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Negative chronotropic and inotropic effects exerted by diadenosine hexaphosphate (AP_6A) via A_1-adenosine receptors

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1 Diadenosine hexaphosphate (AP_6A) exerts vasoconstrictive effects. The purpose of this study was to investigate whether AP_6A has any effect on cardiac function.

2 The effects of AP_6A (0.1 – 100 μM) on cardiac contractility and frequency were studied in guinea-pig and human isolated cardiac preparations. Furthermore, the effects of AP_6A on the amplitude of the L-type calcium current, on the adenosine 3':5'-cyclic monophosphate (cyclic AMP) content and on the phosphorylation of regulatory phosphoproteins, i.e. phospholamban and troponin inhibitor, were investigated in guinea-pig isolated ventricular myocytes.

3 In isolated spontaneously beating right atria of the guinea-pig AP_6A exerted a negative chronotropic effect and reduced the rate of contraction maximally by 35% ($IC_{20} = 35$ μM).

4 In isolated electrically driven left atria of the guinea-pig AP_6A exerted a negative inotropic effect and reduced force of contraction maximally by 23% ($IC_{20} = 70$ μM).

5 In isolated electrically driven papillary muscles of the guinea-pig AP_6A alone was ineffective, but attenuated isoprenaline-stimulated force of contraction maximally by 23% ($IC_{20} = 60$ μM). Furthermore, AP_6A attenuated the relaxant effect of isoprenaline.

6 In human isolated electrically driven ventricular preparations AP_6A alone was ineffective, but attenuated isoprenaline-stimulated force of contraction by maximally 42% ($IC_{20} = 18$ μM). Moreover, AP_6A attenuated the relaxant effect of isoprenaline.

7 All these effects of AP_6A were abolished by the selective A_1-adenosine receptor antagonist 1,3-dipropyl-cyclopentyl-xanthine (DPCPX, 0.3 μM), whereas the M-cholinoceptor antagonist atropine (10 μM) and the P_2-purinoceptor antagonist suramin (300 μM) failed to abolish the effects of AP_6A.

8 AP_6A 100 μM had no effect on the amplitude of the L-type calcium current, but attenuated isoprenaline-stimulated L-type calcium current. The maximum of the current-voltage relationship (*I*-V curve) was shifted to the left by isoprenaline and additional application of AP_6A shifted the *I*-V curve back to the right to the control value. The phosphorylation state of phospholamban and the troponin inhibitor was unchanged by AP_6A alone, but was markedly attenuated by AP_6A in the presence of isoprenaline. Cyclic AMP levels remained unchanged by AP_6A, even after stimulation with isoprenaline.

9 In summary, AP_6A exerts negative chronotropic and inotropic effects in guinea-pig and human cardiac preparations. These effects are mediated via A_1-adenosine receptors as all effects were sensitive to the selective A_1-adenosine receptor antagonist DPCPX. Furthermore, the effects of AP_6A on cyclic AMP levels, protein phosphorylation and the L-type calcium current are in accordance with stimulation of A_1-adenosine receptors.

Keywords: Diadenosine hexaphosphate (AP_6A); diadenosine polyphosphates; A_1-adenosine receptors; guinea-pig heart; human heart; inotropy, chronotropy, cyclic AMP; protein phosphorylation; L-type calcium current

Introduction

Diadenosine polyphosphates are a group of adenosine dinucleotides, which consist of two adenosine molecules linked via their 5' position by a chain of two or more phosphates (abbreviated AP_nA, where n represents the number of phosphates in the connecting chain). Diadenosine polyphosphates are ubiquitous molecules found in prokaryotes and eukaryotes. Moreover, diadenosine polyphosphates are stored in secretory granules of, for example, platelets, thus opening the possibility of diadenosine polyphosphates acting as extracellular signalling molecules (for review see Baxi & Vishwanatha, 1995). In mammalian tissue AP_nAs have been described in hepatocytes (Rapaport & Zamecnik, 1976) and secretory granules of platelets, of adrenal chromaffin cells and of brain synaptosomes (for review see Pintor & Miras-Portugal, 1995). Until now AP_6A has been identified in bovine adrenal chromaffin granules too, (Pintor *et al.*, 1992) and recently in human platelets (Schlüter *et al.*, 1994). The physiological importance of diadenosine polyphosphates has not yet been clearly established. The pleiotropic effects of diadenosine polyphosphates are mediated via P_2-purinoceptors (Hoyle, 1990; Pintor & Miras-Portugal, 1995). AP_6A and other diadenosine polyphosphates are potent antagonists of adenosine 5'-diphosphate (ADP)-induced platelet aggregation at the P_{2T}-purinoceptor (Harrison *et al.*, 1975). Moreover, AP_6A elicited phasic contractions in the vas deferens (Stone, 1981) and in the urinary bladder mediated via P_{2X}-purinoceptors (Stone, 1981; Hoyle *et al.*, 1989). Furthermore, AP_6A constricted rat aortae via an increase in cytosolic calcium concentration (Schlüter *et al.*, 1994). The type of receptor mediating the vasoconstriction has not been characterized.

However, data on the influence of diadenosine polyphosphates on the heart are presently lacking. Thus, the purpose of

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this study was to investigate the effects of AP_6A on the rate and force of contraction in guinea-pig and human isolated heart preparations and the supposed mechanism of action. Thus, the effects of AP_6A on the L-type calcium current, on phosphorylation of regulatory phosphoproteins, i.e. phospholamban (PLB) and the troponin inhibitor (TnI), and on adenosine 3':5'-cyclic monophosphate (cyclic AMP) levels were investigated in guinea-pig isolated ventricular cardiomyocytes.

Methods

Rate and force of contraction experiments

Experiments were performed as described previously (Böhm *et al.*, 1984). In brief, right atria, left atria and papillary muscles from right ventricles were isolated from hearts of reserpine-treated (5 mg kg^{-1}, 16 h before death) male guinea-pigs (300–400 g). Trabeculae carneae were isolated from failing hearts of patients undergoing heart transplantation due to dilated cardiomyopathy. All studies were approved by the local ethics committee and patients gave written informed consent. Patient data are summarized in Table 1. Medical treatment consisted of cardiac glycosides, angiotensin-converting-enzyme-inhibitors and diuretics in all cases. Single patients received dobutamine (Table 1, A) nitrates (Table 1, B) and β_2-sympathomimetics (Table 1, F). The bathing solution contained (in mM): NaCl 119.8, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1.05, NaH_2PO_4 0.42, $NaHCO_3$ 22.6, Na_2 EDTA 0.05, ascorbic acid 0.28 and glucose 5.0, continuously gassed with 95% O_2 and 5% CO_2 and maintained at 35°C resulting in a pH of 7.4. Isometric force of contraction was measured after preloading each muscle to optimal length. Papillary muscles and left atria were electrically stimulated at 1 Hz (Böhm *et al.*, 1984), human ventricular preparations at 0.5 Hz with rectangular pulses 5 ms duration (Steinfath *et al.*, 1992); the voltage was about 10–20% greater than the threshold. Preparations were allowed to contract until equilibrium was reached (at least 30 min) before 1 $\mu g\ ml^{-1}$ adenosine deaminase (ADA) was added for additional 30 min. After addition of either 1,3-dipropyl-8-cyclopentyl-xanthine (DPCPX 0.3 μM) or dimethyl sulphoxide (DMSO; solvent control) for 30 min, AP_6A was added cumulatively. The shape of the isometric contraction curve of papillary muscles was calculated by using twitches recorded at high chart speed.

