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DIADENOSINE TRIPHOSPHATE (Ap_3A) MEDIATES HUMAN PLATELET AGGREGATION BY LIBERATION OF ADP

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Human platelets store considerable amounts of diadenosine 5',5'''-P^1,P^3-triphosphate, which is released together with the homologue diadenosine tetraphosphate (Ap_4A) upon thrombin-induced aggregation (Lüthje,J. & Ogilvie,A. (1983) Biochem. Biophys. Res. Commun. 115, 253-260). We now report that, when added to platelet-rich plasma at 10-20 μM, diadenosine triphosphate gradually induces aggregation. The addition of diadenosine tetraphosphate antagonizes this effect by rapidly disaggregating the platelets. When another physiological but structurally unrelated stimulus, i.e. PAF (Platelet activating factor) is introduced into the system, diadenosine triphosphate drastically enhances and prolongs the aggregatory effect of PAF. Again, Ap_4A is antagonistic in this system. The mechanism of Ap_3A-stimulation can be explained by the slow and continuous liberation of ADP from Ap_3A by the action of a hydrolyzing enzyme which is present in human plasma. Our studies suggest that Ap_3A may be physiologically important in providing a relatively long-lived stimulus that can modulate platelet aggregation.

Diadenosine triphosphate (Ap_3A) has very recently been identified in eukaryotic cells using a specific enzymatic and chromatographic methodology (1,2). Based on in-vitro data it has been suggested that Ap_3A might play an antagonistic role toward the homologue diadenosine tetraphosphate (Ap_4A) which is a potential signal molecule for the initiation of DNA replication (3-6) and might pleiotropically act as an "alarmone" (7).

Human platelets store Ap_3A as well as Ap_4A in a metabolically inactive state (2,8). Both dinucleotides are released on exposure to thrombin which induces platelet aggregation. The possible function of these dinucleotides for the process of platelet aggregation seems of high interest. Ap_4A has been found to act as a potent competitive inhibitor of platelet aggregation induced by ADP (9) which is probably an important physiological stimulus of aggregation (10,11). With Ap_3A, however, these authors were able to influence ADP-induced aggregation only marginally (9). Here we demonstrate that in platelet-rich plasma Ap_3A itself has aggregating potency which can be counteracted by Ap_4A. The molecular mechanism of aggregation induced by Ap_3A has been clarified in showing a slow release of ADP from Ap_3A when incubated in human plasma.

Abbreviations: PAF, platelet activating factor; PRP, platelet-rich plasma

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MATERIALS & METHODS

Reagents. [^{3}H]Ap_3A was from Amersham (labeling service). Ap_4A, Ap_3A, ADP and platelet activating factor (PAF) were from Sigma. Alkaline phosphatase (calf intestine) was from Boehringer, Mannheim.

Preparation of platelet-rich plasma (PRP) and aggregation studies. Fresh blood was collected from volunteers into acid citrate/dextrose anticoagulant and centrifuged at room temperature for 15 minutes at 120 x g to yield PRP. Aggregation of platelets was followed photometrically in a Shimadzu photometer equipped with a mechanical stirring device and with a chart recorder. Standardization of the measurement as well as testing of platelet sensitivity was performed with ADP (1 μM) as initiator of platelet aggregation (10,11).

Repurification of Ap_3A and Ap_4A. The dinucleotides (10 nmol) were applied onto an HPLC system [2] and chromatographed as described (1). The fractions containing Ap_3A (Ap_4A) were pooled and desalted with a Sephadex G-10 column (60 x 2.2 cm) [3] eluted with water. The fractions containing the nucleotides were concentrated by evaporation.

