Identification and Characterization of Adenosine 5'-Tetraphosphate in Human Myocardial Tissue

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Running Title:
Westhoff: AP₄ in human myocardial tissue
Summary

Endocrine functions of the human heart have extensively been studied. Only recently, nucleotidergic mechanisms have been studied in detail. Therefore, an isolation strategy was developed to isolate novel nucleotide compounds from human myocardium. The human myocardial tissue was fractionated by several chromatographies. A substance purified to homogeneity was identified as adenosine 5′-tetraphosphate (Ap₄) by matrix assisted laser desorption/ionization mass spectrometry (MALDI-MS), post-source-decay MALDI-MS (PSD-MALDI-MS) and enzymatic cleavage analysis. Furthermore, Ap₄ was also identified in ventricular specific granules. In the isolated perfused rat heart, Ap₄ elicited dose-dependent vasodilations. Vasodilator responses were abolished in the presence of the P₂Y₁-receptor antagonist, MRS 2179 (1 µM) or the NO-synthase inhibitor, N⁵-nitro-L-arginine methyl ester (L-NAME, 50 µM). After removal of the endothelium by Triton X-100, Ap₄ induced dose-dependent vasoconstrictions. Inhibition of P₂X-receptors by pyridoxal-phosphate-6-azophenyl-2;4-disulfonic acid (PPADS, 30 µM) or desensitization of P₂X-receptors by α,β-methylene ATP (α,β-meATP, 1 µM) diminished these vasoconstrictor responses completely.

In the present study Ap₄ has been isolated from human tissue. Ap₄ was shown to exist in human myocardial tissue and was identified in ventricular specific granules. In coronary vasculature the nucleotide exerted vasodilation via endothelial P₂Y₁-receptors and vasoconstriction via P₂X-receptors on vascular smooth muscle cells. Ap₄ acts as an endogenous extracellular mediator and might contribute to the regulation of coronary perfusion.

Key words: adenosine tetraphosphate, myocardial tissue, coronary vasodilation, purinoceptors
Introduction

The endocrine functions of myocardial tissue have widely been investigated in the past. Well-known myocardium-derived hormonal factors include the atrial natriuretic peptide (1) or angiotensin II (2). Except for the long-known nucleotide, ATP, new myocardium-derived nucleotides like diadenosine polyphosphates have only recently been isolated (3).

As in myocardial tissue a great variety of nucleotidergic receptor subtypes (P1- and P2-receptors) are expressed (4,5), the secretion of nucleotides from myocardial cells represents one of the autocrine functions of the heart (6,7). In many studies it has been demonstrated that nucleotides exert numerous effects on cardiac functions (4,5). One of the important mechanisms of nucleotidergic signalling in the heart is the regulation of coronary perfusion. A2A receptors, P2X1 receptors as well as P2Y1 receptors contribute to the modulation of coronary vascular tone (6-11).

The present study aimed to extend our knowledge on autocrine loops in myocardial tissue. To this purpose, an isolation procedure was constructed, which is suitable to detect further novel nucleotides secreted by human myocardium. The experiments revealed adenosine 5’-tetraphosphate (Ap4) in human myocardial tissue. Moreover, secretory granules contained this nucleotide, and studies in an animal model of coronary circulation revealed potent effects of Ap4 on coronary vascular tone.
Materials and Methods

Materials

All reagents were purchased from Sigma (St. Louis, Mo) unless otherwise specified. Human myocardial tissue was obtained from 2 graft recipients after heart transplantation. The study was approved by the local ethical committee and written consent was obtained from the transplant patients. In the hearts used for isolation of nucleotides, coronary heart disease was the primary disorder leading to transplantation. For isolation of adenosine 5’-tetraphosphate only macroscopically intact tissue from the left ventricle was used.

