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Effect of selective agonists and antagonists on atrial adenosine receptors and their interaction with Bay K 8644 and [^{3}H]-nitrendipine

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1 (−)-N^6-phenylisopropyladenosine (R-PIA) and N^6-cyclohexyladenosine (CHA), highly selective agonists at A_1-adenosine receptors, 5'-N-ethyl-carboxamidoadenosine (NECA), a non-selective agonist at A_1 and A_2 receptors, and 2-phenylaminoadenosine (CV-1808), a selective A_2 agonist, were compared in spontaneously beating and electrically driven atria. R-PIA, CHA and NECA inhibited contraction in both preparations. CV-1808 was not effective up to 500 nM.

2 1,3-Dipropyl-8-cyclopentylxanthine (DPCPX), a new selective A_1 receptor antagonist, competitively inhibited the effects of the adenosine agonists, at low concentrations ($IC_{50} < 1$ nM).

3 CHA and NECA were able to inhibit the positive inotropic effect of Bay K 8644 both in spontaneously beating and in electrically driven atria.

4 R-PIA, CHA and NECA (agonists), 8-phenyltheophylline (PT) and DPCPX (antagonists), failed to influence [^{3}H]-nitrendipine binding on microsomal membranes from guinea-pig atria and ventricles in a range of concentrations from 1 nM to 100 μM.

5 The data support the existence of A_1 receptors in atrial tissue. No evidence for a direct interaction between adenosine analogues and Bay K 8644 was found at the level of slow calcium channels. Adenosine analogues appear to antagonize the effects of Bay K 8644 indirectly by activation of A_1 receptors.

Introduction

Adenosine decreases the beating frequency and the force of contraction in isolated atrial preparations of different species (for reviews see Schütz & Freissmuth, 1985; Brückner *et al.*, 1985; Scholz *et al.*, 1987). The mechanism of these actions is still elusive and debatable. Most of the biological effects of adenosine appear to be mediated by membrane adenosine receptors that are coupled to adenylate cyclase via a G regulatory protein. Two receptors subtypes have been recognized: the A_1 subtype, which inhibits adenylate cyclase, and the A_2 subtype, which activates the enzyme (Van Calker *et al.*, 1979; Londos *et al.*, 1980; Stone, 1984). But this model does not hold regarding the effects of adenosine at the atrial level. It has been suggested that in mammalian atrial tissue a variant adenosine cell surface receptor exists, not detectably coupled to adenylate cyclase (Brückner *et al.*, 1985) but coupled instead to K^+ channels via a G-protein (Böhm *et al.*, 1986). An alternative hypothesis is that an adenosine A_3 receptor is present in cardiac tissue (Ribeiro & Sebastião, 1986). The activation of this A_3 receptor is postulated to induce electrophysiological effects directly by affecting transmembrane ion currents, mainly of calcium.

Recently discovered selective agonists and antagonists for adenosine receptors (see Bruns *et al.*, 1987b) have been used in the present paper to investigate the extracellular purinoceptors involved in atrial function. The recently reported, highly selective, A_1-receptor antagonist 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), (Jacobson *et al.*, 1985; Haleen *et al.*, 1987; Bruns *et al.*, 1987a; Williams, 1987) and the A_2-selective agonist 2-phenylaminoadenosine (CV-1808) (Bruns *et al.*, 1986; 1987a,b) have been used. N^6-cyclohexyladenosine (CHA) and (−)-N^6-phenylisopropyladenosine (R-PIA), both A_1 selective agonists, and 5'-N-ethyl-carboxamidoadenosine (NECA), a non selective A_1 and A_2 agonist, were also studied for comparison. The

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effects of these compounds were evaluated in spontaneously beating atria and in electrically driven isolated atria of the guinea-pig.

