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THE PRESENCE OF DIADENOSINE 5',5'''-P^1,P^3-TRIPHOSPHATE (Ap_3A) IN HUMAN PLATELETS

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Diadenosine triphosphate (Ap_3A) has been identified and quantified in human platelets using a coupled enzymatic assay specific for Ap_3A, after fractionation of acidic extracts with high-performence liquid chromatography. Upon thrombin-induced aggregation, Ap_3A is released together with the homologue diadenosine tetraphosphate (Ap_4A).
Extracts of human platelets do also contain enzymatic activities that degrade diadenosine tetraphosphate as well as diadenosine triphosphate. These enzymes, however, are not released during thrombin-induced aggregation of the platelets.

The intracellular level of the unusual nucleotide diadenosine tetraphosphate (Ap_4A) has been found to be directly related to the proliferative activity of eukaryotic cells (1). A target of the potential signal molecule is DNA polymerase α showing non-covalent binding of Ap_4A (2,3) and catalyzing DNA synthesis with Ap_4A acting as a primer (3,4).
The enzymatic synthesis of Ap_4A has been shown in a reaction mixture containing lysyl-tRNA synthetase, ATP, Mg^{2+}, and lysine (5). With ADP instead of ATP, however, the main reaction product was diadenosine triphosphate (Ap_3A). Ap_3A has been identified in eukaryotic cells by Ogilvie and Jakob (6,7).

It has recently been reported that human platelets contain abundant amounts of Ap_4A which is released during aggregation of the platelets (8). It has been suggested that Ap_4A might play a role as an additional growth factor released from the platelets, thus being involved in the stimulation of smooth-muscle cell proliferation.
Here we describe that the homologue Ap_3A is also present in high amounts in human platelets and that Ap_3A is also released during aggregation. The role of Ap_3A as a potential antagonist of Ap_4A is discussed.

MATERIALS & METHODS

Reagents. [^{3}H] Ap_4A was a gift of Dr. E. Holler, [^{3}H] Ap_3A was from Amersham (labeling service).
Enzymatic assay of Ap_3A and Ap_4A. Ap_4A was measured by a coupled luminescence assay as described by Ogilvie (9). The method for measuring Ap_3A is similar

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to the assay of Ap_4A extended by the addition of phosphoenolpyruvate-pyruvate kinase. The technique has been described in detail (7).

Isolation of platelets and extraction of nucleotides. Human platelets were prepared from fresh blood essentially as described (8), with the modification that the forces of sedimentation were increased (2000 x g, 10 min). After the last sedimentation, the platelets were resuspended in Tyrode buffer (without Ca^{2+}) and quickly mixed with ice-cold TCA (10 % final conc.). Further treatment of the extract and its neutralization has been described (7,9).

Release experiments. Washed platelets (2 x 10^9 cells/ml) were suspended in Tyrode buffer (without Ca^{2+}) and incubated at 37°C for 6 min with thrombin (2.2 U/ml). After rapid cooling, the samples were centrifuged at 12.000 x g for 5 min. The supernatant and, separately, the pellets were extracted with TCA and analyzed as described above.

High-performance liquid chromatography (h.p.l.c.). The reversed phase technique of Schweinsberg and Loo (10) was modified for our intention as described (7). 200 µl of the neutralized extract were mixed with [^{3}H] Ap_3A and [^{3}H]Ap_4A and applied onto the column. Elution rate was constant at 1 ml/min. Fractions of 0.2 ml were collected. The amounts of ADP and ATP were determined using the absorption coefficients obtained from standard runs.

Platelet lysate and measurement of enzyme activities. The sedimented platelets were gently shaken for 15 min at 4°C with an equal volume of Hepes buffer (50 mM pH 7.75, $MgCl_2$ 1 mM) containing 1 % (v/v) Nonidet P40. After centrifugation at 12,000 x g for 15 min, the supernatant was used for measuring enzymatic activities. Hydrolysis of Ap_4A, Ap_3A and of ATP was followed by product analysis with thin layer chromatography as described (17).

