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Diadenosine 5′,5″-P^1,P^5-pentaphosphate harbors the properties of a signaling molecule in the heart

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Abstract Dinucleotide polyphosphates (ApnA) have emerged as signaling molecules in rapidly dividing cells. The presence and role of Ap5A in the heart remain unknown. Here, we report that the myocardium contains abundant amounts of diadenosine 5′,5″-P^1,P^5-pentaphosphate (Ap5A), a member of the ApnA family. Ischemia induced 10-fold decrease in the myocardial concentration of Ap5A. A target of Ap5A action was identified to be the cardiac ATP-sensitive K^+ (K_{ATP}) channel, a metabolism-sensitive ion conductance activated in ischemia. At levels found in hearts prior to ischemia, Ap5A maintained a low probability of K_{ATP} channel opening, but at levels found in harts following ischemia, Ap5A allowed a high probability of K_{ATP} channel opening. Taken together, the present data suggest that Ap5A harbors the properties of a signaling molecule involved in the cardiac response to metabolic stress.

Key words: Diadenosine polyphosphate; Diadenosine pentaphosphate; K_{ATP} channel; Ischemia; Heart

1. Introduction

Diadenosine polyphosphates (ApnA) are dinucleotides composed of two adenosine moieties linked through ribose 5′-carbons to a phosphate group chain of different length ($n = 2$–6). Although biosynthesis of such molecules has been postulated four decades ago [1], the presence and role of ApnA in mammalian tissue still remains controversial [2–4].

More recently, intracellular ApnA have emerged as putative signaling molecules implicated in the maintenance of vital cellular functions [2,3,5–9]. In prokaryotic cells, changes in the concentration of cytosolic ApnA have been reported during oxidative stress and associated with binding to, and inhibition of oxidative stress-related proteins [10,11]. In eukaryotes, the role of ApnA in the response to oxidative stress remains unknown.

Here, we report that a member of the ApnA family, diadenosine 5′,5″-P^1,P^5-pentaphosphate (Ap5A), is present in the heart, senses the metabolic state of the myocardium, and targets a metabolism-sensing ion conductance. This provides the first evidence for a role of a member of the ApnA family in the cardiac response to oxidative stress.

2. Materials and methods

2.1. Measurement of Ap5A in heart muscle

Cardiac ventricles, obtained from adult guinea-pigs (350–450 g; anesthetized with 40 mg/kg sodium pentobarbital, i.p.), were frozen at −70°C. In certain experiments, hearts were first made globally ischemic by shutting-off coronary perfusion for 10 min [12]. Tissue specimens (1 g) were pulverized under liquid nitrogen, the powder added to 2 ml ice-cold perchloric acid (0.7 M), homogenized and the mixture centrifuged (3000×*g*, 15 min, 4°C). The supernatant (2 ml) was removed and mixed with 4 ml freon (1.1.2-trichlorotrifluoroethan and tri-*n*-octylamine in a ratio of 7.81:2.19), and this mixture centrifuged (3000×*g*, 10 min, 4°C). The obtained supernatant was adjusted to pH 7.5 with 5 M KOH, and Ap5A measured by high performance liquid chromatography (HPLC), as previously described [13,14]. HPLC analysis was performed, at room temperature, using a C_{18} reversed phase column (Phenomenex, 250×4.6 mm, 5 μm particles), protected by a Guard Pack module. The chromatographic system consisted of the LKB delivery system (2152 LC Controler), LKB injector (2157 Autosampler), linear UV detector (UVIS 204) and a Hitachi module integrator (D-2500). The injected volume was 100 μl. The mobile phase was composed of 10 mM K_2HPO_4, 2 mM tetrabutylammonium and 17% acetonitrile, at pH 7.5. Detection was monitored at 260 nm. A linear correlation between injected doses (0–12 nmol) of synthetic Ap5A and corresponding peak areas on chromatograms was obtained ($r = 0.999995$). The concentrations of Ap5A in ventricular tissue extract were calculated based upon peak areas of Ap5A standards injected before each experiment.

