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Antagonism between (−)-N^6-phenylisopropyl-adenosine and the calcium channel facilitator Bay K 8644, on guinea-pig isolated atria

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1 Antagonism between (−)-N^6-phenylisopropyladenosine (PIA) and the dihydropyridine calcium channel facilitator Bay K 8644 was investigated in guinea-pig spontaneously beating or electrically driven isolated atria, taken from normal and from reserpine-treated animals.

2 PIA (3–100 nM) produced a dose-dependent decrease in contractile tension and frequency in spontaneously beating atria being more effective in reserpinized preparations.

3 Bay K 8644 (5–200 nM) produced an increase in contractile tension in both normal and reserpinized atria. In electrically driven left atria the positive inotropic effect of Bay K 8644 was similar to that in spontaneously beating preparations. The positive chronotropic effect of Bay K 8644 was slight and variable.

4 PIA produced a rightward parallel shift of the concentration-response curves for the positive inotropic effects of Bay K 8644 in all experimental conditions. In spontaneously beating atria from normal guinea-pigs, the Schild regression plot was linear and its slope near to unity; pA_2 of PIA 8.63 ± 0.05 (IC_{50} 2.35 ± 0.25 nM). In electrically driven atria the antagonism by PIA of the effects of Bay K 8644 was apparently competitive, and the IC_{50} of PIA was 18.6 ± 0.4 nM. PIA antagonized the positive chronotropic effect of Bay K 8644 in spontaneously beating preparations, both from normal and from reserpine-treated animals.

5 Carbachol did not modify the positive inotropic effects of Bay K 8644.

6 These data indicate that PIA may interact with Bay K 8644 at the level of the slow calcium channels, and may decrease the transmembrane calcium flux into the cell.

Introduction

Adenosine is continuously produced by the working heart (Rubio *et al.*, 1979). This nucleoside directly depresses the rate of firing of the sino-atrial pacemaker and the force of atrial contraction thus yielding negative chronotropic and inotropic effects in various animal species (for reviews see: Baer & Drummond, 1979; Burnstock, 1980; Daly *et al.*, 1983; Berne *et al.*, 1983; Schütz & Freissmuth, 1985; Stone, 1985). There is much controversy on the mechanism responsible for the cardiac effects of adenosine (for reviews see: Endoh *et al.*, 1983; Schütz & Freissmuth, 1985). A first hypothesis suggests that adenosine reduces the catecholamine-induced increase in calcium inward current mediated by adenosine 3′:5′-cyclic monophosphate (cyclic AMP) accumulation (Baumann *et al.*, 1981; Dobson, 1983; Dobson & Schrader, 1984; Isenberg & Belardinelli, 1984; Böhm *et al.*, 1985). Alternatively, adenosine reduces the synthesis of cyclic AMP through the activation of inhibitory adenosine receptors (R_i/A_1) coupled to adenylate cyclase (Evans *et al.*, 1982; Collis, 1983; Leung *et al.*, 1983). Data indicating that adenosine may act by changing K^+ and/or Ca^{2+} conductance directly, by a mechanism independent of cyclic AMP have also been presented (De Gubareff & Sleator, 1965; Schrader *et al.*, 1975; Hartzell, 1979; Tuganowski *et al.*, 1980; Belardinelli & Isenberg, 1983; Endoh *et al.*, 1983; Hughes & Stone, 1983). However, the effector of this mechanism is still unknown. Preliminary results suggested a role of adenosine in modulating slow Ca^{2+} channels (Caparrotta *et al.*, 1985). We investigated this hypothesis by studying the interaction of a stable analogue of adenosine, (−)-N^6-phenylisopropyladenosine, with a calcium channel facilitator, Bay K 8644, on guinea-pig isolated atria.

Bay K 8644 is a 1,4-dihydropyridine derivative

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which enhances myocardial contractility and constricts blood vessels (Schramm *et al.*, 1983a,b; Wada *et al.*, 1985) by increasing the transmembrane calcium current through the slow channels. Bay K 8644 binds to the same dihydropyridine binding site as does nifedipine, in or near voltage-operated slow calcium channels (Schramm *et al.*, 1983a,b; Thomas *et al.*, 1984; Ishii *et al.*, 1985). However, in contrast to nifedipine, Bay K 8644 promotes the influx of calcium ions, thus increasing heart contractility.

