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

Jiankai Luo, Vera Jankowski, Nihayrt Güngör, Joachim Neumann, Wilhelm Schmitz, Walter Zidek, Hartmut Schlüter, Joachim Jankowski

Abstract—Diadenosine polyphosphates have been characterized as extracellular mediators controlling numerous physiological effects. In this study, diadenosine tetraphosphate, 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 tetraphosphate, diadenosine pentaphosphate, and diadenosine hexaphosphate 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 tetraphosphate, diadenosine pentaphosphate, and diadenosine hexaphosphate were found in human cardiac-specific granules, and the amount of diadenosine tetraphosphate, diadenosine pentaphosphate, and diadenosine hexaphosphate were found in human cardiac-specific granules, and the amount of diadenosine tetraphosphate, diadenosine pentaphosphate, and diadenosine hexaphosphate were estimated in the range of 500 μmol/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 tetraphosphate, 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. (Hypertension. 2004;43:1055-1059.)

Key Words: myocardium ▪ cardiac function

Diadenosine polyphosphates (ApnA) have attracted growing interest in the past decade with respect to their roles in cardiovascular physiology and pathology.1–5 The actions of the ApnA within the cardiovascular system are mediated by the various purinoceptor subtypes. So far, 14 mammalian purinoceptor subtypes have been cloned,6,7 and 6 ApnAs containing 2 to 7 phosphate groups have been identified in humans.8–11 The affinities of a given ApnA 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 ApnA 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 ApnA actions, it is not surprising that the ApnA actions reported in literature widely differ among various species. Currently, it is difficult to decide to what extent species-dependent differences in ApnA actions are caused by different purinoceptor distribution and to species-specificity of some of the known purinoceptor subtypes.

Obviously, local concentrations of ApnA are important determinants of ApnA-mediated effects.8,9 Hence, the local production of ApnA in the cardiovascular system has been studied recently. In earlier experiments, both Ap2A and Ap3A have been identified in human myocardial tissue.17 Both Ap2A and Ap3A have been characterized as vasodilators,14 whereas Ap4A, Ap5A, and Ap6A also have vasoconstricting properties,10,14 which increase with increasing number of phosphate groups.15 Therefore, it was of interest whether ApnA with >3 phosphate groups also occur in human...
myocardium. Given powerful local effects of these ApnA, because of high local concentrations, the presence of vasoconstrictive ApnA 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 ApnA release from chromaffin granules. Therefore, in the present study, we examined whether ApnA are also present in human myocardium, which activate P2X purinoceptors in low concentrations and hence are powerful vasoconstrictors, ie, ApnA 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 ApnA occur.18–20 The experiments revealed that the ApnA 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; 300 × 8 mm; Merck, Germany). The eluate of the preparative reversed-phase chromatography was fractionated by size-exclusion chromatography, anion-exchange chromatography, and reversed-phase chromatography. The chromatographic conditions were identical to those of authentic ApnA, Ap5A, and Ap6A. The chromatograms showing a significant UV absorption at 254 nm in the chromatographed by reversed-phase chromatography. Each fraction containing 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, Ap5A, and Ap6A. 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, Ap5, or Ap6 (Table). The fragment patterns of the analyzed fractions were identical to those of authentic Ap4A, Ap5A, and Ap6A (Table). Furthermore, the interconnection of phosphate groups to the adenosines was analyzed by enzymatic analysis using alkaline phosphatase, 3’-nucleotide, and 5’-nucleotide hydrolase. Alkaline phosphatase and 3’-nucleotide, and 5’-nucleotide phosphatase yielded AMP and ATP (Figure 2A) after incubation of the fraction labeled in Figure 1A. The 5’-nucleotidase yielded AMP and ATP (Figure 2B) after incubation of the fraction labeled in Figure 1B, and AMP and Ap5 (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, Ap5A, and