Measurement of Ap_3A-hydrolysis in human plasma. [^{3}H]Ap_3A (3.5 μM; 22.7 Ci/mmol) was incubated with PRP at 37°C. Aliquots were withdrawn and pipetted into ice-cold trichloroacetic acid (10% final conc.). After centrifugation (12.000xg; 5 Min.; 4°C) the supernatants were neutralized as described (12). Two microliters were spotted together with nucleotide markers on poly(ethyleneimine)-cellulose thin layers. The plates were first developped in water and, after drying, in 0.5 M LiCl. The spots visible under ultraviolet light were marked with a pencil and cut out. The thin layer pieces were overlayed with scintillation cocktail (toluene containing 0.5% 2,5-diphenyloxazole; 0.03% 1,4-bis-(4-methyl-5-phenyl-oxazol-2-yl)benzene) and counted in a Packard liquid scintillation counter. Incubation of [^{3}H]Ap_3A with water instead of PRP was run as a control.

RESULTS & DISCUSSION

Ap_3A induces aggregation of human platelets. To investigate the effect of diadenosine triphosphate (Ap_3A) on human platelets, we used freshly prepared platelet-rich plasma from healthy donors for bioassay (10, 13). Addition of Ap_3A at 20 μM induced, after a lag of several minutes, a slowly increasing aggregation of the platelets (Figure 1). In contrast, aggregation

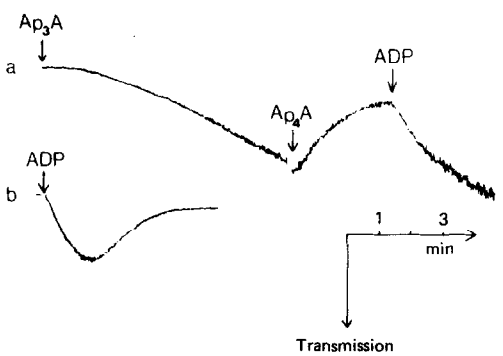

Figure 1: Aggregation of human platelets induced by Ap_3A. Platelet-rich plasma (PRP) was freshly prepared from the blood of healthy donors. Aggregation of platelets was followed photometrically. a) Diadenosine triphosphate (Ap_3A) was added to give a concentration of 20 μM. Diadenosine tetraphosphate (Ap_4A) was then added at 100 μM as indicated by the second arrow. b) Aggregation induced by ADP (0.5 μM).

induced by ADP at low concentrations (less than 2 μM) started immediately and showed spontaneous reversion. A contamination of Ap_3A with ADP as the possible stimulus was definitely excluded by chromatographing 10 nmol of Ap_3A with an HPLC system, which showed no traces of ADP. To remove any other aggregating component from our Ap_3A sample, we repurified the dinucleotide, and obtained identical results. Figure 1 also demonstrates that diadenosine tetraphosphate (Ap_4A) had strong disaggregating potency. This result extends the finding of Harrison et al. that Ap_4A can competitively inhibit ADP-induced aggregation (9). Using Ap_3A up to 0.5 mM in our system, we always observed aggregating effects which could be counteracted by Ap_4A. The stimulation of aggregation was maximal with Ap_3A at concentrations between 10 and 30 μM.

Ap_3A enhances the aggregating effect of platelet activating factor (PAF). We also investigated the effect of Ap_3A on platelet aggregation induced by the platelet activating factor, a lipid released from platelets and leucocytes which may play a regulatory role in aggregation under various conditions such as thrombosis, inflammation, etc. (14-16). PAF added at 3.8×10^{-8} M caused immediate aggregation followed by spontaneous reversion after several minutes (Figure 2). When added at 5 μM before PAF, Ap_3A caused a dramatic enhancement of platelet aggregation whereas the homologue Ap_4A at the same concentration showed no effect. Furthermore, Ap_3A inhibited the spontaneous disaggregation observed with PAF alone (Figure 2). Ap_4A, when added later at high concentration (50 μM), rapidly dissociated the platelets.
Fig. 2 also demonstrates that ADP again overrode the disaggregating effect of Ap_4A. This is consistent with a competitive mechanism for Ap_4A-inhibition of

