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Abundant amounts of diadenosine 5′,5‴-P^1,P^4-tetraphosphate are present and releasable, but metabolically inactive, in human platelets

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Diadenosine 5′,5‴-P^1,P^4-tetraphosphate (Ap_4A) may be formed in the back-reaction of the amino acid-activation reaction [Zamecnik, Stephenson, Janeway & Randerath (1966) *Biochem. Biophys. Res. Commun.* **24**, 91–98]. On the basis of a number of observations of the properties of Ap_4A it has been suggested that it may have a signal function for the initiation of DNA replication in eukaryotic cells [Grummt (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 371–375]. In the present paper human platelets have been shown to contain relatively large amounts of Ap_4A. The compound is apparently metabolic inactive in platelets, but it is almost quantitatively released when platelets are activated to aggregate by treatment with thrombin. The results are discussed in connection with the known growth-stimulating activity of platelets.

Ap_4A was first identified as a product of the back-reaction of the amino acid-activation reaction (Zamecnik *et al.*, 1966). It was later shown to be present in several types of eukaryotic cells and the level was found to vary widely with the proliferative activity of the cells (Rapaport & Zamecnik, 1976; Plesner *et al.*, 1979). The compound binds to DNA polymerase-α as a very specific ligand and it affinity-labels the 57000-M_r subunit of the enzyme (Grummt *et al.*, 1979). Tryptophanyl-tRNA synthetase (EC 6.1.1.2) activity has been shown to be tightly associated with DNA polymerase-α activity and to Ap_4A-binding activity, suggesting a link between the amino acid-activation process and DNA replication in mammalian cells (Rapaport *et al.*, 1981). Experiments with permeabilized G_1-phase-arrested BHK (baby-hamster kidney) cells have shown that Ap_4A stimulates the initiation of DNA synthesis, and it has been suggested that the nucleotide functions as a signal molecule for the cellular transition from the resting state to the growing state by triggering initiation of DNA replication (Grummt, 1978). It has recently been found that Ap_4A may act as a primer for DNA synthesis in a system *in vitro* containing purified HeLa-cell DNA polymerase-α, deoxy-ATP and a synthetic double-stranded octadecamer oligodeoxyribonucleotide. The oligonucleotide is part of the origin region of DNA replication in simian virus 40 (Zamecnik *et al.*, 1982). In the present paper, Ap_4A has been identified in human blood platelets and shown to be present in amounts relative to protein and to ATP which are considerably larger than the amounts present in proliferating mammalian cells. The compound does not seem to be metabolized in platelets and most of it is secreted during thrombin-induced aggregation of platelets. Ap_4A is also present in platelets from rabbit and rat.

Abbreviation used: Ap_4A, diadenosine 5′,5‴-P^1,P^4-tetraphosphate.

Experimental procedures

High-pressure liquid chromatography

The instrument used was a Waters LCS-IV automated microprocessor-based system fitted with a 25 cm Partisil-10 SAX strong-anion-exchanger.

Column packing. A 45 cm stainless-steel funnel with a capacity of 50 ml was connected to a 25 cm stainless-steel column with an internal diameter of 4.5 mm. Partisil-10 SAX (2.5 g, enough to fill the column) was suspended in 50 ml of 0.25 M-KH_2PO_4, pH 4.5, containing 0.5 M-KCl. After treatment for 5 min in an ultrasonic bath, the slurry was rapidly packed into the funnel and a flow of 30 ml/min was applied, the same buffer as for the suspension being used. Three combined Waters chromatographic pumps maintained the flow against a constant back-pressure of 41.4 MPa (6000 lbf/in^2). The flow was decreased gradually in such a way that a back-pressure of 41.4 MPa could be maintained. The pressure was usually constant within 1 min with a flow of 12–15 ml/min. The high flow rate was maintained for 15 min and the column was thereafter ready for use.