Purification of the adenosine 5’-tetraphosphate from human myocardial tissue

After excision from the heart transplant recipient, human myocardial tissue (10 g) was immediately placed in ice-cooled physiological saline solution and processed within 30 min. The following isolation procedure was designed to exclusively isolate adenosine 4’-tetraphosphate from myocardial tissue. The coronary tissue was cut into small pieces (about 1 cm³), frozen in liquid nitrogen and stored at -80°C for one night. Then the tissue was lyophilized and powdered (step 1). The powder was suspended in 200 ml 0.6 M ice-cold perchloric acid and homogenized at 25,000 rpm three times for 1 min. The homogenate was ultracentrifuged at 30,000 rpm for 60 min at 4°C. The supernatant was adjusted with KOH to pH 8.5 and stored at 4°C for 30 min to precipitate KClO₄. After centrifugation at 4,000 rpm for 10 min at 4°C, the supernatant was titrated to pH 6.5 with HCl and centrifuged again as above (step 2).
A reversed-phase column (Lichroprep RP-18, 310 x 25 mm, Merck) was used to concentrate the nucleotide (step 3). The column was equilibrated with 40 mM triethylammonium acetate (TEAA) in water. The supernatant with 40 mM TEAA added (final concentration) was pumped to the column, washed with 40 mM TEAA and eluted with 40% acetonitrile (ACN) in water at a flow rate of 1 ml/min. The 40% ACN-eluate was collected, frozen at -80°C and lyophilized.

Size exclusion chromatography was performed according to Schlüter et al. (12) (step 4). Size exclusion gel Sephacryl S-100 High Resolution (1000 x 16 mm, S100 HR, Pharmacia BioTech, Piscataway, N.J.) was equilibrated with water. The dried sample from the preparative reversed-phase column resolved in 5 ml water was loaded onto the column. The eluent (water) was pumped with a flow rate of 1 ml/min. The effluent was monitored with a UV-detector at 254 nm.

An anion exchange column (Fractogel EMD DEAE-650, 300 x 25 mm, Merck) was equilibrated with 10 mM ammonium acetate (pH 7.4) (step 5). After adding 10 mM ammonium acetate (final concentration), pH 7.4, to the fraction from the size exclusion chromatography, it was pumped through the column. Nucleotides were eluted by 1 M ammonium acetate (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 (step 6) was purified further with an affinity chromatography. The affinity chromatography gel, phenyl boronic acid coupled to a cation-exchange resin (BioRex 70, BioRad, Richmond Calif.), was synthesized according to Barnes et al.(13). The affinity resin was packed into a glass column (150 x 20 mm) and equilibrated
with 1 M ammonium acetate (pH 9.5). The pH of the eluate from the anion-exchange column was adjusted to pH 9.5 and loaded to the affinity column. The column was washed with 1 M ammonium acetate (pH 9.5) with a flow rate of 1 ml/min. Binding substances were eluted with 1 mM HCl. The eluate was frozen and lyophilized. Fractions were monitored with a UV-detector at 254 nm.

The fractions from the affinity-chromatography were desalted by reversed-phase high-performance liquid chromatography (HPLC) (Superspher 100 RP C18 endcapped, 250 x 4 mm, Merck) (step 7). The fractions dissolved in eluent A (40 mM TEAA) were injected to the HPLC. After a washing period of 10 min with eluent A the nucleotide-containing fraction was eluted with 30 % acetonitrile in water. The UV-absorbing fraction was collected.

The desalted fraction from the affinity chromatography was fractionated by an anion-exchange chromatography (step 8). The anion exchange column (50 x 5 mm, Mono-Q HR 5/5, Pharmacia Biotech) was equilibrated with eluent A (10 mM K$_2$HPO$_4$, pH 8.0). The sample dissolved in eluent A was injected to the column at a flow rate of the mobile phase of 0.5 ml/min. Binding substances were eluted using a linear gradient with increasing concentration of eluent B (50 mM K$_2$HPO$_4$ + 1 M NaCl, pH 8.0). The time program of the gradient was: 0 - 10 min 0 - 5 % B, 10 - 100 min 5 - 35 % B, 100 - 105 min 35 - 40 % B, 105 - 110 min 40 - 100 % B. The wavelength of the UV-detector was fixed to 254 nm. Fractions were collected every 2 min.