Isolation of cardiomyocytes

Cardiomyocytes were isolated as described previously (Neumann *et al.*, 1989) with minor modifications. Guinea-pigs (180–220 g) were stunned by cervical dislocation. Hearts were rapidly excised, mounted on a modified Langendorff perfusion system and perfused retrogradely via the cannulated aorta in a non-recirculating manner at a constant rate of 10 ml min^{-1} for 3 min with a calcium-free buffer (solution A) containing (in mM): NaCl 100, KCl 10, KH_2PO_4 1.2, $MgSO_4$ 5, glucose 20, taurine 50, 3-(N-morpholino)propanesulphonic acid (MOPS) 10, pH was adjusted to 6.9 at 37°C. After washing to remove blood cells, the hearts were perfused with the same medium containing 0.1% collagenase in a recirculation fashion at a rate of 30 ml min^{-1} for 30 min. Following enzyme perfusion atria were cut off and ventricles were minced and incubated for one hour in solution B, which consisted of (in mM): KCl 70, KH_2PO_4 30, $MgSO_4$ 20, taurine 20, succinic acid 5, creatine 5, 1,2-cyclohexylenedinitriltetraacetic acid monohydrate (EGTA) 1, β-hydroxybutyric acid 7.3, pyruvic acid 5, Na_2ATP 5, pH was adjusted to 7.4, temperature was maintained at 37°C. The cell suspension was passed through 200 μm mesh nylon gauze (Heidland, Gütersloh, Germany). Calcium concentration was gradually increased to 0.38 mM and the cell suspension was centrifuged for 3 min at $25 \times g$. The resulting cell pellet was resuspended in phosphate-free solution C consisting of (in mM): NaCl 132, KCl 4.8, $MgSO_4$ 1.2, glucose 10, N-(2-hydroxyethyl)piperazine-N'-(2-ethansulphonic acid (HEPES) 10, sodium pyruvate 2.5, pH was adjusted to 7.4, temperature was maintained at 37°C. This cell suspension was centrifuged for 3 min at $25 \times g$. The final cell suspension was resuspended in solution C.

Table 1 Haemodynamic data from patients used to obtain the results depicted in Figure 4

Patient	Sex	Age (years)	NYHA	PCW (mmHg)	EF (%)	CI ($l\,min^{-1}\,m^2$)
A	M	45	IV	18	10	1.4
B	M	67	IV	22	16	1.9
C	M	57	IV	16	19	2.1
D	M	57	IV	23	15	1.3
E	M	37	IV	15	17	2.3
F	M	40	IV	10	12	1.5

All patients had undergone heart transplantation due to dilated cardiomyopathy. NYHA: functional class of the New York Heart Association; PCW: pulmonary capilary wedge pressure; EF: radionuclide-determined left ventricular ejection fraction; CI: cardiac index.

Labelling of cardiomyocytes and protein phosphorylation

A volume of 3 ml of a gravity settled suspension of freshly isolated cardiomyocytes was incubated at 37°C with 5 mCi of ^{32}P-labelled orthophosphate in 5 ml of solution C. After 60 min, cardiomyocytes were washed with 10 ml of solution C by allowing cells to settle by gravity. Finally, gravity-settled cardiomyocytes were diluted five fold in solution C and the resulting cell suspension was used in phosphorylation experiments.

Drug solutions were prepared in solution C containing 200 μM of sodium metabisulphite in order to protect isoprenaline from oxidation and 10 units ml^{-1} adenosine deaminase. The drug solution (150 μl) was preincubated at 37°C for 2 min before it was mixed with 150 μl of the diluted cardiomyocytes, kept at 37°C. After 5 min, reaction was stopped by adding 150 μl sodium dodecyl sulphate (SDS) stop solution (Laemmli, 1970), which consisted of tris(hydroxymethyl)aminomethane (Trizma base) 62.5 mM, SDS 10% (w/v), glycerol 10% (v/v), DL-dithiothreitol (DL-DTT) 0.6% (w/v) and a trace of bromophenol blue, pH was adjusted to 6.8. Samples were frozen at −20°C.

SDS polyacrylamide gel electrophoresis

SDS polyacrylamide gel electrophoresis was performed as follows: samples were thawed and then heat-treated for 10 min at 95°C. An aliquot of 100 μl corresponding to 70 μg protein was applied on each lane. Gels were run according to Lindemann and Watanabe (1985) in a Hoefer SE 600 vertical slab gel unit (HSI, San Francisco, U.S.A.) with 10% polyacrylamide separating gels (1.6% cross-linked) and 4% stacking gels. Electrophoresis was initially run at 40 mA per gel unit until the dye entered the separating gel. Then, the current was increased to 60 mA per gel unit and electrophoresis was finished when the dye left the separating gel. Gels were stained with coomassie blue R-250 and dried after destaining. Changes in the phosphorylation state of phospholamban and troponin-inhibitor were visualized and quantitated by use of Phosphor Imager SF and Image Quant software (Molecular Dynamics, Sunny Vale, CA, U.S.A.) as described previously (Neumann *et al.*, 1995a). Apparent molecular weights were determined by low molecular weight calibration kit (Pharmacia LKB, Piscataway, NJ, U.S.A.) consisting of rabbit muscle phosphorylase b (94 kDa), bovine serum albumin (67 kDa), egg white ovalbumin (43 kDa), bovine erythrocyte carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and bovine milk α-lactalbumin (14.4 kDa).

Determination of cyclic AMP

Determination of cyclic AMP content was performed as described previously (Neumann *et al.*, 1989). Cardiomyocytes were isolated as described above. After the calcium concentration had been increased, the resulting cell pellet was resuspended in solution C (containing 200 μM of sodium metabisulphite and 10 units ml^{-1} adenosine deaminase). This cell suspension was centrifuged at $25 \times g$ for 3 min through a gradient of solution C containing 6% bovine serum albumin. The resulting cell pellet was washed five times with solution C. The incubation with drugs was performed as follows: 30 min with either 0.3 μM DPCPX or DMSO, then 10 min with 0.1 μM isoprenaline and then 10 min with 100 μM AP_6A or solution C. The reactions were stopped by adding 150 μl of 0.1 M HCl. Samples were heat-treated for 10 min at 95°C and were centrifuged for 15 min at $14.000 \times g$. Protein was determined according to Bradford (1976) with bovine serum albumin as standard and cyclic AMP was measured by radioimmunoassay as described previously (Neumann *et al.*, 1989).

Electrophysiological experiments

Electrophysiological experiments were performed as described previously (Herzig *et al.*, 1995). Guinea-pig ventricular myocytes were isolated by a collagenase/protease digestion of Langendorff-perfused hearts (37°C, 52 mmHg) obtained from male animals (250–350 g). After 5 min of perfusion with calcium-free Tyrode solution (composition in mM: NaCl 135, KCl 4, NaH_2PO_4 0.3, $MgCl_2$ 1, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES) 10, dextrose 10, pH 7.4), enzymes were added to this solution at a flow-independent dose rate of 1.4 mg min^{-1} collagenase (type 1, Worthington, Freehold, New Jersey, U.S.A.) and 0.6 mg min^{-1} protease (type XIV, Sigma, Deisenhofen, Germany) over a period of 5 min, by use of an infusion pump. Afterwards the hearts were perfused for 10 min with enzyme-free Tyrode solution containing 0.2 mM calcium. Cells were harvested after the hearts had been minced with fine scissors, gentle agitation of the tissue and filtering through a nylon mesh. Cells were plated in petri dishes which served as recording chambers (volume approx. 1 ml) on the stage of an inverted microscope (Leica, Köln, Germany).