2.2. Single channel recordings

Ventricular cardiomyocytes were dissociated from pentobarbital-anesthetized guinea-pigs using an established enzymatic procedure [15,16]. In brief, hearts were retrogradely perfused (at 37°C) with medium 199 for 2–3 min, followed by Ca^{2+}-EGTA-buffered low-Ca^{2+} medium ($pCa = 7$) for 80 s, and finally low-Ca^{2+} medium containing pronase E (8 mg per 100 ml), proteinase K (1.7 mg per 100 ml), bovine albumin (0.1 g per 100 ml, fraction V) and 200 μM $CaCl_2$. Ventricles were cut into small fragments (6–10 mm^3) in the low-Ca^{2+} medium enriched with 200 μM $CaCl_2$. Single cells were isolated by stirring the tissue (at 37°C) in a solution containing pronase E and proteinase K supplemented with collagenase (5 mg per 10 ml). After 10 min, the first aliquot was removed, filtered through a nylon sieve, centrifuged for 60 s (at 300–400 rpm), and washed twice. Remaining tissue fragments were re-exposed to collagenase, and isolation continued for 2–3 such cycles. Isolated cardiomyocytes were stored in low-Ca^{2+} medium with 200 μM $CaCl_2$. Rod-shaped cardiomyocytes with clear striations and smooth surface were used for electrophysiological recordings. Cells were superfused with (in mM): KCl 140, $MgCl_2$ 1, EGTA 5, HEPES-KOH 5 (pH 7.4). Fire-polished pipettes, coated with Sylgard (resistance 5–7 mΩ), were filled with (in mM): KCl 140, $CaCl_2$ 1, $MgCl_2$ 1, HEPES-KOH 5 (pH 7.3). The gigaohm seal patch-clamp technique was applied in the inside-out configuration [17,18]. Recordings were made at room temperature (22°C), using a patch-clamp amplifier (Axopatch-1C). Single channel activity was monitored on-line on a high-gain digital storage oscilloscope (VC-6025; Hitachi) and stored on tape with the aid of a PCM converter system (VR-10, Instrutech). Data were reproduced, low-pass filtered at 4 kHz (−3 dB) by a Bessel filter (Frequency Devices 902), sampled at 80 μs rate, and further analyzed using the 'BioQuest' software

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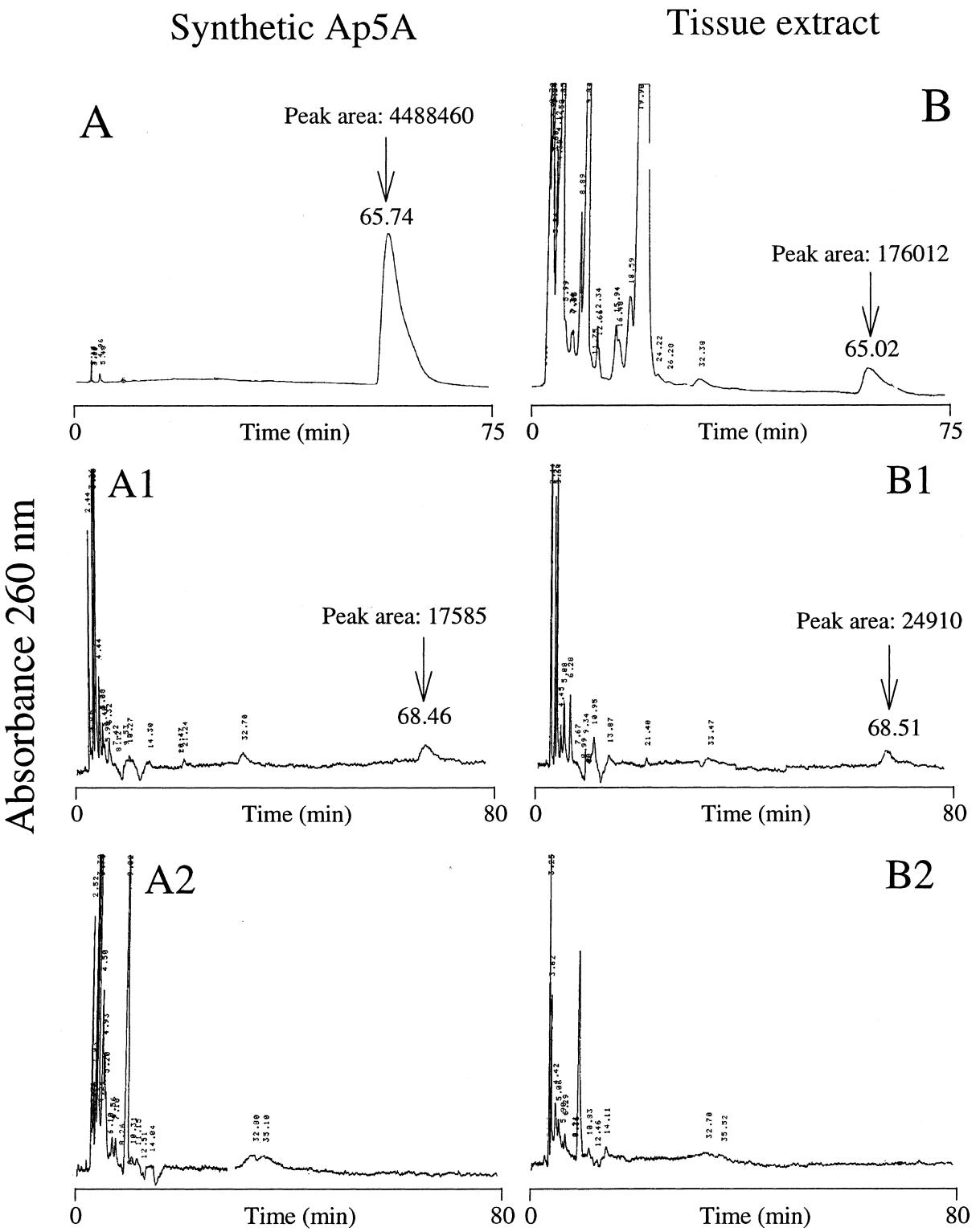

