Endogenous Diadenosine Tetrrophosphate, Diadenosine Pentaphosphate, and Diadenosine Hexaphosphate in Human Myocardial Tissue

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Abstract—Diadenosine polyphosphates have been characterized as extracellular mediators controlling numerous physiological effects. In this study, diadenosine tetrrophosphate, diadenosine pentaphosphate, and diadenosine hexaphosphate were isolated and identified in human myocardial tissue. Human myocardial tissue was homogenized and fractionated by affinity chromatography, displacement chromatography, anion-exchange chromatography, and reversed-phase chromatography. In fractions purified to homogeneity, diadenosine polyphosphates were revealed by matrix-assisted laser desorption/ionization mass spectrometry and ultraviolet spectroscopy. These diadenosine polyphosphates were further identified by enzymatic analysis, which demonstrated an interconnection of the phosphate groups with the adenosines in the 5′ positions of the riboses. Furthermore, diadenosine tetrrophosphate, diadenosine pentaphosphate, and diadenosine hexaphosphate were found in human cardiac-specific granules, and the amount of diadenosine tetrrophosphate, diadenosine pentaphosphate, and diadenosine hexaphosphate was estimated in the range of \( \approx 500 \mu \text{mol/L} \). In conclusion, the experiments show that the diadenosine polyphosphates with 2 and 3 phosphate groups occur in human myocardial tissue, and so do the diadenosine polyphosphates with 4 to 6 phosphate groups. After being released by cholinergic stimulation, which is known to affect diadenosine polyphosphate release from secretory granules, diadenosine tetrrophosphate, diadenosine pentaphosphate, and diadenosine hexaphosphate activate P2X purinoceptors in vascular smooth muscle; hence, they can act as vasoconstrictors. It may be inferred that the differential action of both predominantly vasodilator and vasoconstrictor diadenosine polyphosphates allow a fine-tuning of myocardial blood flow by locally released diadenosine polyphosphates. (\textit{Hypertension.} 2004;43:1055-1059.)

Key Words: myocardium • cardiac function

Diadenosine polyphosphates (Ap_n A) have attracted growing interest in the past decade with respect to their roles in cardiovascular physiology and pathology.\(^1\)\(^-\)\(^5\) The actions of the Ap_n A within the cardiovascular system are mediated by the various purinoceptor subtypes. So far, 14 mammalian purinoceptor subtypes have been cloned,\(^6\),\(^7\) and 6 Ap_n As containing 2 to 7 phosphate groups have been identified in humans.\(^8\)\(^-\)\(^11\) The affinities of a given Ap_n A to the various purinoceptor subtypes depends on the number of phosphate groups linking both adenosine moieties.\(^9,12,13\) Moreover, the purinoceptor subtypes are very differently distributed within the cardiovascular system. Depending on the purinoceptor subtypes activated in a given tissue, the Ap_n A are both vasoconstrictors and vasodilators,\(^14,15\) inhibitors and stimulators of platelet aggregation,\(^8,9,11\) and modulators of cell proliferation.\(^9,11,16\) Given this diversity of Ap_n A actions, it is not surprising that the Ap_n A actions reported in literature widely differ among various species. Currently, it is difficult to decide to what extent species-dependent differences in Ap_n A actions are caused by different purinoceptor distribution and to species-specificity of some of the known purinoceptor subtypes.

Obviously, local concentrations of Ap_n A are important determinants of Ap_n A-mediated effects.\(^8,9\) Hence, the local production of Ap_n A in the cardiovascular system has been studied recently. In earlier experiments, both Ap_2 A and Ap_3 A have been identified in human myocardial tissue.\(^17\) Both Ap_2 A and Ap_3 A have been identified as vasodilators,\(^14\) whereas Ap_4 A, Ap_5 A, and Ap_6 A also have vasoconstricting properties,\(^10,14\) which increase with increasing number of phosphate groups.\(^15\) Therefore, it was of interest whether Ap_n A with \( \geq 3 \) phosphate groups also occur in human myocardial tissue.
myocardium. Given powerful local effects of these Ap₅A, because of high local concentrations, the presence of vasoconstrictive Ap₅A in human myocardium might have important consequences with respect to pathologic conditions such as myocardial infarction or excess sympathetic nerve stimulation, which is known to stimulate Ap₅A release from chromaffin granules. Therefore, in the present study, we examined whether Ap₅A are also present in human myocardium, which activate P2X purinoceptors in low concentrations and hence are powerful vasoconstrictors, ie, Ap₅A containing 4 to 6 phosphate groups. From earlier findings in the literature, this hypothesis appeared likely, because in chromaffin granules isolated from various tissues, several or all known types of Ap₅A occur.¹⁸–²⁰ The experiments revealed that the Ap₅A acting predominantly as vasoconstrictors are also detectable in human myocardium.