Whole-cell patch-clamp recordings were performed in 'physiological' solutions in order to avoid any confounding effects of artificial conditions on intracellular signal transduction. Tyrode solution (see above, but containing 2 mM $CaCl_2$, 22–24°C) served as the extracellular solution and recording pipettes (soft glass coated with Sylgard, 1.2–2.5 MΩ) were filled with (in mM): K-aspartate 80, KCl 50, KH_2PO_4 10, $MgCl_2$ 0.5, MgATP 3, HEPES 5, ethylene glycol *bis*(β-aminoethyl ether) *N,N',N'*-tetraacetic acid (EGTA) 1, pH 7.4.

L-type calcium-currents were elicited by voltage steps from a holding potential of −40 mV to a test potential of +10 mV for 300 ms, applied every 10 s. Current voltage relationships were determined by clamping for 300 ms from the holding potential to different test potentials. Current was recorded by an L/M-PC amplifier (LIST-Electronic, Darmstadt, Germany) connected to a 486 computer which was equipped with the ISO2 software (version 1.2, MFK, Niedernhausen, Germany). Currents were evaluated as the difference between peak inward current and the current level at the end of the test pulse. Series resistance was compensated to the maximum possible extent, using the feedback circuitry of the amplifier.

Isoprenaline (10 mM) was dissolved in distilled water containing 1 mg ml^{-1} ascorbic acid to prevent oxidation. After an equilibration period of at least 5 min, cells were superfused with Tyrode solution containing 0.01 μM isoprenaline at a rate of 60 ml h^{-1}. After stabilisation of maximal isoprenaline effect cells were superfused with Tyrode containing 0.01 μM isoprenaline and 100 μM AP_6A. After stabilisation of AP_6A effect, cells were superfused again with Tyrode solution containing only 0.01 μM isoprenaline. In blocking experiments the same protocol was used except all solutions contained 0.3 μM DPCPX and cells were incubated with 0.3 μM DPCPX for at least 30 min before further superfusion.

Chemicals

Substances used in all experiments were AP_6A purchased from Sigma and purified chromatographically before use as described (Heidenreich *et al.*, 1995), 1,3-dipropyl-8-cyclopentylxanthine (DPCPX, Research Biochemicals International, Natick, MA, U.S.A.) dissolved in DMSO (1 mM), adenosine deaminase (Boehringer Mannheim, Germany), (±)-isoprenaline-HCl (Boehringer Ingelheim, Germany), (−)-N^6 (2-phenylisopropyl)-adenosine (**R**-PIA, Sigma, Deisenhofen, Germany), suramin sodium (ICN Biomedicals, Eschwege, Germany).

2'-O-succinyladenosine 3',5'-monophosphate tyrosine methylester (Sigma, München, Germany) was iodinated with ^{125}I-labelled sodium iodide (Amersham Buchler, Braunschweig, Germany) as described previously (Böhm *et al.*, 1984). Bio-Rad protein assay, gamma globulin standard and all materials for SDS-PAGE were purchased from Bio-Rad (München, Germany). All other chemicals were of analytical or best commercial grade available. Deionized and bidistilled water was used throughout.

Statistics

The experimental data given in text and figures are means ± s.e.mean of n experiments. The significance of differences of data of contraction and frequency were estimated by means of 2-sided Student's t test for unpaired observations. All other data (time parameters , cyclic AMP content, phosphorylation, I_{CaL}) were compared by one-way analysis of variance followed by Bonferroni's correction for multiple comparisons. A P value less than 0.05 was regarded as significant. As no maximal effects in reduction of force of contraction and beating frequency could be achieved with the concentrations investigated, IC_{20} values were determined. For this calculation the prestimulation value was set as 100% and the IC_{20} value is the concentration which produces a reduction to 80% of the prestimulation value. The IC_{20} values were determined by linear interpolation.

Results

Effects of AP_6A on force and rate of contraction

In isolated electrically driven left atria of the guinea-pig AP_6A exerted a negative inotropic effect starting at 10 μM and reduced force of contraction by maximally 23% ($n=8$, Figure 1a,b). The IC_{20} value was 70 μM. DPCPX 0.3 μM abolished the negative inotropic effect ($n=8$, Figure 1a,b). The selective A_1-adenosine receptor agonist **R**-PIA (1 μM) reduced force of contraction to 21.6±8.1% of control, further application of 100 μM AP_6A did not change the inotropic response (15.3±3.2%, $n=3$). The negative inotropic effect of AP_6A was reversible upon washout.

In isolated spontaneously beating right atria of the guinea-pig, AP_6A exerted a negative chronotropic effect starting at 3 μM, reducing the frequency by maximally 35% ($n=6$, Figure 2). This effect was abolished by 0.3 μM DPCPX ($n=7$, Figure 2). The IC_{20} value was 35 μM. The negative chronotropic effect of AP_6A was reversible upon washout. In isolated electrically driven papillary muscles of the guinea-pig, 100 μM AP_6A alone had no effect on force of contraction (data not shown), whereas after stimulation with 0.01 μM isoprenaline additionally applied AP_6A elicited a negative inotropic effect starting at 1 μM and reduced force of contraction by maximally 23% ($n=6$, Figure 3a,b), i.e. AP_6A reduced the isoprenaline-induced stimulation (i.e. difference between control