Fig. 1. Identification of diadenosine 5′,5″-P[1],P[5]-pentaphosphate (Ap5A) in the heart by HPLC. A and B: Elution profiles of synthetic Ap5A (5 nmoles, Sigma) (A) or ventricular tissue extract (100 μl) from normoxic heart (B). A1 and B1: Elution profiles of fractions recovered from peaks of interest in A (1/200; A1) and B (1/7; B1), lyophilized and rechromatographed. A2 and B2: Elution profiles of the fraction recovered from peaks of interest in A (1/200; A1) and B (1/7; B1), lyophilized, incubated with phosphodiesterase I (Sigma, EC 3.1.4.1. at 3 mU/ml during 10 min; 37°C) and rechromatographed. Each peak of interest is labeled by a vertical arrow, and the retention time (in min) and peak area (arbitrary units) indicated. Note similar elution profiles for synthetic Ap5A and the peak of interest in ventricular tissue extract under various experimental conditions.

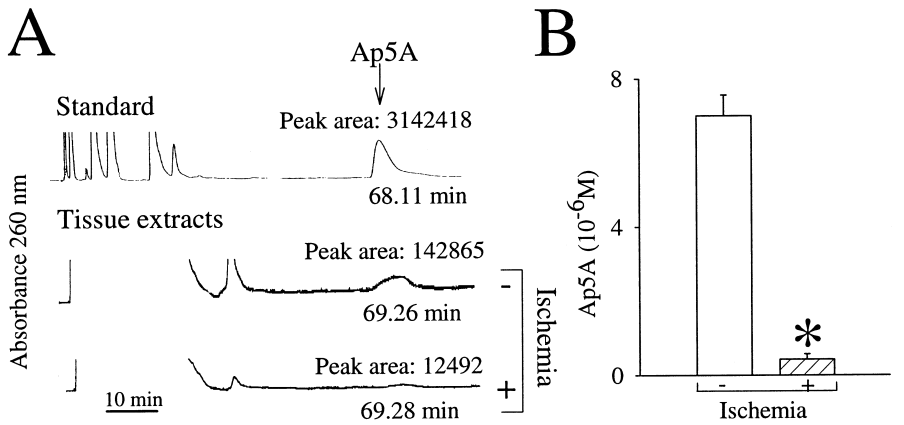

Fig. 2. Ischemia decreased the levels of Ap5A in cardiac tissue. A: Elution profile of synthetic Ap5A (4 nmol) in the presence of a cocktail of synthetic nucleotides (upper panel). Elution profile of ventricular tissue extract (100 μl) from normoxic (middle panel) and 10-min ischemic (lower panel) hearts. The peak corresponding to Ap5A is indicated by an arrow. For each peak of interest the retention time (in min) and peak area (arbitrary units) are indicated. B: Average values of the estimated levels of Ap5A in normoxic (open bar; $n=6$) and ischemic (hatched bar; $n=4$) hearts. Vertical bars represent mean ± S.E.M. ($*P<0.001$).

[19,20]. The threshold for judging the open state was set at half of the single channel amplitude. Channel activity, assayed by digitizing segments of current recordings and forming histograms of base line and open level data points, were expressed as NPo (N, number of channels in the patch; Po, probability of each channel to be open).

2.3. Statistics

Results are expressed as mean ± standard error of the mean; n refers to the number of animals. Significant differences between two means were determined with the Student's t-test ($P<0.05$ was considered significant).

