Identification of Diadenosine Hexaphosphate in Human Erythrocytes

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Abstract—Diadenosine polyphosphates have been identified as important regulators of vascular tone and blood pressure. In reference to the background of the well-known vasoconstriction induced by hemolysate, we questioned whether this action may be due in part to the presence of diadenosine polyphosphates in human erythrocytes. Therefore, erythrocytes were separated from other blood cells and deproteinated. To concentrate and purify nucleotides, the extract was chromatographed by anion exchange, affinity, and reversed-phase columns. In one of the purified fractions, diadenosine hexaphosphate (diadenosine 5′, 5′-P₆, P₆ hexaphosphate [AP₆A]) was identified by matrix-assisted laser desorption/ionization mass spectrometry, ultraviolet spectroscopy, and enzymatic analysis. Hemolysate of erythrocytes injected into the isolated perfused rat kidney induced a dose-dependent vasoconstriction, which was partially inhibited by P₂₇-purinoceptor antagonist. The data document the existence of AP₆A in erythrocytes. AP₆A may be involved in the pathogenesis of vasospasm induced by free hemoglobin. (Hypertension. 1999;34[part 2]:872-875.)

Key Words: diadenosine polyphosphates ■ erythrocytes ■ vasoactivity

Diadenosine polyphosphates were isolated from platelets, adrenal gland, and heart.¹⁻⁴ The presence of diadenosine phosphates in granules releasable into the circulation suggests an important role in the control of local perfusion or blood pressure.¹⁻⁵,⁶ Vasoactive action of the diadenosine polyphosphates was demonstrated in numerous vascular models, including most of the physiologically important elements of blood pressure regulation. Diadenosine polyphosphates have different actions on the vasculature, depending on the number of phosphate groups. Diadenosine 5′, 5′-P₆, P₆ diphosphate (AP₂A), diadenosine 5′, 5′-P₆, P₆ triphosphate (AP₃A), and diadenosine 5′, 5′-P₆, P₆ tetraphosphate (AP₄A) are potent vasodilators in the mesenteric and coronary vasculature,³⁻⁵,⁶ whereas diadenosine 5′, 5′-P₆, P₆ pentaphosphate (AP₅A) and diadenosine 5′, 5′-P₆, P₆ hexaphosphate (AP₆A) appear to have vasoconstrictor properties.¹

The vasoconstrictive effect of hemolysate of dog erythrocytes on dog basilar artery in vitro is caused by ATP.⁷ Furthermore, a hypertensive factor that induced a prolonged elevation of blood pressure in normotensive rats has been reported in the erythrocytes of spontaneously hypertensive rats. It appeared that the substance occurred in normotensive as well as hypertensive rats, albeit at a lower concentration in normal animals.⁸ Diadenosine tetraphosphate was found to be synthesized within avian erythrocytes.⁹ Therefore, in the present study we examined whether diadenosine polyphosphates also exist in human erythrocytes.

Methods

Blood from normotensive subjects (men aged 26±4 years) was collected in tubes containing sodium heparin (1%), and the erythrocyte number was counted. The blood was centrifuged at 2000g for 10 minutes, and the plasma together with the buffy coat was discarded. The erythrocytes were washed twice with ice-cold saline. After centrifugation, 5 volumes of ice-cold high-performance liquid chromatography (HPLC)–grade water were added and placed in the refrigerator at 4°C for 20 minutes to hemolyze the erythrocytes. This hemolysate was centrifuged at 6000g for 10 minutes

