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Resistance to antagonism of atrial P_1 purinoceptor responses in the presence of K^+ channel blockade

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Abstract

The rate of onset of the negative inotropic responses of guinea-pig isolated paced atria to the adenosine receptor agonist, N^6-cyclopentyladenosine, was significantly slowed by the K^+ channel inhibitor, 4-aminopyridine (10 mM). The concentration-dependent inhibition of developed tension by N^6-cyclopentyladenosine, however, was unaffected by 4-aminopyridine (10 mM). Thus, K^+ efflux only governs the speed of onset of the negative inotropic response and does not appear to be a major component in the negative inotropy produced by the adenosine A_1 receptor agonist. The P_1 purinoceptor antagonist, 8(*p*-sulfophenyl)theophylline (1×10^{-5} M) significantly shifted the concentration–response curve for N^6-cyclopentyladenosine to the right (concentration-ratio, 7.1 ± 1.5). In the presence of 4-aminopyridine (10 mM), 8(*p*-sulfophenyl)theophylline caused a non-parallel rightwards shift of the curve. At the IC_{35} there was no significant shift, whereas at the IC_{75} there was a small significant displacement of the curve. The adenosine A_1/A_3 receptor agonist, N^6-2-(4-aminophenyl)ethyladenosine (APNEA) yielded a biphasic concentration–response curve which was significantly shifted to the right by 8(*p*-sulfophenyl)theophylline (1×10^{-5} M). In the presence 4-aminopyridine, however, there was no shift of the APNEA concentration–response curve by 8(*p*-sulfophenyl)theophylline. These results show that when K^+ channels are blocked by 4-aminopyridine, the residual response is resistant to antagonism by the P_1 purinoceptor antagonist, 8(*p*-sulfophenyl)theophylline. This residual component may involve L-type Ca^{2+} channels, the adenosine A_1 receptor being possibly coupled to the two transduction pathways for negative inotropism via the different components of the G protein (receptor-transducer promiscuity).

Keywords: Atrium, left, paced, guinea pig; Adenosine receptor agonist; APNEA (N^6-2-(4-aminophenyl)ethyladenosine); 4-Aminopyridine; N^6-Cyclopentyladenosine; K^+-channel; 8(*p*-Sulfophenyl)theophylline

1. Introduction

Stimulation of the adenosine A_1 receptors in the left atria induces an efflux of K^+ (Belardinelli and Isenberg, 1983). This stimulation of K^+ efflux promotes a shortening of the action potential duration and hence allows less time for Ca^{2+} influx into the cardiac myocytes. Since contraction of the cardiac myocytes is dependent on the intracellular Ca^{2+} level, a fall in contraction size is observed.

Studies to investigate the role of K^+ efflux in mediating the negative inotropic responses of adenosine receptor agonists have normally involved the quantification of rubidium-86 (Rb^{86}) efflux (Urquhart et al., 1991), since the use of radioactive K^+-42 is impracticable. Rb^{86} efflux has been shown to be qualitatively equivalent to K^+ movements (Van Zwieten, 1968; Smith et al., 1986; Quast and Baumlin, 1988). Urquhart et al. (1991) showed that the K^+ channel blocker 4-aminopyridine at a concentration of 10 mM antagonised the efflux of Rb^{86} from left atria induced by adenosine. At the same concentration, 4-aminopyridine inhibited the negative inotropic response to the selective adenosine A_1 receptor agonist, (−)-N^6-(2-phenylisopropyl)adenosine (Urquhart et al.,1993). The latter result was at variance with that of other observers who have reported that 4-aminopyridine at a concentration of 3 mM failed to shift the concentration–response curves (De Biasi et al., 1989). Ford and Broadley (1999) suggested that the differences in the apparent ability of 4-aminopyridine to block the (−)-N^6-(2-phenylisopropyl)adenosine concentration–

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response curve was due to insufficient time being allowed for each (−)-N^6-(2-phenylisopropyl)adenosine response to plateau. In the presence of 4-aminopyridine, the negative inotropic response to (−)-N^6-(2-phenylisopropyl)adenosine is slowed and takes approximately 10 min to reach its plateau compared with about 3 min in the absence of 4-aminopyridine. This study, however, failed to consider that 4-aminopyridine has the ability to release neuronal catecholamine stores (Glover, 1981). The results observed could therefore have been due to a mixture of direct and indirect anti-adrenergic effects of the adenosine receptor agonist employed.

The present study was therefore undertaken to evaluate the effect of 4-aminopyridine on the response of the adenosine A_1 receptor selective agonist N^6-cyclopentyladenosine in left atria from animal pretreated with reserpine to deplete neuronal catecholamine stores. Since reserpine may have adverse effects on cardiac tissue (Torphy et al., 1982), the β-adrenoceptor antagonist propranolol was also used as an alternative means of blocking the effects of any noradrenaline released by 4-aminopyridine. We have previously shown that the antagonism of the negative inotropic responses of guinea-pig left atria to N^6-cyclopentyladenosine by 8(*p*-sulfophenyl)theophylline (Gardner and Broadley, 1999) and to (−)-N^6-(2-phenylisopropyl)adenosine by cyclopentyltheophylline (Ford and Broadley, 1997) is atypical. There was a limit to the shift of the concentration–response curves at higher concentrations of antagonist and therefore a plateauing of the Schild plots. The 8(*p*-sulfophenyl)theophylline- or cyclopentyltheophylline-resistant component could have arisen from a duality of coupling of the A_1 receptor through either different G proteins or to independent transduction pathways. This has been referred to as receptor-transducer promiscuity (Kenakin, 1993). One of these pathways could be via K^+ efflux and the other could be through Ca^{2+} channel closure. A role for L-type Ca^{2+} channel blockade in the atrial negative inotropic response to adenosine A_1 receptor stimulation has been suggested by several authors (Visentin et al., 1990; Fassina et al., 1991; Jahnel et al., 1992). However, in atrial myocytes, adenosine has been shown to cause negative inotropy by activation of the inwardly rectifying K^+-current (I_{KAch}) and not by a direct decrease in L-type Ca^{2+} inward current (Wang and Belardinelli, 1994). When cAMP levels are raised in atrial myocytes, adenosine can lower cAMP and promote a decrease in Ca^{2+} channel activity via dephosphorylation of the channel protein (Jahnel et al., 1992). The present study examines the hypothesis that the atypical blockade by 8(*p*-sulfophenyl)theophylline arises because two transduction pathways may be involved in the atrial negative inotropic response to adenosine A_1 receptor stimulation. The antagonism of N^6-cyclopentyladenosine by 8(*p*-sulfophenyl)theophylline was therefore examined in both the absence and presence of 4-aminopyridine to block the K^+ channel component.