**Methods**

**Purification Procedures**

Diadenosine polyphosphates were extracted from human myocardial tissue as described previously.¹⁷ The extract was concentrated by preparative reversed-phase chromatography (Lichroprep RP-18; 310×25 mm; Merck, Germany). The eluate of the preparative reversed-phase chromatography was fractionated by size-exclusion gel, preparative anion-exchange chromatography, preparative affinity chromatography, and reversed-phase chromatography. The chromatographic conditions were identical to the methods described previously;¹⁷ these conditions are also available in an online supplement at www.hyper.ahajournals.org. Next, the desalted and lyophilized eluate of the affinity chromatography was dissolved in 40 mmol/L TEAA solution and was chromatographed by 2 connected reversed-phase columns (Supersphere 100 RP-18 end-capped; 300X8 mm; Merck) in the displacement mode.²¹ These reversed-phase columns were equilibrated with 40 mmol/L TEAA. The carrier (40 mmol/L TEAA) was pumped through the system at a flow rate of 100 μL/min during injection of the sample. After the injection, n-butanol (100 mmol/L in 40 mmol/L TEAA) was used as displacer (flow rate 100 μL/min). Each fraction (1 mL) was lyophilized and was further fractionated by anion-exchange chromatography.

The eluate of the displacement chromatography was fractionated by analytical anion-exchange chromatography (TSK DEAE-5PW; 75×7.5 mm; Tosoh, Japan) and desalted by analytical reversed-phase chromatography (Supersphere 100 RP-18 end-capped; 100X2.1 mm, 4 μm; Merck).²⁷ The lyophilized eluate of analytical reversed-phase chromatography was analyzed by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry and postsource decay MALDI mass spectrometry using the conditions as described in Jankowski et al.²² UV spectroscopy,²⁷ and enzymatic cleavage experiments.²³

**Identification of Ap₅A From Human Cardiac-Specific Granules**

Diadenosine polyphosphates were isolated from specific granules of human left ventricular tissue according to the method of Lao et al.¹⁷

**Results**

The extracts of human myocardial tissues were fractionated by size exclusion chromatography, anion-exchange chromatography, affinity chromatography, displacement chromatography, and analytic anion-exchange chromatography. Each fraction showing a significant UV absorption at 254 nm in the analytic anion-exchange chromatography was further chromatographed by reversed-phase chromatography. Each fraction with a significant absorbance at 254 nm of the reversed-phase high-performance liquid chromatography (HPLC) was further analyzed by mass spectrometric methods, UV spectroscopy, and enzymatic analysis.

Figure 1 shows a typical chromatogram of reversed-phase HPLC. The purified substances underlying the fractions labeled by arrows in the Figure were analyzed. By MALDI mass spectrometry, the molecular masses of these fractions (Figure 1) were revealed as 837.2 Da, 917.8 Da, and 997.4 Da [M+H]⁺, which correspond to Ap₄A, Ap₃A, and Ap₅A. The UV spectra of these substances were characteristic of adenosine, with a maximum at 259 nm and minimum at 230 nm (data not shown). Moreover, the fragmentation patterns of the isolated substances were analyzed by postsource decay (PSD)-MALDI mass spectrometry.²²,²⁴

The results show that the analyzed substances contained phosphate groups, adenosine, AMP, ADP, ATP, Ap₃A, or Ap₅A (Table). The fragment patterns of the analyzed fractions were identical to those of authentic Ap₄A, Ap₃A, and Ap₅A (Table). Furthermore, the interconnection of phosphate groups to the adenosines was analyzed by enzymatic analysis using alkaline phosphatase, 3'-nucleotidase, and 5'-nucleotidase hydrodase. Alkaline phosphatase and 3'-nucleotidase had no effect on these molecules (data not shown). The 5'-nucleotidase yielded AMP and ATP (Figure 2A) after incubation of the fraction labeled in Figure 1A, AMP and Ap₅A (Figure 2B) after incubation of the fraction labeled in Figure 1B, and AMP and Ap₃A (Figure 2C) after incubation of the fraction labeled in Figure 1C. The retention time in cleavage patterns is in accord with those of authentic Ap₄A, Ap₃A, and...
Ap₆A. These results demonstrate that all adenosines in the purified molecules are interconnected via 5'-phosphodiester bonds of the riboses with the phosphates. In summary, by MALDI mass spectrometry, UV absorption analysis, PSD-MALDI mass spectrometry, and enzymatic analysis, the substances underlying the UV absorption peaks in the reversed-phase HPLC shown in Figure 1 were identified as Ap₄A, Ap₅A, and Ap₆A.