Purification Procedures

The erythrocytes of ~160 mL blood from normotensive subjects were prepared as described above and hemolyzed by addition of 5 volumes of ice-cold HPLC-grade water containing 10 mmol/L EDTA; 5 volumes of ice-cold perchloric acid (final concentration, 0.6 mol/L) were then added and mixed. The solution was centrifuged at 6000g for 20 minutes at 4°C. The supernatant was neutralized with KOH to pH 8.5 and placed in a refrigerator for 20 minutes to precipitate KClO₄. After the supernatant was centrifuged at 6000g for 10 minutes at 4°C, it was titrated to pH 6.5 with HCl and centrifuged again at the same conditions as described above. After addition of 40 mmol/L triethylammonium acetate (TEAA) (final concentration), the supernatant was pumped through a preparative reversed-phase column (Lichroprep RP-18, 310×25 mm, Merck). The nucleotide-containing fraction was eluted with 40% acetonitrile (in water) at a flow rate of 1 mL/min. The 40% acetonitrile eluate was collected, frozen in the refrigerator (~0°C), and lyophilized. The lyophilizate was further separated by an anion exchange column (Fracogel EMD DEAE-650, 300×25 mm, Merck). The column was equilibrated with 10 mmol/L NH₄Ac (pH 7.4), then the lyophilizate was dissolved in 200 mL of 10 mmol/L NH₄Ac and pumped through the column. The sample was eluted with 1 mol/L NH₄Ac (pH 7.4) at a flow rate of 3.0 mL/min. The effluent was detected with a UV detector at 254 nm.

The eluate from the anion exchange column containing a final concentration of 1 mol/L NH₄Ac (pH 9.5) was passed through an affinity column (150×20 mm) and equilibrated with 1 mol/L NH₄Ac...
(pH 9.5). The affinity column was prepared from a cation exchange resin (Bio-Rex 70, Bio-Rad) by binding phenyl boronic acid to the gel. Binding substances were eluted with 1 mmol/L HCl and monitored with a UV detector at 254 nm. The eluate was frozen and lyophilized for the next step.

Fractions from affinity chromatography were desalted by reversed-phase HPLC (Superspher 100 RP-18 end capped, 250×4 mm, Merck). The fractions dissolved in 5 mL TEAA (40 mmol/L) were injected into the HPLC. After a washing period of 10 minutes with 40 mmol/L TEAA, the nucleotide-containing fraction was eluted with 30% acetonitrile in water. The absorbing fraction was collected.

An anion exchange column (Mono Q HR 5/5, 50×5 mm, Pharmacia) was used for the next chromatographic step. The column was equilibrated with eluent A (10 mmol/L K2HPO4, pH 8.0). The desalted sample dissolved in eluent A. The flow rate of the mobile phase was 0.5 mL/min. Binding substances were eluted by a linear gradient (1 to 10 minutes 0% to 5% B, 10 to 100 minutes 5% to 35% B, 100 to 105 minutes 35% to 40% B, and 105 to 110 minutes 40% to 100% B) of eluent B (50 mmol/L K2HPO4, 1 mol/L NaCl, pH 8.0). The wavelength of the UV detector was fixed at 254 nm.

The fractions from the anion exchange chromatography were further separated by reversed-phase HPLC (Superspher 100 RP-18 end capped, 250×4 mm, Merck). The fractions with a final concentration of 40 mmol/L TEAA (eluent A) were injected into the column. The flow rate was 0.5 mL/min, and the following gradient of eluent B (100% acetonitrile, 0 to 4 minutes 0% to 2% B, 4 to 50 minutes 2% to 7% B, 50 to 56 minutes 7% to 60% B) was used to elute the binding sample. The wavelength of the UV detector was 254 nm.


The molecular masses of the molecules in the fractions from reversed-phase HPLC were determined by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS). A reflectron-type time-of-flight (RETOF) mass spectrometer (Reflex III, Bruker), equipped with nitrogen laser (337 nm; pulse length, 3 ns) was used for ion generation and mass analysis. In MALDI-MS, large fractions of the desorbed analyte ions undergo postsource decay (PSD) during flight in the field free drift path. With the use of a RETOF setup, sequence information from PSD fragment ions of precursors produced by MALDI was obtained. For MALDI-MS and PSD MALDIMS, a speed-vacuum-dried sample was dissolved in 10 μL water. Then 1.0 μL of the 3-hydroxy–picolinic acid matrix solution (50 g/L) in water was mixed with 0.5 μL of the sample on a flat metallic support and dried in a stream of cold air. Desorption of analyte ions was performed by laser shots of irradiances in the range of 10 to 107 W/cm2 focused to spot sizes of typically 50 to 100 μm in diameter. The ions were generated with an energy of 28.5 keV for detection. The spectra were registered by a recorder.

