, thrombin, or 5HT. ATP alone caused a small but significant reduction of the AEC which is at present unexplained.

Effect of Aggregating Agents on PGE$_1$-stimulated Adenylate Cyclase

Addition of PGE$_1$ to platelets causes a rapid increase in the cyclic AMP content of the cells. This can be measured as the formation of labeled cyclic AMP in platelets which have been allowed to incorporate radioactive adenine into their metabolic nucleotides. This increase is inhibited by the simultaneous addition of ADP or adrenaline. The results in Fig. 8 show that the inhibition
Fig. 8. Effect of ATP on the inhibition by aggregating agents of PGE₁-stimulated $^3$H cyclic-AMP synthesis in platelets. Samples of $^3$H adenine-labeled citrate PRP (0.5 ml) were stirred at 37°C for 15 sec with 10 μl of purified ATP (final concentration, 0.2 mM) or Tris-saline. Then a mixture of PGE₁ (final concentration, 0.25 mM) and the various aggregating agents as indicated was added in 10 μl. After a further 10 sec perchloric acid was added and $^3$H cyclic AMP determined as described (see Materials and Methods). The figure shows the increase in the percentage of total intracellular $^3$H as cyclic AMP. The basal level of $^3$H cyclic AMP in unstimulated platelets was 0.044% and was not affected by ATP.

of cyclic AMP formation by ADP was abolished by ATP, whereas ATP had no effect on the inhibition by adrenaline. In this experiment no detectable inhibition of cyclic AMP formation was observed with either thrombin or vasopressin. ATP by itself had no effect on cyclic AMP levels, but it caused a small increase in the response to PGE₁.

**DISCUSSION**

Inhibition of aggregation by ATP was first described more than 10 yr ago, and the structural similarity between ATP and ADP immediately suggested the possibility of a competitive antagonism at the membrane receptor for ADP.⁵ No detailed investigations of the early effects of ATP have been published. This may be due in part to the fact that relatively high concentrations of ATP must be used to cause inhibition, and the normal level of contamination of ATP with ADP precludes the use of commercially available material for this type of experiment. The simple purification method used here yields ATP as the acid free of ADP; this can be neutralized and used directly.

Several claims have been made that ATP itself causes aggregation.⁶⁻⁷ However, the aggregation is inhibited by an ADP-removing system (pyruvate kinase and phosphoenol pyruvate), suggesting that the effect is due to contaminating ADP.¹ In our experiments, the addition of freshly purified ATP at concentrations up to 300 μM induced neither aggregation nor a shape change in citrate PRP.

Investigation of the kinetics of inhibition of aggregation is complicated by the fact that there is no clear relationship between the light transmission of a platelet suspension and its degree of aggregation. The maximum rate of change of light transmission, although giving the closest approximation to Michaelis-Menton kinetics, still shows substantial deviations from theory at low concentrations of aggregating agents.¹¹ By observing the degree of inhibition of aggre-
gation at high concentrations of ADP, we clearly show that the kinetics of inhibition of ADP-induced aggregation by ATP are different from those observed with adenosine, 2-chloroadenosine, AMP, PGE₁, and compound RA233. These latter are now all known to act through effects on the metabolism of cyclic AMP. PGE₁, adenosine, and 2-chloroadenosine, which stimulate adenylyl cyclase, all showed partially competitive kinetics, although competitive inhibition of ADP-induced shape change by 2-chloroadenosine has been reported; the effects of these compounds on cyclic AMP are inhibited by ADP. RA233, which inhibits cyclic AMP phosphodiesterase, inhibited aggregation noncompetitively; ADP has little effect on the cyclic AMP level in platelets exposed to RA233. The inhibition of ADP-induced aggregation by ATP was almost completely overcome by increasing the concentration of ADP, which is clearly consistent with a competitive mode of action. This is further supported by the competitive kinetics for the inhibition of ADP-induced shape change by ATP reported before.

The experiments in which the time of preincubation with the different inhibitors was varied also revealed a difference between ATP and the other compounds. Inhibition of aggregation by PGE₁, 2-chloroadenosine, adenosine, AMP, and RA233 all increased with time, whereas full inhibition by ATP was achieved without preincubation. These results suggest that ATP competes with ADP for a receptor site on the platelet, and this view is supported by the failure of ATP to inhibit aggregation induced by low concentrations of thrombin, vasopressin, 5HT, or adrenaline.

Among the other known effects of ADP on human platelets are the induction of the shape change, stimulation of consumption of intracellular metabolic ATP, and the inhibition of the effect of PGE₁ on the synthesis of cyclic AMP. Some of these effects are also induced by other aggregating agents. The shape change and ATP consumption can be observed in response to thrombin, 5HT, and vasopressin, although not with adrenaline; inhibition of cyclic AMP synthesis occurs with adrenaline, while 5HT, thrombin, and vasopressin have little or no effect on this system in citrate PRP.

When ATP was added in sufficient quantity, the effects of ADP on platelet shape, ATP consumption, and on adenylate cyclase were abolished. The same concentrations of ATP did not inhibit these effects when induced by thrombin, 5HT, vasopressin, or adrenaline.

Although prolonged incubation of platelets with ATP may cause inhibition by indirect mechanisms, our experiments indicate that ATP acts immediately as a weak specific competitive inhibitor of the actions of ADP on platelets, and as such it can be used to study the involvement of released ADP in the induction of aggregation by other agents. The experiments that established the specificity of ATP also show that released ADP is not involved in the induction of shape change, the associated consumption of metabolic ATP, and the induction of aggregation by 5HT, vasopressin, or thrombin, nor in the induction of aggregation and inhibition of cyclic AMP production by adrenaline. These agents therefore must induce their effects, including aggregation, by some means other than by the release of intracellular ADP to the outside of the cell.

Several other facts also challenge the central role of ADP. Adrenaline induces
aggregation without the characteristic change in light transmission associated with the ADP-induced shape change. 5HT and vasopressin do not inhibit cyclic AMP production, whereas ADP does, and thrombin will aggregate platelets in circumstances in which they will not aggregate to ADP.

The best evidence implicating ADP in aggregation induced by other agents is derived from the experiments using pyruvate kinase and phosphoenol pyruvate or other ADP-removing systems. The aggregation tracings from these experiments show that these systems principally accelerate disaggregation and have little effect on the rate of primary aggregation, suggesting that released ADP is involved in later stages of aggregation induced by these agents. Our experiments were specifically designed to minimize the influence of the release reaction: the concentration of vasopressin, thrombin, 5HT, and adrenaline used induced only the first phase of aggregation. Thus, the evidence presented here does not bear on the mediation of secondary aggregation. However, the results indicate that primary aggregation can be brought about by several agents without the involvement of ADP.

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