RESULTS

Identification and quantitation of diadenosine triphosphate (Ap_3A) in human platelets. The neutralized extract of human platelets was chromatographed with an h.p.l.c. system (Fig. 1). The complex elution profile allowed identification of some major nucleotides (i.e. ATP, ADP), as compared with the parallel run of a mixture of standard nucleotides (Fig. 2). The enzymatically determined as well as the radioactive peaks of Ap_3A and Ap_4A, respectively, coeluted with two distinct optical peaks suggesting the presence of both dinucleotides in the extract of the platelets (Fig. 1). Quantitation with the coupled luminescence assays revealed Ap_3A as well as Ap_4A being present in human blood platelets in almost similar high amounts (Table 1). When applying the appropriate extinction coefficient, the quantitation of Ap_3A from the optical profile yielded almost the same value as the enzymatic determination. The analogous measurement of Ap4A, however, revealed an overestimation by the optical integrator suggesting that the corresponding optical peak in the h.p.l.c. chromatogram also contained unidentified material. All quantitative results concerning Ap_4A as well as Ap_3A were therefore from the enzymatic analyses in the fractions of the h.p.l.c. chromatograms. The contents of Ap_3A, Ap_4A, ATP, and ADP in platelets of six different donors are listed in Table 1.

Release of Ap_3A upon thrombin-induced aggregation. When treated with thrombin, platelets release nucleotides from their dense storage granules (11). Table 2

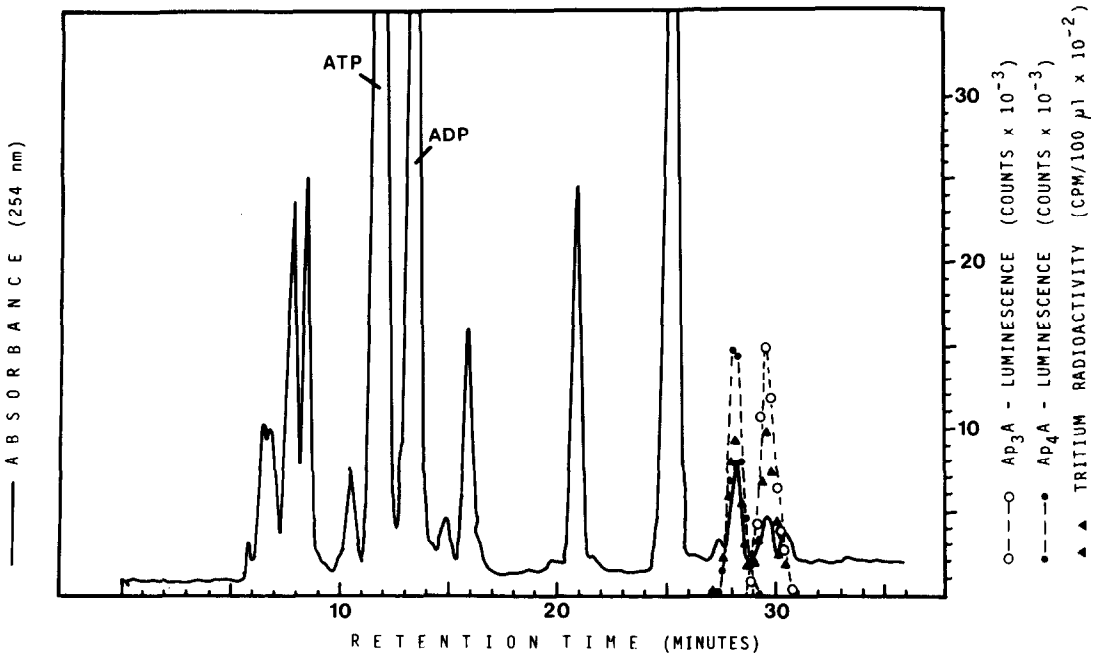

Figure 1: High performance liquid chromatography of an acidic extract from human platelets. [^{3}H] Ap_3A and [^{3}H] Ap_4A were added as internal markers. Aliquots of the collected fractions (0.2 ml) were measured for tritium radioactivity (▲), for Ap_3A-luminescence (○), and for Ap_4A-luminescence (●) as described under MATERIALS & METHODS.

shows the results of two independent experiments with platelets being treated with thrombin. Almost all Ap_3A and Ap_4A is released during aggregation. Both dinucleotides are extruded to a relatively higher extent than ATP and even

