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Identification of Adenosine 5'-Tetraphosphate in Rabbit Platelets and its Metabolism in Blood

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=ABSTRACT=

Adenosine 5'-tetraphosphate (ATPP) was identified and quantified in extracts of rabbit platelets by elution of extracts containing authentic adenosine 5'-tetraphosphate and comparison of retention time with nucleotide standards using high-performance liquid chromatography technique. The amount of adenosine 5'-tetraphosphate was 0.62 nmoles/10^9 cells which was 62-fold lower than that of ATP but only 10-fold lower than that of ADP. During platelet aggregation induced by thrombin, adenosine 5'-tetraphosphate was released to a relatively high extent. The degradation rates and half-lives of adenosine 5'-tetraphosphate were measured during incubation of platelets in whole blood, erythrocyte suspension and plasma, respectively. The results suggest that plasma contributes more than blood cells to the catabolism of adenosine 5'-tetraphosphate. The pattern of degradation indicates that ATPP may be degraded mainly to AMP by soluble enzymes in plasma and very slowly to ADP and/or AMP by ectoenzymes on blood cells such as erythrocyte. The nature of the enzymes responsible for the degradation of adenosine 5'-tetraphosphate is yet to be identified.

Key Words: Adenosine 5'-tetraphosphate, Platelet, Nucleotides, ATP, Thrombin

INTRODUCTION

An unusual nucleotide, adenosine 5'-tetraphosphate (ATPP) has been first detected as a contaminant in isolation of ATP from ox muscle (Marrian, 1953). The presence of ATPP has later been reported in other mammalian skeletal muscles as well (Lieberman, 1955, Small & Cooper, 1966a) and in isolated rat liver mitochondria incubated with inorganic orthophosphate (Heldt & Klingenberg, 1965).

It has been demonstrated that the yeast 3-phosphoglycerate kinase catalyzes the ATPP synthesis from ATP and 1, 3-diphosphoglycerate (Small & Cooper, 1966a), whereas the skeletal muscle adenyl kinase catalyzes the synthesis from ATP and ADP (Kupriyanov et al, 1986) in vitro. A recent study has shown that 5' nucleotidase from snake venom yields ATPP from AP_5A in vitro (Schluter et al, 1994). Small and Cooper (1966b) have purified a non-specific hydrolase from rabbit muscle which cleaves only the terminal phosphate from ATPP.

Although the biological roles of ATPP are not entirely identified, some biochemical and pharmacological functions have been suggested. ATPP has been claimed to split actomyosin into actin and myosin in carp muscle (Winand-Devigne et al, 1967). Lobaton et al (1975) has proposed that ATPP has a regulatory role by an inhibition of dinucleoside tetraphosphatase. A functional study has suggested

that exogenous ATPP inhibits platelet aggregation prompted by ADP (Harrison & Brossmer, 1976). A possible role of exogenous ATPP in the regulation of blood pressure has recently been suggested (Kong et al, 1991).

Among various candidates, the activated platelet is the most obvious source of extracellular adenine nucleotides (Ugurbil & Holmsen, 1981; Pearson & Olsson, 1990). Although in a number of studies on platelets, adenine nucleotides, such as ATP, ADP and diadenosine polyphosphates, have been identified (Agarwall & Parks, 1975; Flodgaard & Klenow, 1982; Luthje & Ogilvie, 1983; Schlutter et al, 1994), the presence of ATPP has never been demonstrated. In the present study, however, we identified and quantified ATPP from rabbit platelets using HPLC technique. To study the biological significance of ATPP in circulation, thrombin-induced release of ATPP from platelets was examined. In addition, the time course of ATPP degradation was followed in three different conditions: whole blood, erythrocyte suspension and plasma.

MATERIALS AND METHODS

Isolation of platelets

Rabbit platelets were isolated at 4℃ by Flodgaard and Klenow's method (1982). Microscopic examination of the preparation revealed no contamination of erythrocytes and leukocytes. Total number of platelets was counted by Coulter counter (S-plus IV, Coulter).