The fractions from anion exchange chromatography were further separated by reversed-phase HPLC (Superspher 100 RP C18 endcapped, 250 x 4 mm, Merck) (step 9). The fractions dissolved in eluent A (40 mM TEAA) were injected to the HPLC. The following gradient was
programmed: 0-4 min 0 - 2 % B, 4 - 79 min 2 - 7 % B, 79 - 85 min 7 - 60 %. The flow rate was 0.5 ml/min. The wavelength of the UV-detector was 254 nm. 1 ml fractions were collected.

**Purification of adenosine 5’-tetraphosphate from porcine myocardial specific granules**

Specific granules were obtained from porcine left ventricular tissue according to De Bold and Bencosme (14). Briefly, immediately after sacrifice the heart was removed, washed in ice cold 0.25 M sucrose (containing 0.2 % glycogen, 1 mM EDTA, pH 7) and placed in a container with ice cold 0.25 M sucrose. After removing large vessels and fat, the tissue was washed for a second time in ice cold 0.25 M sucrose. After collecting 10 g myocardial tissue it was homogenized at 4 °C. The resulting pulp was washed into a glass homogenizer with 10 volumes of 0.25 M sucrose. The suspension was homogenized by 20 strokes of a Teflon pestle. The resulting homogenate was filtered by a cheesecloth. This filtrate was centrifuged at 1,900 g for 10 min. The supernatant was filtered again by a cheesecloth, at 1,900 g for 10 min, and centrifuged at 32,000 g for 10 min. The pellet containing specific granules was resuspended in 0.25 M sucrose and centrifuged again at 32,000 g for 10 min. The pellet was resuspended by addition of 4 ml 1.6 M sucrose (containing 0.2 % glycogen, 1 mM EDTA, pH 7). The remaining pellet was further resuspended by homogenizing in a glass homogenizer by a Teflon pestle. The suspension was pipetted on top of 0.5 ml of 2 M sucrose (containing 0.2 % glycogen, 1 mM EDTA, pH 7) and topped with 1 ml of 0.25 M sucrose. The resulting gradient was centrifuged immediately at 154,000 g for 60 min. After centrifugation 5 fractions were obtained. The protein concentration was measured according to Bradford (15). The fraction with less than 10% of the total protein amount contained the specific granules. This fraction was collected, diluted to a 10-fold volume with water, and centrifuged at 32,000...
MALDI-MS and PSD MALDI-MS

The molecular masses of the molecules in the fractions from reversed-phase HPLC (step 9) were determined by matrix assisted laser desorption / ionization mass spectrometry (MALDI-MS). A reflectron type time-of-flight (RETOF) mass spectrometer, equipped with a nitrogen-laser (337 nm, pulse length 3 ns) was used for ion generation and mass analysis (12). In MALDI-MS large fractions of the desorbed analyte ions undergo postsource decay (PSD) during flight in the field free drift path. Using a RETOF set-up, sequence information from PSD fragment ions of precursors produced by MALDI were obtained (16). For MALDI-MS and PSD MALDI-MS speed-vacuum-dried samples were dissolved in 10 µl bidistilled water. 1.0 µl of the 3-hydroxyl-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 $10^6$-$10^7$ W/cm$^2$ focused to spot sizes of typically 50 to 100 µm in diameter. The ions generated were accelerated with an energy of 12 KeV for detection. The spectra were recorded by a LeCroy 9400 recorder.