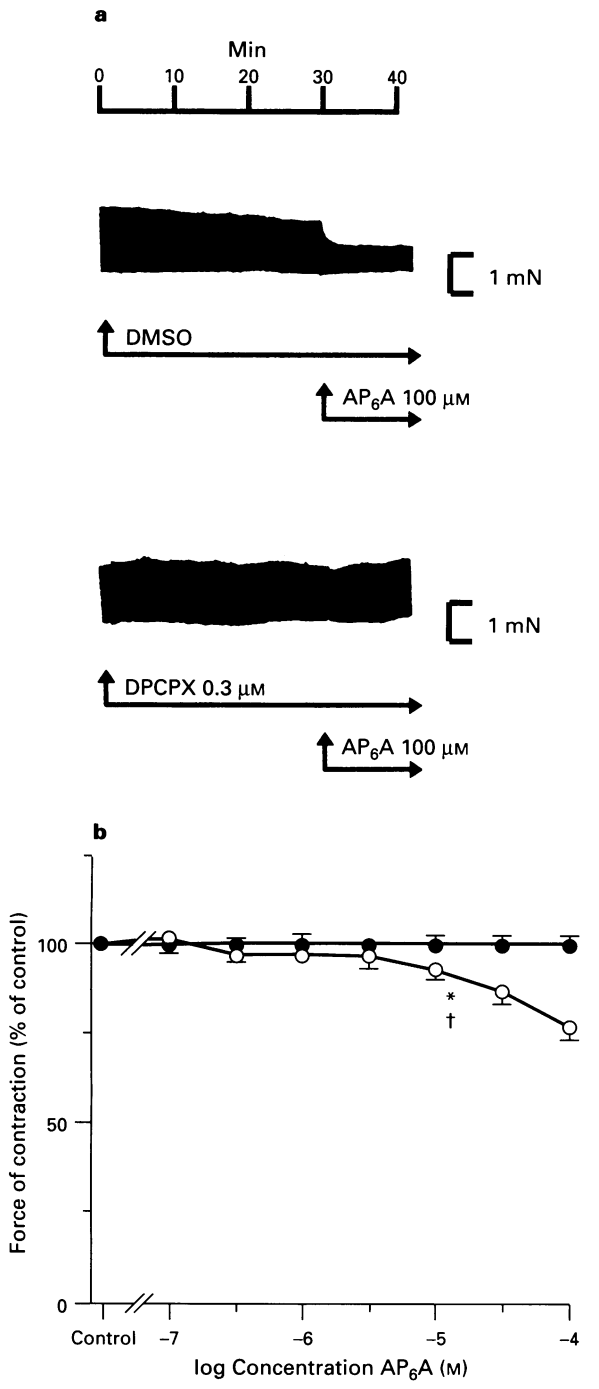

Figure 1 Effects of AP_6A on the contractile response in isolated, electrically driven left atria of the guinea-pig. (a) Original recordings illustrating the effect of 100 μM AP_6A on force of contraction of guinea-pig left atria (upper trace) and in the presence of 0.3 μM DPCPX (lower trace). (b). Concentration-response curve for the effects of AP_6A on the force of contraction in guinea-pig left atria. Effects of AP_6A alone (○, $n=8$) and of AP_6A in the presence of 0.3 μM DPCPX (●, $n=8$). Ordinate scale: force of contraction as % of predrug values, vertical lines show s.e.mean. Predrug values amounted to 2.1 ± 0.3 mN ($n=16$). * and † denote the first significant difference versus predrug and DPCPX values, respectively.

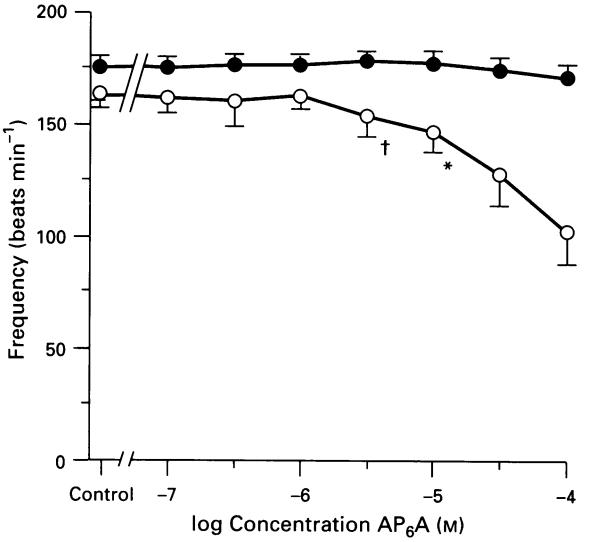

Figure 2 Concentration-response curve for the effects of AP_6A on frequency in isolated spontaneously beating right atria from guinea-pigs. Effects of AP_6A alone (○, $n=6$) and of AP_6A in the presence of 0.3 μM DPCPX (●, $n=7$). Ordinate scale: beats min^{-1}, vertical lines show s.e.mean. * and † denotes first significant difference versus predrug and DPCPX values, respectively.

and isoprenaline values) by 74%. The IC_{20} value was 60 μM. The selective A_1-adenosine receptor agonist **R**-PIA (1 μM) decreased isoprenaline-stimulated force of contraction to 60.2 ± 3.1% of the maximum, further application of 100 μM AP_6A did not change the inotropic response (68.8 ± 4.2%, $n=4$). Furthermore, the effects of AP_6A on contraction time parameters were investigated (Figure 3c). Time to peak tension was reduced from 97.1 ± 2.9 ms to 90.4 ± 3.6 ms by isoprenaline ($n=13$) and remained unchanged in the additional presence of AP_6A (91.7 ± 4.6 ms, $n=6$). Time of relaxation was shortened from 137.9 ± 2.7 ms to 125 ± 2.5 ms by isoprenaline ($n=13$) and was increased by AP_6A to 145.8 ± 2.7 ms ($n=6$). Total contraction time was reduced from 234.6 ± 3.3 ms to 217.1 ± 4.9 ms by isoprenaline ($n=13$) and increased to 237.5 ± 5.1 ms by AP_6A ($n=6$). These effects of AP_6A were attenuated in the presence of DPCPX (0.3 μM, $n=7$).

In human isolated electrically driven trabeculae carneae AP_6A elicited a negative inotropic effect only after prestimulation with isoprenaline (3 μM). AP_6A reduced force of contraction starting at 10 μM with maximal reduction by 42% ($n=7$); in the presence of 0.3 μM DPCPX the negative inotropic effect was abolished ($n=9$, Figure 4a,b). The IC_{20} value was 18 μM. Furthermore, the effects of AP_6A on contraction time parameters were investigated (Figure 4c). Time to peak tension was decreased from 177.9 ± 8.0 ms to 139.3 ± 3.1 ms by isoprenaline ($n=16$) and remained unchanged in the additional presence of AP_6A (142.9 ± 5.9 ms, $n=7$). Time of relaxation was shortened from 320.8 ± 8.4 ms to 192.7 ± 6.2 ms by isoprenaline ($n=13$) and was increased by AP_6A to 230.8 ± 11.0 ms ($n=7$). Total contraction time was shortened from 487.9 ± 12.5 ms to 331.1 ± 6.9 ms by isoprenaline ($n=13$) and increased by AP_6A to 369.3 ± 14.0 ms ($n=7$). These effects of AP_6A were attenuated in the presence of DPCPX (0.3 μM, $n=9$).

Effects of AP_6A on phosphorylation and cyclic AMP content

The effects of AP_6A were investigated in guinea-pig isolated ventricular cardiomyocytes. In control cells the cyclic AMP level amounted to 4.8 ± 0.6 pmol mg^{-1} protein and was not changed by application of 100 μM AP_6A (5.4 ± 0.3 pmol mg^{-1} protein, $n=12$). Isoprenaline (0.1 μM) increased cyclic AMP levels to 10.9 ± 0.8 pmol mg^{-1} protein and subsequently applied 100 μM AP_6A did not change the cyclic AMP value, either alone (10.0 ± 0.7 pmol mg^{-1} protein) or in the presence of 10 μM DPCPX (9.5 ± 0.5 pmol mg^{-1} protein, $n=12$). Also in cells only treated with 10 μM DPCPX alone cyclic AMP content was not changed compared to control