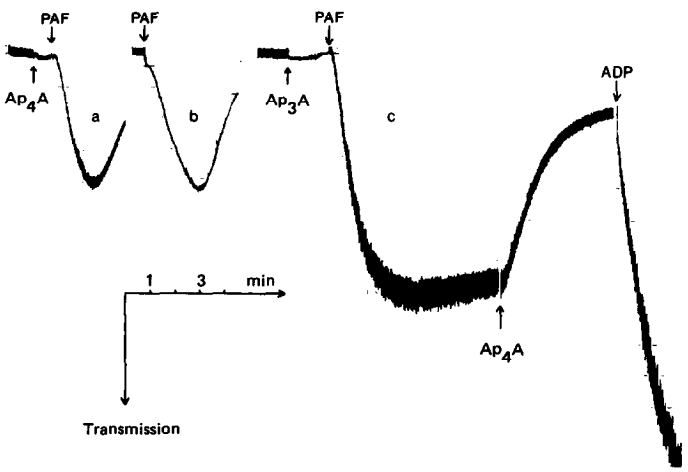

Figure 2: Effects of Ap_3A and Ap_4A on platelet aggregation induced by platelet activating factor (PAF; 3.8×10^{-8}M). a) Ap_4A (5 μM) was added before PAF. b) Aggregation by PAF alone. c) Effect of Ap_3A (5 μM) added before PAF. Ap_4A was then added at a conc. of 50 μM. Lastly, ADP was pipetted at a conc. of 50 μM.

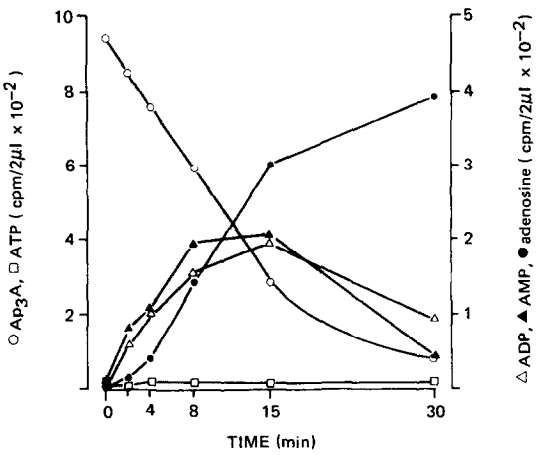

Figure 3: Liberation of ADP from Ap_3A in human plasma. [^{3}H]Ap_3A (3.5 µM; 22.7 Ci/mmol) was incubated with PRP at 37°C. Aliquots were withdrawn and treated as described under MATERIALS & METHODS.

ADP-induced aggregation (9). Our experiments clearly show that the dinucleotides have an antagonistic influence on platelet aggregation. Moreover, both effects proved to be completely reversible.

Ap_3A liberates ADP in human plasma. The time course of aggregation induced by Ap_3A suggested the possibility that the slow generation of a stimulatory molecule, namely ADP, might be responsible for the growing of platelet complexes. To confirm this, tritium-labelled Ap_3A was incubated in PRP (Fig. 3). The slow degradation of Ap_3A was reflected in the simultaneous formation of ADP and AMP, which were then further degraded to adenosine. Incubation of [^{3}H]Ap_3A with platelet-poor plasma (obtained after 12,000 x g centrifugation of PRP) yielded identical results, suggesting that the enzyme activities are in the plasma and are not associated with the platelets. It is not yet known whether the diadenosine triphosphatase activity in the plasma is an unspecific phosphodiesterase, or whether it is a specific enzyme. Specific hydrolases have been described for Ap_3A (17,18) as well as for Ap_4A (18-20). Our experiments prove that in platelet-rich plasma Ap_3A slowly liberates ADP, which is a potent aggregation stimulus. Assuming a rate of hydrolysis of Ap_3A of less than 5% per minute (Fig. 3), Ap_3A should continuously generate sufficient ADP to mediate the slow aggregation shown in Fig. 1. These considerations prompted us to simulate the time course of aggregation by adding ADP in constant portions every minute. These experiments yielded aggregation curves that were very similar to those obtained from a single application of Ap_3A (Fig. 4).