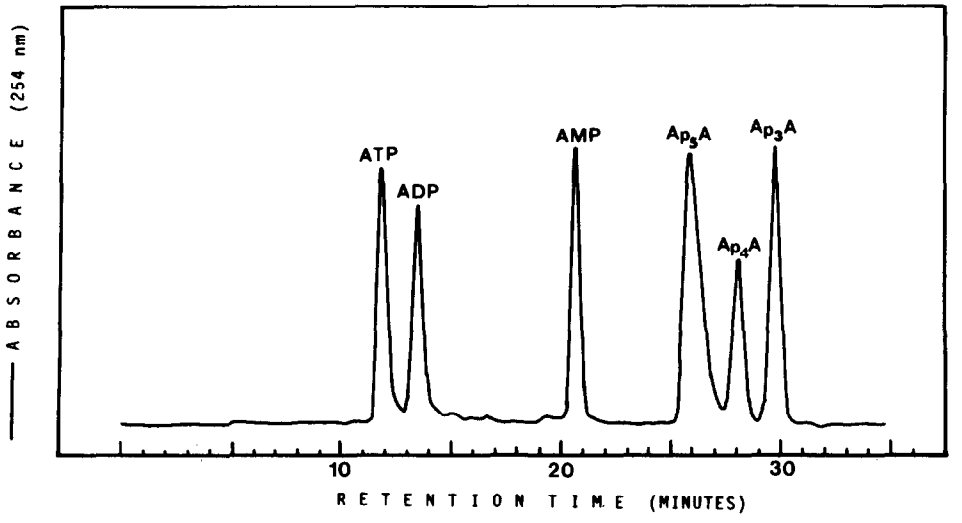

Figure 2: Separation of ATP, ADP, AMP, Ap_5A, Ap_4A and Ap_3A on a reversed phase column by high-performance liquid chromatography. Details of the procedure are given under MATERIALS & METHODS.

Table I

Contents of Diadenosine triphosphate (Ap_3A), Diadenosine tetraphosphate (Ap_4A), ATP and ADP in Human Platelets

Donor	Ap_3A	Ap_4A	ATP	ADP
	(pmol/10^6 platelets)			
E.R.	0.52	0.53	40.50	30.10
Y.W.	0.41	0.36	32.48	18.12
E.H.	0.83	0.43	67.23	42.33
J.L.	0.63	0.39	36.95	24.50
H.B.	0.63	0.56	48.70	31.50
E.A.	0.94	0.51	19.50	13.40

Neutralized acidic extracts from human platelets were measured for Ap_3A, Ap_4A, ATP and ADP, respectively, by means of high performance liquid chromatography and of enzymatic assays as described under MATERIALS & METHODS.

Table II

Release of diadenosine triphosphate and other nucleotides from human platelets after thrombin-induced aggregation

Experiment	Treatment	Ap_3A	Ap_4A	ADP	ATP
		(Extracellular content, % of total amount)			
I	Buffer	5.2	n.m.	1.3	1.3
	Thrombin	98	88	63	29
II	Buffer	31.5	14.1	16.7	12.6
	Thrombin	78.5	90.5	76.7	48.1

Washed human platelets (about 2 x 10^9/ml) suspended in Tyrode buffer (without Ca^{2+}) were treated with thrombin as described under MATERIALS & METHODS. A buffer-treated platelet suspension served as a control. After incubation, the probes were cooled and centrifuged. The adenine nucleotides of the pellet (cellular content) and of the supernatant (extracellular content) were determined. The sum of the corresponding pellet and supernatant contents was set at 100 %.

ADP (Table 2). The partial release of nucleotides from buffer-treated platelets of another individual in experiment II was due to partial aggregation of the platelets, as realized by microscopic examination.