Extraction of nucleotides

Washed platelets were mixed with 0.5 ml of 5% (v/v) ice-cold TCA. After 20 min at 4℃, the extract was neutralized and isolated as described by Flodgaard and Klenow (1982). Nucleotides in the extract were analyzed directly using HPLC.

Release of ATPP from platelets

ATPP release from platelet was examined as described by Luthje and Ogilvie (1983). Briefly, washed platelets(2×10^9 cells/ml) were suspended in Ca^{++}-free Tyrode buffer, and then incubated at 37℃ for 6 min with thrombin(2.2 units/ml of suspension). After rapid cooling, the mixture was centrifuged at $12,000\times g$ for 5 min at 4℃. In both the supernatant and the pellet, the amounts of released and remaining nucleotides were determined using HPLC. The nucleotides in the pellet were extracted before analysis, as described above.

Metabolism of ATPP in blood

Commercial ATPP (500 μM) was added to 20 ml of blood(Hct of 30%) which was then incubated for 2 hr at 37℃. During incubation, aliquots of blood were sampled at timed intervals, and centrifuged at $10,000\times g$ for 20 sec. The resulting plasma was mixed with 2 volumes of 5% TCA for enzyme inactivation. Following centrifugation for 2 min, the supernatant was neutralized as described above. The amount of ATPP remaining was determined using HPLC. The average rate of ATPP degradation and the half life were determined by regression analysis. The experiment was also carried out on erythrocyte suspension (Hct of 30%) and cell-free plasma.

Analysis of nucleotides

ATPP in the neutralized extract was identified and quantified using the reversed phase techniques of Schweinsberg and Loo (1980) with slight modifications. The extract (10 μl) was appliedonto a reversed-phase μ Bondapak C_{18} column (3.9×300 mm, Waters) from Water Assoc. The column was equilibrated by an isocratic elution of 0.1 mM potassium phosphate buffer (pH 5.0) for 20 min at 1 ml/min. For complete analysis of adenine nucleotide pools, a 30-min linear gradient from 0% to 100% was run at 1 ml/min by mixing 0.1 mM phosphate

buffer and the same buffer containing 25% methanol. The eluent was monitored at 254 nm, and concentration of nucleotides was determined by compairing the retention time and peak areas with those of known nucleotide standards, respectively.

Chemicals

All chemicals except AP_4A and AP_5A were purchased from Sigma(St. Louis, Mo., U.S.A.). The diadenosine polyphosphates were purchased from Amersham (Buckinghamshire, U.K.).

Statistical analysis

Results are presented as means ± SEM. Statistical significance was determined using Student's t-test for paired or unpaired experiments. $p<0.05$ was considered significant.

RESULTS

Identification of adenosine 5'-tetraphosphate (ATPP)

Fig. 1A shows a typical chromatogram of 12 nucleotide standards of which complete elution was allowed within a 30 min by using a linear gradient system. ATPP was eluted with a retention time of about 7.5 min. The chromatographic profile of neutralized extracts contained a peak with an identical retention time, indicating the presence of ATPP (Fig. 1B). Addition of exogenous ATPP to the extract further confirmed the ATPP presence in rabbit platelets (Fig. 1C). Contents of adenine nucleotides and nucleoside including ATPP in the platelet are given in Table 1. The amount of ATPP was 0.62 nmoles/10^9 cells. The ratio of ATPP to ATP and to ADP was 1/62 and 1/10, respectively. ATP was the most dominant nucleotide. The high ratio of ATP to ADP indicates a difference between rabbit and human platelets in which it is approximately 0.8 (Flodgaard & Klenow, 1982). A very small amount of adenosine (0.07 nmoles/10^9