(−)-N^6-phenylisopropyladenosine (PIA, Londos *et al.*, 1980) was used as a stable analogue of adenosine, acting on the cell surface R adenosine receptors functionally linked to adenylate cyclase.

The effect of PIA on the positive inotropic and chronotropic effects of Bay K 8644 was studied on guinea-pig isolated atria, spontaneously beating or electrically driven, taken from normal and from reserpine-treated animals in order to exclude any interference by endogenous catecholamines.

Methods

Hearts were removed from guinea-pigs of either sex (400–600 g) and placed in physiological solution (29°C) of the following composition (mM): NaCl 120 KCl 2.7, $CaCl_2$ 1.36, $MgCl_2$ 0.9, $NaHCO_3$ 11.9, NaH_2PO_4 0.4, glucose 5.5, bubbled with 95% O_2 and 5% CO_2. The K^+ concentration was increased without isotonic compensation to 22 mM to depolarize electrically driven left atria, in order to inactivate the fast sodium channels and to generate responses supported by slow inward Ca^{2+} fluxes.

The atria were dissected, suspended in a 30 ml organ bath and connected to a high sensitive transducer (Basile, type DYO). An initial tension of 1 g was applied to the tissues and changes in isometric tension were recorded by a writing oscillograph (Basile, Unirecord System, mod 7050). The control developed tension ranged from 0.8 to 1.3 mN. Left atria were mounted on punctate electrodes with a load of 0.5 g and stimulated by square wave electrical pulses of 3 ms duration and a voltage 10% to 20% greater than threshold by a Grass stimulator (Mod. 24KR). The control developed tension ranged from 0.09 to 0.20 mN.

An equilibration period of 60 min was allowed before experiments were started. Concentration-response curves were constructed by cumulative addition of Bay K 8644, 5 nM to 200 nM. Responses were allowed to stabilize before the concentration was increased. 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).

PIA 3 to 50 nM was added to the bath and allowed to equilibrate, unless otherwise stated, for 6–8 min before a cumulative Bay K 8644 concentration-response curve was performed.

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 at the plateau. The responses of Bay K 8644 in the presence of PIA were related to the maximum effect of Bay K 8644 (100%) in the absence of the inhibitor. Only one concentration of PIA was tested in a single preparation, due to the prolonged period of washing required after exposure to Bay K 8644.

The concentration-response curves for the positive chronotropic effects of Bay K 8644 were expressed as percentage increase from the basal level and not as percentage of the maximum effect, as the effect of Bay K 8644 on the atrial rate was low (+ 30%) and never reached a plateau at the concentrations used.

Reserpinized atria were obtained by treating guinea-pigs with reserpine 2.5 mg kg^{-1} i.p. twice, 48 and 12 h before the experiment.

Drugs and compounds used

Methyl-1, 4-dihydro-2, 6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate, Bay K 8644, was generously supplied by Dr G. Franckowiak from Bayer AG (Wuppertal, FRG). Bay K 8644, 1 mM, was freshly dissolved in absolute ethanol. This stock solution was diluted in appropriate amounts in pre-warmed and pre-aerated bathing solution to achieve the desired final concentration. As the drug was sensitive to light, all experiments were carried out in a dark room using red light.

(−)-N^6-phenylisopropyladenosine (PIA; Boehringer, Mannheim) was dissolved in ethanol to produce a stock solution of 10 mM and subsequently diluted with 50% ethanol-50% bathing solution to achieve the desired concentration. The total volume of ethanol added never exceeded 30 µl in a 30 ml organ bath.

Other drugs used: reserpine (Ciba-Geigy); isoprenaline chloride (Boehringer Ingelheim); noradrenaline

Table 1 Effects of (−)-N^6-phenylisopropyladenosine (PIA) on contractile tension and frequency in spontaneously beating atria from normal and reserpine-treated guinea-pigs

	PIA IC_{50} (nM)	
Group	*Contractile tension*	*Frequency*
Normal	15 ± 2.7	21 ± 3.0
Reserpine-treated	9 ± 0.8	13 ± 1.2
	($P < 0.01$)	($P < 0.01$)

Means ± s.e. mean of 10 preparations are shown.