Human platelets contain enzymatic activities that degrade Ap_3A and Ap_4A to AMP. Total cellular lysates were prepared from washed human platelets using buffer containing 0.5 % Nonidet P 40, a detergent that rapidly dissolves the cellular membrane (12). Figures 3 - 5 show the kinetics of degradation of Ap_3A, Ap_4A and ATP, respectively, in lysates from human platelets. Each nucleotide was tested at two concentrations, 3.6 and 100 µM for Ap_3A and Ap_4A, 0.25 and 100 µM in case of ATP. The major product of degradation was in all cases AMP. With Ap_3A as the substrate, we observed a remarkable effect of substrate concentration. Almost complete degradation was obtained with Ap_3A being present at a concentration of 3.6 µM (Fig. 3a) whereas Ap_3A at 100 µM was only partially metabolized (Fig. 3b). The results obtained for the hydrolysis of Ap_4A are different from the report of Flodgaard & Klenow who did not find degradation of Ap_4A in extracts from human platelets using a different method for the preparation of the crude enzyme (8). Measuring the wash liquid of the platelets, no hydrolyzing activities could be detected with the three substrates (data not shown). This result suggests that the enzymatic activities in the lysates are intracellular or membrane-bound and not a contamination with plasma. We also measured the hydrolysing activities after thrombin-induced aggregation.

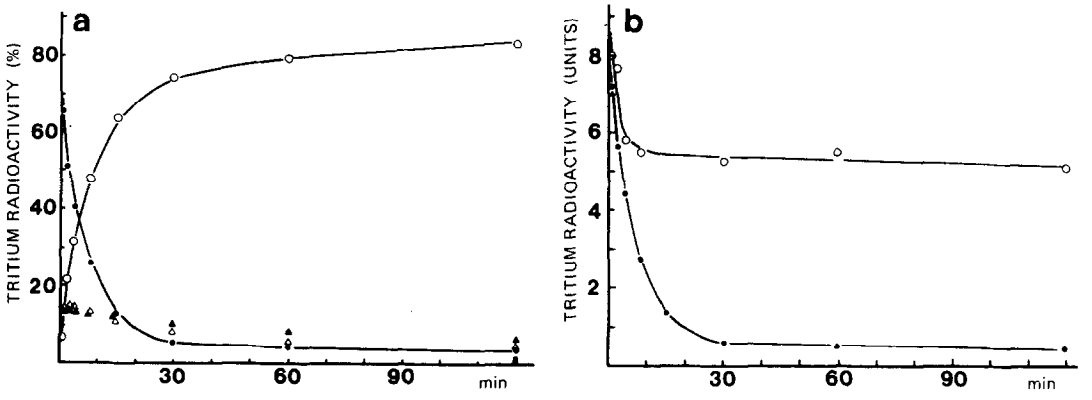

Figure 3a: Kinetics of Ap_3A hydrolysis by a lysate of human platelets and product analysis. The degradation of [^{3}H] Ap_3A (3.6 µM) by a lysate of human platelets was followed by withdrawal of aliquots at different times and chromatography on poly(ethylene-imine)-cellulose thin layers. The development of the plates was performed with 0.5 M lithium chloride. The sum of radioactivity found in Ap_3A (●), ATP (▲), ADP (△) and AMP (○) was set at 100 %.

Figure 3b: Kinetics of Ap_3A hydrolysis by a lysate of human platelets at two different substrate concentrations. The method was identical with that described for Fig. 3a. The Ap_3A concentrations were 3.6 µM (●) or 100 µM (○). One unit of tritium radioactivity was equivalent to 10^3 cpm/µl assay.

