Characterization of the adenosine receptor in porcine coronary arteries

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- 1 Relaxant responses of ring preparations from porcine ventricular coronary arteries to adenosine and various stable adenosine analogues were investigated *in vitro*.
- 2 The adenosine analogues did not produce contraction but elicited almost complete relaxation of coronary arteries precontracted with 3 μ M prostaglandin F_{2 α} (PGF_{2 α}), even after removal of the endothelium.
- 3 The order of potency, was 5'-N-ethylcarboxamide-adenosine (NECA) > 2-(2-phenylethylamino)5'-N-ethylcarboxamide-adenosine (2-PEA-NECA) > 2-phenylamino-adenosine (CV-1808) > N⁶-[R(-)-1-phenyl-2-propyl]adenosine (R-PIA) > N⁶-[S(+)-1-phenyl-2-propyl]adenosine (S-PIA) > N⁶-cyclopentyladenosine (CPA) > adenosine > ATP = ADP which suggested the presence of adenosine A₂-receptor subtypes.
- 4 There was an excellent correlation between the calculated pD₂ values on coronary arteries and the pK_D values at adenosine A₂ binding sites, whereas no correlation was obtained when the pD₂ values were compared to the pK_D values at adenosine A₁-binding sites on membranes from porcine striata.
- 5 The relaxant effects of adenosine and its analogues were competitively antagonized by 8-(*p*-sulphophenyl)-theophylline (8-SPT), producing pA₂ values similar to the respective pK_D value of the antagonist at adenosine A₂ binding sites.
- 6 It is suggested that the porcine coronary artery possesses adenosine A₂ receptors which seem to be similar to the adenosine A₂ binding site in pig striatum, whereas no evidence was obtained for the presence of adenosine A₁ receptors.

Introduction

Adenosine receptors mediate several physiological effects, the most important of which may be the restoration of a balance between metabolic demand and supply (Newby, 1984). According to the classification proposed by Burnstock (1978) the pharmacological responses to adenosine are mediated through extracellular adenosine (P₁) receptors which have been further subdivided into two types of membrane bound adenosine receptors, namely adenosine A₁ and A₂ receptors both of which are coupled to adenylate cyclase via a G regulatory protein. While the A₁ subtype of adenosine receptor mediates inhibition of adenylate cyclase, the A₂ subtype induces activation of the enzyme (Van Calcar *et al.*, 1978; Londos *et al.*, 1980). Although antagonists with adenosine A₁ receptor selectivity, and only little effect on phosphodiesterase, have become available, classification of adenosine receptor subtypes is still based predominantly on the relative potencies of various adenosine analogues (Stone, 1984; Bowmer & Yates, 1989). At the A₁ receptor subtype, N⁶ substituted analogues, e.g., N⁶-[1-phenyl-2-propyl]adenosine (PIA), are more potent than 5' substituted analogues, such as 5'-N-ethylcarboxamide-adenosine (NECA), with the reverse being true at the A₂ receptor subtype. By use of this classification, the presence of A₂ subtype receptors has been demonstrated in canine (Kusachi *et al.*, 1983; Nakazawa & Mustafa, 1988), bovine (Mustafa & Askar, 1985) and human coronary arteries (Ramagopal *et al.*, 1988). The results of the present study add support to the contention that responses to adenosine of porcine coronary arteries are also mediated mainly, if not exclusively through A₂ adenosine receptors while there is no evidence for the presence of A₁ adenosine receptors in this vascular smooth muscle.

Methods

Binding assay

A crude membrane preparation was prepared from porcine striata as described by Bruns *et al.* (1986). Briefly, striata were

disrupted in 10 volumes of ice-cold 50 mM Tris HCl buffer (pH 7.4) for 30 s with a Polytron PT-10/35 homogenizer (Brinkmann) at setting 5–6 and centrifuged at 50,000 *g* for 10 min. The supernatant was discarded and the pellet was resuspended with a Polytron in 10 volumes of ice-cold Tris HCl buffer as above and recentrifuged. The final pellet was resuspended at 1 g 5 ml⁻¹, and stored in plastic vials at –70°C until used. All incubations were carried out in triplicate for 45 min at 37°C in TRF 95 vials. The final volume of 1 ml contained: Tris HCl buffer (50 mM Tris HCl + 10 mM MgCl₂, pH 7.4), 4 nM [³H]-NECA, 1 μ g ml⁻¹ adenosine deaminase, 50 nM N⁶-cyclopentyladenosine (CPA, to eliminate A₁ receptor binding), substance under investigation and pig striatum membranes (5–6 mg of original tissue weight). CPA was omitted from those studies where both A₁ and A₂ components of binding were determined. The incubation was terminated by addition of about 5 ml ice-cold buffer (50 mM Tris HCl + 154 mM NaCl, pH 7.4) and filtration through Whatman GF/B filter sheets, which were then further rinsed with about 5 ml ice-cold buffer. The damp filters were put into scintillation vials, scintillation fluid (5 ml ControGel) was added and the vials incubated at 37°C for 30 min and shaken. After the contents of the vials had been cooled down to room temperature the radioactivity was measured in a beta counter.