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Gradient system. A modification of the KH_2PO_4/KCl system described by Hartwick & Brown (1975) was used. The chromatography was initiated by an isocratic period of 25 min by using 7 mM-KH_2PO_4, pH 4.0, at a flow of 1 ml/min. The flow was increased to 2 ml/min and a 40 min linear gradient generated by two microprocessor-controlled Waters chromatographic pumps mixing 0.25 M-KH_2PO_4/0.5 M-KCl, pH 4.5, with the abovementioned buffer was applied. An isocratic period of 10 min was then allowed and the column was finally re-equilibrated with the dilute starting buffer for 25 min before a new sample was automatically injected and the cycle repeated.

Measurement of nucleotide concentration

The quantification was obtained by electronic integration (Waters data module). The integrator was calibrated by nucleotide standards, and nucleotide amounts down to 50 pmol could be measured with an error of less than 10%.

Isolation of platelets

Citrated blood (10 mM-citrate final concn.) was centrifuged at 22°C for 10 min at 125 *g*. To the resulting platelet-rich plasma was added 1% (v/v) of 0.1 M-EDTA and the platelets were sedimented by centrifugation at 1700 *g* at 22°C for 10 min. The platelets were resuspended in STEG buffer (0.148 M-NaCl/0.02 M-Tris/6 mM-EDTA/5 mM-glucose), pH 7.4, and washed twice in the same buffer. Microscopic examination of the preparations showed a high purity with regard to platelets and the almost complete absence of erythrocytes and leucocytes.

Isolation of the acid-soluble fraction

Isolated washed platelets from 30–50 ml of blood were sedimented by centrifugation at 2100 *g* and extracted with 0.5 ml of 5% (w/v) ice-cold trichloroacetic acid containing about 30 nmol of the internal standard adenosine 5′-[$\beta\gamma$-methylene]triphosphate (p[CH_2]ppA). After 10 min at 0°C the precipitate was removed by centrifugation and the supernatant was treated by shaking it three times with 1 vol. of 20% tri-n-octylamine in Freon (Chen *et al.*, 1977). After centrifugation the aqueous phase was freeze-dried and redissolved in 200 μl of 20 mM-Tes (2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]-amino}ethanesulphonic acid) buffer, pH 8.0. The protein present in the acid-insoluble precipitate was determined as described by Lowry *et al.* (1951), with bovine serum albumin as standard.

Materials

Partisil-10 SAX was from Whatman. Ap_4A, p[CH_2]ppA and alkaline phosphatase (EC 3.1.3.1) were purchased from Boehringer. Thrombin from human plasma (3000 National Institutes of Health units per mg of protein) and Tes were from Sigma. 5′-Nucleotide phosphodiesterase was purified from pig small-intestine brush border. The enzyme contains less than 0.1% alkaline phosphatase activity and was shown to be a 5′-exonuclease catalysing the cleavage of both 2′,5′ and 3′,5′ phosphodiester bonds. One unit of enzyme is the amount hydrolysing 1 μmol of p[CH_2]ppA per min at pH 8.0 and 25°C (H. Flodgaard, C. Torp-Pedersen, O. Norén & H. Sjöström, unpublished work).