The mechanism of action of adenosine analogues at the atrial level was also investigated. We have recently shown that R-PIA antagonizes the positive inotropic effect of Bay K 8644 (Caparrotta *et al.*, 1985; 1987), a dihydropyridine slow calcium channel activator (Schramm *et al.*, 1983). The interaction of CHA and NECA with Bay K 8644 was studied and compared to that of R-PIA. In order to investigate if the antagonism by adenosine analogues to Bay K 8644 was dependent on A_1 receptors, or, instead, on a possible interaction with slow calcium channels, two approaches were used: (i) the interaction between CHA, R-PIA, NECA and Bay K 8644 was studied in the presence of the A_1-selective antagonist DPCPX; (ii) radioligand binding assays were carried out by use of [^{3}H]-nitrendipine to label the dihydropyridine binding site on microsomal fractions from guinea-pig atria and ventricles.

Methods

Tissue preparations for recording mechanical response

The hearts were removed from guinea-pigs of either sex (300–500 g) and placed in a physiological solution (29°C) of the following composition (mM): NaCl 120, KCl 2.7, $CaCl_2$ 1.36, $MgCl_2$ 0.09, $NaHCO_3$ 11.9, NaH_2PO_4 0.42, glucose 5.5 and gassed with 95% O_2 plus 5% CO_2. The atria were dissected, suspended in a 30 ml organ bath and connected to a transducer (Basile type DY0). An initial tension of 1 g was applied to the tissues and changes in isometric tension were recorded using an oscillograph (Basile, Unirecord System, Mod. 7050).

Left atria were mounted on punctate electrodes with a load of 0.5 g and stimulated by square wave electrical pulses (0.5–2 Hz, 3 ms, 0.5–1.2 V) provided by a Grass stimulator (Mod. S4 KR). The voltage was about 20% greater than threshold. The control developed tension ranged from 0.8 to 1.3 mN.

An equilibration period of 60 min was allowed before experiments were started. Concentration-response curves were constructed by a cumulative addition of agonist after the force had reached a new equilibrium. The effect of Bay K 8644 was slow in onset (20–30 min) and required a prolonged period of washing for reversal (at least 2 h). The effect of the stable analogues of adenosine reached equilibrium after 6–8 min. Reversibility of the effect of a single concentration of adenosine analogues was investigated by a 15 min washing with drug-free bathing solution at the end of experiments. The cumulative concentration-response curves to adenosine analogues were performed before and after treatment with Bay K 8644 and DPCPX. The concentration-response curves for the negative inotropic and chronotropic effects of adenosine analogues, were expressed as percentage decrease from the basal level. Cumulative concentration-response curves for the positive inotropic effects of Bay K 8644 were expressed as percentage of the maximum increase over control tension induced by Bay K 8644. The response to Bay K 8644 in the presence of adenosine antagonists was related to the maximum effect of Bay K 8644 (100%) in the absence of the inhibitor.

[^{3}H]-nitrendipine binding

Atria and ventricles were dissected, minced, and homogenized in 50 vol of 50 mM Tris HCl buffer, pH 7.4, with a Brinkman Polytron, PTA-10 probe at setting 6 for 30 s. After discarding a 3,500 g × 15 min pellet, a 48,000 g × 45 min pellet was prepared and resuspended in the Tris buffer to 0.5 mg protein ml^{-1}. The membranes were frozen in aliquots until use (−25°C) at which time they were washed once more. Two ml of the suspension was incubated with [^{3}H]-nitrendipine (78 Ci mmol^{-1}) 0.1 nM (a concentration close to its equilibrium K_d) in triplicate, and with increasing concentrations of R-PIA, CHA, NECA, 8-phenyltheophylline (PT), DPCPX. R-PIA, CHA and NECA were also tested in the presence of 10 μM PT. After 60 min at 23°C, the assays were terminated by filtration through Whatman GF/C glass filters under suction. The filters were washed four times with ice-cold buffer, dried and counted in 5 ml of acidified Instagel (Packard) by liquid scintillation counting. Non specific binding was determined by incorporation of 1 μM nifedipine in the assays and routinely represented about 30% of total binding. Our binding assay was similar to that employed by other groups (Bolger *et al.*, 1982). Different incubation media were also used, i.e., Tris HCl buffer pH 7.4 with the addition of 1.5 mM $MgCl_2$ or 10 mM $CaCl_2$.