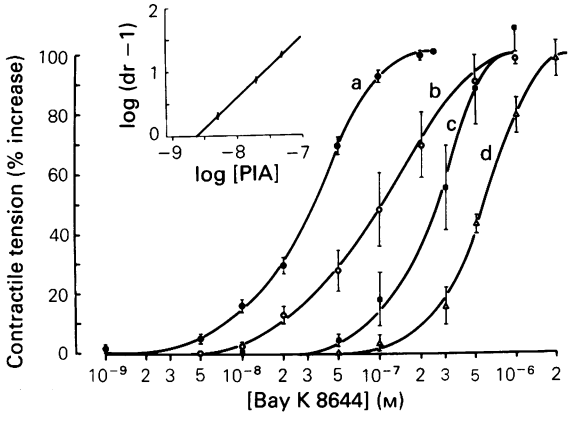

Figure 1 Inhibitory effect of (−)-N^6-phenylisopropyladenosine (PIA) on the positive inotropic effect of Bay K 8644 in normal atria. Cumulative concentration-response curves for Bay K 8644 in the absence (a, ●) (n = 14–21) and in presence of PIA 5×10^{-9}M (b, ○) (n = 7–9), 2×10^{-8}M (c, ■) (n = 4-6), 5×10^{-8}M (d, △) (n = 4–6). The Bay K 8644-induced contraction in the presence of PIA is related to the maximum effect of Bay K 8644 (100%) in the absence of the inhibitor. Each point is the mean and vertical lines indicate s.e. mean. Inset: Schild regression for PIA with Bay K 8644 as the agonist (dr = dose-ratio). The slope of the regression line was 0.92 ± 0.02 (r = 0.99), and the apparent pA_2 value was 8.63 ± 0.05.

bitartrate (Sigma); carbamylcholine chloride (carbachol, Sigma).

Analysis of results

Values presented are means ± s.e. mean. Statistical differences between mean values were analysed by use of Student's t test.

The Schild plot (Arunlakshana & Schild, 1959) was constructed by the calculated regression of log (dose-ratio − 1) on −log (concentration of PIA). Dose-ratios were calculated by using the points of the concentration-response curve from 20% to 80% level. The pA_2 extrapolated from the Schild plot gave the 'empirical' K_i of the antagonist PIA. The K_i thus obtained was compared with the IC_{50} calculated by the equation $K_i = IC_{50}/(1 + [C]/K_a)$.

Results

Effects of PIA on the basal contractile tension and frequency

PIA, the R-site adenosine receptor agonist produced a concentration-dependent decrease in contractile tension and frequency in spontaneously beating atria at concentrations 3–100 nM. The IC_{50} values for PIA, shown in Table 1, indicate that PIA was slightly but significantly more effective in atria from reserpine-treated animals. The maximal inhibition of both atrial parameters was 95 ± 3% at 100 nM PIA. This is in accordance with data of Evans *et al.*, (1982).

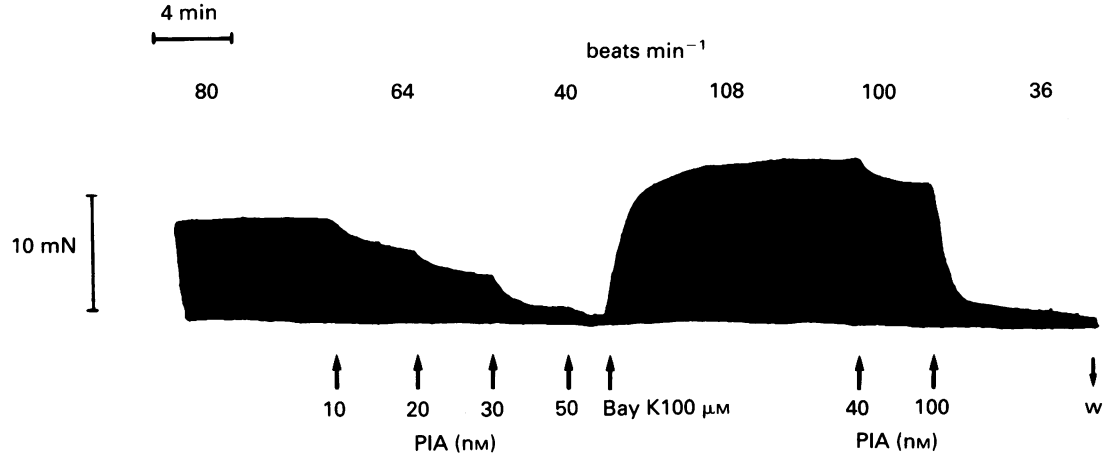

Figure 2 Depressant effects of (−)-N^6-phenylisopropyladenosine (PIA) on the contractile tension and frequency in spontaneously-beating atria from the guinea-pig. The effects of PIA were reversed by Bay K 8644. PIA was much less effective in the presence of Bay K 8644. Increasing concentrations of PIA were added cumulatively.