Results

Platelets are known to contain high levels of ribonucleotides. Most of them are sequestered at high concentrations in a metabolically inactive state within the dense storage granules (Ugurbil & Holmsen, 1981). In the present chromatographic analyses of the acid-soluble fraction of human platelets the previously shown content of 5′-phosphorylated derivatives of adenosine, guanosine, cytidine and uridine has been confirmed (Rao *et al.*, 1974; D'Souza & Glueck, 1977). In addition, a hitherto-unidentified u.v.-absorbing peak with the retention time (about 67 min) of synthetic Ap_4A was observed (Fig. 1*a*). In contrast with, e.g., ATP and GTP, the peak was completely resistant to treatment of the acid-soluble fraction with alkaline phosphatase (Fig. 1*b*). After re-isolation, the compound co-chromatographed with authentic Ap_4A (Fig. 1*c*) and was found to be degraded by treatment with 5′-nucleotide phosphodiesterase (Fig. 1*d*). During degradation of Ap_4A with phosphodiesterase, ATP should appear as an intermediate, and the end product should be 1 mol of PP_i and 2 mol of AMP for each mol of Ap_4A. This means that, at any time during the enzymic reaction, the two sums, $Ap_4A + ATP + PP_i$ and $Ap_4A + \frac{1}{2}ATP + \frac{1}{2}AMP$ respectively, should be constant. Analyses of incubation mixtures of two different concentrations of phosphodiesterase with the isolated compound showed that it had been partially and completely degraded, respectively. In both cases the data obtained are in full agreement with those expected for Ap_4A (Table 1). On the basis of these properties of the isolated compound, it is concluded that human platelets contain Ap_4A. Analyses of the acid-soluble fraction of washed platelets from five different persons showed only small variations in the ratio of Ap_4A to ATP and to protein (Table 2). Both ratios are considerably higher (about 10-fold) than those previously reported for rapidly growing eukaryotic cells (Rapaport & Zamecnik, 1976). The ratios of nucleotides to protein based on cell counting agreed within 5% with those based on protein determination when it was assumed that 10^{11} platelets contain 189 mg of protein (Holmsen & Robkin, 1980). The ratios of the different nucleotides to