Organ bath studies

Porcine hearts were obtained from a local slaughter house within 30 min after death and transported to the laboratory where the ventricular coronary arteries were carefully removed and placed in Krebs-Henseleit solution (composition in mM: NaCl 118, KCl 4.7, MgSO₄ 1.2, CaCl₂ 1.2, KH₂PO₄ 1.2, NaHCO₃ 25, glucose 11, EDTA 0.03) gassed with 5% CO₂ in oxygen. The arteries were cut into rings (2–3 mm) and stainless steel hooks were inserted through the lumen, allowing them to be suspended for isometric tension recording in 10 ml organ baths containing Krebs-Henseleit solution at 37°C, bubbled continuously with 5% CO₂ in O₂. Changes in the tone of the preparations were recorded isometrically with electromechanical transducers (Statham model UC3) and a potentiometric recorder. At the beginning of the experiments,

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the rings were stretched to an initial tension of about 1.5 g and allowed to equilibrate and establish baseline tension for 2 to 3 h in the bathing medium, which was changed every 15 min. During this time the preparations were stimulated twice with KCl (30 mM) and the baseline tension was adjusted to about 1 g. After repeated changes of the bathing solution the tissues were exposed to $3 \mu\text{M}$ prostaglandin $\text{F}_{2\alpha}$, which induced a stable submaximal contraction of about 80% of maximum response. Concentration-response curves to adenosine analogues were then determined in the absence and presence of antagonists. Inhibitors were added 30 min before the agonists to the organ baths and remained in contact with the tissue when the responses to agonists were tested. At the end of each concentration-response curve papaverine ($300 \mu\text{M}$) was added to induce complete relaxation of the arterial rings. This effect was taken as 100% relaxation. In each experiment 6 rings of the same artery were investigated at the same time, one of which was used as a control preparation, to correct for any sensitivity changes during the experiment.

Data analysis

Non-specific binding of [^3H]-NECA was defined as binding in the presence of $100 \mu\text{M}$ NECA. Specific binding was about 85–90% of the total binding. The experimental data were fitted to a two-site model yielding Hill coefficients of 1.0. The regression algorithm applied was derived from the Downhill Simplex Method of Multidimensions (Press *et al.*, 1987). The 95% confidence limits for the estimated parameters $B_{\text{max}1}$, $B_{\text{max}2}$, $\text{IC}_{50}(1)$, $\text{IC}_{50}(2)$ and non-specific binding were obtained by maximizing and minimizing each of the 5 parameters, with the restriction that the Chi square value of the residuals was kept within the 95% confidence limits, by use of the same algorithm mentioned above.

The confidence limits for the calculated curves depicted in the figures were obtained from the envelopes of all calculated curves obtained in the process of estimating the confidence limits for the 5 parameter estimates. They depict the '95% confidence channel', that implies the course of the 'true' curve, assuming the two-site model to be applicable.

Concentration-response curves on coronary arteries were analysed with a non-linear computer programme, and E_{max} (maximal effect) and pD_2 values (negative logarithm of the molar concentration of the agonist producing 50% of E_{max}) were derived from this analysis. Apparent pA_2 values were calculated according to the equation $\text{pA}_2 = \text{pA}_x + \log(x - 1)$, where pA_x is the negative logarithm of the molar concentration of the antagonist used and x is the ratio of equieffective concentrations of the agonist in the presence and absence of the antagonist (Arunlakshana & Schild, 1959; Furchgott, 1972). Data are given as means $\pm$ s.e.mean, statistical analysis was performed by Student's *t* test.