Bay K 8644, in our experimental conditions, displaced [^{3}H]-nitrendipine with an IC_{50} of the order of 10 nM in accordance with binding data obtained by Janis *et al.* (1984a,b) in rabbit ventricular microsomes. Competition between nitrendipine and [^{3}H]-Bay K 8644 indicated a common high affinity binding site for Ca^{2+} channel activators and antagonists (Janis *et al.*, 1984a,b).

Drugs and compounds

The following were used: N^6-cyclohexyladenosine (CHA) (a kind gift of Prof. Luiz Belardinelli, Dept of Physiology, University of Virginia, U.S.A.); 1,3-

Table 1 Effects of stable analogues of adenosine on contractile tension and frequency in spontaneously beating and electrically driven guinea-pig isolated atria

Agonist	*Spontaneous*		*Electrically driven*
	Contractile tension $-\log EC_{50}$	*Frequency* $-\log EC_{50}$	*Contractile tension* $-\log EC_{50}$
R-PIA (A_1-agonist)	7.82 ± 0.07 ($EC_{50} = 15$ nM)	7.68 ± 0.06 ($EC_{50} = 20$ nM)	7.66 ± 0.06 ($EC_{50} = 21$ nM)
CHA (A_1-agonist)	7.64 ± 0.06 ($EC_{50} = 23$ nM)	6.93 ± 0.11 ($EC_{50} = 118$ nM)	7.12 ± 0.06 ($EC_{50} = 75$ nM)
NECA (A_1 and A_2 agonist)	7.63 ± 0.3 ($EC_{50} = 24$ nM)	6.94 ± 0.10 ($EC_{50} = 114$ nM)	7.46 ± 0.08 ($EC_{50} = 34$ nM)
CV-1808 (A_2-agonist)	ND	ND	ND

ND = not detectable.
Data are the mean ± s.e.mean of 5–10 preparations.
R-PIA = (−)-N^6-phenylisopropyladenosine; CHA = N^6-cyclohexyladenosine; NECA = 5′-ethyl-carboxamido-adenosine; CV-1808 = 2-phenylaminoadenosine.

dipropyl-8-cyclopentylxanthine (DPCPX) (Research Biochemical Inc.); 5′-N-ethyl-carboxamidoadenosine (NECA) (Sigma). Methyl-1,4,-dihydro-2,3-dimethyl-3-nitro-4-(2-trifluoro-methylphenyl)-pyridine-5-carb - oxylate (Bay K 8644), was generously supplied by Dr G. Franckowiak from Bayer AG (Wuppertal, F.R.G.). Bay K 8644, 1 mM, was freshly dissolved in absolute ethanol. This stock solution was diluted in appropriate amounts in bathing solution to achieve the desired final concentration. All experiments were carried out in a dark room using red light as the drug was sensitive to light. Nifedipine (4-(2′-nitrophenyl)-2,6-dimethyl-3,5-dicarbomethoxy-1,4 - dihy - dropyridine) (Pfizer). [^{3}H]-nitrendipine (2,6-dimethyl-3,5-dicarbomethoxy-4-(3-nitro)phenyl-1,4-dihydropyridine) (New England Nuclear, Boston, Ma) purity >97%, was stored protected from light at −20°C. 2-Phenylaminoadenosine (CV-1808) (Research Biochemical Inc.). (−)-N^6-phenylisopropyladenosine (R-PIA) (Boehringer, Mannheim, F.R.G.), was dissolved and diluted 50% ethanol-50% bathing solution. 8-Phenyltheophylline (PT) (Sigma) was dissolved in dimethylsulphoxide (DMSO).