UV-spectroscopy

The substances in the fractions of the reversed-phase HPLC (step 9), dissolved in 100 µl (pH 6.5), were analyzed by a UV-spectrometer (UV / Vis-Spectrophotometer, DU-600, Beckman). The UV absorption was scanned from 400 nm to 190 nm with a scan speed of 400 nm/min.
Enzymatic cleavage experiments

Aliquots of the fractions from the reversed-phase column (step 9) were incubated with enzymes as follows. The samples were dissolved a) in 20 µl 200 mM Tris buffer (pH 8.9) and incubated with 5'-nucleotide hydrolase 3 mU; from *Crotalus durissus*, EC 3.1.15.1, from Boehringer Mannheim, purified according to Sulkowski & Laskowski(18) for 9 min at 37 °C; b) in 20 µl 200 mM Tris and 20 mM EDTA buffer (pH 7.4) and incubated with 3'-nucleotide hydrolase (1 mU; from calf spleen, EC 3.1.16.1, from Boehringer Mannheim) 1 h at 37 °C and c) in 20 µl 10 mM Tris, 1 mM ZnCl₂ and 1 mM MgCl₂ buffer (pH 8) and incubated with alkaline phosphatase (1 mU; EC 3.1.3.1, from calf intestinal mucosa, from Boehringer Mannheim) 1 h at 37 °C. The reaction was terminated by an ultrafiltration with a centrifuge filter (exclusion limit 10 kDa, Millipore). After filtration of the enzymatic cleavage products the filtrate, dissolved in 980 µl eluent A (10 mM K₂HPO₄, pH 7), was subjected to an anion exchange chromatography (MiniQ PC 3.2/3, Pharmacia). The gradient was: 0-3 min: 0 % B (50 mM K₂HPO₄, pH 7 with 1 M NaCl), 3-20 min: 0-50 % B, 20-21 min: 50-100 % B. The flow rate: was 100 µl/min.

Isolated perfused heart

Isolated perfused rat hearts were taken from male Wistar-Kyoto rats aged 3 months weighing 250 – 300 g and were prepared according to van der Giet et al.(11).

Responses of preparations to Ap₄, α,β-methyleneadenosine 5'-triphosphate (α,β-meATP), 2-methylthioadenosine 5'-triphosphate (2-meSATP), 2-chloroadenosine 5'-triphosphate (2-ClATP) were assessed at basal tone. For each substance dose-response curves were
constructed, with 5 min being allowed to elapse between consecutive doses. This procedure allowed dose-response curves for several agonists to be constructed for the same preparation. Desensitisation was not detected when 5 min were allowed to elapse between consecutive doses. Single doses of sodium nitroprusside (10 nmol) and acetylcholine (10 nmol) were used as controls for endothelium-independent or endothelium-dependent vasodilations mediated via NO.

Further experiments were done after endothelium was removed by Triton X-100 to investigate endothelium-independent effects of all used agonists. The endothelium was removed by perfusion of the isolated heart for 5 s with 0.1% Triton X-100. Successful removal was checked by the acetylcholine response. Unaffected K⁺-induced contractions (130 mM bolus) indicated intact vascular smooth muscle cell function. In some experiments with removed endothelium P₂X receptors were desensitised by permanent perfusion with α,β-meATP (1 µM) to block P₂X-mediated vasoconstrictions.

The specific P₂Y₁ receptor antagonist 2'-deoxy-N⁶-methyl-adenosine 3',5'-diphosphate diammonium (MRS2179, 1 µM), the P₂X receptor antagonist pyridoxal-phosphate-6-azophenyl-2,4-disulphonic acid (PPADS, 10 µM), and the nitric oxide (NO)-synthase inhibitor N⁶-nitro-L-arginine methyl ester (L-NAME, 10µM) were added to the perfusate 30 min before challenge with mono- or dinucleotides. MRS2179 and PPADS did not significantly influence vascular tone, whereas L-NAME significantly (P<0.05) increased perfusion pressure. Permanent perfusion with α,β-meATP showed a rapid and completely desensitising vasoconstriction.