2. Materials and methods

Experimental animals were housed and used according to the guidelines of the Animals (Scientific Procedures) Act 1986.

2.1. Isolated tissue preparation

Male Dunkin–Hartley guinea-pigs (250–300 g) were killed by a blow to the back of the head followed by exsanguination under running water. The rib cage was removed to expose the heart. Left atria were removed by a means of cotton threads attached to the tip of the left atrial appendage and atrioventricular junction. The latter thread attached the atrium to the electrode tips of a Harvard bipolar platinum electrode and the first thread was attached to an isometric transducer (type UFI, 57 g sensitivity range). A resting diastolic tension of 0.5–1.0 g was then applied.

The tension developed by the atria was displayed on an 8 channel Devices MT8P polygraph. Left atria were electrically stimulated at 2 Hz using square pulse waves, of 5 ms duration at threshold voltage +50%, delivered by a Harvard 50–72 stimulator. The parameters used for electrical stimulation have previously been shown to drive cardiac tissue without causing significant autonomic transmitter releases, when delivered by electrodes in direct contact with the tissue (Koch-Weser and Blinks, 1963).

The tissues were suspended in 50 ml organ baths containing Krebs–bicarbonate solution maintained at 37 ± 0.5°C by a Grant FH15 circulator and gassed with 5% CO_2 in oxygen. The Krebs–bicarbonate solution was made up in double distilled water and had the following composition in mM: NaCl 118.0, KCl 4.7, $CaCl_2.2H_2O$ 2.5, $MgSO_4.7H_2O$ 1.2, $NaHCO_3$ 24.9, $KH_2PO_4.2H_2O$ 1.2, glucose 11.6. An equilibration period of approximately 60 min was allowed before addition of the adenosine receptor agonist. During this period, the bathing Krebs solution was replaced with fresh Krebs every 15 min.

2.2. Drug administration

After the 60-min equilibration period, cumulative concentration–response curves were constructed by additions of half-logarithmic increases in concentration of agonist. Each response was allowed to plateau (about 6 min in the presence of 4-aminopyridine) before a subsequent agonist concentration was added. Wherever possible, the volume of individual bolus doses of agonists added to the bath did not exceed 0.3 ml. In experiments involving 8(*p*-sulfophenyl)theophylline, it was added 30 min into the equilibration period and remained present throughout the experiment. Agonist concentration–response curves were then constructed in the presence of the antagonist as described above.

In some experiments, 4-aminopyridine (10 mM) was added 45 min into the 60-min equilibration period and was present throughout the experiment. Where propranolol was used to block the effects of any catecholamines released by 4-aminopyridine, a concentration of 1×10^{-6} M was added 15 min prior to the addition of 4-aminopyridine. In experiments to observe the effect of a single concentration of N^6-cyclopentyladenosine, a single bolus dose (3×10^{-8} M) was added after the 60 min equilibration period and the effects observed over a 5 min period. Each tissue received only one concentration–response curve or single concentration exposure, thus eliminating the need for time controls.

In experiments involving tissue from reserpinised animals, the guinea-pigs received reserpine (5 mg/kg i.p.) 18–24 h prior to sacrifice of the animal. To validate the success of the reserpinisation, a dose (1×10^{-4} M) of the indirectly acting sympathomimetic amine, tyramine (Broadley, 1996) was added prior to the commencement of any experimental protocol. The positive inotropic response to tyramine in the reserpine pretreated tissues ($106 \pm 0.8\%$ of the pre-drug developed tension) was virtually abolished compared with the control ($146 \pm 7.9\%$), indicating substantial depletion of the endogenous noradrenaline stores.

2.3. Data analysis and statistics

Responses to each concentration of agonist were measured as the reduction of tension from the resting developed tension immediately preceding the start of the concentration–response curve. These were then expressed as the percent of the resting developed tension (systolic–diastolic tension). Values are reported as mean ± standard error of the mean (S.E.M.) ($n \geq 4$). Maximum responses of each concentration–response curve were expressed as the maximum inhibition of the resting developed tension and mean ± S.E.M. calculated.

IC_{50} (molar concentration producing 50% of the maximum inhibition of initial tension) or IC_{35} and IC_{75} values were calculated from the individual concentration–response curves. Geometric mean values were then calculated as the anti-log of the mean log IC_{50}, IC_{35} or IC_{75} and presented with the 95% confidence limit.

Tension responses from the single concentration experiments were compared by means of an unpaired Student's *t*-test. The IC_{35}, IC_{50} and IC_{75} of cumulative-concentration response curves were compared by means of a one way analysis of variance (ANOVA) followed by a post hoc Duncan multiple range test. Maximal responses for the concentration–response curves were compared by an unpaired Student's *t*-test. Results were taken to be statistically different if probability (P) values of less than 0.05 were returned.

2.4. Drugs and solutions

N^6-2-(4-aminophenyl)ethyladenosine (APNEA) (Semat), 4-aminopyridine (Sigma, Poole, Dorset, UK), N^6-cyclopentyladenosine (Sigma), propranolol (Inderal™ injection 1 mg/ml, Zeneca Pharmaceuticals, Macclesfield, UK), reserpine (Sigma), 8-(*p*-sulfophenyl)theophylline (Semat) and tyramine (Sigma) were obtained commercially.

4-aminopyridine, N^6-cyclopentyladenosine and APNEA were initially dissolved in water:PEG 400 (50:50). Further dilution of N^6-cyclopentyladenosine and APNEA solutions were made using double distilled water. 8(*p*-sulfophenyl)theophylline was dissolved in double distilled water. Reserpine was dissolved by adding benzyl alcohol (0.08 ml) to reserpine (10 mg) and citric acid (10 mg). To this, Tween 80 (0.4 ml) was added and the concentration adjusted to 2.5 mg of reserpine per ml using distilled water.