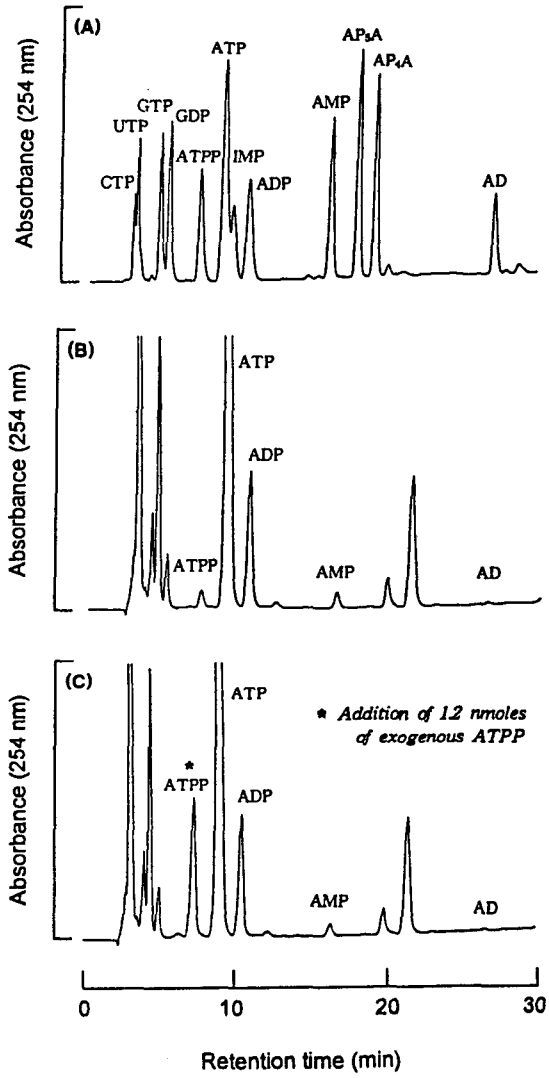

Fig. 1. Identification of adenosine 5'-tetraphosphate (ATPP). (A) Separation of 11 nucleotides and 1 nucleoside standards. The amount of each standard was 100 μM. (B) Typical chromatographic profile of the acid-soluble extract obtained from rabbit platelets. The putative ATPP was indicated as a peak with an average retention time of 7.5 min. (C) Verification of the putative ATPP by including authentic ATPP in the extract. The amount of the exogenous ATPP was 1.2 nmoles. Note the increased absorbance at same retention time as the putative ATPP peak. In B and C, two peaks were truncated due to high sensitivity of detector. Details of chromatographic conditions were described in MATERIALS and METHODS.

Table 1. Contents of adenine nucleotides and nucleoside in rabbit platelets

Adenine compounds	Concentration (nmoles/10^9 platelets)
ATPP	0.62±0.08
ATP	38.11±2.35
ADP	5.83±0.57
AMP	0.78±0.04
Adenosine	0.07±0.01

The acid-soluble extracts obtained from rabbit platelets were analyzed by HPLC and the content of each nucleotide was determined by measuring a peak area. Values represent mean±SEM of 9 experiments.

cells) indicates that degradation of nucleotides during extraction was very low. Diadenosine polyphosphate, AP_4A and AP_5A were included in the mixture of nucleotide standards, but they are not detected in the extract. An unidentified peak with retention time of 20 min in chromatograms (Fig. 1) was thought to be putative AP_3A, although there was no further investigation.

Release of adenine nucleotide from platelets

To examine if ATPP is localized in and can be released from dense storage granules in platelets, like other nucleotides (Ugubil and Holmsen, 1981), washed platelets were treated with thrombin. All adenine nucleotides except ATPP leaked out spontaneously during the incubation (Fig. 2). ATPP was released to a relatively high extent (0% for control and 27.6±3.6% for thrombin-treated platelets, n=6) as compared with other nucleotides (in the case of ADP, 4.3±0.9% for control and 15.8±2.4% for thrombin-treated platelets, n=6) during platelet aggregation.

Fate of adenosine 5'-tetraphosphate

The fate of ATPP released from platelets was followed after incubating the authentic ATPP in three different conditions: whole blood, erythrocyte