UV Spectroscopy

The substances in the fractions of reversed-phase HPLC were analyzed by a UV spectrometer (UV/Vis-Spectrometer, FA JASCO V-530). The UV absorption was scanned from 400 to 190 nm with a scan speed of 400 nm/min.

Enzymatic Cleavage Experiments

Dried fractions of the reversed-phase HPLC were dissolved in 10 μL water. A 1.0-μL sample was mixed with (1) 20 μL 200 mmol/L Tris buffer (pH 8.9) and 3 μU 5'-nucleotidase (EC 3.1.15.1, from Crotaulus durissus, Boehringer Mannheim) and incubated for 9 minutes at 37°C; (2) 20 μL 200 mmol/L Tris, 20 mmol/L EDTA buffer (pH 7.4), and 1 μU 3'-nucleotidase (EC 3.1.16.1, from calf spleen, Boehringer Mannheim) and incubated for 60 minutes at 37°C; and (3) 20 μL 10 mmol/L Tris, 1 mmol/L ZnCl2, 1 mmol/L MgCl2 buffer (pH 8), and 1 μU alkaline phosphatase (EC 3.1.3.1 from calf intestinal mucosa, Boehringer Mannheim) and incubated for 60 minutes at 37°C. After the enzyme was removed with a centrifuge filter (5-kDa cutoff), the enzymatic cleavage products were chromatographed on an anion exchange column (Mono-Q HR 5/2, 50×2 mm, Pharmacia). The enzymatic cleavage products dissolved in eluent A (10 mmol/L K2HPO4, pH 8.0) were injected to the column at a flow rate of 0.3 mL/min. Binding substances were eluted by a linear gradient of eluent B (50 mmol/L K2HPO4, 1 mol/L NaCl, pH 8.0, 1 to 2 minutes 0% to 5% B, 2 to 22 minutes 5% to 40% B, and 22 to 22.5 minutes 100% B). The wavelength of the UV detector was fixed at 254 nm.

Isolated Perfused Rat Kidney

Isolated perfused rat kidney was prepared according to Hofbauer et al. Briefly, adult male Wistar-Kyoto rats (weight, 300 to 400 g) were anesthetized, and the kidney was isolated. The kidney was perfused at a constant flow of 8 mL with Tyrode's solution of the following composition (mmol/L): NaCl 137, KCl 2.7, CaCl2 1.8, MgCl2 1.1, NaHCO3 12, NaH2PO4 0.42, d-glucose 5.6 equilibrated with 5% CO2/95% O2 and maintained at 37°C. Perfusion pressure was monitored with a pressure transducer (Statham Transducer P23GB, Siemens) connected to a bridge amplifier (Hugo Sachs) and recorded on a pen writer. Preparations were allowed to equilibrate for 30 minutes before experimentation. The erythrocyte hemolysates were injected in boluses of 100 μL each.

Results

To investigate the existence of diadenosine polyphosphates, human erythrocytes were hemolized and deproteinated. The concentrate was purified by anion exchange and affinity chromatography. The desalted eluate of the affinity chromatography was fractionated by anion exchange chromatography with a linear gradient. The chromatogram is shown in Figure 1A. Every fraction with a significant UV absorption at 254 nm was chromatographed by reversed-phase HPLC. A typical chromatogram is presented in Figure 1B. All fractions with UV absorption at 254 nm from reversed-phase HPLC were analyzed by MALDI-MS. In the fraction labeled AP6A in Figure 1B, the molecular mass of 996.6 Da ([M+H]+ = 997.6 Da) was measured (Figure 2A). At pH 7.0, the UV spectrum of this fraction has maximum and minimum absorbances at 259 and 230 nm, respectively, which is similar to that of adenosine. Figure 2B represents the PSD MALDI mass spectrum from the fraction of the reversed-phase HPLC.
labeled AP₆A. In the Table, the masses obtained by PSD MALDI-MS of the molecule are assigned to the respective fragment ions. The observed fragments are almost the same as that of commercially available AP₆A. The connection of phosphate groups to adenosine in the molecule underlying the fraction of the reversed-phase HPLC labeled AP₆A was evaluated by enzymatic analysis. We observed that 5'-nucleotidase yielded AMP plus adenosine 5'-pentaphosphate (Figure 3C), whereas 3'-nucleotidase (Figure 3B) and alkaline phosphatase (Figure 3A) had no effect on the molecule. These results of enzymatic cleavage suggest that in the molecule the adenosines are bridged by 2 phosphates via 5'-phospho-ester bonds, and there is no terminal phosphate group.