Drugs

The following pharmacological agents were used: CHA (N^6 -cyclohexyladenosine, Boehringer, Mannheim, F.R.G.), NECA ($5'$ - N -ethylcarboxamide-adenosine, Byk-Gulden, Konstanz, F.R.G.), adenosine (Fluka, Buchs, Switzerland), CPA (N^6 -cyclopentyladenosine), R-PIA (N^6 -[$\text{R}(-)$ -1-phenyl-2-propyl]adenosine), S-PIA (N^6 -[$\text{S}(+)$ -1-phenyl-2-propyl]adenosine, Research Biochemicals, Incorp., Wangen, Switzerland), ADP (adenosine diphosphate), ATP (adenosine triphosphate), $\text{PGF}_{2\alpha}$ (prostaglandin $\text{F}_{2\alpha}$), forskolin, isoprenaline sulphate, papaverine hydrochloride, (Sigma, Munich, F.R.G.), 8-SPT (8-(*p*-sulphophenyl)-theophylline-monohydrate), CV-1808 (2-phenylamino-5'- N -ethylcarboxamide-adenosine, European Patent No. 277 917, 1988) were synthesized at Sandoz Ltd., Basel, Switzerland.

Both prostaglandin $\text{F}_{2\alpha}$ ($\text{PGF}_{2\alpha}$) and forskolin were dis-

solved in ethanol and diluted in distilled water to give a 1 mM solution containing 10% ethanol. Samples of this solution were stored at -20°C until use. CV-1808 was dissolved in 50% ethanol containing 25% N -methylpyrrolidone (v/v), to give a 1 mM solution containing 1.5% ethanol and 1% N -methylpyrrolidone. CPA and 2-PEA-NECA were dissolved in polyethylene glycol (PEG) 400 and diluted with distilled water to give a 1 mM solution containing 60% PEG 400. CHA, R-PIA and S-PIA were dissolved in dimethyl sulphoxide (DMSO) and diluted in distilled water to give a 1 mM solution containing 10% DMSO. Further dilutions were performed in either 5% glucose or physiological salt solution. All compounds, except papaverine, forskolin and $\text{PGF}_{2\alpha}$, were dissolved just before use. Drug concentrations are given as molar concentrations throughout.

Results

Agonism

Under control conditions neither adenosine nor CPA elicited a contractile response of porcine coronary arteries. Even after activation of adenylate cyclase by isoprenaline ($1 \mu\text{M}$) or forskolin (0.1 and $1 \mu\text{M}$), CPA like adenosine elicited no contractile response but induced an additional relaxation (Figure 1). Consequently, only for relaxant responses were concentrations-response curves to the various adenosine analogues determined, using arterial preparations precontracted with $3 \mu\text{M}$ $\text{PGF}_{2\alpha}$ (Figure 2). In preliminary experiments it was observed that removal of the endothelium from the arterial preparations, by rubbing the interior of the ring with a wooden matchstick, did not modify the relaxant response to adenosine. Therefore no special attention was paid to preserve the endothelium when the relaxant activities of the various adenosine analogues were investigated. All the compounds tested induced nearly complete relaxation of rings from porcine ventricular coronary arteries. Comparison of the calculated pD_2 values on coronary vascular smooth muscle with the pK_D values determined on membranes from pig striatum

Figure 1 Representative tracing of responses to adenosine of rings from porcine ventricular coronary arteries without and after activation of adenylate cyclase with isoprenaline (Iso, $1 \mu\text{M}$) or forskolin (For, 1 and $0.1 \mu\text{M}$).

Figure 2 Cumulative concentration-response curves for 5'-N-ethylcarboxamide-adenosine (NECA) (●—●, $n = 7$), 2-(2-phenylethylamino) NECA (2-PEA-NECA) (▽—▽, $n = 10$), 2-phenylamino-adenosine (CV-1808) (■—■, $n = 8$), N⁶-[R(-)-1-phenyl-2-propyl]adenosine (R-PIA) (□—□, $n = 5$), N⁶-[S(+)-1-phenyl-2-propyl]adenosine (S-PIA) (○—○, $n = 6$), N⁶-cyclopentyladenosine (CPA) (▼—▼, $n = 13$), N⁶-cyclohexyladenosine (CHA) (△—△, $n = 4$), adenosine (○—○, $n = 13$), ATP (△—△, $n = 8$) and ADP (□—□, $n = 10$) on rings from porcine ventricular coronary arteries stimulated with 3 μ M PGF_{2 α} . The effects are expressed as percentages of the relaxant response to 300 μ M papaverine. The points represent means and vertical lines s.e.mean.

Figure 3 Correlation between drug potencies for relaxation of porcine coronary arteries (pD_2 values, abscissa scale) and (pK_D values, ordinate scale) at adenosine A₁ (○) and A₂ (●) binding sites in pig striatum. Data were compared by linear regression analysis. The correlation coefficient and regression line for the correlation of pD_2 values and A₂ binding are indicated; $P < 0.001$.