All chemicals were of analytical or best commercial grade available. Deionized and twice-distilled water was used throughout.

Mathematical and statistical analysis of results

Values presented are the mean ± s.e.mean. The analysis of concentration-response curves was carried out from data in the region between 20% and 80% of maximal response. A linear least squares regression was constructed from individual values of effect versus log concentration.

The −log of concentration that produced half-maximal effect ($-\log EC_{50}$) and its standard error were determined by interpolation according to Tallarida & Murray (1987).

The log (dose ratio − 1) was plotted against −log antagonist concentration and a least squares regression analysis was used to determine pA_2 (Arunlakshana & Schild, 1959). Alternatively, K_i was determined according to the formula K_i = [inhibitor]/(dose ratio − 1) (Furchgott, 1967).

Results were tested for significance by Student's t test for unpaired data. A P value less than 0.05 was considered as significant.

Results

Effect of adenosine analogues on the contractile tension and frequency

CHA, NECA and R-PIA produced a concentration-dependent decrease of contractile tension and frequency in spontaneously beating atria at concentrations 3–300 nM (Table 1). CV-1808 did not show significant effects on isolated atria up to 500 nM. R-PIA, CHA and NECA were also effective in decreasing contractile tension in electrically driven atria (Table 1). The order of potency was R-PIA >= NECA > CHA.

Effect of a highly selective A_1 receptor antagonist, DPCPX, on the actions of R-PIA, CHA and NECA

In spontaneously beating atria, 1 to 50 nM DPCPX inhibited the negative effects of R-PIA on contractile

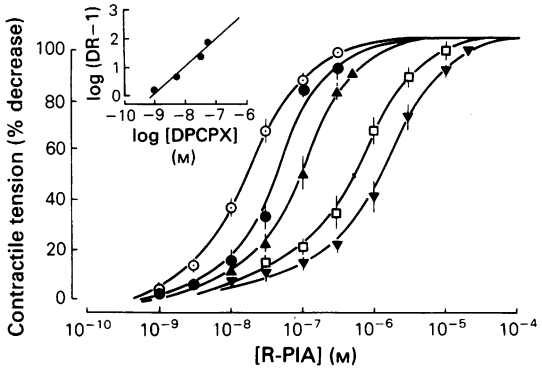

Figure 1 Inhibition by 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) of the negative inotropic effect of N^6-phenylisopropyladenosine (R-PIA) in spontaneously beating atria. Cumulative concentration-response curves for R-PIA alone (○) or in the presence of DPCPX 1 nM (●), 5 nM (▲), 30 nM (□) and 50 nM (▼). Each point is the mean of 4–6 experiments. Vertical lines indicate s.e.mean. Inset: Schild plot.

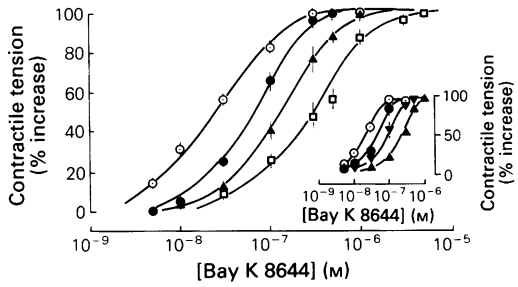

Figure 2 Inhibition by N^6-cyclohexyladenosine (CHA) of the positive inotropic effect of Bay K 8644 in electrically driven atria (larger figure) and in spontaneously beating atria (inset). Cumulative concentration-response curves for Bay K 8644 in the absence (○) and in presence of CHA 50 nM (●), 80 nM (▼), 100 nM (▲) and 500 nM (□). Each point is the mean of 6–10 experiments. Vertical lines indicate s.e.mean.

tension in a concentration-dependent manner and shifted the concentration-response curve of R-PIA to the right, indicating a competitive antagonism (Figure 1). The Schild plot (Figure 1, inset) was linear with a slope 0.96 ± 0.12 ($r = 0.98$). The pA_2 value of the antagonism of R-PIA by DPCPX was 9.18 ± 0.16 corresponding to an apparent K_i of 0.66 nM.