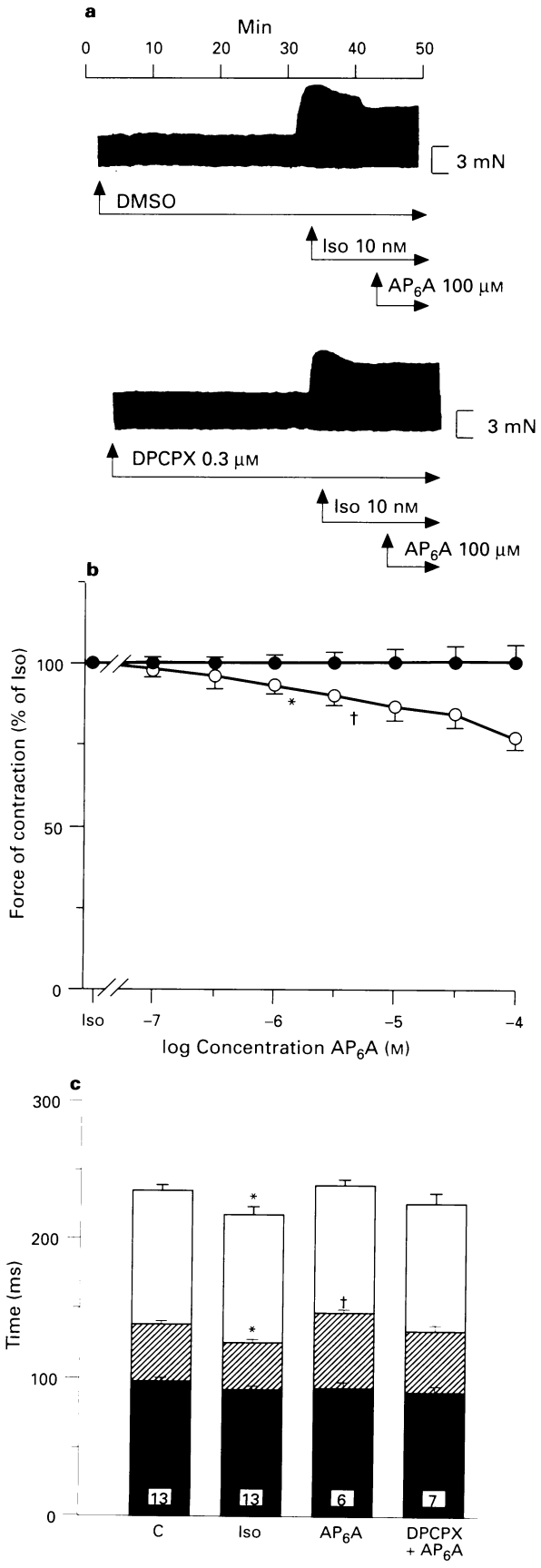

Figure 3 Effects of AP_6A on contractile response in isolated, electrically driven right papillary muscles of the guinea-pig. (a) Original recordings illustrating the effect of 100 μM AP_6A on force of contraction of guinea-pig right papillary muscle prestimulated with 0.01 μM isoprenaline (Iso), alone (upper trace) and in the presence of 0.3 μM DPCPX (lower trace). (b) Concentration-response curve for the effects of AP_6A on force of contraction in the presence of 0.01 μM isoprenaline. Effects of AP_6A in the absence (○, $n=6$) and in the presence of 0.3 μM DPCPX (●, $n=7$). Ordinate scale: force of contraction as % of isoprenaline values, vertical lines show s.e.mean. Isoprenaline values amounted to 2.0 ± 0.5 mN ($n=13$). * and † denote the first significant difference versus isoprenaline and DPCPX values, respectively. (c) Effects of AP_6A on time parameters in guinea-pig right papillary muscles. Effects of AP_6A in the absence and presence of 0.3 μM DPCPX compared to isoprenaline (Iso) and predrug values (C) on time to peak tension (solid part of columns), time of relaxation (cross-hatched part of columns) and total contraction time (open part of columns). Vertical lines show s.e.mean. The numbers in the columns denote the number of experiments. * and † denote significant differences versus predrug and isoprenaline values, respectively.

(5.9 ± 0.4 pmol mg^{-1} protein, $n=12$). Furthermore, the effects of AP_6A on the phosphorylation pattern of isolated [^{32}P]-labelled phosphoproteins in cardiomyocytes were investigated. Phospholamban (PLB), myosin light chains (MLC) and troponin I (TnI) have been immunologically identified previously (Neumann *et al.*, 1994). The effects of AP_6A on phosphorylation of PLB are depicted in Figure 5b: 100 μM AP_6A alone did not change the phosphorylation state of PLB ($n=8-11$). Isoprenaline (0.1 μM) increased the phosphorylation state of PLB to $178.5\pm15.5\%$ of control ($n=10$), whereas in the presence of additional 100 μM AP_6A, the increase by isoprenaline was attenuated to $104.3\pm10.6\%$ of control ($n=10$). The effects of AP_6A were diminished by 10 μM DPCPX ($145.1\pm8.2\%$ of control). DPCPX (10 μM) alone did not affect phosphorylation of PLB ($109.7\pm3.9\%$ of control). The effects of AP_6A on PLB-phosphorylation in a typical autoradiogram are shown in Figure 5c. The autoradiogram does not depict changes in TnI phosphorylation as PLB is best seen with long exposure whereas TnI is best seen on short exposure (Neumann *et al.*, 1993). However, the phosphorylation of TnI was affected in the same way as PLB: isoprenaline (10 μM) increased TnI-phosphorylation to $128.1\pm5.9\%$ of control, further application of 100 μM AP_6A attenuated the stimulated phosphorylation to $105.1\pm6.7\%$ of control, but not in the presence of 10 μM DPCPX ($123.4\pm6.6\%$ of control, $n=10$). DPCPX alone (10 μM) did not change TnI-phosphorylation ($106.1\pm8.4\%$ of control, $n=10$).

Effects of AP_6A on the L-type calcium current

Application of 100 μM AP_6A to guinea-pig isolated ventricular myocytes did not affect the amplitude of L-type calcium current ($n=4$, data not shown). Stimulation with 0.01 μM isoprenaline increased the calcium current to $508.3\pm66.7\%$ of control ($n=9$, Figure 6a). Additional application of 100 μM AP_6A attenuated the stimulated calcium current to $180.5\pm18.8\%$ of control ($n=5$), i.e. the effect of isoprenaline was reduced by 80%. This effect was markedly attenuated in the presence of 0.3 μM DPCPX (346.3 ± 59.2 of control, $n=4$).

A typical experiment illustrating original current traces and the time course of the amplitude of the L-type calcium current are depicted in Figure 6b. Original current traces correspond to the time points as indicated in the lower trace. Isoprenaline 0.01 μM induced a pronounced increase of the calcium current. This effect was attenuated by the additional application of 100 μM AP_6A. The effect of AP_6A could be reversed by washout.

The current to voltage relationship (Figure 6c) was shifted to the left by 10 μM isoprenaline compared to control ($n=3$). Further application of 100 μM AP_6A shifted the current-to-voltage relation to the right reaching a peak maximum similar to control.