3. Results

3.1. Negative inotropic responses to a single concentration of N^6-cyclopentyladenosine

In control untreated guinea-pig left atria, a single bolus concentration (3×10^{-8} M) of N^6-cyclopentyladenosine

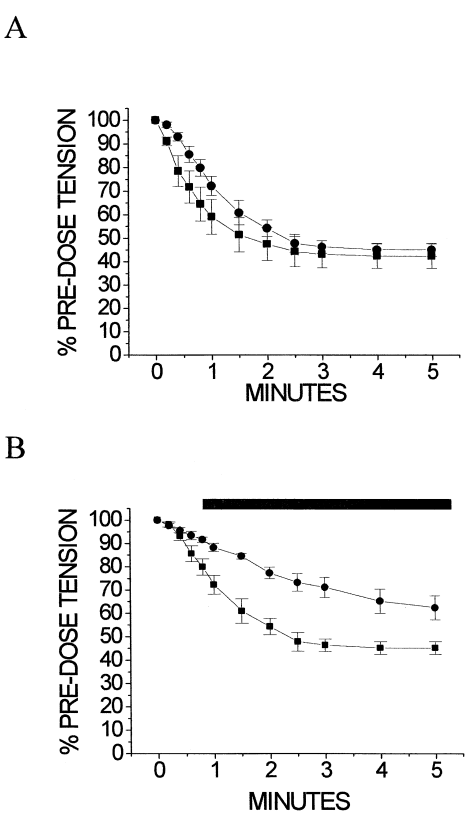

Fig. 1. Mean time-courses for the negative inotropic responses of guinea-pig left atria to a single concentration (3×10^{-8} M) of N^6-cyclopentyladenosine. (A) Comparison of atria from untreated (■, $n = 6$) and reserpinised guinea-pigs (●, $n = 4$). No points were significantly different. (B) Effect of N^6-cyclopentyladenosine in atria from reserpinised guinea-pigs in the absence (■, $n = 4$) and presence (●, $n = 4$) of 4-aminopyridine (10 mM). The horizontal bar indicates that points over this time period are significantly different. Error bars represent S.E.M.

caused a time-dependent negative inotropy (Fig. 1). The onset of the response to N^6-cyclopentyladenosine was rapid, occurring 12 s after the addition of N^6-cyclopentyladenosine and plateauing after 3 min.

The time-course to a single concentration of N^6-cyclopentyladenosine was not significantly affected by either reserpinisation (Fig. 1A) or the addition of propranolol (1×10^{-6} M) (Fig. 2A).

3.2. Effects of 4-aminopyridine on the negative inotropic responses to a single concentration of N^6-cyclopentyladenosine

In reserpinised tissue, the addition of 4-aminopyridine to the bathing solution caused a significant ($P < 0.05$) transient positive inotropy ($142.0 \pm 4.0\%$ of the initial resting tension, $n = 4$) which then fell to a level not significantly ($P > 0.05$) different from the pre-4-aminopyridine resting tension. The peak increase in tension ($142.0 \pm 4.0\%$ of resting developed tension)and the duration of this positive inotropy (3.1 ± 0.4 min, $n = 4$) were significantly less ($P < 0.05$) than the peak ($199.2 \pm 10.4\%$,

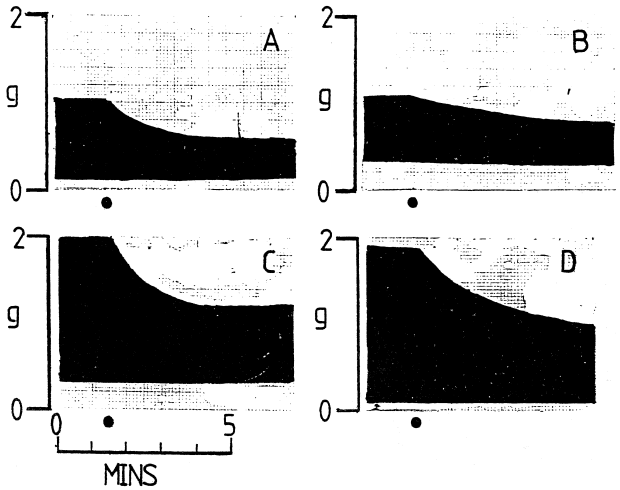

Fig. 3. Typical recordings of individual time-courses for the negative inotropic response of guinea-pig left atria to a single concentration (3×10^{-8} M) of N^6-cyclopentyladenosine, added to the bath at ●. (A) In reserpinised tissue. (B) In the presence of 4-aminopyridine (10 mM) in reserpinised tissue. (C) In the presence of propranolol (1×10^{-6} M). (D) In the presence of 4-aminopyridine (10 mM) and propranolol (1×10^{-6} M).

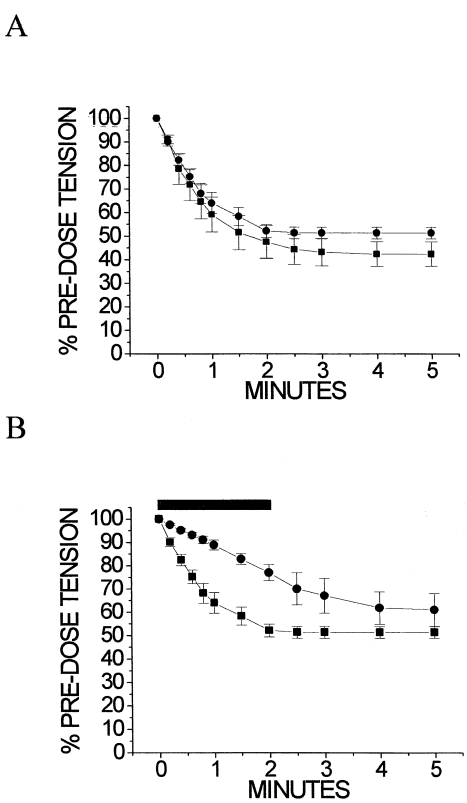

Fig. 2. Mean time-courses for the negative inotropic response of guinea-pig left atria to a single concentration (3×10^{-8} M) of N^6-cyclopentyladenosine. (A) Comparison of atria in the presence (●) ($n = 4$) and absence (■) ($n = 6$) of propranolol (1×10^{-6} M). No points were significantly different. (B) Effects of N^6-cyclopentyladenosine in the presence of propranolol (1×10^{-6} M) in the presence (●) ($n = 4$) and absence (■) ($n = 4$) of 4-aminopyridine (10 mM). The horizontal bar indicates that points over this time period were significantly different. Error bars represent S.E.M.