Identification of ApnA 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 was analyzed by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry and postsource decay 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, Ap5A, and Ap6A. 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, Ap5, or Ap6 (Table). The fragment patterns of the analyzed fractions were identical to those of authentic Ap4A, Ap5A, and Ap6A (Table). Furthermore, the interconnection of phosphate groups to the adenosines was analyzed by enzymatic analysis using alkaline phosphatase, 3’-nucleotide, and 5’-nucleotide hydrolase. 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 Ap5 (Figure 2B) after incubation of the fraction labeled in Figure 1B, and AMP and Ap6 (Figure 2C) after incubation of the fraction labeled in Figure 1C.
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. The concentrations of Ap₄A, Ap₅A, and Ap₆A (see Results) in specific granules are not very different from those of Ap₂A and Ap₃A found earlier. Why had Ap₄A, Ap₅A, and Ap₆A not been identified in our previous study? 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?
answer this question, the cardiovascular effects of Ap4A have to be considered. Although presently our knowledge of the role of these molecules is still quite incomplete, animal experiments have given some hints as to potential physiological functions of these molecules. In the coronary vasculature of isolated hearts, Ap4A, Ap5A, and Ap6A induce species-dependent and dose-dependent vasodilation, which are mediated by release of either nitric oxide (NO) or prostacyclin (PGL). These vasodilations are partially prevented or converted to a vasoconstriction after inhibition of NO or PGI2 production.27–29 Regarding electrophysiological effects on isolated hearts, Ap4A, Ap5A, and Ap6A increase action potential duration and refractory period, both effects being mediated by release of NO and PGI2.28 In guinea pig left atria, Ap4A, Ap5A, and Ap6A inhibited the positive inotropic response elicited by electrical stimulation.30,31 After beta-adrenergic stimulation, Ap4A and Ap6A exert negative chronotropic and inotropic effects in animals and human ventricular preparations. In contrast, Ap4A alone can exert positive inotropic effects.31,32 In physiologically relevant concentrations, Ap4A, Ap5A, and Ap6A may serve as endogenous modulators of ryanodine receptor-gated Ca2+ release channels in membranes prepared from rat cardiac muscle.33 Furthermore, Ap4A, Ap5A, and Ap6A are potent inhibitors of myocardial KATP channels.34–37

The concentration of Ap4A in coronary venous blood is increased during ischemia and reperfusion of heart, whereas it cannot be detected with normal oxygen supply.35 This increase of Ap4A during ischemia is probably caused by the release of Ap4A stored in myocardial-specific granules and activated platelets in blood.36 Ap4A reduces cardiac infarct size and reperfusion injury in the ischemic canine heart. Furthermore, Ap4A 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.37 Ap4A has cardioprotective effects on hypothermic heart storage and cardioplegia.10,41 Some of these effects of Ap4A appear to be mediated by activating protein kinase C and mitochondrial KATP channels via P2 years mimicking, in part, the effects of ischemic preconditioning.42 Moreover, Ap4A is an intracellular regulator ligand of the sarcosomal KATP channel.43 Therefore, the intracellular Ap4A may directly interact with the mitochondrial KATP channel or is released to extracellular spaces and interacts with adjacent cells.

Ap4A was shown to bind to the nucleotide-binding domain of the myocardial KATP channel,43 thus decreasing channel opening probability. The ischemia-induced decreased Ap4A levels may thus contribute to open the KATP channel under ischemic conditions. The KATP 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 KATP channels appears to be only a small segment of the potential regulatory functions of myocardial Ap4A. It may be assumed that Ap4A may have further effects on myocardial ion channels and purinoceptors, and so may the other ApnAs show regulatory effects, although differing according to the number of phosphate moieties. Taken together with the present findings, it may be inferred that the Ap4A 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 Ap4A is at its very beginning, it is likely that changes in the patterns of ApnA levels associated with different metabolic or vascular disorders may have further still unknown effects in the pathophysiology of heart disease.

Acknowledgments

This work was supported by a grant of the DFG (Schl 406/1–2).

References

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

Jiankai Luo, Vera Jankowski, Nihayrt Güngär, Joachim Neumann, Wilhelm Schmitz, Walter Zidek, Hartmut Schlüter and Joachim Jankowski

Hypertension. 2004;43:1055-1059; originally published online April 5, 2004;
doi: 10.1161/01.hyp.0000126110.46402.dd

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2004 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://hyper.ahajournals.org/content/43/5/1055

Data Supplement (unedited) at:
http://hyper.ahajournals.org/content/suppl/2004/04/30/43.5.1055.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/