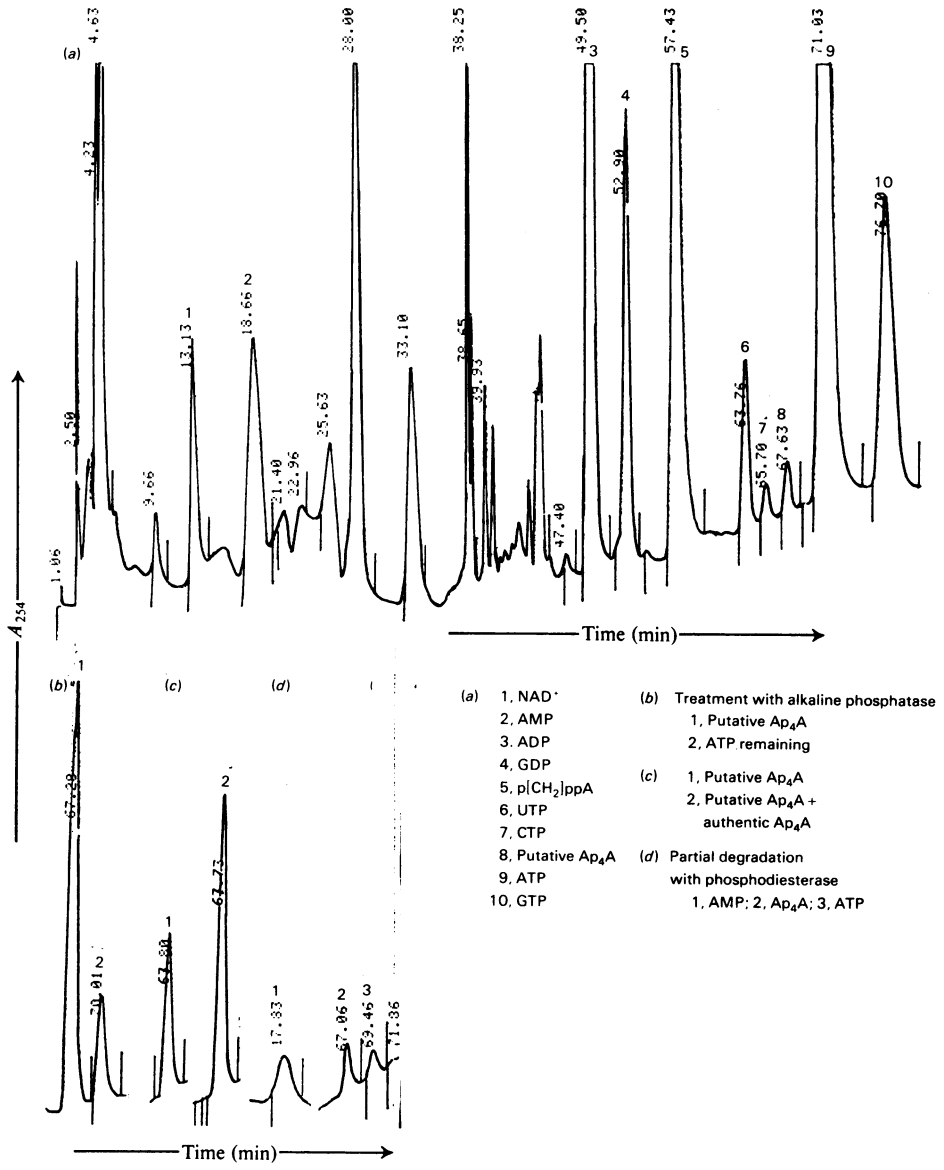

Fig. 1. *High-pressure-liquid-chromatographic analyses of ribonucleotides from the acid-soluble fraction of human platelets*

The sideways-on values against the peaks are retention times in minutes. The larger upright peak numbers are identified on the Figure. (*a*) The freeze-dried residue from the extract from about 1×10^{10} platelets was dissolved in 200 μl of 20 mM-Tes buffer, pH 8.0, and 20 μl were chromatographed; the u.v. detector attenuation was 0.02 a.u.f.sc. (absorbance units full scale). (*b*) The remaining 180 μl of dissolved residue from (*a*) was treated for 2 h at 37°C with 20 μg of alkaline phosphatase and the whole sample chromatographed. Part of this chromatogram is shown; the u.v. detector attenuation was 0.1 a.u.f.sc. (*c*) The putative Ap_4A (67 min) peak from (*b*) was recovered, and the nucleotide adsorbed on charcoal, eluted with pyridine/ethanol/water (10:40:50, by vol.), freeze-dried and redissolved in 20 mM-Tes buffer, pH 8.0. Peak 1: rechromatography of 324 pmol of the isolated compound; quantification was based on the assumption that putative Ap_4A has an ε_{260} value of 30.8 litre · mol · cm^{-1}. U.V. detector attenuation in the chromatogram was 0.02 a.u.f.sc. Peak 2: chromatography of a mixture of 324 pmol of authentic Ap_4A and 324 pmol of isolated putative Ap_4A. The integrator calibrated with authentic Ap_4A calculated that the peak contained 622 pmol of Ap_4A. (*d*) Partial degradation of putative Ap_4A with 5′-nucleotide phosphodiesterase; the u.v. detector attenuation was as in (*c*); only part of the chromatogram is shown.

Table 1. *Stoichiometry of products formed by treatment of putative Ap_4A from human platelets with 5'-nucleotide phosphodiesterase*

The incubations were carried out in volumes of 100 μl of 20 mM-Tes buffer/2 mM-orthophosphate, pH 8.0, at 37°C for 20 min. Measurements of the ribonucleotide concentrations were performed by chromatography as described in the Experimental procedures section, and PP_i was measured with an error less than 6% as previously described (Flodgaard & Fleron, 1974). The putative Ap_4A was assumed to have an ε_{mM}^{260} of 30.8 litre·mol^{-1}·cm^{-1} at neutral pH. Abbreviation used: PDE, 5'-nucleotide phosphodiesterase.

		Content (pmol in assay mixture)					
Expt.	PDE (units)	Putative Ap_4A	ATP	AMP	PP_i	$Ap_4A + \frac{1}{2}AMP + \frac{1}{2}ATP$	$Ap_4A + ATP + PP_i$
1	0	581	0	0	—	581	—
	0.02	107	362	450	—	513	—
2	0	753	0	0	78	753	831
	0.2	0	193	1176	616	785	809

Table 2. *Ribonucleotide content of washed platelets from man*

Results are means ± S.E.M., with the numbers of donors given in parentheses.