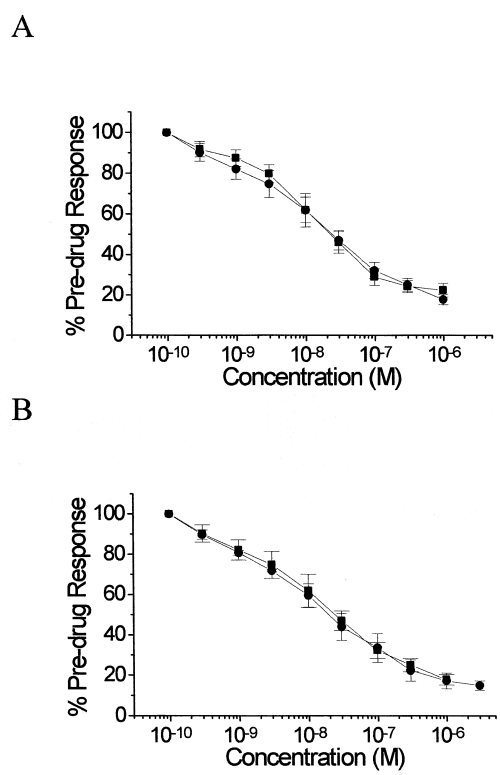

Fig. 4. Mean ($n = 4$) concentration–response curves for the negative inotropic responses to N^6-cyclopentyladenosine in guinea-pig isolated left atria. (A) Comparison of atria from untreated (■) and reserpinised (●) guinea-pigs. (B) N^6-cyclopentyladenosine in left atria from reserpinised guinea-pigs in the absence (■) and presence (●) of 4-aminopyridine (10 mM). No points were significantly different. Error bars denote S.E.M.

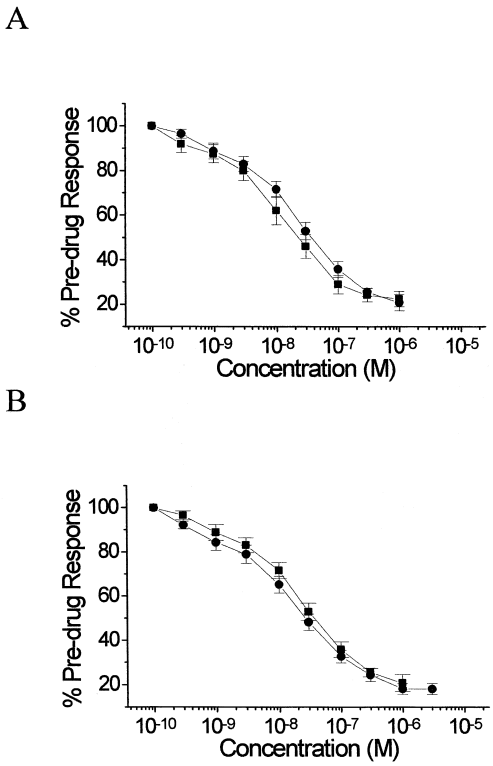

Fig. 5. Mean ($n = 4$) concentration–response curves for the negative inotropic responses to N^6-cyclopentyladenosine in guinea-pig isolated left atria. (A) Comparison of atria in the presence (●) and absence (■) of propranolol (1×10^{-6} M). (B) Effects of N^6-cyclopentyladenosine in the presence of propranolol (1×10^{-6} M) in the presence (●) and absence (■) of 4-aminopyridine (10 mM). Error bars denote S.E.M. No points were significantly different.

$n = 4$) and duration (9.9 ± 1.1 min) of the untreated control.

In the presence of propranolol (1×10^{-6} M), 4-aminopyridine caused a significant ($P < 0.05$) positive inotropy ($167.0 \pm 4.8\%$ of resting developed tension, $n = 4$) which was significantly less ($P < 0.05$) than in the untreated control atria. The tension then fell to a level ($105.0 \pm 7.0\%$) that was not significantly different from the pre-4-aminopyridine resting developed tension ($P > 0.05$). The duration of this positive inotropy (5.1 ± 0.4 min, $n = 4$) was significantly less ($P < 0.05$) than the untreated control (9.9 ± 1.1 min, $n = 4$), but not significantly different ($P > 0.05$) from that in the reserpinised tissue.

In reserpinised tissue, 4-aminopyridine significantly ($P < 0.05$) slowed the onset of the negative inotropy produced by a single concentration of N^6-cyclopentyladenosine (Figs. 1B and 3B). The maximal effect, measured at 5 min after addition of the N^6-cyclopentyladenosine, was also significantly attenuated ($P < 0.05$).

In the presence of propranolol (1×10^{-6} M), 4-aminopyridine significantly ($P < 0.05$) slowed the onset of the negative inotropy produced by a single concentration of N^6-cyclopentyladenosine, but the maximum extent of the negative inotropy was not significantly ($P > 0.05$) reduced (Figs. 2B and 3D).

3.3. Negative inotropic effects of N^6-cyclopentyladenosine added cumulatively

N^6-cyclopentyladenosine caused a concentration-dependent negative inotropy (IC_{50} $1.5(1.0–2.0) \times 10^{-8}$ M) with a $77.5 \pm 3.4\%$ ($n = 6$) maximal inhibition of resting tension (Fig. 4A). The mean IC_{50} values and maximum inhibition of resting developed tension for N^6-cyclopentyladenosine were not significantly ($P > 0.05$) different in either reserpinised tissue ($0.9(0.3–2.2) \times 10^{-8}$ M, $82.1 \pm 4.0\%$) (Fig. 4A), or in the presence of 1×10^{-6} M propranolol ($1.9(1.4–2.6) \times 10^{-8}$ M, $79.0 \pm 4.0\%$) (Fig. 5A).

3.4. Effect of 4-aminopyridine on concentration–response curves to N^6-cyclopentyladenosine in reserpinised tissue

4-aminopyridine (10 mM) did not shift the concentration–response curve to N^6-cyclopentyladenosine in reserpinised tissues (Fig. 4B). Neither the mean IC_{35} nor IC_{75} values were significantly different ($P > 0.05$) from the reserpine control values (Table 1). The maximum inhibition of resting tension by N^6-cyclopentyladenosine was also not significantly different ($P > 0.05$) compared to the reserpinised control (Fig. 4B, Table 1).

3.5. Effect of 4-aminopyridine on concentraton–response curves to N^6-cyclopentyladenosine in the presence of propranolol

4-aminopyridine (10 mM) did not shift the concentration–response curve to N^6-cyclopentyladenosine (Fig. 5B)

Table 1
Effects of 8(*p*-sulfophenyl)theophylline (1×10^{-5} M) and 4-aminopyridine (10 mM) on the IC_{35} and IC_{75} values and maximum responses for the negative inotropic responses to N^6-cyclopentyladenosine in reserpinised guinea-pig left atria
ns = not significant.