3. Results and discussion

Synthetic Ap5A, subjected to ion-pair chromatography, had a retention time of 67.99 ± 2.27 min ($n=8$; Fig. 1A). This retention time was significantly different from that of other synthetic nucleotides, i.e. NAD (3.24 ± 0.03 min, $n=6$), AMP (4.95 ± 0.16 min, $n=6$), diadenosine triphosphate (10.01 ± 0.29 min, $n=6$), ADP (10.02 ± 0.29 min, $n=6$), FAD (12.51 ± 0.35 min, $n=6$), ATP (21.65 ± 0.60 min, $n=6$), diade-

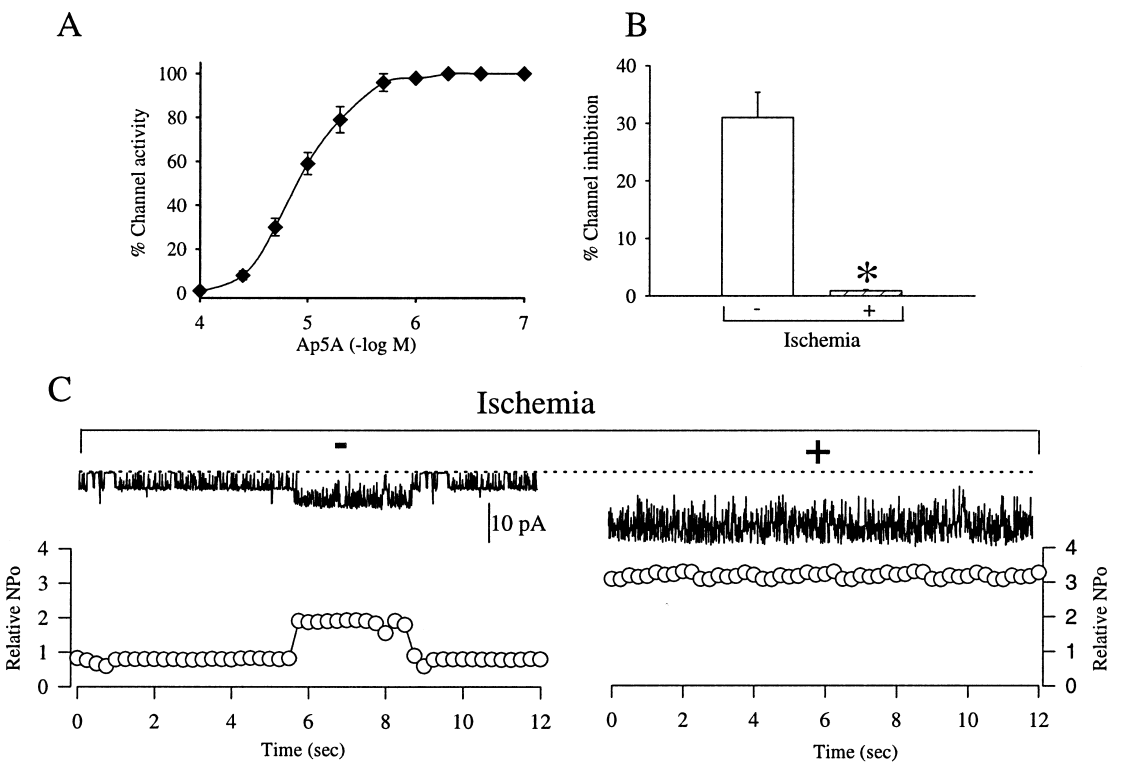

Fig. 3. Ap5A regulates the opening of cardiac K_{ATP} channels. A: Concentration-response curve of Ap5A versus K_{ATP} channel activity. Data points represent mean ± S.E.M. ($n=3$–6). Results are expressed as percentage of maximal channel activity determined in the absence of a ligand. B: Effect of normoxic and ischemic levels of Ap5A on K_{ATP} channel opening. Vertical bars represent mean ± S.E.M. ($n=4$–6). $*P<0.001$ (unpaired t-test). Results are expressed as percentage of complete channel inhibition (100% when NPo = 0). C: Upper traces: continuous recording of K_{ATP} channel activity in the presence of normoxic (left panel) and ischemic (right panel) levels of Ap5A. Lower traces: corresponding NPo values calculated over 0.25-s long intervals. Dotted line represents the zero current level. Holding potential: −60 mV.

nosine tetraphosphate (28.07 ± 0.92 min, $n = 6$) and diadenosine hexaphosphate (140.56 min, $n = 1$). Thus, the used experimental procedure allowed separation of Ap5A from other nucleotides.