Nucleotide	Content (nmol/mg of protein)
Ap_4A	0.42 ± 0.04 (5)
ATP	46 ± 3.5 (5)
ADP	41 ± 3.9 (5)
NAD	1.9 ± 0.3 (5)
GTP	6.8 ± 0.6 (5)
GDP	5.2 ± 0.5 (5)
CTP	1.1 ± 0.4 (4)
UTP	2.6 ± 0.4 (5)

protein given in Table 2 are, however, for most nucleotides severalfold higher than those calculated from previously reported nucleotide content per platelet as determined by high-pressure liquid-chromatographic methods (Rao *et al.*, 1974; Pross *et al.*, 1977; D'Souza & Glueck, 1977). This discrepancy may partly be accounted for by the fact that, in the present case, platelets were analysed immediately after isolation and not after storage in the cold. This explanation is supported by the fact that, in contrast with others, we do not find platelets to contain nucleosides, suggesting the absence of degradation of nucleotides in our case. In addition, our values for ATP content are in agreement with those obtained by others using enzymic methods (Holmsen *et al.*, 1972; Mills & Thomas, 1969).

Chromatograms of the acid-soluble fractions of washed platelets from rat and rabbit were also found to contain alkaline phosphatase-resistant peaks with the retention time of about 67 min. By these criteria Ap_4A is, therefore, also present in the platelets of these two species. The amounts of the compound relative to protein are, however, almost ten-fold lower in these two species than in man.

Ap_4A, which may be formed in the back reaction of the amino acid-activation reaction, has been shown to have a higher metabolic activity than ATP in BHK (baby-hamster kidney) cells (Rapaport & Zamecnik, 1976). Since platelets are almost devoid of protein-synthetic activity, it was considered worthwhile to compare the metabolism of ATP and Ap_4A in them. Platelets were incubated with [^{3}H]adenosine for various lengths of time and the acid-soluble fractions chromatographed. The peaks corresponding to Ap_4A and ATP were recovered and, after re-chromatography, the specific radioactivities of the two compounds were determined. It appeared that after incubation of suspensions of platelets with [^{3}H]adenosine for, e.g., 4 h, ATP was heavily labelled (2670 c.p.m.·nmol^{-1}), whereas Ap_4A was devoid of any significant radioactivity (50 c.p.m.·nmol^{-1}). This suggests that Ap_4A is not metabolized in platelets. Similar conclusions were drawn from experiments where equimolar concentrations of Ap_4A or ATP were incubated with homogenates of platelets. ATP was rapidly degraded under conditions where no significant decrease in the concentration of Ap_4A could be detected (Fig. 2).

The metabolic inactivity of Ap_4A in platelets suggested that it, in common with the metabolically inactive pool of other ribonucleotides, is present in the dense storage granules. This possibility was supported by experiments where platelets were treated with thrombin. It was previously shown that, under such conditions, platelets aggregate and release the metabolically inactive pool of the ribonucleotides (Holmsen *et al.*, 1969). The present experiments confirm these observations. Washed platelets suspended in EDTA-containing buffer

Table 3. *Effect of thrombin-induced aggregation of washed human platelets on the release of ribonucleotides and protein*
Washed platelets were suspended in Tyrode (1910) buffer without Ca^{2+} (about 2.5×10^9 platelets/ml). Samples were incubated at 37°C for 6 min with or without addition of thrombin (1.2 units/ml). After rapid cooling the samples were centrifuged in the cold at 12000 *g* for 5 min and the ribonucleotide and protein contents of the precipitate (cellular fraction) and the supernatant (extracellular fraction) were determined.