Human erythrocytes were hemolyzed, and the effect on isolated perfused rat kidney was tested. In the isolated rat kidney, the human erythrocyte hemolysates caused a dose-dependent vasoconstrictive effect, which was partially inhibited by the P₂-purinoceptor antagonist suramin (Figure 4).

**Discussion**

The findings document that AP₆A, which is released from human platelets during platelet aggregation, also exists in human erythrocytes. The physiological actions of AP₆A have been demonstrated in earlier reports. Briefly, AP₆A increases systemic blood pressure, contracts aortic rings and renal vasculature, and elevates cytosolic free Ca²⁺ concentration in rat aorta and in vascular smooth muscle cells. Furthermore, AP₆A directly stimulates the proliferation of cultured mesangial cells and augments mesangial cell growth induced by other mitogens released from platelets. These observations demonstrate that AP₆A may play a role in the pathogenesis and maintenance of hypertension.

Because hemolysate is known to induce vasospasm in a variety of experimental and pathological conditions, these results suggest that not only ATP but also AP₆A may contribute to the vasoconstriction that occurs in vasospasm. Furthermore, AP₆A elicits an allosteric activation of Ca²⁺-ATPase and increases its activity. The activity of Ca²⁺-ATPase, which is the only calcium transporting system found in the human erythrocyte membrane, is increased in essential hypertension. This is compatible with a higher concentration of AP₆A, at least in erythrocytes of essential hypertension.

In human erythrocytes, diadenosine polyphosphates bind preferentially with high affinity to deoxyhemoglobin. The affinity increases with the number of phosphates of the diadenosine polyphosphates. Binding of diadenosine

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**Figure 2.** MALDI mass spectrum (A) and PSD MALDI mass spectrum (B) of the fraction labeled AP₆A from reversed-phase HPLC in Figure 1B.

**Figure 3.** Anion exchange chromatograms of the fraction labeled AP₆A from reversed-phase HPLC in Figure 1B after incubation with alkaline phosphatase (A), 3'-nucleotidase (B), and 5'-nucleotidase (C). AP₅ indicates adenosine 5'-pentaphosphate.

**Figure 4.** Changes in perfusion pressure (ΔP) in the rat isolated perfused kidney induced by erythrocyte hemolysate in the absence (●) and presence (▲) of 100 μmol/L suramin. Each point is the mean of 5 determinations (mean±SEM values, n=5). *P<0.05.
polyphosphates stabilizes the low-oxygen affinity conformation of hemoglobin. This aspect may be interesting according to the development of hypertension, because it was reported that oxyhemoglobin increased the tension of aortic rings of spontaneously hypertensive rats, whereas the effect on normotensive rats was negligible.

AP₆A was reported to be synthesized in chicken erythrocytes. The synthesis and distribution of AP₆A in erythrocytes are still unknown. To our knowledge, no in vivo synthesis of diadenosine polyphosphates larger than AP₄A has been described. The concentration of AP₆A in erythrocytes is difficult to estimate. One reason is that AP₆A binds to hemoglobin with an affinity that is 10-fold higher than the affinity of 2,3-diphosphoglycerate to hemoglobin. During the deproteination step, AP₆A bound to hemoglobin may be precipitated, resulting in a low recovery of AP₆A.

In summary, AP₆A was identified in human erythrocytes, where it may have a direct effect on vascular tone after being released by hemolysis. How the Ca²⁺-ATPase–stimulating effect and the oxygen affinity–lowering effect of AP₆A to hemoglobin may be involved in the regulation of vascular tone should be clarified in future studies.

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References

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