In ventricular tissue extracts, subjected to the same procedure, a peak occurred at 67.4 ± 2.0 min ($n = 10$; Fig. 1B), a retention time not significantly different from that recorded for synthetic Ap5A ($P > 0.05$). This peak was isolated from other peaks (13.7 ± 4.4 min from the closest peak; $n = 10$) on the chromatogram of ventricular tissue extracts (Fig. 1B). On rechromatography, the purified ventricular fraction, corresponding to the peak of interest, appeared with a retention time (Fig. 1B1) that was also indistinguishable from rechromatographed, synthetic, Ap5A (Fig. 1A1). The purified ventricular fraction and synthetic Ap5A were both degraded by phosphodiesterase I (EC 3.1.4.1. at 3 mU/ml for 10 min; 37°C; Fig. 1A2 and B2), an enzyme known to cleave ApnA [12,13], but not by alkaline phosphatase (EC 3.1.3.1. at 1 mU/ml for 1 h; 37°C; data not shown), an enzyme to which ApnA are resistant [13,14]. These findings fulfil the criteria established for detection of Ap5A [13,14], suggesting that the myocardium contains this member of the ApnA family.

The estimated level of Ap5A found in the normoxic myocardium was 7.0 ± 0.6 μM ($n = 6$, Fig. 2B, open bar). In this regard, the myocardium apparently possesses levels of Ap5A higher than those previously found in the pancreas or liver [21,22], but lower than levels found in secretory tissues such as the adrenal medulla or platelets [13,23].

Little is known regarding the regulation of tissue levels of Ap5A. After mechanical injury or a challenge with a nutrient, tissue levels of certain ApnA, in particular diadenosine triphosphate and diadenosine tetraphosphate, rise from nanomolar to micromolar levels [21,22]. Here, in the heart, we found that global 10-min ischemia, which is known to produce moderate metabolic stress [12], produced a significant decrease in ventricular Ap5A which dropped from micro- to submicromolar levels ($P < 0.001$; Fig. 2A and B).

The significance of ischemia-induced decrease in cardiac Ap5A levels is at present unknown. Previously, in rapidly dividing cells, stress-regulated dynamics of other ApnA have been associated with regulation of proteins with nucleotide binding domains involved in cellular signaling coupled to DNA replication, growth and cell division [2,3,5–9,24]. However, the adult myocardium is a terminally differentiated tissue, for which the role of intracellular Ap5A remains to be determined.

In cardiac cells, a major sensor of metabolic stress is the ATP-sensitive K^+ (K_{ATP}) channel, an ATP-gated ion conductance which opening promotes cellular survival under ischemic injury [25–28]. The nucleotide binding domains of the K_{ATP} channel protein complex may serve as targets for Ap5A [29–35]. We and others have previously shown that moderate metabolic stress, including a 10-min long ischemia, increases the probability of opening of cardiac K_{ATP} channels [25–28,36]. In principle, a ligand of K_{ATP} channels which fluctuate around its EC_{50}, during ischemia, would profoundly affect channel opening under this condition. So far, no single endogenous K_{ATP} channel ligand with such property has been identified [25–28,31,37]. Ap5A antagonized the opening of cardiac K_{ATP} channels, in excised membrane patches, with an EC_{50} of 12.6 ± 1.7 μM ($n = 6$), a value close to the levels of Ap5A found in the myocardium (Fig. 3A). Concentrations of Ap5A, found in hearts prior to metabolic stress (range: 5.41 to 9.17 μM), maintained a low probability of K_{ATP} channel opening (Fig. 3B, C). Concentrations of Ap5A found in hearts following global ischemia (range: 0.13 to 0.79 μM) were associated with a high probability of channel opening (Fig. 3B, C). Thus, Ap5A fluctuates during ischemic stress within a range of concentrations which closely correspond to the EC_{50} that defines the action of this ApnA on the probability of K_{ATP} channel opening. Such property distinguishes Ap5A from ATP, which also regulates the probability of K_{ATP} channel opening, but which concentrations far exceed the EC_{50} value for channel inhibition, regardless of the metabolic state of the myocardium [25–28,31,37,38]. Therefore, Ap5A should be considered as a potential, ischemia-sensing, regulator of cardiac K_{ATP} channels.

In summary, the present study has identified Ap5A in the myocardium, determined the regulation of Ap5A levels by ischemic stress, and provided evidence for ischemia-dependent regulation of K_{ATP} channels by Ap5A. Taken together, the present data suggest that Ap5A harbors the properties of a signaling molecule in the heart which may transduce the effect of ischemia on the activity of a metabolism-sensitive ion conductance.

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