Effects of PIA on the positive inotropic actions of Bay K 8644 in different atrial preparations

In spontaneously beating atria, taken from normal guinea-pigs, Bay K 8644 produced a concentration-dependent increase in contractile tension (Figure 1). The positive inotropic effect was evident at 5 nM and reached a plateau at 100 nM, in accordance with data of Schramm *et al.*, (1983a) in guinea-pig isolated perfused hearts. The EC_{50} was 33 ± 3 nM. The maximum increase was 159 ± 14% over control level. The effect of Bay K 8644 was slow in onset and reached equilibrium within 10–30 min.

The effect of various concentrations of PIA on the dose-response curves for the positive inotropic effect of Bay K 8644 are shown in Figure 1. The three concentrations of PIA used, 5 nM, 20 nM and 50 nM, reduced the control tension by 15 ± 2% (n = 10), 55 ± 6% (n = 10) and 85 ± 7% (n = 5), respectively. PIA inhibited the positive inotropic effect of Bay K 8644 in a concentration-dependent manner. At all concentrations of PIA, the antagonism was fully surmountable by increasing the Bay K 8644 concentration to 1000 nM or more. Hence, PIA produced parallel rightward shifts in the Bay K 8644 concentration-response curves, indicative of a possible competitive antagonism. The IC_{50} of PIA was 2 ± 0.4 nM.

The Schild regression plot (Figure 1) was linear with a slope of 0.92 ± 0.02 (r = 0.99); 99% confidence limits = 0.88–0.96. The pA_2 value of the antagonism of Bay K 8644 by PIA was determined to be 8.63 ± 0.05 and the K_i 2.35 ± 0.25 nM, corresponding to the IC_{50} calculated according to the equation $K_i = IC_{50}/(1 + [C]/K_a)$. The negative effects of PIA on guinea-pig isolated atria were also antagonized by Bay K 8644 (Figure 2).

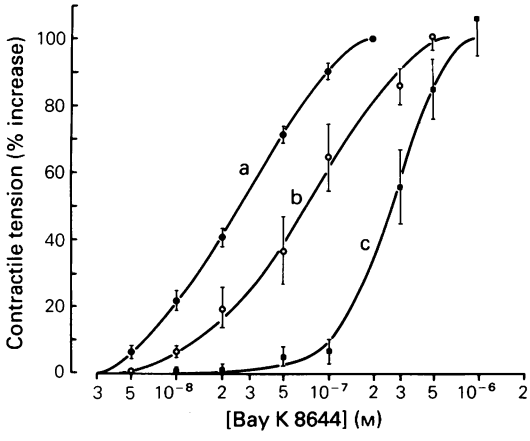

Figure 3 Inhibition by (−)-N⁶-phenylisopropyladenosine (PIA) of the positive inotropic effect of Bay K 8644 on spontaneously beating atria from reserpine-treated guinea-pigs. Cumulative concentration-response curves for Bay K 8644 in the absence (a, ●) (n = 14) and presence of PIA 4 × 10^{-9}M (b, ○) (n = 5–7) and 10^{-8}M (c, ■) (n = 5–6). Responses are expressed as in Figure 1. Each point is the mean and vertical lines show s.e. mean.