All monoadenosinephosphates, sodium nitroprusside and acetylcholine were applied as 100 µl bolus into a valve proximal to the preparation. Drug dilutions were performed daily from
stock solutions of 10 mM (concentrates stores frozen in bidistilled water) with Krebs-Henseleit-buffer unless indicated otherwise. Heparin (sodium salt), PPADS, MRS2179, α,β-meATP, 2-meSATP, 2ClATP were from Research Biochemicals Inc., Deisenhofen Germany. Responses were measured as changes in perfusion pressure (mmHg) and results were presented as the means ± standard error of the mean (S.E.M.). Two-way repeated measures of variance, followed by Bonferroni’s multiple comparison test (for changes in coronary perfusion pressure) were used to identify where statistically significant differences had occurred. The effects of L-NAME, MRS2179, PPADS, and α,β-meATP on coronary perfusion pressure were compared with drug-free control conditions using Mann-Whitney test. All P values presented are two-tailed. P values <0.05 were considered significant.
Results

Human ventricular myocardial tissue from transplant recipients was lyophilized and powdered (step 1). Homogenized powder was deproteinated by perchloric acid (step 2). Nucleotides were concentrated from the supernatant by reversed-phase chromatography (step 3). The concentrate was fractionated with size exclusion chromatography (step 4). The resulting chromatogram is presented in Figure 1A. Anionic substances with a molecular mass between 300 - 2000 Da (indicated by solid line in Figure 1A) were concentrated by an anion exchange HPLC (step 5). The resulting chromatogram is given in Figure 1B. The fraction indicated by a solid line in Figure 1B was further fractionated by reversed-phase HPLC (step 6 & 7) and by anion exchange chromatography (step 8). The corresponding chromatogram is shown in Figure 1C. The arrow in Figure 1C labels the fraction which was further screened for nucleotides. This fraction was passed through an affinity column to concentrate mononucleoside polyphosphates. The resulting eluate was desalted by a reversed phase column and fractioned for the last time by anion exchange chromatography.

The fraction labeled by an arrow in Figure 1C was analyzed by MALDI-MS: The UV-absorbing fraction revealed a molecular mass of 588.2 Da [M+H]+. The UV-spectrum of this fraction at pH 7.0 showed maximum and minimum absorbances at 259 nm and 230 nm, respectively, indicating the presence of an adenine moiety. By PSD-MALDI-MS the fraction labeled in Figure 1C was analyzed to obtain fragment ion masses (Figure 2). The fragment ions showed masses identical with those of phosphate, adenosine, AMP and ADP. The PSD-MALDI-MS fragment spectrum of authentic AP₄ showed an identical fragment ion pattern (Table 1). In table 1 the measured fragment ions of the fraction labeled in Figure 1C and of authentic AP₄ are listed together with an interpretation of the fragments. The above measurements therefore revealed a molecule containing adenosine together with 4 phosphate moieties. In a further series of experiments the connection of the adenosine to the phosphate...
moieties was studied. To this purpose enzymatic cleavage experiments were done. 3’-exonuclease had no effect on the purified nucleotide, whereas alkaline phosphatase yielded AMP, ADP, and ATP. After incubation of the fraction with 5’-exonuclease, AMP was detected (Figure 3). The results suggest that in the molecule a chain of 4 phosphate groups is bound to adenosine via a 5’-phosphodiester bond.

In order to determine the localization of Ap₄ in ventricular myocardium, specific granules were examined for the presence of Ap₄. As shown in Figure 4, Ap₄ was found in ventricular specific granules. The amount of Ap₄ was estimated 4.3 µmol/g wet tissue according to the UV absorption signal in the chromatogram.

In isolated perfused rat heart experiments the mean coronary flow rate as measured in a total of 51 experiments amounted to 8.3±1.8ml/min to achieve a constant coronary perfusion pressure of 60±1mmHg. MRS2179 and PPADS did not significantly change vascular tone while L-NAME significantly (P<0.05) increased perfusion pressure. Permanent perfusion with α,β-meATP elicited a rapidly desensitizing vasoconstriction.

Figure 5 demonstrates the action of 2-meSATP, 2-ClATP, α,β-meATP and Ap₄ on coronary vasculature. At baseline perfusion pressure the nucleotides except for α,β-meATP caused dose-dependent vasodilation. The dose-response curves were not parallel and the maximal contractions induced varied considerably, which makes calculation of potency ratios difficult, but based on estimation of concentrations equi-effective to 1 nmol 2-ClATP the rank order of potency was Ap₄ ≥ 2-ClATP ≥ 2-meSATP.