Treatment	*n*	IC_{35} (M)	IC_{75} (M)	Maximum response (% inhibition of resting tension)
Control	4	$3.5\ (0.4–28.0) \times 10^{-9}$	$5.6\ (3.6–8.8) \times 10^{-8}$	82.1 ± 3.0
4-Aminopyridine	4	$4.4\ (1.5–13.0) \times 10^{-9}$ ns	$5.9\ (1.1–30.0) \times 10^{-8}$ ns	82.6 ± 4.0
8(*p*-sulfophenyl)theophylline	4	$4.5\ (0.5–49.0) \times 10^{-9}$ ns	$34.0\ (19.0–61.0) \times 10^{-8}$ [a]	83.2 ± 2.0

[a] Denotes significant ($P < 0.05$) difference from control.

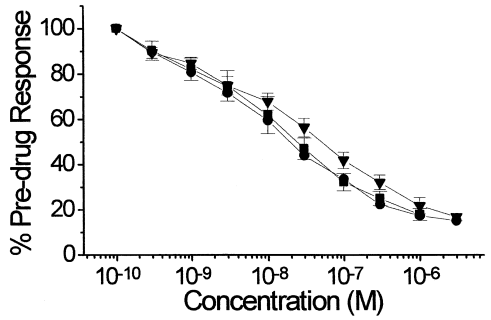

Fig. 6. Mean ($n = 4$) concentration–response curves for the negative inotropic responses to N^6-cyclopentyladenosine in isolated left atria from reserpinised guinea-pigs. Control left atria (■), in the presence of 4-aminopyridine 10 mM (●) and in the presence of 8(*p*-sulfophenyl)theophylline 1×10^{-5} M (▼). Error bars denote S.E.M.

in the presence of propranolol (1×10^{-6} M). Neither the mean IC_{35} nor IC_{75} values were significantly different ($P > 0.05$) from the control values in the absence of propranolol (Table 1). The maximum inhibition of resting tension by N^6-cyclopentyladenosine was also not significantly different ($P > 0.05$) compared to the control (Fig. 5B, Table 1).

3.6. Effect of 8(p-sulfophenyl)theophylline on concentraton–response curves to N^6-cyclopentyladenosine in reserpinised tissue

8(*p*-sulfophenyl)theophylline (1×10^{-5} M) caused a non-parallel rightward shift of the N^6-cyclopentyladenosine concentration–response curve in reserpinised tissues (Fig. 6). The non-parallel nature of this shift was emphasised by the fact that the mean IC_{35} value in the presence of 8(*p*-sulfophenyl)theophylline was not significantly different ($P > 0.05$) from the value in its absence, while the mean IC_{75} in the presence of 8(*p*-sulfophenyl)theophylline was significantly greater ($P < 0.05$) than in the reserpine control in its absence (Table 2). At the IC_{50} value, the mean shift of the N^6-cyclopentyladenosine concentration–response curve produced by 8(*p*-sulfophenyl)theophylline (1×10^{-5} M), measured as the concentration ratio, was 4.1 ± 1.3. In untreated control atria, 8(*p*-sulfophenyl)theophylline (1×10^{-5} M) caused a 12.5 ± 4.6 fold shift of the concentration–response curve, which was significantly more ($P < 0.05$) than obtained in the reserpinised tissues. The non-parallel shift of the N^6-cyclopentyladenosine concentration–response curve produced by 8(*p*-sulfophenyl)theophylline, and its decreased ability to antagonise the N^6-cyclopentyladenosine concentration–response curves in reserpinised tissues, led to reserpinisation being abandoned as a method to prevent catecholamine release by 4-aminopyridine in further studies.

3.7. Effect of 8(p-sulfophenyl)theophylline on concentraton–response curves to N^6-cyclopentyladenosine in the presence of propranolol and 4-aminopyridine

In the absence of 4-aminopyridine but presence of propranolol, 8(*p*-sulfophenyl)theophylline (1×10^{-5} or 1×10^{-4} M) caused significant ($P < 0.05$) parallel rightward shifts of N^6-cyclopentyladenosine concentration–response curves (Fig. 7). The mean IC_{35} and IC_{75} values were significantly different ($P < 0.05$) from the control values in the absence of 8(*p*-sulfophenyl)theophylline. The shift of the curve (concentration ratio at the IC_{50}, 7.1 ± 1.5) caused by 1×10^{-5} M 8(*p*-sulfophenyl)theophylline in the presence of propranolol was not significantly different ($P > 0.05$) from that obtained in the absence of propranolol (concentration-ratio, 12.5 ± 4.6).

In the presence of 4-aminopyridine (10 mM) and propranolol, 8(*p*-sulfophenyl)theophylline (1×10^{-5} and 1×10^{-4} M) caused rightward shifts of the N^6-cyclopentyladenosine concentration–response curve (mean IC_{50} values, $5.4(2.8–10.4) \times 10^{-8}$ and $11.4(4.0–88.0) \times 10^{-8}$ M), the latter significantly ($P < 0.05$), compared to the control in the presence of 4-aminopyridine and propranolol ($0.83(0.51–1.36) \times 10^{-8}$ M) (Fig. 7). The N^6-cyclopen-

Table 2
Effect of 4-aminopyridine (10 mM) and 8(*p*-sulfophenyl)theophylline (8-sPT, 1×10^{-5}or 1×10^{-4} M) on the IC_{35} and IC_{75} values and maximum responses for the negative inotropic responses to N^6-cyclopentyladenosine in guinea-pig left atria in the presence of propranolol (1×10^{-6} M)
ns = not significantly different from propranolol control.

Treatment	*n*	IC_{35} (M)	IC_{75} (M)	Maximum response (% inhibition of resting tension)
Control	6	$8.3\ (1.5–45.4) \times 10^{-9}$	$3.9\ (0.7–20.0) \times 10^{-8}$	77.5 ± 3.4
Propranolol	4	$8.5\ (4.1–18.0) \times 10^{-9}$	$6.9\ (4.5–11.0) \times 10^{-8}$	79.0 ± 4.0
4-Aminopyridine + propranolol	4	$7.0\ (2.4–20.0) \times 10^{-9}$ ns	$6.0\ (3.6–10.0) \times 10^{-8}$ ns	81.7 ± 2.0
8-sPT (1×10^{-5} M) + propranolol	4	$77.0\ (36.0–200.0) \times 10^{-9}$ [a]	$53.0\ (28.0–100.0) \times 10^{-8}$ [a]	82.0 ± 3.0
8-sPT (1×10^{-5} M) + 4-aminopyridine + propranolol	4	$11.0\ (1.8–72.0) \times 10^{-9}$ ns	$26.0\ (8.7–77.0) \times 10^{-8}$ [a]	80.2 ± 3.0
8-sPT (1×10^{-4} M) + propanolol	4	$1600\ (870–3160) \times 10^{-9}$ [a]	$900\ (820–999) \times 10^{-8}$ [b]	77.6 ± 4.3
8-sPT (1×10^{-4} M) + 4-minopyridine + propranolol	4	$20.0\ (3.0–151.0) \times 10^{-9}$ ns	$1200\ (80–1800) \times 10^{-8}$ [c]	79.9 ± 1.3

[a] Denotes significant ($P < 0.05$) difference from propranolol control.
[b] ($n = 2$, because not all tissues reached 75% inhibition).
[c] ($n = 3$, because not all tissues reached 75% inhibition).