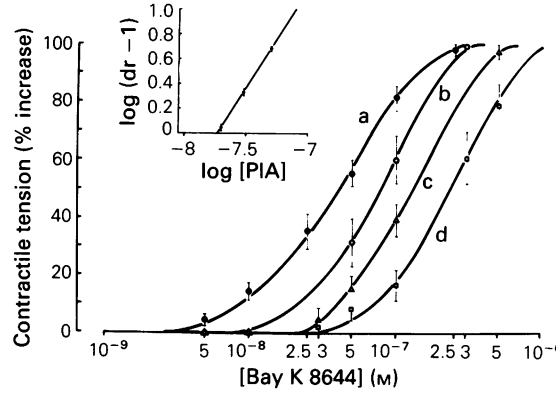

Figure 4 Inhibition by (−)-N⁶-phenylisopropyladenosine (PIA) of the positive inotropic effect of Bay K 8644 on electrically driven left atria. Cumulative concentration-response curves for Bay K 8644 in the absence (a, ●) (n = 8–11) and in the presence of PIA 2 × 10^{-8}M (b, ○) (n = 4–5); 3 × 10^{-8}M (c, △) (n = 4) and 5 × 10^{-8}M (d, □) (n = 8–9). Responses expressed as in Figure 1. Each point is the mean and vertical lines show s.e. mean. Inset: Schild regression for PIA with Bay K 8644 as the agonist (dr = dose-ratio). The slope of the regression line was 1.58 ± 0.02 (r = 0.99) and apparent pA_2 value 7.73 ± 0.02.

In spontaneously beating atria from reserpine-treated guinea-pigs, the positive inotropic effect of Bay K 8644 was very similar to that in atria from untreated animals (Figure 3). Here too, increasing concentrations of PIA produced a parallel shift to the right of the Bay K 8644 concentration-response curve. The IC_{50} of PIA was 1.5 ± 0.4 nM, not significantly different ($P > 0.20$) from that in untreated atria.

In electrically driven left atria, we eliminated the frequency parameter, that was strongly affected by PIA (Table 1). The positive inotropic effect of Bay K 8644 proportionally increased with the rate of stimulation (0.5 to 3 Hz). Thus we used 1.5 Hz as a fixed rate of stimulation.

The positive inotropic effect of Bay K 8644 was similar to that previously shown (Figure 4). PIA inhibited the positive inotropic effect of Bay K 8644 in a concentration-dependent manner and shifted the concentration-response curve of Bay K 8644 to the right. But, in these experimental conditions, the IC_{50} of PIA was 8.5 ± 0.2 nM, about 4 times higher than in spontaneously beating atria.

Table 2 pA_2 for (−)-N^6-phenylisopropyladenosine (PIA) as an antagonist of the positive inotropic effect of Bay K 8644 in guinea-pig atria

		Developed tension	
	Slope[1]	*pA_2*	*IC_{50}* (nM)
Spontaneously beating	0.92 ± 0.02	8.63 ± 0.05	2.35 ± 0.25
Electrically driven	1.58 ± 0.02*	7.73 ± 0.02*	18.60 ± 0.40*

*$P < 0.01$. [1]Slope of log (dose-ratio − 1) against log molar concentration of the antagonist, PIA.

The Schild regression plot (Figure 4) was linear with a slope of 1.58 ± 0.02 ($r = 0.99$); 99% confidence limits 1.54–1.64. The apparent pA_2 value, extrapolated from the Schild regression plot, was 7.73 ± 0.02 and the K_i 18.6 ± 0.4 nM (Table 2), higher than the IC_{50} calculated according to the equation $K_i = IC_{50}/(1 + [C]/K_a)$. This behaviour of the Schild regression may indicate a temporal or a thermodynamic disequilibrium. The diffusion, or drug-receptor interaction, may be rate-limiting (Kenakin, 1985). However, the slope was not modified by changing the incubation time (5 to 20 min) of PIA with the left atria (not shown).

Effect of PIA on the positive chronotropic effect of Bay K 8644.

The interaction between PIA and Bay K 8644 on the atrial frequency was difficult to investigate as the positive chronotropic effect of Bay K 8644 was very low and variable. The frequency increased over the control level of 14 ± 2% in normal and of 31 ± 4% in reserpinized atria but never reached a maximum at the concentrations used. Consequently, concentration-response curves for the chronotropic effect of Bay K 8644 in the absence and presence of PIA were incomplete and difficult to analyse. The positive chronotropic effect of Bay K 8644 was apparently antagonized by PIA, but the type of antagonism was difficult to define.

Lack of interaction between carbachol and Bay K 8644

The specificity of the antagonism of Bay K 8644 by PIA was tested by using carbachol which, like PIA, has negative inotropic and chronotropic effects on atrial preparations.