	Extracellular content (% of total amount)					
Treatment	Ap_4A	ATP	ADP	GTP	GDP	Protein
Buffer	6.2	4.4	10	6.3	11	4
Thrombin	89	53	75	36	66	11

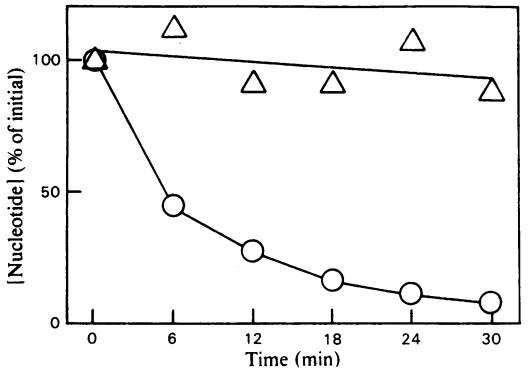

Fig. 2. *Stability of ATP and Ap_4A in homogenates of human platelets*
Washed platelets were resuspended in 10 mM-Tris buffer (pH 7.6)/5 mM-$MgCl_2$ and treated in a Virtis homogenizer for 1 min at 45000 rev./min. The homogenate (4.4 mg of protein · ml^{-1}) was incubated at 37°C in the presence of 0.1 mM concentrations of ATP (○) or Ap_4A (△). Samples were treated with trichloroacetic acid, and the acid-soluble fractions were analysed by high-pressure liquid chromatography.

released only a small part of their ribonucleotide pool, including Ap_4A. Treatment with thrombin, however, gave rise to complete aggregation of platelets in less than 6 min (according to aggregometer measurements; results not shown) and to simultaneous release of part of the content of ATP, ADP, GTP and GDP, in accordance with previous findings (D'Souza & Glueck, 1977). For Ap_4A, however, almost all was released, supporting the notion that most, if not all, of it is present as a metabolically inactive pool in the dense storage granules of human platelets (Table 3).

Discussion

The content of Ap_4A in platelets constitutes probably most if not all of the whole pool present in the circulating human blood. We have not been able to detect any Ap_4A in erythrocytes, although preparations enriched in lymphocytes and monocytes always showed the presence of small amounts of Ap_4A. This might, however, be accounted for by a small number of platelets that always contaminated these preparations. The contaminating platelets often adhered to the leucocytes. With an average content of about 0.3×10^6 platelets per μl, the circulating blood of an adult person would contain about 1 μmol of Ap_4A. The presence of Ap_4A in platelets means that the determination of this compound in whole organs such as liver should be corrected for the contribution from the blood present in the particular organ.

Platelets are formed from precursors which again are derived from cytoplasmic fragments of megakaryocytes. The megakaryocytes participating in this process are represented by cells of a number of ploidy classes. The apparent metabolic inactivity of Ap_4A in platelets suggests that the biosynthesis of this compound and its organization in the dense storage granula takes place in the megakaryocytes. There is no evidence of a function for the Ap_4A released during aggregation of platelets. It may be important in the process of coagulation or it may function as a cell growth factor. It is known that platelets may interact with damaged vessel walls and that platelets release a factor that is mitogenic for smooth-muscle cells. This factor has been identified as a polypeptide (Ross & Vogel, 1978). It has been suggested that endothelial injury, platelet interaction with subendothelium, release of a mitogenic polypeptide and resulting smooth-muscle cell proliferation could be a major cause of intimal thickening (Mustard *et al.*, 1977). In view of the present knowledge about the possible function of Ap_4A for initiation of DNA replication, it is tempting to speculate that Ap_4A released from platelets may function as an additional growth factor. It may be of importance in this context that platelets do not seem to contain enzymes capable of hydrolysing Ap_4A. During platelet aggregation a local high concentration of released Ap_4A may be obtained. Norm-

ally nucleotides such as Ap_4A would not pass a cell membrane, but in this connection it may also be of significance that platelets release proteins that enhance the permeability of cell membranes (Nachman *et al.*, 1972).

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