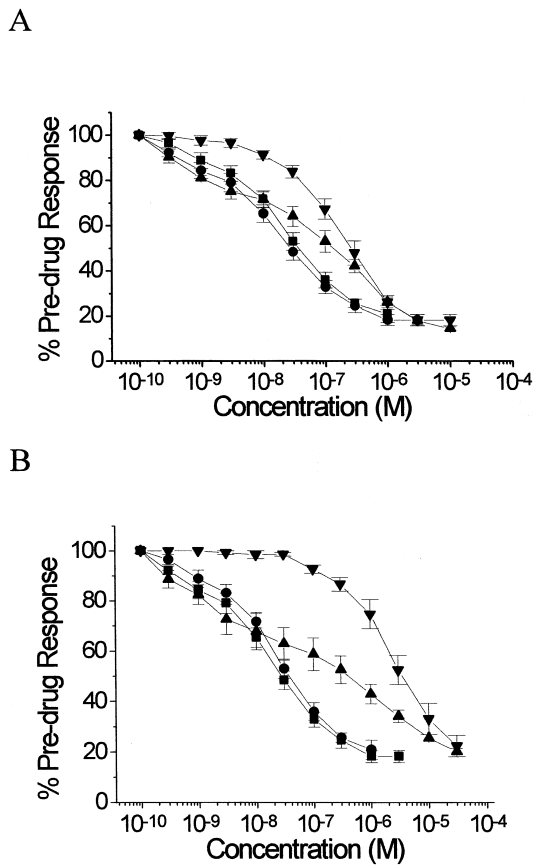

Fig. 7. Effects of 8(*p*-sulfophenyl)theophylline on the mean ($n = 4$) concentration–response curves for the negative inotropic responses to N^6-cyclopentyladenosine in guinea-pig isolated left atria. Control curves are in the presence of propranolol (1×10^{-6} M) (■) or propranolol (1×10^{-6} M) and 4-aminopyridine (10 mM) (●). (A) Effect of 8(*p*-sulfophenyl)theophylline at 1×10^{-5} M, curves in the presence of propranolol (1×10^{-6} M), 4-aminopyridine (10 mM) and 8(*p*-sulfophenyl)theophylline (1×10^{-5} M) (▲), propranolol and 8(*p*-sulfophenyl)theophylline (1×10^{-5} M) alone (▼). (B) Effect of 8(*p*-sulfophenyl)theophylline at 1×10^{-4} M, curves in the presence of propranolol (1×10^{-6} M), 4-aminopyridine (10 mM) and 8(*p*-sulfophenyl)theophylline (1×10^{-4} M) (▲), propranolol and 8(*p*-sulfophenyl)theophylline (1×10^{-4} M) alone (▼). Error bars denote S.E.M.

tyladenosine concentration–response curve was not, however, shifted in a parallel fashion. This non-parallel shift of the N^6-cyclopentyladenosine concentration–response curve by 8(*p*-sulfophenyl)theophylline in the presence of 4-aminopyridine is shown by the fact that the IC_{35} values for N^6-cyclopentyladenosine in the combined presence of 4-aminopyridine and 8(*p*-sulfophenyl)theophylline (1×10^{-5} and 1×10^{-4} M) were not significantly ($P > 0.05$) different from the 4-aminopyridine and propranolol control. In contrast, the mean IC_{75} value for N^6-cyclopentyladenosine in the presence of both 8(*p*-sulfophenyl)theophylline (1×10^{-5} M) and 4-aminopyridine was significantly different ($P < 0.05$) from the 4-aminopyridine and propranolol control (Table 2). In the case of 1×10^{-4} M 8(*p*-sulfophenyl)theophylline, not all tissues reached 75% inhibition and therefore the IC_{75} values were incomplete, however, a substantial increase in the IC_{75} value was still evident (Table 2).

3.8. Effect of APNEA on left atrial tension

APNEA added to the left atria caused a concentration-dependent negative inotropy (Fig. 8). The concentration–response curve to APNEA appears to be biphasic with a shallow phase of negative inotropy over the concentration range of 10^{-9} M to 3×10^{-7} M followed by a steeper phase to the maximal effect at 3×10^{-5} M (Fig. 8).

3.9. Effect of 4-aminopyridine on concentraton–response curves to APNEA in the presence of propranolol

In the presence of 4-aminopyridine (10 mM), the concentration–response curve to APNEA was significantly ($P < 0.05$) shifted to the left, i.e., the responses were potentiated (Fig. 8). The IC_{50} value was significantly less ($P < 0.05$) than that in the presence of only propranolol (Table 3). The maximum response to APNEA was not significantly ($P > 0.05$) different in the presence of 4-aminopyridine compared to controls Table 3).

3.10. Effect of 8(p-sulfophenyl)theophylline on concentraton–response curves to APNEA in the presence of propranolol and 4-aminopyridine

In the absence of 4-aminopyridine but presence of propranolol, 8(*p*-sulfophenyl)theophylline caused a significant ($P < 0.05$) rightward shift of the APNEA concentration–response curve. The IC_{50} value in the presence of 8(*p*-sulfophenyl)theophylline was significantly ($P < 0.05$) greater than in its absence (Table 3). Although only an incomplete concentration–response curve could be constructed in the presence of 8(*p*-sulfophenyl)theophylline,

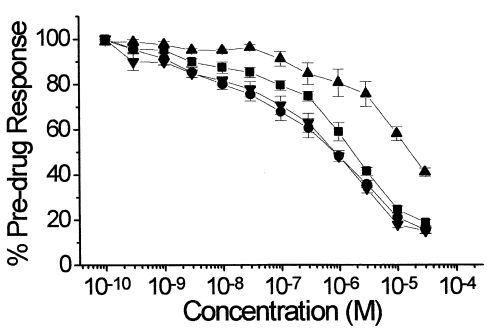

Fig. 8. Mean ($n = 4$) concentration–response curves for the negative inotropic responses to APNEA in guinea-pig isolated left atria in the presence of propranolol (1×10^{-6} M). Control (propranolol alone) (■), propranolol and 4-aminopyridine (10 mM) (●), propranolol, 4-aminopyridine (10 mM) and 8(*p*-sulfophenyl)theophylline (1×10^{-5} M) (▼), propranolol and 8(*p*-sulfophenyl)theophylline alone (▲). Error bars denote S.E.M.