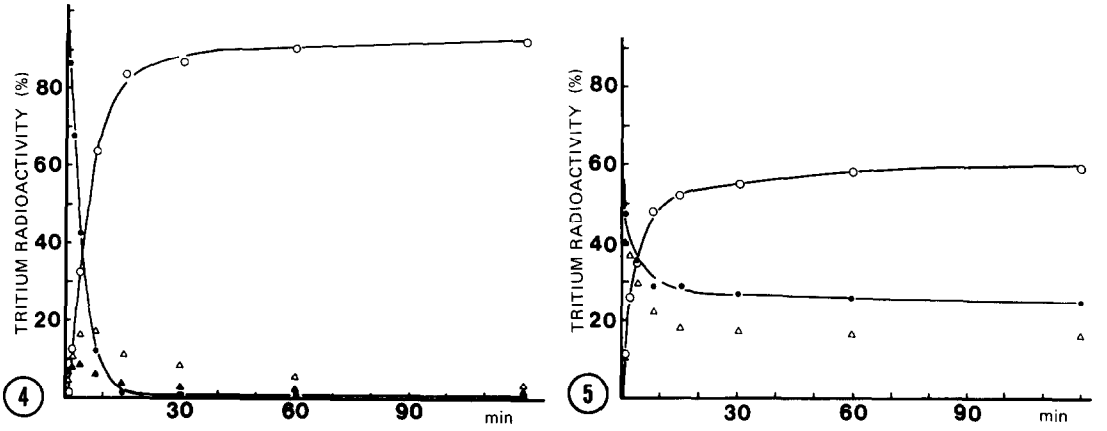

Figure 4: Kinetics of Ap_4A hydrolysis by a lysate of human platelets and product analysis. The degradation of [^{3}H] Ap_4A (3.6 µM) in a lysate of human platelets was measured analogously to the procedure described for Figure 3a. The sum of the radioactivity found in Ap_4A (●), ATP (▲), ADP (△), and AMP (○) was set at 100 %.

Figure 5: Kinetics of ATP hydrolysis in a lysate of human platelets and product analysis. The degradation of [^{3}H] ATP (0.25 µM) by a lysate of human platelets was determined as described for Figure 3a. The sum of the radioactivity found in ATP (●), ADP (△), and AMP (○) was set at 100 %.

No activity on Ap_3A, Ap_4A or ATP could be detected in the supernatant. Almost all activity, however, remained extractable from the pellets. Therefore, the experiment gives no evidence for release of hydrolyzing enzymes that are able to degrade the extruded nucleotides (data not shown).

DISCUSSION

Human platelets contain diadenosine triphosphate (Ap_3A) in similarly high amounts as its homologue diadenosine tetraphosphate (8). The identification and quantitation of Ap_3A was performed using a specific methodology developed for this purpose (7). The concentration of ATP and ADP found in human platelets is in accordance with published data (13,14). Flodgaard and Klenow have raised the question as to whether the complete pool of Ap_4A that is present in the circulating blood is located in the platelets. Our data, however, show that whole blood extracts contain 2 - 3 times more Ap_4A and Ap_3A than can be extracted from platelets (results not given). This suggests that the dinucleotides are also present in other blood constituents.

Diadenosine triphosphate was almost completely released from the platelets upon thrombin-induced aggregation suggesting that Ap_3A is stored in dense granules which also contain other releasable constituents (11). Concerning other releasable nucleotides, our results are in good accordance with published data, i.e. almost total extrusion of Ap_4A (8) and partial release of ADP and ATP (15).

Human platelets contain enzymes that hydrolyze Ap_3A as well as Ap_4A. Highly specific hydrolases have been described for Ap_3A (16) and for Ap_4A (17 - 20) from different tissues. Besides those specific enzymes, less specific phosphodiesterases could be responsible for the degradation of the dinucleotides (20). The kinetics of degradation of Ap_4A and Ap_3A at variable concentrations suggest that both nucleotides are degraded by different enzymes. If the dinucleotides are stored in metabolically inactive granules (11) the function of those enzymes in human platelets remains unclear. We have investigated the possibility that during aggregation those enzymes are released together with the corresponding nucleotides in order to limit possible effects of the nucleotides by degradation. The aggregation experiments, however, gave no evidence for a release of those enzymatic activities into the extracellular space.

From studies with purified aminoacyl-tRNA synthetases the dinucleotides are believed to be side-products of the protein synthetic machinery (5). Platelets, however, are known to be almost devoid of protein synthetic activity. Flodgaard & Klenow therefore suggested that the biosynthesis of Ap_4A might occur in precursor cells, i.e. megakaryocytes.

Since Ap_3A has been found to inhibit the binding of Ap_4A to DNA polymerase α (21), and since Ap_3A interferes with the metabolism of Ap_4A (22), diadenosine triphosphate might act as an antagonist of Ap_4A. Furthermore, Ap_3A itself has been shown to be a poor primer of DNA synthesis when compared to Ap_4A (4). It will be necessary to look for further physiological functions of diadenosine triphosphate in platelets.

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