Alkaline phosphatase prevents aggregation mediated by Ap_3A. Additional proof that ADP released from Ap_3A is responsible for aggregation was obtained from experiments involving the use of alkaline phosphatase at a concentration that

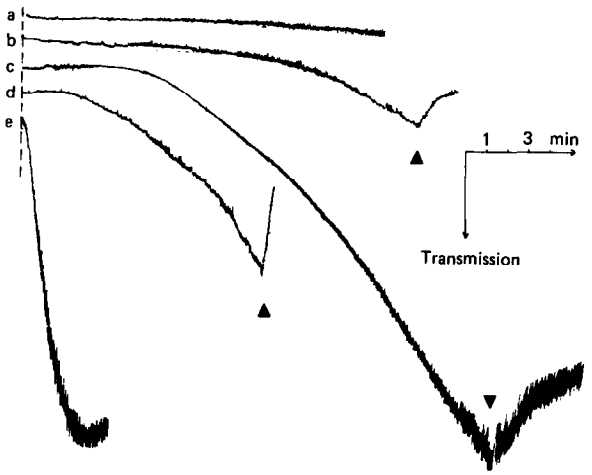

Figure 4: Aggregation induced by repeated additions of small amounts of ADP or by a single application of Ap_3A. Aggregating nucleotides were added to 1 ml of PRP at the time marked by the dashed line. a) PRP without any additions. b) Repeated additions of ADP. At the time indicated by the dashed line 1 µl of ADP was first pipetted to give a concentration of 0.01 µM. This addition was repeated every minute. c) Single addition of Ap_3A to give a final conc. of 10 µM. d) Repeated additions of ADP as in exp.b but with ten-fold higher amounts of ADP (0.1 µM). e) Control aggregation after a single addition of ADP (1 µM). The triangles indicate the addition of Ap_4A (0.1 mM).

immediately destroyed ADP liberated from Ap_3A (Fig. 5). Ap_3A itself is resistant to the enzyme. Heat-denatured phosphatase did not prevent aggregation induced by Ap_3A. The results support the conclusion that Ap_3A itself does not act as the stimulatory agent, but as a source of an aggregating product that is phosphatase-sensitive, such as ADP.

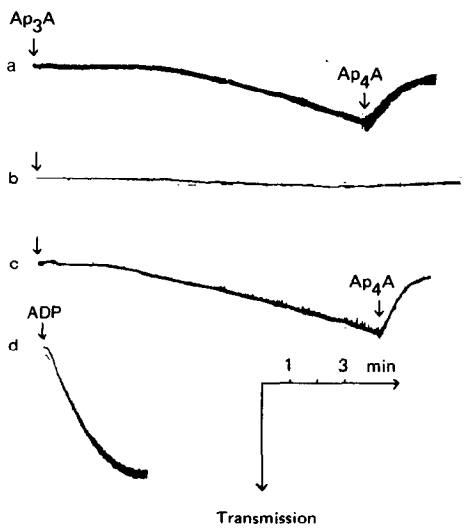

Figure 5: Effect of alkaline phosphatase (5 µg/ml) on platelet aggregation induced by Ap_3A. a) Aggregation induced by Ap_3A (10 µM). b) Alkaline phosphatase was added shortly before Ap_3A. c) Heat-denatured alkaline phosphatase was added before Ap_3A. d) Control aggregation induced by ADP (1 µM). In exp. a and c, Ap_4A was added at 0.1 mM.

The aggregatory potency of Ap_3A in PRP from a variety of human donors was also measured, When the same concentration of Ap_3A was applied, we observed quantitative differences, as can be seen by comparing corresponding traces in Figs. 4 and 5. These differences were, however, always reflected in a different sensitivity of the platelets towards ADP, which can also be seen in Figs. 4 and 5. The reasons for such varying "intrinsic" sensitivities of the platelets are not yet known (13).

In conclusion, the unusual dinucleotides Ap_3A and Ap_4A, both of which are stored in human platelets and are released during activation, can elicit antagonistic effects on the process of aggregation. Ap_3A mediates aggregation by liberating ADP, which in turn depends on an enzymatic activity in the plasma. Ap_4A has disaggregating properties. Our results provide evidence for possible extracellular functions of diadenosine polyphosphates which have mainly been considered to act as intracellular signal nucleotides (7). Further work should be done to confirm that these in-vitro effects also operate in-vivo to modulate platelet function.

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