Table 3
Effect of 4-aminopyridine (10 mM) and 8(*p*-sulfophenyl)theophylline (8-sPT, 1×10^{-5} M) on the IC_{50} values and maximum responses for the negative inotropic responses to APNEA in guinea-pig left atria all in the presence of propranolol (1×10^{-6} M)

	n	IC_{50}	Maximum response (% inhibition of resting tension)
Propranolol	4	$9.7\ (6.6–14.2) \times 10^{-7}$	81.3 ± 1.2
4-Aminopyridine + propranolol	4	$3.2\ (1.4–7.5) \times 10^{-7}$ [a]	84.4 ± 1.5
8-sPT + propranolol	4	$28.0\ (9.0–83.0) \times 10^{-7}$ [a]	58.9 ± 1.8 [a,b]
8-sPT + 4-aminopyridine + propranolol	4	$4.3\ (2.8–6.5) \times 10^{-7}$ [a]	84.9 ± 1.3

[a] Denotes significant ($P < 0.05$) difference from propranolol control.
[b] Note the maximum effect was measured at the highest concentration (3×10^{-5} M) used, higher concentration may have produced a greater effect.

the inhibition of resting developed tension produced by the maximum concentration of APNEA used was significantly ($P < 0.05$) reduced (Fig 8, Table 3). In the presence of 4-aminopyridine, the shift of the APNEA concentration–response curve by 8(*p*-sulfophenyl)theophylline was abolished (Fig. 8). The APNEA concentration–response curve and IC_{50} were not significantly different ($P > 0.05$) from the control (4-aminopyridine and propranolol) (Table 3).

4. Discussion

Interactions of adenosine receptor ligands with the adenosine A_1 receptor have been associated with activation of K^+ efflux through the same channel that is linked to the atrial muscarinic receptor (I_{Ach}) (Belardinelli and Isenberg, 1983; Cerbai et al., 1988; Visentin et al., 1990; Urquhart et al., 1993). The effect of the inactivation of this channel by the K^+ channel blocker 4-aminopyridine was therefore examined on the negative inotropic response of guinea-pig paced left atria to the adenosine A_1 receptor agonist, N^6-cyclopentyladenosine. Addition of 4-aminopyridine (10 mM) to the bathing medium caused a transient positive inotropy in the guinea-pig left atria. This is consistent with its K^+ channel blocking properties. Although only blockade of the receptor operated I_{Ach} channel was desired, 4-aminopyridine also possess the ability to block voltage operated K^+ channels involved in the cardiac action potential (Hille, 1984). This leads to a prolongation of action potential duration which allows more time for Ca^{2+} entry and hence positive inotropy. In fact, 4-aminopyridine has been widely reported as a positive inotrope in both atrial (Glover, 1981; Furukawa et al., 1985) and ventricular muscle (Yanagisawa and Taira, 1979: Wollmer et al., 1981). The transient nature of this inotropy in the guinea-pig left atria may be related to the fact that in this species the action potential only transiently increases (Glover, 1982).

Since 4-aminopyridine is known to release catecholamines (Glover, 1981, 1982), studies involving this antagonist were carried out in tissues where these stores were depleted by reserpinisation. Since reserpinisation requires pretreatment of the guinea-pigs 24 h beforehand and reserpine is potentially cardiotoxic (Torphy et al., 1982), 4-aminopyridine was also assessed in the presence of propranolol to block the β-adrenoceptor-mediated effects of any catecholamines released. The positive inotropy to 4-aminopyridine in both reserpinised tissue and in the presence of propranolol was significantly smaller and of shorter duration than that seen in untreated tissue. Reserpinisation was effective in depleting catecholamine stores since the positive inotropy to the indirectly acting sympathomimetic, tyramine (Broadley, 1996), was significantly reduced compared to that in untreated tissue. In the case of propranolol, the concentration employed, 10^{-6} M, caused over a thousand-fold shift of isoprenaline concentration–response curves in guinea-pig left atria (Broadley, 1996) and hence should have effectively abolished the effects of any catecholamine released on the addition of 4-aminopyridine. The positive inotropy to 4-aminopyridine that remained in reserpinised tissue and in the presence of propranolol could therefore be attributed to K^+ channel blockade and not to any residual catecholamine release.

In either reserpinized tissues or in the presence of propranolol and in the absence of 4-aminopyridine, N^6-cyclopentyladenosine caused a negative inotropic response consisting of a rapid initial phase followed by a slower more prolonged reduction of tension. The maximum effect was no different from that in untreated atria. In the presence of 4-aminopyridine, however, the initial rapid phase of negative inotropy was abolished, giving way to a slow-onset sustained negative inotropy which reached the same maximum effect in untreated and propranolol treated tissues. There was a small but significant reduction of the maximum effect in tissue from reserpinised animals. This reduction in maximum effect of N^6-cyclopentyladenosine in reserpinised tissues is probably due to the responses being slowed to such a degree that the maximum effect was not reached in the 5 min recording period. It therefore appears that blockade of the primary effect of K^+ efflux by 4-aminopyridine is to slow the onset of the negative inotropy produced after adenosine A_1 receptor activation without any effect on the maximal response. This confirms earlier observations from this laboratory using non-reserpinised or non-β-adrenoceptor blocked tissues (Ford and Broadley, 1999) or using (−)-N^6-(2-phenylisopropyl)

adenosine and adenosine as the agonists (Urquhart et al., 1993).

The lack of effect on the ultimate magnitude of the response to N^6-cyclopentyladenosine is emphasised by the failure of 4-aminopyridine to antagonise the cumulative-concentration response curve to N^6-cyclopentyladenosine in both the presence of propranolol and in tissue from reserpinised animals. Since in construction of concentration–response curves, the effect of each concentration of N^6-cyclopentyladenosine on left atrial tension was allowed to plateau, the speed of onset the negative inotropy was not of importance. The failure of 4-aminopyridine to antagonise the concentration–response curves to another adenosine A_1 receptor agonist (−)-N^6-(2-phenylisopropyl)adenosine has previously been reported (De Biasi et al., 1989). Since 4-aminopyridine has been shown to completely block the efflux of Rb^{86} by (−)-N^6-(2-phenylisopropyl)adenosine (Urquhart et al., 1991), it would appear that adenosine A_1 receptor-mediated K^+ efflux only governs the speed of onset of the negative inotropy produced by N^6-cyclopentyladenosine and not the maximal effect achieved by each concentration.