In the electrically driven left atria, DPCPX inhibited the negative inotropic effect of R-PIA and shifted the concentration-response curve of R-PIA to the right. In these experimental conditions, the apparent K_i of DPCPX was 0.67 nM (Table 2) as in spontaneously beating atria. DPCPX antagonized the negative effect on contraction by CHA and NECA in spontaneously beating and in paced atria with a similar potency (Table 2).

Table 2 Effects of (−)-N^6-phenylisopropyladenosine (R-PIA), N^6-cyclohexyladenosine (CHA) and 5′-N-ethyl-carboxamidoadenosine (NECA) on contractile tension in electrically driven atrial preparations alone or with 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) (5 nM)

Compound	[1]*Contractile tension* −log EC_{50} (M)	[2]*Apparent* K_i (nM)
R-PIA	7.66 ± 0.06	
R-PIA + DPCPX	6.75 ± 0.10*	0.67
CHA	7.12 ± 0.06	
CHA + DPCPX	6.04 ± 0.14*	0.45
NECA	7.46 ± 0.08	
NECA + DPCPX	6.46 ± 0.05*	0.54

[1] Data are the mean ± s.e.mean of 4–6 experiments.
[2] Apparent K_i was determined according to the formula: K_i = [Inhibitor]/(dose ratio − 1) (Furchgott, 1967).
* $P < 0.001$.

Effect of CHA and NECA on the positive inotropic effect of Bay K 8644

Bay K 8644 induced a concentration-dependent positive inotropic effect on spontaneously beating atria (−log EC_{50} = 7.68 ± 0.02 M) (Figure 2, inset). The positive chronotropic effect of Bay K 8644 became evident only at concentrations inducing the maximum increase in contractility, being very small (about 15% above control).

Stimulation frequency was found to modify the effect of Bay K 8644 on contraction. In electrically driven left atria, the positive inotropic effect of Bay K 8644 increased proportionally with the rate of stimulation from 0.5 to 1.5–2 Hz; at higher rates, over 2 Hz, the positive inotropic effect of Bay K 8644 decreased. Thus we used 1.5 Hz as a fixed rate of stimulation, when the effect of Bay K 8644 was at its maximum. In such experimental conditions, the −log EC_{50} was 7.61 ± 0.03 M.

CHA inhibited the positive inotropic effect of Bay K 8644 in a concentration-dependent manner, both in spontaneously beating and in paced atria (Figure 2). CHA shifted the concentration-response curve of Bay K 8644 to the right.

In both conditions the Schild plot was linear but the slope was different: 3.21 ± 0.75 in spontaneously

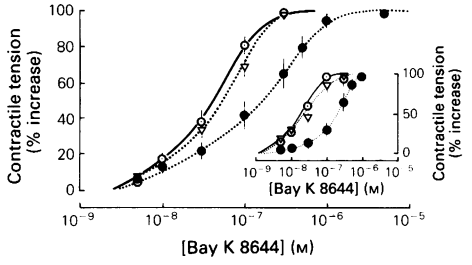

Figure 3 Inhibition by 5'-N-ethylcarboxamidoadenosine (NECA) on the positive inotropic effect of Bay K 8644 in electrically driven atria (larger figure) and in spontaneously beating atria (inset). Cumulative concentration-response curves for Bay K 8644 in the absence (○) and in presence of NECA 50 nM (▽) and 100 nM (●). Each point is the mean of 4–6 experiments. Vertical lines indicate s.e.mean.

beating atria and 0.79 ± 0.20 in paced atria. The pA_2 was 7.29 ± 0.02 in spontaneously beating and 7.72 ± 0.24 in paced atria. The antagonism by CHA of Bay K 8644 is qualitatively similar to that obtained previously with R-PIA (Caparrotta *et al.*, 1985; 1987). NECA also inhibited responses to Bay K 8644 (Figure 3) but was less potent than CHA.