Carbachol was tested at concentrations of 7 nM, 10 nM and 20 nM, which reduced the atrial rate and contractility by 15%, 30% and 60% respectively. Carbachol did not modify the positive inotropic action of Bay K 8644 (Table 3), nor the chronotropic effect (not reported).

Effect of PIA in depolarized atria

We studied the effects of PIA on Ca^{2+}-dependent contraction in potassium-depolarized electrically stimulated left atria (Figure 5). PIA was able to counteract the contractile responses generated in these experimental conditions by the inward slow calcium current.

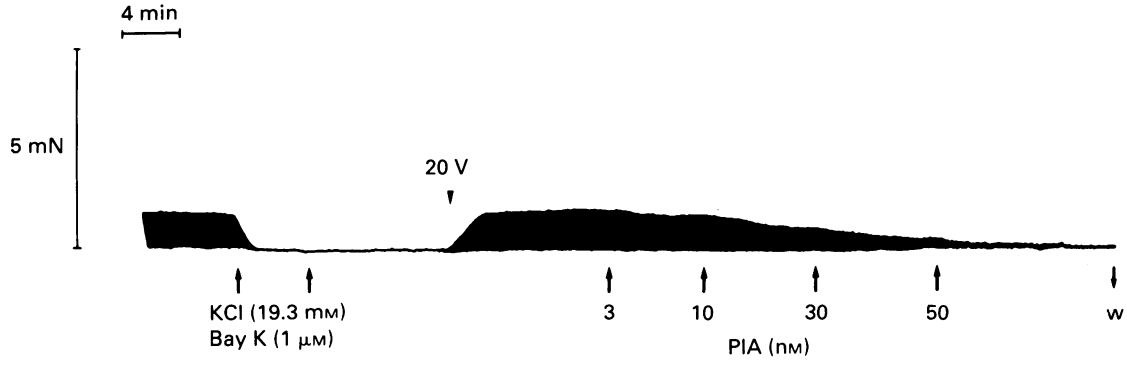

Figure 5 The effects of (−)-N^6-phenylisopropyladenosine (PIA) on the contractions induced in the potassium-depolarized, electrically-driven (1.5 Hz, 3 ms, 0.6 V) left atria of the guinea-pig. The medium contained a final K^+ concentration of 22 mM in order to inactivate the fast sodium channels (KCl 19.3 mM was added to obtain a final concentration of 22 mM). PIA was added cumulatively and the final concentrations in the medium are indicated.

Table 3 Lack of an interaction between carbachol and Bay K 8644

Carbachol	n	*Bay K 8644* 5 nM	10 nM	50 nM	100 nM	500 nM
—	12	8.3 ± 2.6	23.1 ± 4.1	74.9 ± 5.8	93.7 ± 3.5	97.9 ± 2.1
7 nM (IC_{15})	2	14	31	75	100	—
10 nM (IC_{30})	5	14.2 ± 5.7	29.4 ± 9.0	72.7 ± 6.5	93.2 ± 4.9	99.4 ± 0.6
20 nM (IC_{60})	5	18.6 ± 5.4	26.7 ± 5.2	76.8 ± 13.4	88.8 ± 8.0	100 ± 0

Carbachol was introduced in the medium 10 min before Bay K 8644. Data are expressed as percentage of the maximum developed tension to Bay K 8644. Mean ± s.e. mean of the number of preparations indicated (*n*) are shown.

Discussion

PIA, a stable analogue of adenosine, inhibits at nanomolar concentrations the positive inotropic effect of Bay K 8644, a calcium channel facilitator, in guinea-pig isolated atria. The antagonism is evident both in spontaneously beating preparations, taken from normal and from reserpine-treated animals, as well as in electrically driven left atria.

The antagonism by PIA of the effects of Bay K 8644 is specific as carbachol had no effect on the response to Bay K 8644. Cholinoceptor agonists are known (Belardinelli & Isenberg, 1983) to increase conductance of the membrane to K^+ in atrial myocytes, with a consequent shortening of the action potential and inhibition of the slow calcium current. The lack of interaction between carbachol and Bay K 8644 indicates that PIA acts by a mechanism different from the cholinoceptor agonists. It is also known (Schramm *et al.*, 1983a; Ishii *et al.*, 1985) that the positive inotropic effect of Bay K 8644 is neither the result of α- or ß-adrenoceptor stimulation nor of an interaction with dopamine, 5-hydroxytryptamine, histamine or opiate receptors.