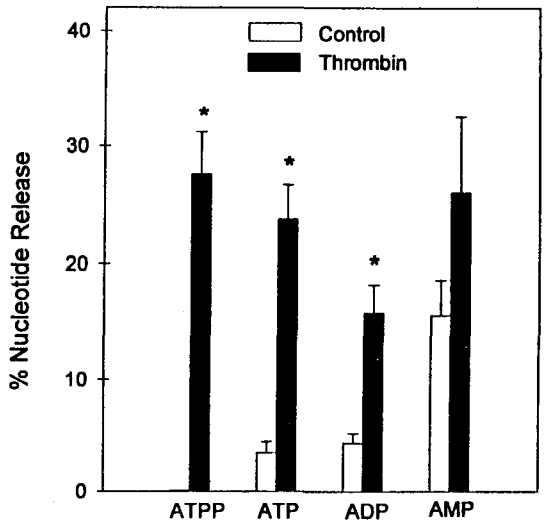

Fig. 2. Release of adenosine 5'-tetraphosphate and other adenine nucleotides from platelets by thrombin. Washed rabbit platelets(2×10^9 cells/ml) was suspended in Ca^{++}-free Tyrode buffer and incubated in the absence (control) and presence of thrombin(2.2 untis/ml) for 6 min at 37℃. Further procedures were described in MATERIALS and METHODS. Each column represents mean % release±SEM(n=6) of total amount of each nucleotide within platelets. Statistically significant release by thrombin was indicated in a comparison with control($p < 0.01$). Note no spontaneous release of ATPP in the control condition.

suspension and plasma. In whole blood, ATPP was degraded at a rate 4.16 nmoles/ml/min with the half-life of 114 min. Interestingly, throughout the experimental period, the level of ATP in the blood were kept low without any increment. Adenosine was not detected in metabolites, probably due to a rapid uptake by blood cells (Catravas, 1984). To identify the portion of the blood responsible for the metabolism of ATPP, the degradation rate and half-life of ATPP were determined in erythrocyte suspension and in plasma. In erythrocyte suspension ATPP was very slowly degraded (rate, 1.72 nmoles/ml/min; a half-life, 600 min). In contrast, ATPP in plasma was degraded with a relatively high rate of 5.16 nmoles/ml/min and a half-life of 141 min (Fig.

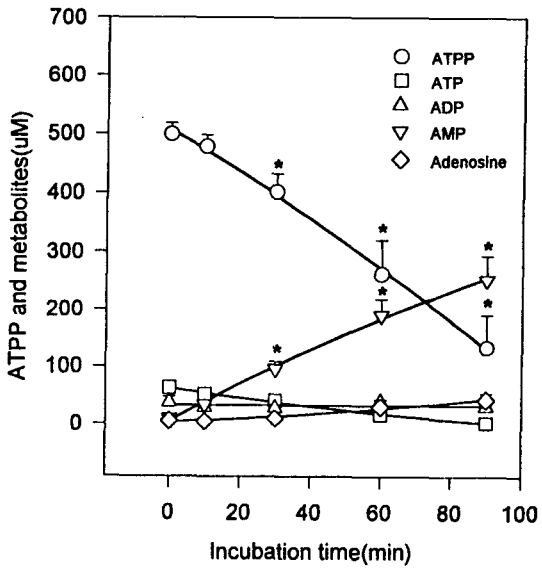

Fig. 3. Catabolism pattern of adenosine 5'-tetraphosphate by plasma. Exogenous ATPP(500 uM) was incubated in cell-free plasma for 90 min at 37℃. At timed intervals, aliquots of blood were sampled and centrifuged. The supernatants were treated with TCA and acid-soluble fractions were analyzed by HPLC. Values represent the changes in concentration of nucleotides including ATPP at a given time(n=6). Note that the concentration of AMP was only significantly ($p<0.01$) increased as ATPP was degraded.

3). In both erythrocyte suspension and plasma, the amount of ATP were not changed as in whole blood. The concentrations of AMP and ADP in whole blood were significantly increased with the rate 3.51 nmoles/ml/min and 2.28 nmoles/ml/min, respectively. However, the major metabolite in plasma appeared to be AMP, and its concentration increased symmetrically as that of ATPP decreased (4.88 nmoles/ml/min for AMP vs. 5.28 nmoles/ml/min for ATPP). In erythrocyte suspension, the concentrations of AMP and ADP were slightly increased.

These results indicate that ATPP is primarily degraded to AMP by soluble enzymes in plasma and in a small extent, to ADP and/or AMP by ectoenzymes on blood cells including erythrocytes. A higher rate of ATPP degradation in plasma than in whole blood may imply little contribution of blood cells in the degradation of ATPP.