The effects of Bay K 8644 were not antagonized in electrically driven atria by carbachol at concentrations of 50 nM and 100 nM, which reduced the atrial contractility by 40% and 70% respectively.

Effect of adenosine agonists and antagonists on [^3H]-nitrendipine binding in microsomal membrane fractions

In order to investigate the possibility of a direct interaction between adenosine analogues and Bay K 8644 at the level of dihydropyridine sites, studies were carried out with [^{3}H]-nitrendipine, a ligand for dihydropyridine binding sites in slow calcium channels of cardiac membranes (Janis *et al.*, 1984; Sarmiento *et al.*, 1987).

R-PIA, CHA and NECA (agonists), PT and DPCPX (antagonists), failed to influence [^{3}H]-nitrendipine binding up to a 100 μM concentration, both in atrial and ventricular membrane fractions. Similar negative results were also found when using Tris HCl buffer to which 1.5 mM $MgCl_2$ or 10 mM $CaCl_2$ was added, in order to affect the affinity status of extracellular adenosine receptors or dihydropyridine receptor in slow calcium channels (Yeung *et al.*, 1985; Ptasienski *et al.*, 1985).

Effect of DPCPX on the inhibition by adenosine agonists to Bay K 8644

The interaction of R-PIA and CHA with Bay K 8644 was studied in the presence of the antagonist

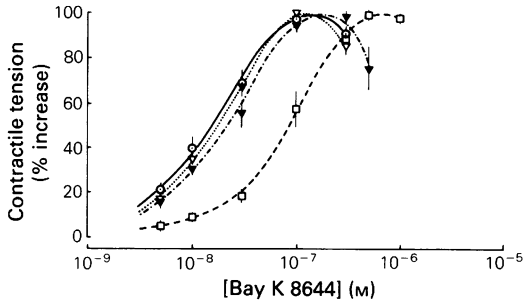

Figure 4 Effects of 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) on the antagonism between N^6-cyclohexyladenosine (CHA) and Bay K 8644 in spontaneously beating atria. Cumulative concentration-response curves for Bay K 8644 alone (○——○) in the presence of DPCPX 5 nM (▼–·–·–▼), CHA 80 nM (□– – –□), and both DPCPX 5 nM plus CHA 80 nM (▽···▽). Each point is the mean of 4–6 experiments. Vertical lines indicate s.e.mean.

DPCPX. The isolated atria, after stabilization, were incubated for 10 min in the presence of DPCPX 5 nM. This concentration of DPCPX did not modify the Bay K 8644 positive inotropism (Figure 4). The inhibition exerted by R-PIA 50 nM (IC_{50}) and by CHA 80 nM (IC_{50}) of the positive inotropic effect of Bay K 8644 was abolished in the presence of DPCPX (Figure 4).

Discussion

A major finding of this study was that data obtained by the use of stable adenosine agonists and antagonists support the existence of A_1 adenosine receptors in atrial tissue and their involvement in the negative effects of adenosine at atrial level. This is in accordance with previous studies (Evans *et al.*, 1982; Collis, 1983; Brückner *et al.*, 1985) on guinea-pig atria and in rat atria (Paton, 1983).

The data show that CHA and R-PIA, highly selective A_1 receptor agonists, and NECA, a non selective A_1 and A_2 agonist, are all able to decrease contractile tension and frequency in a concentration-dependent manner (3–300 nM). In contrast, CV-1808, an A_2-selective agonist, does not show significant effects on isolated atria up to 500 nM. The highly selective A_1 receptor antagonist DPCPX, competitively antagonized the negative inotropic and chronotropic effects of R-PIA, CHA and NECA at low concentrations. The apparent K_i values for contraction were 0.67, 0.45 and 0.54 nM, respectively, in accordance with the K_i (0.46 nM) found in central nervous system binding studies (Bruns *et al.*, 1987b).