Discussion

Diadenosine polyphosphates are stored in secretory granules of, for example, platelets, chromaffin adrenomedullar cells and

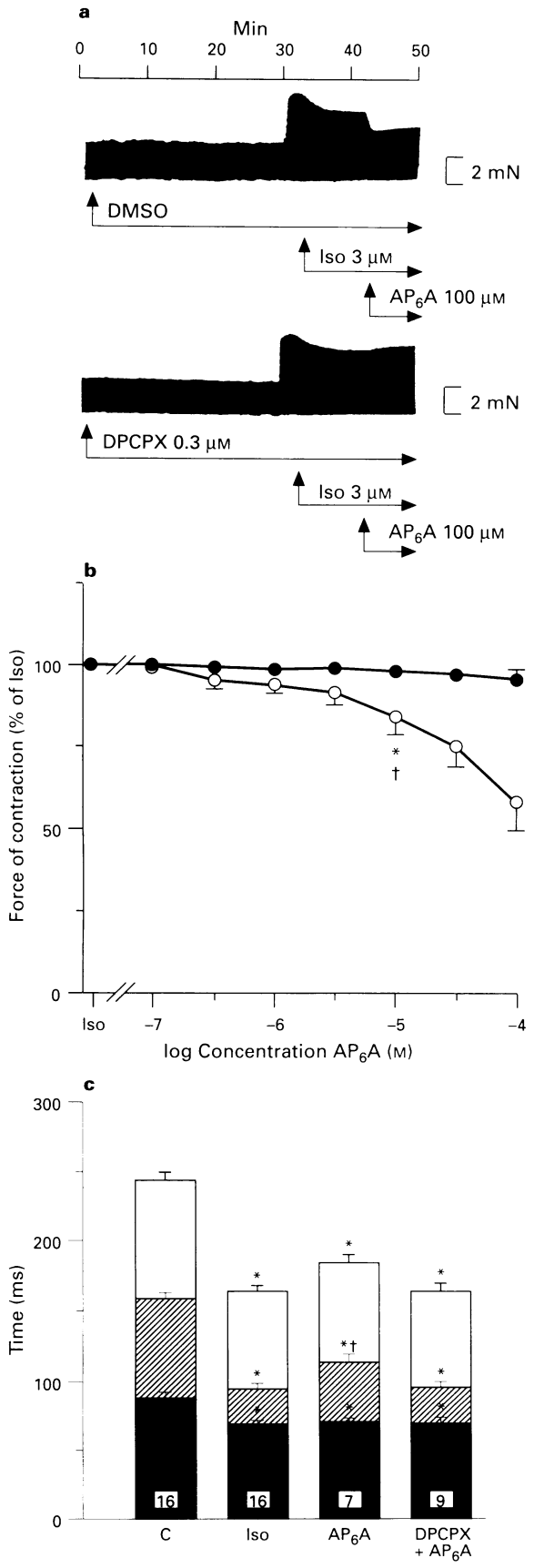

Figure 4 Effects of AP_6A on contractile response in human isolated, electrically driven ventricular preparations. (a) Original recordings illustrating the effect of 100 μM AP_6A on force of contraction in human ventricular preparations prestimulated with 3 μM isoprenaline, (Iso), alone (upper trace) and in the presence of 0.3 μM DPCPX (lower trace). (b) Concentration-response curve for the effects of AP_6A on force of contraction in human ventricular preparations after prestimulation with 3 μM isoprenaline. Effects of AP_6A in the absence (○, $n=7$) and in the presence of 0.3 μM DPCPX (●, $n=9$). Ordinate scale: force of contraction as % of isoprenaline values, vertical lines show s.e.mean. Isoprenaline values amounted to 8.0 ± 1.5 mN ($n=16$). * and † denote the first significant difference versus isoprenaline and DPCPX values, respectively. (c) Effects of AP_6A on time parameters in human ventricular preparations. Effects of AP_6A in the absence and presence of 0.3 μM DPCPX compared to isoprenaline (Iso) and predrug values (C) on time to peak tension (solid part of columns), time of relaxation (cross-hatched part of columns) and total contraction time (open part of columns). Vertical lines show s.e.mean. The numbers in the columns denote the number of experiments. * and † denote significant differences versus predrug and isoprenaline values, respectively.

synaptosomes and they exert a variety of effects as extracellular signalling molecules (for review see Baxi & Vishanatha, 1995). The diadenosine polyphosphate AP_6A has been detected in human platelets, too, and exerts vasoconstrictive effects (Schlüter *et al.*, 1994). Therefore, we wanted to investigate whether AP_6A has any effect on cardiac contractility. AP_6A decreased force and rate of contraction in guinea-pig atrial preparations, whereas in guinea-pig papillary muscles and human ventricular preparations AP_6A decreased force of contraction only in the presence of the cyclic AMP increasing β-adrenoceptor agonist isoprenaline. As it is known that diadenosine polyphosphates activate different P_2-purinoceptors (Hoyle, 1990; Pintor & Miras-Portugal, 1995) we hypothesized that AP_6A also mediates its cardiac effects via purinoceptors. However, the combination of a direct negative chronotropic and inotropic effect in atrial tissue with an 'indirect' negative inotropic effect in ventricular tissue, i.e. only after stimulation with cyclic AMP increasing agonists, is observed after stimulation of cardial M-cholinoceptors (Löffelholz & Pappano, 1985) or A_1-adenosine receptors (Olsson & Pearson, 1990). In our experiments neither the cardiac M-cholinoceptor antagonist atropine (10 μM) nor the P_2-purinoreceptor antagonist suramin (300 μM) altered the effects of AP_6A (data not shown). Only the selective A_1-adenosine receptor antagonist DPCPX (0.3 μM) abolished the effects of AP_6A on rate and force of contraction. The possibility that AP_6A did activate A_1-adenosine receptors after degradation to adenosine is excluded as all experiments were performed in the presence of adenosine deaminase (ADA). ADA converts adenosine to inosine which does not attenuate catecholamine-induced contractile responses (Rockhoff & Dobson, 1980).

All the effects of AP_6A described here are consistent with stimulation of A_1-adenosine receptors. Stimulation of A_1-adenosine receptors of cardiac atrial preparations causes a negative chronotropic effect by slowing of the pacemaker rate in the sinus node (West *et al.*, 1987) and a direct negative inotropic effect due to activation of a potassium outward current by shortening the action potential duration and hence reducing calcium influx and force of contraction (Belardinelli & Isenberg, 1983; Wang & Belardinelli, 1994; Brückner *et al.*, 1985). As AP_6A exerted negative chronotropic and inotropic effects in guinea-pig atrial preparations, the effects of AP_6A found here are consistent with A_1-adenosine receptor stimulation.

In guinea-pig papillary muscles and human ventricular preparations AP_6A exerted negative inotropic effects only after isoprenaline stimulation. Stimulation of A_1-adenosine receptors leads to a reduction of isoprenaline-induced phosphorylation of phosphoproteins (Gupta *et al.*, 1993a; Neumann *et al.*, 1994) and is accompanied by a reduction of isoprenaline-induced force of contraction and rate of relaxation (Dobson, 1983; Brückner *et al.*, 1985; Neumann *et al.*, 1995b). In agreement with these observations we detected a reduction of isoprenaline-induced phosphorylation of PLB and TnI by AP_6A and an accompanying reduction in the isoprenaline-induced force of contraction and rate of relaxation. The reduction of isoprenaline-stimulated force of contraction by adenosine is accompanied by an inhibition of the L-type