The slow monophasic negative inotropy produced by N^6-cyclopentyladenosine in the presence of 4-aminopyridine must therefore be brought about by a mechanism other than K^+ efflux. In the left atria, a role for L-type Ca^{2+} channel inhibition in the adenosine A_1 receptor-mediated negative inotropy has been proposed (De Biasi et al., 1989; Fassina et al., 1991; Jahnel et al., 1992). In the presence of 4-aminopyridine, N^6-cyclopentyladenosine is unable to cause negative inotropy through K^+ efflux and the adenosine A_1 receptor-mediated closure of L-type Ca^{2+} channels may then be responsible for the termination of the plateau phase of the action potential and the negative inotropy.

In tissue from reserpinised animals, the non-selective adenosine receptor antagonist, 8(*p*-sulfophenyl)theophylline, displayed a reduced ability to antagonise the N^6-cyclopentyladenosine concentration–response curves when compared to its ability in tissues from untreated animals. Hence it would appear that reserpinisation itself, impairs the ability of 8(*p*-sulfophenyl)theophylline to antagonise the effects of N^6-cyclopentyladenosine, the reason for which is unclear at present. In the presence of propranolol, however, 8(*p*-sulfophenyl)theophylline produced a rightward shift of the N^6-cyclopentyladenosine concentration–response curves similar to that seen in untreated tissues.

In the presence of 4-aminopyridine and propranolol, 8(*p*-sulfophenyl)theophylline produced significantly smaller non-parallel shifts of the N^6-cyclopentyladenosine concentration–response curves than in the absence of 4-aminopyridine. Hence, the portion of negative inotropy remaining in the presence of 4-aminopyridine is less susceptible to antagonism by 8(*p*-sulfophenyl)theophylline. The concentration–response curves in the presence of both concentrations of 8(*p*-sulfophenyl)theophylline were clearly biphasic, the lower concentrations of N^6-cyclopentyladenosine being virtually unblocked while there was some antagonism at the higher concentrations. This decreased ability of 8(*p*-sulfophenyl)theophylline to antagonise the negative inotropy produced by N^6-cyclopentyladenosine could account for our previous findings that there is a plateauing to the antagonism of N^6-cyclopentyladenosine by 8(*p*-sulfophenyl)theophylline (Gardner and Broadley, 1999). It may be the case that this plateau is brought about by the switching of the mechanism producing the negative inotropy from K^+ efflux to another mechanism such as closure of L-type Ca^{2+} channels.

The final objective of this study was to characterise the effect of the adenosine A_1/A_3 receptor selective agonist APNEA. In the presence of propranolol alone, APNEA caused a concentration-dependent negative inotropy presumably through activation of adenosine A_1 receptors. The activation of adenosine A_3 receptors is unlikely since we have shown that the adenosine A_3 receptor selective agonist, N^6-(3-iodobenzyl)adenosine-5′-*N*-methyuromide (IB-MECA), fails to produce negative inotropy up to 10^{-6} M (Gardner and Broadley, 1999). The concentration–response curve to APNEA appeared to be biphasic with a shallow initial component followed by a second steeper phase of the negative inotropy. This biphasic appearance of the APNEA concentration–response curve may itself indicate dual mechanisms mediating the negative inotropy. Unlike in the case of N^6-cyclopentyladenosine, 4-aminopyridine produced a significant potentiation (leftward shift) of the APNEA concentration–response curve. This potentiation indicates that APNEA is more potent at causing negative inotropy in the left atria when the receptor-mediated K^+ efflux is blocked. Also, the negative inotropy caused by APNEA in the presence of 4-aminopyridine was completely resistant to blockade by 8(*p*-sulfophenyl)theophylline. In fact, the concentration–response curves for APNEA in the presence of 4-aminopyridine alone and 4-aminopyridine and 8(*p*-sulfophenyl)theophylline lie on top of each other over the full concentration range studied. This observation adds weight to the idea that the negative inotropic response produced when K^+ efflux is blocked is resistant to antagonism by 8(*p*-sulfophenyl)theophylline.

The question remains as to the mechanism of the remaining negative inotropy in the presence of K^+ channel blockade by 4-aminopyridine and the nature of the second messenger system(s) linking the adenosine A_1 receptor to the effector. A primary candidate is through the closure of Ca^{2+} channels. In support of this, (−)-N^6-(2-phenylisopropyl)adenosine has been shown to still exert negative inotropy in atria partially depolarized by raising the K^+ so that the contractions are entirely due to slow inward Ca^{2+} currents, and this was not blocked by 4-aminopyridine (Ford and Broadley, 1993). Evidence in atrial myocytes, however, has shown that adenosine causes negative inotropy by activation of the inwardly rectifying K^+ current (I_{KAch}) and not by a direct decrease in L-type Ca^{2+} inward

current (Wang and Belardinelli, 1994). When cAMP levels are raised in guinea-pig ventricular cells (Kato et al., 1990) and atrial myocytes (Jahnel et al., 1992), adenosine can lower these levels and promote decreased Ca^{2+} channel activity through dephosphorylation of the Ca^{2+} channel protein. Furthermore, negative inotropy occurs in papillary muscles only when basal levels of cAMP have been raised through agents such as forskolin and catecholamines (Bohm et al., 1984; Urquhart and Broadley, 1992). (−)-N^6-(2-phenylisopropyl)adenosine does not appear to affect basal levels of cAMP in guinea-pig atrial and ventricular preparations (Bruckner et al., 1985). However, it is possible that in the presence of K^+ channel blockade, basal levels of cAMP may rise, thus allowing N^6-cyclopentyladenosine to exert an inhibitory effect.

The 8(*p*-sulfophenyl)theophylline-resistance of the negative inotropic response of left atria to N^6-cyclopentyladenosine and APNEA in the presence of 4-aminopyridine could have arisen from a duality of coupling of the adenosine A_1 receptor to the two transduction pathways. This has been referred to as receptor-transducer promiscuity (Kenakin, 1993). One of these pathways could be via K^+ efflux and the other could be through Ca^{2+} channel closure. Two- or three-state receptor models may predict that the same adenosine A_1 receptor exists in more than one conformational state and that these link with the two pathways for signal transduction (Leff et al., 1997; Clarke and Bond, 1998). It is possible that the antagonist displays different affinities for these conformations and thus explains the resistance to blockade when the K^+ channels are blocked by 4-aminopyridine.

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