In spontaneously beating atria, the antagonism of Bay K 8644 by PIA was apparently competitive. The IC_{50} of PIA was 2.3 ± 0.2 nM. The Schild plot for PIA against Bay K 8644 was linear with a slope near to unity, which suggests simple competition between an agonist and an antagonist with a homogeneous receptor population (Kenakin, 1985). These results indicate that PIA and Bay K 8644 may compete for a common receptor site in or near the slow calcium channel. Alternatively, the observed antagonism may be the result of an allosteric competition between PIA and Bay K 8644, i.e, the two drugs may interact with topologically distinct sites, but the binding of one drug induces a conformational change that prevents the binding of the other drug.

In electrically driven atria, PIA also produced parallel rightward shifts in the Bay K 8644 concentration-response curves; but the IC_{50} of PIA was 10 times higher than in spontaneously beating atria (18.6 ± 0.4 nM) and the Schild regression had a slope higher than unity (1.58 ± 0.02). This behaviour may indicate a temporal or a thermodynamic disequilibrium (Kenakin, 1985). The drug-receptor interaction may be rate-limiting, as the slope was not modified by varying the time (5 to 20 min) of equilibration of PIA with left atria.

Bay K 8644 apparently binds to the same binding site as nifedipine (Schramm *et al.*, 1983a, b; Thomas *et al.*, 1984; Ishii *et al.*, 1985) but these receptor sites can exist in low- and high-affinity states (Glossmann *et al.*, 1984). Further, the channel can exist in different conformations through which it can cycle in the intact cell (open, closed, inactivated) (Mestre *et al.*, 1985). The channel blocking drugs often do not interact with closed, or resting, channels. The functional kinetics of channel blocking drugs are thus difficult to define (Spedding, 1985). Recent findings (Thomas *et al.*, 1986) with Bay K 8644 in voltage-clamp experiments showed that Bay K 8644 binds only to the open state of the calcium channel preventing its closing as long as the compound is bound.

Adenosine (Belardinelli & Isenberg, 1983) and PIA (Böhm *et al.*, 1985) shorten the normal action potential in atrial preparations and hence reduce the entry of Ca^{2+} into the cell. Adenosine was also found to produce a dose-dependent slowing of the rate of sinus node cells, mainly by decreasing the rate of diastolic depolarization (West & Berlardinelli, 1985). This may decrease the probability for Bay K 8644 finding calcium channels in the open state, which is the only state responsive to Bay K 8644. In electrically driven atria, where the rate of depolarization is constant, and not decreased by the presence of PIA, the probability that Bay K 8644 binds to the open channel and develops its facilitator effect is increased. In these experimental conditions, the only action component of PIA is the one shortening action potential. This allows a simple explanation for the lower IC_{50} of PIA in electrically driven atria compared with the IC_{50} in spontaneously beating atria, and could also provide an explanation for the disequilibrium in drug-receptor interaction suggested by a slope higher than unity of

the Schild regression in electrically driven atria.

Although the present data do not allow us to choose between the various molecular mechanisms involved in the PIA-Bay K 8644 interaction, they indicate that this interaction takes place at the level of slow calcium channels.

Further evidence is obtained from the inhibitory effect of PIA on the contractile response generated by electrical stimulation in K^+-depolarized atria. This conclusion supports the hypothesis that adenosine acts in atria by a mechanism independent of cyclic AMP and indicates that the effector of this mechanism is the slow calcium channel. It is also substantiated by results showing a direct action of 2-chloroadenosine on the voltage-activated calcium currents in cultured rat dorsal root ganglia neurones (Dolphin & Scott, 1986).

A general consideration suggested by our data is whether an analogous situation may also occur *in vivo*. If so, adenosine might be considered as a physiological endogenous factor modulating slow calcium channel function.

In summary, the results presented here suggest that in guinea-pig atria PIA is able to interact at a site in or near the slow calcium channels, and that occupation of these sites by PIA may decrease the transmembrane calcium flux into the cell. This supports the hypothesis of an involvement of slow Ca^{2+} channels in the 'direct' action of adenosine, independent of cyclic AMP. If so, adenosine has to be considered as a putative endogenous factor modulating Ca^{2+} channel function *in vivo*.

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