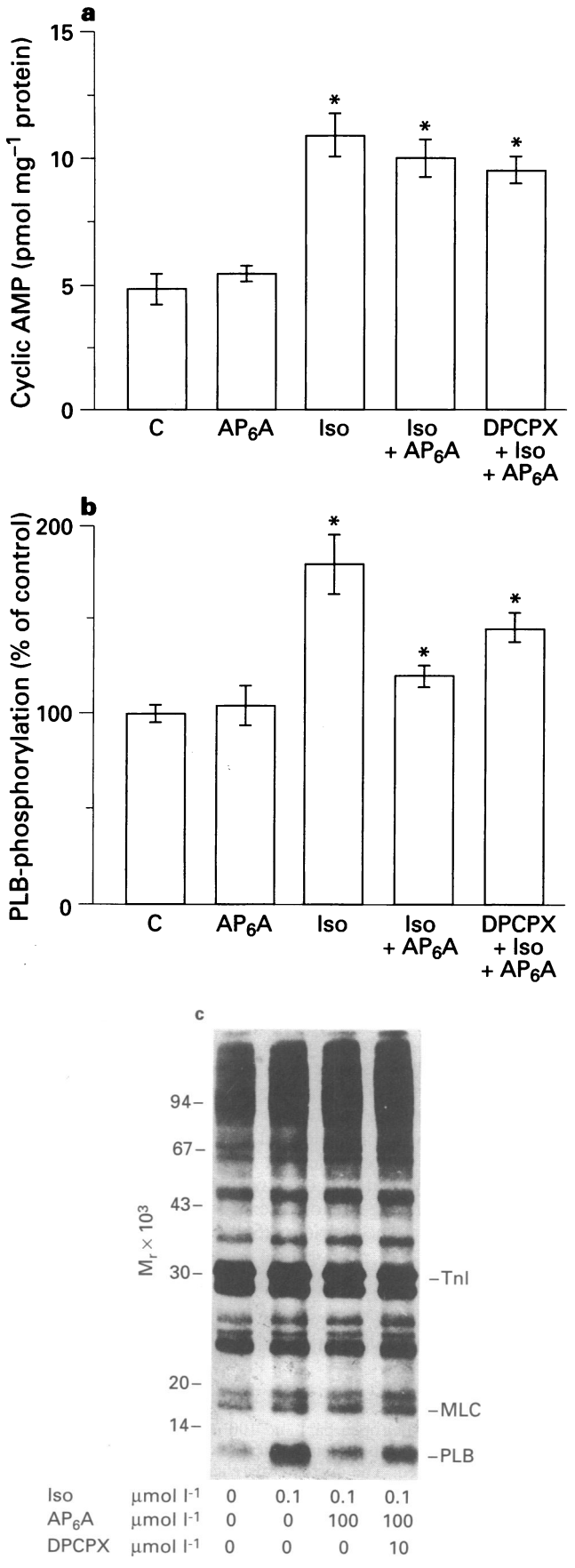

Figure 5 Effects of AP_6A on protein phosphorylation in guinea-pig isolated ventricular myocytes. (a) Effects of AP_6A on the cyclic AMP levels in guinea-pig isolated ventricular myocytes. Effects of 100 μM AP_6A, alone ($n=12$), after stimulation with 0.1 μM isoprenaline ($n=12$) and in the presence of 10 μM DPCPX ($n=12$), compared to control ($n=12$) and isoprenaline ($n=12$). Ordinate scale: cyclic AMP content in pmol mg^{-1} protein. *Significant difference versus control. (b) Effects of AP_6A on the phosphorylation of phospholamban (PLB). Effects of 100 μM AP_6A, alone ($n=12$), after stimulation with 0.1 μM isoprenaline ($n=12$) and in the additional presence of 10 μM DPCPX ($n=12$), compared to control ($n=12$) and isoprenaline ($n=12$). Ordinate scale: PLB-phosphorylation as % of control. * and † denote significant difference versus control and isoprenaline, respectively. (c) Autoradiogram of a 10% polyacrylamide gel depicting the effect of AP_6A on the phosphorylation of phospholamban (PLB). ^{32}P-labelled cardiomyocytes were incubated at 37°C for 5 min in the presence of isoprenaline (lane 2), isoprenaline and AP_6A (lane 3), isoprenaline + DPCPX + AP_6A (lane 4). Molecular weight standards are indicated on the left. MLC, myosin light chains; TnI, troponin 1.

calcium current (Isenberg & Belardinelli, 1984; Kato *et al.*, 1990). As AP_6A markedly attenuated the amplitude of isoprenaline stimulation L-type calcium current, this effect, too, is consistent with stimulation of A_1-adenosine receptors.

In the present study neither AP_6A alone nor AP_6A in the presence of isoprenaline changed the cyclic AMP levels in guinea-pig isolated ventricular myocytes. Although in some species e.g. rats, A_1-adenosine *agonists do* decrease isoprenaline-elevated cyclic AMP content (Dobson, 1978; 1983; Martens *et al.*, 1987), in guinea-pig ventricular preparations only marginal (Neumann *et al.*, 1989; Behnke *et al.*, 1990) or no decreases (Gupta *et al.*, 1993a; Neumann *et al.*, 1994; 1995b) of cyclic AMP content were detected after A_1-adenosine receptor stimulation. Thus, our findings support the hypothesis that AP_6A acts on A_1-adenosine receptors. It is a subject of ongoing research efforts to decide which mechanism mediates the negative inotropic effect on the isoprenaline-stimulated force of contraction in ventricular tissue by A_1-adenosine receptor agonists without affecting the cyclic AMP levels. The signal transduction pathway beyond the A_1-adenosine receptor involves one or more pertussis toxin-sensitive guanine nucleotide-binding protein(s) (Isenberg *et al.*, 1987; Neumann *et al.*, 1994) and the effects of adenosine receptor agonists are at least in part mediated by activation of protein phosphatases (Gupta *et al.*, 1993b; Neumann *et al.*, 1996).

The threshold of the effects of AP_6A on inotropy and chronotropy in guinea-pig cardiac preparations was 1–10 μM. The unspecific adenosine receptor agonist adenosine had the same threshold for its effects on guinea-pig cardiac preparations (Scholz *et al.*, 1987). However, adenosine is more efficacious than AP_6A if tested in the same experimental system (data not shown). The selective A_1-adenosine receptor agonist (−)-N^6-phenyl-isopropyladenosine (**R**-PIA) with a threshold concentration of 0.01–0.1 μM is much more potent than adenosine and AP_6A. Thus, we tested the effects of combined application of **R**-PIA and AP_6A on force of contraction. The effects of the selective A_1-adenosine receptor agonist **R**-PIA (1 μM) were neither enhanced nor reduced by subsequent application of 100 μM AP_6A (see results). This supports the hypothesis that AP_6A acts via A_1-adenosine receptors.

The effects of AP_6A were observed with concentrations greater than 1 μM. However, these effects might play a role in physiological or pathophysiological processes in the heart. Diadenosine polyphosphates are synthesized during protein synthesis in a reaction involving ATP and amino acids catalysed by amino acid tRNA-synthetase (for review see Plateau & Blanquet, 1992). The release of diadenosine polyphosphates into the blood is brought about by release from secretory granules of platelets and chromaffin adrenomedullar cells under certain physiological conditions (e.g. platelet aggregation and stimulation by carbachol, respectively). Enzymes cleaving diadenosine polyphosphates, namely ectohydrolases are also released into the blood by various cell types (for review see Baxi & Vishwanatha, 1995; Guranowski & Sillero, 1992). Interestingly, the metabolism of APnAs is slow in comparison to the nucleotides ATP and AMP (Rodriguez-Pascual *et al.*, 1992), thus providing a longer time for APnAs to reach the target tissues and organ sites.