After removal of the endothelium by Triton X-100, vasodilator responses to 10 nmol bolus of Ap₄, 2-ClATP and 2-meSATP were abolished, and vasoconstrictor responses arose as
presented in Figure 6B. Vasodilations induced by SNP did not change after removal of the endothelium. With intact endothelium both inhibition of P2Y1-receptors by MRS2179 (1µM) and inhibition of NO generation by L-NAME (50µM) abolished vasodilator responses to Ap4, 2-ClATP and 2-meSATP (Figure 6A), vasodilations induced by SNP and vasoconstrictions induced by α,β-meATP remained unaffected (Figure 6A). In the absence of intact endothelium, vasoconstrictor responses to Ap4, 2-ClATP, 2-meSATP and α,β-meATP disappeared after adding either the P2X-receptor antagonist PPADS (30µM, Figure 6B) or the P2X-receptor agonist α,β-meATP (1µM, Figure 6B). In this experimental setting Ap4, 2-ClATP and 2-meSATP did not affect basal tone significantly.
Discussion

In the present study the presence of \( \text{Ap}_4 \) in human myocardial tissue has been demonstrated. Furthermore, \( \text{Ap}_4 \) was also identified in ventricular specific granules. \( \text{Ap}_4 \) exerts \( \text{P}_{2\text{Y}1} \)-receptor mediated vasodilation and \( \text{P}_{2\text{X}} \)-receptor mediated vasoconstriction on coronary vasculature. Although to the best of our knowledge \( \text{Ap}_4 \) has not been demonstrated in human metabolism yet, its presence in mammalian tissues has been documented in a number of thorough experiments mostly performed several decades ago (19-23).

Like most nucleotides \( \text{Ap}_4 \) may have either intracellular or extracellular functions. On the one hand, \( \text{Ap}_4 \) may be involved in intracellular metabolic pathways serving either for cellular energy supply or for intracellular signal transduction. On the other hand, \( \text{Ap}_4 \) may indeed serve as an extracellular messenger, as suggested by its vascular effects. An extracellular action cannot be inferred from the mere existence of \( \text{Ap}_4 \) in myocardial tissue. However, the isolation of this compound from myocardial secretory granules strongly suggests that \( \text{Ap}_4 \) is produced in cardiomyocytes to act as an extracellular messenger.

The characterization of \( \text{Ap}_4 \)'s vasoactive properties revealed several interesting findings:

1) Although the structure of \( \text{Ap}_4 \) is very similar to that of ATP, \( \text{Ap}_4 \) has a longer biological half-life time than ATP. ATP is degraded to more than 95% during a single coronary passage. ATP levels in the coronary circulation increase during conditions of hypoxia or ischemia, suggesting a possible role of nucleotides as extracellular mediators of pathological processes in atherosclerotic vessels. The ATP content in human myocardial tissue is about 40 \( \mu \text{mol/g wet tissue} \) (24). Compared to ATP, the concentration of \( \text{Ap}_4 \) is about 10 times lower, but \( \text{Ap}_4 \) has a longer half-life time in the circulation, as it is more resistant to extracellular nucleotidases than ATP (25). Therefore the findings suggest that \( \text{Ap}_4 \) may exert a least equivalent actions to those of ATP from a quantitative point of view.
2) The findings reveal a profound dependence of Ap4’s actions on endothelial integrity. Ap4 is a potent vasodilator in intact coronary vasculature. In the absence of endothelial P2Y1 receptors, Ap4 is a vasoconstrictor. This endothelium-dependent action may be relevant with respect to coronary pathology. It may be speculated that in severely atherosclerotic coronary vessels Ap4 may be a mediator of vasospastic processes.