It has been proposed that additional or variant

adenosine cell surface receptors exist in atrial tissue which are not coupled to adenylate cyclase, but are coupled instead via G-proteins to other effector systems such as K^+ channels (Böhm *et al.*, 1986) or Ca^{2+} currents (Ribeiro & Sebastião, 1986). A possible role of adenosine in modulating slow calcium channels was further suggested by results obtained in guinea-pig atria (Caparrotta *et al.*, 1987) showing that R-PIA inhibits the positive inotropic effect of Bay K 8644, a dihydropyridine slow calcium channel activator (Schramm *et al.*, 1983). This observation, which indicates a role for adenosine in the regulation of Ca^{2+} conductance and hence force of contraction of the atria, remained to be elucidated. Therefore, we investigated if CHA and NECA could also antagonize Bay K 8644. CHA and NECA were found to inhibit the Bay K 8644 positive inotropism both in spontaneously beating and in electrically driven atria. CHA was more potent than NECA at lower concentrations (50 nM) but equally potent with NECA at higher concentrations (100 nM). CHA produced, like R-PIA (Caparrotta *et al.*, 1987) a parallel rightward shift in the Bay K 8644 concentration-response curves; but in spontaneously beating atria the slope of the Schild regression was very high, indicating a temporal or thermodynamic disequilibrium (Kenakin, 1987) or more simply the influence of the reduced rate of contraction.

A possible competition between adenosine analogues and Bay K 8644 for a common receptor site in or near the slow calcium channel was investigated in radioligand binding studies. The failure of R-PIA, CHA and NECA (agonists), and of PT and DPCPX (antagonists) to influence [^{3}H]-nitrendipine binding in microsomal membrane fractions, provided no evidence for a competitive antagonism between adenosine analogues and the dihydropyridine calcium channel ligand in guinea-pig atria and ventricles. A possible interaction between adenosine analogues and Bay K 8644 at the level of a common receptor site is thus unlikely.

The antagonism by R-PIA and CHA of the effects of Bay K 8644 was eliminated by DPCPX. This indicates that the interaction between adenosine analogues and the slow calcium channel activator is dependent on the A_1 receptor.

An obvious question is how the signal sent by adenosine analogues through the A_1 receptor is transmitted to the final effector, the site of interaction with Bay K 8644. A likely hypothesis is that adenosine analogues send an inhibitory signal from the A_1 receptors by the involvement of a G-protein, not coupled to adenylate cyclase, directly to the site of action of Bay K 8644. This is still to be investigated, but it would be in agreement with results showing that a nucleotide binding protein is involved in the regulation of the receptor-mediated change in K^+ conductance and force of contraction by adenosine and R-PIA (Böhm *et al.*, 1986; Kurachi *et al.*, 1986). However, it should be pointed out that in atrial cells both adenosine (and R-PIA) and acetylcholine (and carbachol) share the same action and mechanism in increasing outflow K^+ currents (for reviews see, Nawrath *et al.*, 1985; Sperelakis, 1987; Isenberg *et al.*, 1987; West *et al.*, 1987). But the interaction of adenosine analogues with Bay K 8644 is apparently distinct from the above mentioned effect, as carbachol does not modify the positive inotropic effect of Bay K 8644 in spontaneously beating (Caparrotta *et al.*, 1987) and in paced atria.

In summary, the present data indicate that A_1 receptors are present in atrial tissue and mediate a negative inotropic response. The antagonism by R-PIA and CHA of the effects of Bay K 8644 is also mediated by A_1 receptors. It remains to be elucidated if the signal sent from A_1 receptors to the site of action of Bay K 8644 is mediated by the activation of a membrane bound G-protein.

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