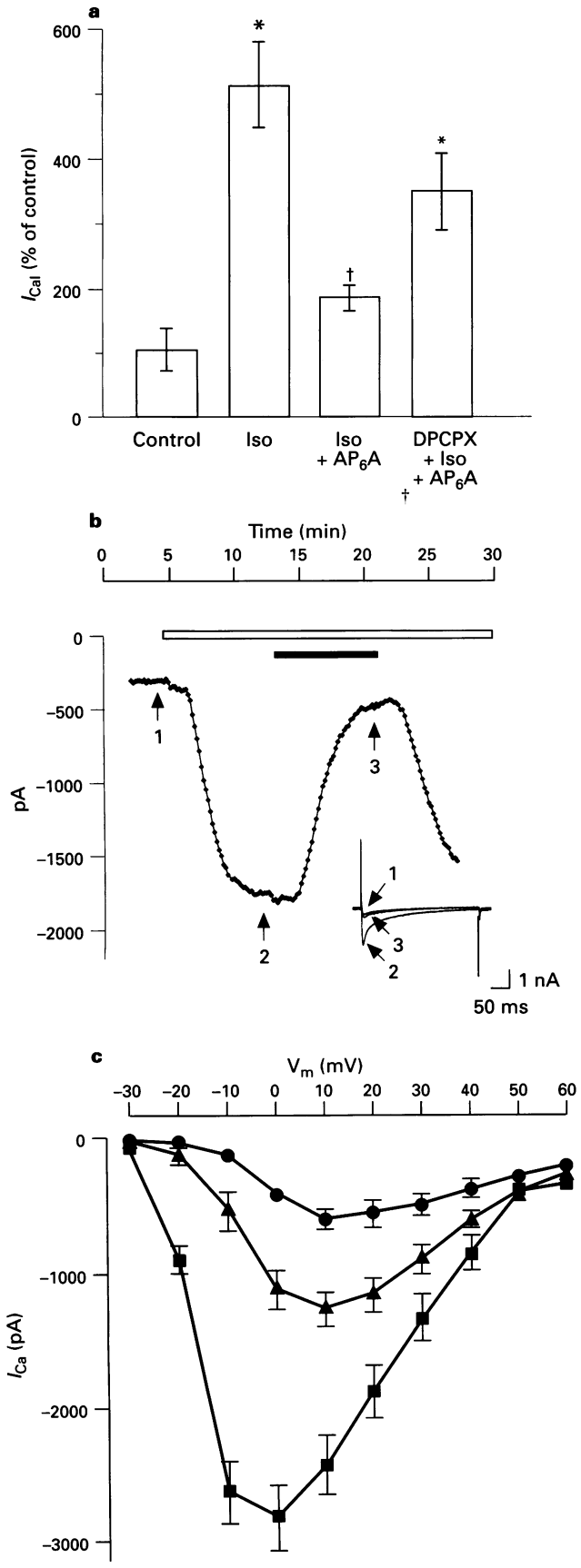

Figure 6 Effects of AP_6A on the L-type calcium current (I_{CaL}) in guinea-pig isolated ventricular myocytes. (a) Effects of AP_6A on the amplitude of L-type calcium current. Effects of 100 μM AP_6A after stimulation with 0.01 μM isoprenaline ($n=5$) and in the presence of 0.3 μM DPCPX ($n=5$), compared to control ($n=9$) and isoprenaline alone values (0.01 μM, $n=9$). Ordinate scale: amplitude of I_{CaL} as % of control. * and † denote significant difference versus control and isoprenaline, respectively. (b) The time course for the effects of 0.01 μM isoprenaline (open bar) and 100 μM AP_6A (solid bar) on the amplitude of the L-type calcium current of a guinea-pig ventricular myocyte is depicted. The current was elicited by voltage steps from −40 mV as holding potential to +10 mV for 300 ms at a frequency of 0.1 Hz. Inset: original traces showing the effect of AP_6A on the amplitude of isoprenaline-stimulated L-type calcium current obtained at the time points 1–3 as indicated by arrows. (c) Effects of AP_6A on the current to voltage relationship. Effects of 100 μM AP_6A on the isoprenaline (0.01 μM) stimulated I_{CaL} (▲, $n=3$) compared to isoprenaline (■, $n=3$) and control current (●, $n=3$). Ordinate: amplitude of L-type calcium current (I_{CaL}) in pA, abcissae: testing potential (V_m) in mV. The current was elicited by voltage steps from −40 mV as holding potential to different testing potentials for 300 ms at a frequency of 0.1 Hz.

Diadenosine polyphosphates have been suggested to act as 'alarmones', which alert the cell to the onset of metabolic stress and subsequently regulate gene or enzyme activities within the cell (Bochner *et al.*, 1984). Furthermore, diadenosine polyphosphates appear after heat shock or exposure to a wide variety of oxidants with several fold increased concentrations, e.g. the level of AP_4A in the whole blood of heat-stressed birds has been shown to increase about 10 fold (Bonaventura & Cashon, 1992). Furthermore AP_nAs obviously have antithrombotic function. During thrombin-induced aggregation almost the whole platelet-content of AP_nAs is released. The AP_nAs are potent antagonists of ADP-induced platelet aggregation and inhibit the release of ADP from platelets (Harrison *et al.*, 1975). In human platelets AP_6A ranged in concentrations from 0.3 to 3 μM of which more than 80% could be released during platelet aggregation (Agha *et al.*, 1992). Beside platelets AP_6A is stored in adrenomedullar chromaffin cells and can be released by certain stimuli, e.g. carbachol (Pintor *et al.*, 1992). Moreover, APnAs are present in the heart tissue (unpublished observation by H. Schlüter) and might be stored here in secretory granules as well, thus raising the possibility of being released by stimulation of, for example, cardiac M-cholinoceptors.

However, up to now it is not evident how and to what extent AP_6A could be liberated from the different sources and the final concentrations that could be achieved *in vivo* in the heart tissue. But, it is conceivable that under certain pathophysiological circumstances 'the alarmone' AP_6A will be formed and released to a greater extent than normal. Further work is required to investigate the physiological or potential pathophysiological role of AP_nAs in cardiac function.

In summary, our investigation shows that AP_6A exerts 'direct' negative inotropic and chronotropic effects in guinea-pig atrial preparations and attenuates the ventricular inotropic response to adrenergic stimulation in guinea-pig and human heart preparations. Furthermore, AP_6A abolished the relaxant effect of isoprenaline and attenuated the isoprenaline-stimulated L-type calcium current. These effects were accompanied by a decrease of the isoprenaline stimulated phosphorylation of regulatory phosphoproteins, whereas the cyclic AMP levels remained elevated. These effects are similar to those observed on stimulation of A_1-adenosine receptors and were sensitive to the selective A_1-adenosine receptor antagonist DPCPX. Hence, our data provide strong evidence that AP_6A mediates its cardiac effects via an action on A_1-adenosine receptors. Thus, diadenosine polyphosphates can stimulate P_1- in addition to P_2-purinoceptors. To our knowledge this is the first account of AP_6A exerting effects on the heart. Although the physiological and potential pathophysiological role of AP_6A remains to be elucidated, it is conceivable that AP_6A may protect the myocardium from sympathetic overstimulation in the same way as adenosine. However, adenosine exerts vasodilatation, whereas AP_6A acts as a vasoconstrictor (Schlüter *et al.*, 1995).

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