3) The actions of Ap4 are mediated by several of the known purinoceptor subtypes. The Ap4-induced vasoconstriction depends on activation of an $\alpha,\beta$-meATP sensitive, completely desensitizable P2X-receptor subtype. Only mRNA for P2X1, P2X2 and P2X4 have been detected in coronary vasculature so far (26). Additionally the P2X5-receptor was cloned in rat heart(27). In accordance with pertinent literature the P2X2-receptor and the P2X5-receptor are not activated by $\alpha,\beta$-meATP(27,28). The P2X4-receptor cannot be inhibited by PPADS(29). The P2X1-receptor is inhibitable by PPADS, sensitive to $\alpha,\beta$-meATP and completely desensitizable(28). Therefore it can be speculated the P2X1-receptor subtype is underlying the Ap4-induced vasoconstriction. P2X-receptor subtypes are known to form hetero-oligomeric channels with new pharmacological properties. Among P2X1, P2X2, P2X4, P2X5, only P2X1 and P2X5 are reported to be able to assemble (30). It cannot finally be excluded that a hetero-oligomeric form of P2X1 and P2X5 contributed to the Ap4-induced vasoconstriction in addition to the P2X1-receptor. On the other hand, the Ap4-induced vasodilation is mediated by the P2Y1-receptor subtype as evidenced by specific inhibition of P2Y1-receptor with MRS2179. From literature, it is known that P2Y1-receptors is expressed abundantly on endothelial cell surfaces, and therefore is responsible for the vasodilator action of Ap4 (31).

Beyond the isolation and characterization of Ap4, the experiments suggest that endogenous myocardium-derived nucleotides may be an important part of the spectrum of myocardial
endocrine functions. Using the isolation procedure established in the present study, it will be possible to screen extracts from myocardial tissue for further functionally relevant novel nucleotides. Since several other vasoactive nucleotides have been described in human myocardial tissue recently (3), the speculation may be allowed, that the group of myocardium-derived nucleotide hormones may be a larger and more complex family of extracellular messengers than was known in the last decades.
References


Legends

Figure 1: Purification of AP$_4$ from ventricular heart tissue

1) Size exclusion chromatography (purification step 4) of the human heart tissue extract. Column: S100 HF (1000 x 16 mM, Pharmacia). Eluent: water. Flow rate: 1 ml/min. Detector: UV Photometer, 254 nm, 0.5 AUFS. Black bar: fraction collected for further purification. B) Anion exchanger HPLC (Purification step 8) of the eluated from the affinity chromatography. Column: Mono-Q HR 5/5 (50 x 5 mM, Pharmacia). Eluent A: 10 mM K$_2$HPO$_4$, pH 8.0; eluent B: 50 mM K$_2$HPO$_4$ + 1 M NaCl, pH 8.0. Gradient: 0-10 min 0-5% B, 10 – 100 min 5-35% B, 100 – 105 min 35 – 40%, 105 – 110 min 40 – 100% B. Flow rate: 0.5 ml/Min. Detector: UV photometer, 254 nm, 0.5 AUFS. The fraction, which was further purified by reversed-phase HPLC, was indicated by the solid line (at 10 min retention time). The fraction containing anionic low-molecular-mass substances in Figure 1A is indicated by a solid line. This fraction was chromatographed by anion-exchange chromatography. The fraction, which was further purified by reversed-phase HPLC, was indicated by the solid line (at 70 min retention time). C) Reversed-phae HPLC (purification step 9) of the desalted fraction 1 from anion exchange chromatography. Column: Superspher 100 RP C18 end-capped. (250 x 4 mM, Merck). Eluent A: 40 mM TEAA in water; eluent B: 100% CAN. Gradient: 0-4 min 0-2% B, 4-79 min 2-7% B, 79 – 85 min 7-60%. Flow rate: 0.5 ml/min detector: UV photometer, 154 nm, 0.5 AUFS.