DISCUSSION

The profile of adenine nucleotides in rabbit platelets observed in the present study was different from that in human platelets in at least two aspects: first, the molar ratio of ATP to ADP in rabbit platelets (6. 5) was much higher than that in human platelets (1.3, Luthje & Ogilvie 1983). Second, diadenosine polyphosphates such as AP_4A and AP_5A were not detected in rabbit platelets, in contrast to the report that human platelets contain AP_3A, AP_4A, AP_5A and AP_6A (Flodgaard & Klenow, 1982; Schluter et al, 1994). Although ATPP may be produced by the action of 5' nucleotidase on AP_5A (Schlutter et al, 1994), the ATPP in rabbit platelets is not likely to be a metabolic product of AP_5A, because in platelets adenine nucleotides, especially, diadenosine polyphosphates, are sequestered into a metabolically inactive state within the dense storage granules (Holmsen et al, 1969; Flodgaard & Klenow, 1982). The possibility of the spontaneous degradation of AP_5A to ATPP is also scanty, since AP_5A is resistant to even alkaline phosphatase and 3' nucleotidase (Schlutter et al, 1994) and there was no 5' nucleotidase in the experimental solutions.

A very small amount of adenosine in the extract indicates that degradation of nucleotides during extraction was negligible. It is, therefore, apparent that rabbit platelets contain ATPP in addition to other nucleotides.

The release experiment indicated that a relatively high amount of ATPP can be released into circulation during platelet aggregation. In the absence of a stimulation by thrombin, nucleotides other than ATPP were spontaneously extruded from platelets. It is unlikely that adenine nucleotides, such as ATP and ADP, having high negative charges get through the plasma membrane by simple diffusion (Cohn,

1990). One possibility of the spontaneous release comes from a partial platelet aggregation in a buffer without thrombin (Floolgaard & Klenow, 1982). If so, however, ATPP must also be released to some extent during partial aggregation. Holmsen et al (1969) have demonstrated that adenine nucleotides are present in two compartments within platelets. Thus, the lack of ATPP release in the present study may be explained by assuming a unique storage which can be released only by stimulations such as thrombin.

Although ATPP is hydrolyzed to ATP and inorganic phosphate by a non-specific enzyme, hydrolase from rabbit muscle (Small & Cooper, 1966b), the enzyme is not likely to be present in the blood. Furthermore, the present experiments revealed an increase in AMP and/or ADP but not in ATP at different sampling points. This pattern of degradation suggests that in blood ATPP is not degraded by a sequential dephosphorylation from ATPP→ATP→ADP→AMP→adenosine but is changed directly to AMP plus tripolyphosphate by soluble enzymes in plasma and very slowly to ADP plus pyrophosphate by ectoenzymes bound to the erythrocyte membrane. For the latter process, the erythrocyte must contain ectoenzymes specific for ATPP which are distinguishable from ATPase. However, the enzymes in plasma seem to be more important than those in the erythrocyte as judged by the rate of degradation. The leukocyte may not contribute to the catabolism of ATPP, although it has high activity of ecto-ATPase specific for ATP (Coade & Pearson, 1989) as evidenced by an equal degradation of ATPP in whole blood and plasma plus erythrocytes.

It is therefore apparent that, erthyrocytes are primarily responsible for the catabolism of ATPP, while leukocytes contribute to that of ATP (Coade & Pearson, 1989). The nature of the enzymes responsible for the ATPP degradation, specially in plasma, remains unknown. By comparing the half life, ATPP seems to be more resistant than ATP which has the half life of 30 min in plasma and 5 min in whole blood (Jorgensen, 1956; Trams et al 1980). This fact may suggest that ATPP is not readily removed from the blood. It has been suggested that vascular endothelial cells contribute to the catabolism of adenine nucleotides, which provides a mechanism for the regulation of circulating vasoactive purine level (Pearson & Gorden 1985; Coade & Pearson, 1989). Whether vascular endothelial cells are also involved in ATPP catabolism is yet to be determined.

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