To examine if Ap₄A, Ap₅A, and Ap₆A are stored in granules, human ventricular-specific granules were isolated from myocardial tissue and the nucleotides were purified to homogeneity. Fractions from the reversed-phase HPLC were analyzed by MALDI mass spectrometry. Fractions with molecular masses of 837.8 Da, 917.3 Da, and 996.9 Da were analyzed by PSD-MALDI mass spectrometry. The fragmentation patterns were identical to authentic Ap₄A, Ap₅A, and Ap₆A. Based on the UV absorption at 254 nm, and using Ap₈A as internal standard and calibration curves of Ap₄A, Ap₅A, and Ap₆A, the amount of Ap₄A, Ap₅A, and Ap₆A in human myocardial-specific granules were estimated at 500 μmol/L.

Discussion

Whereas in earlier studies only Ap₂A and Ap₃A had been isolated from human myocardium, the present study shows that Ap₄A containing 4 to 6 phosphate moieties are also found in human myocardial tissue. Moreover, Ap₄A, Ap₅A, and Ap₆A were identified in myocardial-specific granules, which are known to release their contents into the extracellular space after stimulation, eg, by cholinergic agonists. Most likely, methodological reasons may be responsible. The purification procedure in this study has been considerably improved with respect to the recovery of ApₙA compared with the previous study. In this study, but not in the previous one, we used displacement chromatography before the analytic anion-exchange chromatography and reversed-phase HPLC. This procedure may be more effective to separate ApₙA from myocardial tissue, because displacement chromatography has been shown to be a powerful method for separation of dinucleotide polyphosphates.

From the presence of at least 5 different ApₙAs in human myocardial tissue, it can be inferred that ApₙA may have specific functions in human heart. What is the significance of these findings for cardiac physiology and pathology? To
The concentration of ApA in coronary venous blood is increased during ischemia and reperfusion of heart, whereas it cannot be detected with normal oxygen supply. This increase of ApA during ischemia is probably caused by the release of ApA stored in myocardial-specific granules and activated platelets in blood. ApA reduces cardiac infarct size and reperfusion injury in the ischemic canine heart. Furthermore, ApA mimics cardioprotective effect of ischemic preconditioning in the rat heart and significantly improves the postischemic recovery of cardiac function, reducing the leakage of serum creatine kinase. ApA has cardioprotective effects on hypothermic heart storage and cardiopreservation.

Some of these effects of ApA appear to be mediated by activating protein kinase C and mitochondrial K<sub>ATP</sub> channels via P2 years mimicking, in part, the effects of ischemic preconditioning. Moreover, ApA is an intracellular regulator ligand of the sarcomemmal K<sub>ATP</sub> channel. Therefore, the intracellular ApA may directly interact with the mitochondrial K<sub>ATP</sub> channel or is released to extracellular spaces and interacts with adjacent cells.

ApA was shown to bind to the nucleotide-binding domain of the myocardial K<sub>ATP</sub> channel, thus decreasing channel opening probability. The ischemia-induced decreased ApA levels may thus contribute to open the K<sub>ATP</sub> channel under ischemic conditions. The K<sub>ATP</sub> channel is known to be a sensor of metabolic stress, especially of cellular hypoxia: With decreasing ATP concentrations, the opening probability of this channel increases. This may be an important mechanism for cells undergoing hypoxia to maintain membrane potential despite decreased function of transmembrane ion pumps such as the energy-dependent Na-K-ATPase.

The regulation of K<sub>ATP</sub> channels appears to be only a small segment of the potential regulatory functions of myocardial ApA. It may be assumed that ApA may have further effects on myocardial ion channels and purinoceptors, and so may the other Ap<sub>n</sub>A s show regulatory effects, although differing according to the number of phosphate moieties. Taken together with the present findings, it may be inferred that the ApA represent a new class of messengers in human myocardial cells, exhibiting cellular protective functions in metabolic or ischemic stress. Also, there are other still unknown effects that are dependent on their binding to purinoceptors and ion channels.

**Perspectives**

Given that the research on myocardial ApA is at its very beginning, it is likely that changes in the patterns of ApA levels associated with different metabolic or vascular disorders may have further still unknown effects in the pathophysiology of heart disease.

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**References**


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