Figure 2: Identification of Ap$_4$ by PSD-MALDI mass spectrometry

PSD-MALDI-mass spectrum of the fraction labelled Ap$_4$ from reversed-phase HPLC (Figure 1C)
Figure 3: MALDI mass spectrum of the reaction products after incubation of the fraction, labeled by an arrow in Fig. 1C, with 3’-exonuclease (A), alkaline phosphatase (B), 5’-exonuclease (C), respectively.

Figure 4: Identification and quantification of Ap₄ in myocardial granules
A) Anion exchange HPLC-chromatogram of an extract from myocardial granules. Column: Mono-Q HR 5/2 (50 x 2 mM, Pharmacia). Eluent A: 10 mM K₂HPO₄, pH 8.0; eluent B: 50 mM K₂HPO₄ + 1 M NaCl, pH 8.0. Gradient: 0 – 2 min 0-5% B, 2-22 min 5-40% B, 22-22.5 min 40-100% B, 22.5-23.5 min 100% B. Flow rate: 0.1 ml/min. Detector: UV photometer, 254 nm, 0.5AUFS. B) Electropherogram of an extract from myocardial granules. Detector: UV photometer, 254 nm. Samples injection: hydrodynamic (1 s at 55 psi). Capillary: untreated fused-silica (50 µM I.D., 37 cm length, 30 cm to the detector). -20kV (65-70 µA; 20°C; inlet: cathode, outlet: anode). Buffer: 50 mM citric acid (pH 4.75).

Figure 5: Changes in perfusion pressure in the isolated perfused rat heart induced by 2-meSATP ( ■ ), 2-ClATP ( □ ), α,β-meATP ( ● ) and Ap₄ ( ○ ). Each point is the mean of at least eight determinations and error bars show s.e.mean. Where error bars do not occur, they are within the symbol size. Significant differences from baseline perfusion pressure 2-ClATP ≥ 10⁻¹¹ mol, 2-meSATP and Ap₄ ≥ 10⁻¹⁰.⁵ mol, α,β-meATP ≥ 10⁻⁹.⁵ mol. ED₅₀ (in –log mol) was 10.9 ± 0.4 for 2-ClATP, 10.1 ± 0.2 for 2-meSATP, and 9.7 ± 0.2 for Ap₄ (each n=7). For abbreviations see text.

Figure 6: Changes in perfusion pressure in the isolated perfused rat heart induced by bolus injection of 1 nmol of each agonist before (A) and after endothelium removal (B). A: open bars, in the absence of any antagonist (control), filled bars, with MRS2179 (1µM), hatched
bars, with L-NAME (50µM); B: open bars, in the absence of any antagonist (control), filled bars, with α,β-methylene ATP (1µM), hatched bars, with PPADS (10µM) in the perfusate.

Each column is the mean of at least nine determinations and error bars show the s.e.mean.

*P<0.05 antagonist vs. control. For abbreviations see text.
Table 1. Fragment ions masses (in Da) measured by PSD-MALDI-MS of the fraction from the reversed-phase chromatography (Fig. 1C, labeled with an arrow) and of authentic AP$_4$\textsuperscript{a}.

<table>
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<tr>
<th>Interpretation</th>
<th>Masses of fragment ions of AP$_4$ in Fig. 1C</th>
<th>Masses of fragment ions of authentic AP$_4$</th>
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<td>Phosphate</td>
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<td>AP$_4$</td>
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<td>587.83</td>
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</tbody>
</table>

\textsuperscript{a}Table showing the masses of the fragment ions (in Da) obtained by PSD-MALDI mass spectrometry of the fractions labeled Ap$_4$ (Fig 1 C). From the characteristic fragmentation patterns, the structure of Ap$_4$ can be deduced. The left column, labeled fragment ions, shows the fragments that could be assigned to the masses obtained after fragmentation. M = Protonated parent ion.
Fig. 1
Westhoff et al.
Figure 2, Westhoff et al
Figure 3, Westhoff et al.
Figure 4, Westhoff et al.
Figure 5 Westhoff et al.
Figure 6, Westhoff et al.
Identification and characterization of adenosine 5'-tetraphosphate in human myocardial tissue

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