Table 1 Affinity parameters (pD_2 values) and intrinsic activity for various adenosine analogues of rings from pig ventricular coronary arteries and pK_D values for binding at A₁ and A₂ binding sites in membranes from pig striatum

	pD_2 values	Intrinsic activity	pK_D values	
			A ₁	A ₂
Adenosine	5.51 $\pm$ 0.13	94.2 $\pm$ 1.3 (13)	*	*
ADP	5.38 $\pm$ 0.05	95.8 $\pm$ 1.0 (10)	*	*
ATP	5.39 $\pm$ 0.10	94.6 $\pm$ 1.3 (8)	*	*
CHA	5.46 $\pm$ 0.11	99.3 $\pm$ 0.5 (4)	8.91	5.95
CPA	5.77 $\pm$ 0.07	98.5 $\pm$ 0.5 (13)	9.13	6.03
CV-1808	6.53 $\pm$ 0.07	98.5 $\pm$ 0.2 (8)	5.61	6.57
2-PEA-NECA	7.54 $\pm$ 0.08	95.8 $\pm$ 1.0 (10)	4.81	7.72
NECA	7.74 $\pm$ 0.08	96.5 $\pm$ 1.3 (4)	8.25	7.64
R-PIA	6.29 $\pm$ 0.07	97.0 $\pm$ 1.0 (5)	8.99	6.34
S-PIA	5.85 $\pm$ 0.07	98.0 $\pm$ 1.1 (6)	7.69	5.57

Data are given as mean $\pm$ s.e.mean, number of determinations in parentheses.

* Since binding studies were performed in the presence of adenosine deaminase, pK_D values for adenosine, ADP and ATP were not determined.

Table 2 Apparent pA_2 values for antagonism of various adenosine analogues by 8-(*p*-sulphophenyl)-theophylline (8-SPT) on rings from porcine ventricular coronary arteries

	pA_2 values
Adenosine	4.91 $\pm$ 0.21 (6)
ADP	4.60 $\pm$ 0.14 (7)
ATP	5.13 $\pm$ 0.11 (8)
CHA	4.94 $\pm$ 0.13 (8)
CPA	5.02 $\pm$ 0.11 (8)
CV-1808	5.35 $\pm$ 0.15 (8)
2-PEA-NECA	5.08 $\pm$ 0.09 (5)
NECA	5.31 $\pm$ 0.07 (8)
R-PIA	5.03 $\pm$ 0.08 (8)
S-PIA	4.93 $\pm$ 0.16 (8)

Data shown are means $\pm$ s.e.mean, number of determinations in parentheses.

revealed a significant correlation between pD_2 values and the pK_D values at the A₂ binding sites ($P < 0.001$), whereas there was no correlation when the pD_2 values were compared to the pK_D values for A₁ binding sites (Figure 3, Table 1).

Antagonism

The non-selective adenosine receptor blocker 8-SPT consistently produced a parallel displacement of the concentration-responses curves of all adenosine analogues tested. The calculated pA_2 values for 8-SPT (Table 2) were statistically not different from the pK_D value of 5.55 ($n = 3$) determined for binding of 8-SPT at A₂ binding sites, suggesting that all agonists acted through stimulation of A₂-receptors.

Discussion

The aim of these experiments was to characterise the subtype of adenosine receptor(s) present in porcine coronary arteries by use of adenosine and different stable adenosine analogues. The order of potencies for the relaxant activities of the agonists used in our study, NECA > 2-PEA-NECA > CV-1808 > R-PIA > S-PIA > CPA > adenosine > ATP = ADP, suggested that the responses in this tissue are mediated by adenosine A₂ receptors. Further evidence for this was obtained when the calculated pD_2 values were compared to the pK_D values determined at A₁ and A₂ binding sites on membranes from porcine striata. There was an excellent correlation between the pD_2 values on coronary arteries and the pK_D values at A₂ binding sites. Whereas no correlation could be obtained when the pD_2 values were compared to the pK_D values at A₁ binding sites. In agreement with results obtained from other studies on coronary arteries of various species, such as dog, bovine and man (Kusachi *et al.*, 1983; Mustafa & Askar, 1985; Nakazawa & Mustafa, 1988; Ramagopal *et al.*, 1988), the present results suggest that the adenosine-induced dilatation of pig coronary arteries is also mediated through adenosine A₂ receptors. However, in contrast to that found for canine coronary arteries (Houston *et al.*, 1985), the adenosine-mediated relaxation of pig coronary arteries was not endothelium-dependent. Similar findings have been obtained for human (Ramagopal *et al.*, 1988), bovine (Mustafa & Askar, 1985) and monkey coronary arteries (Toda, 1983). The adenosine receptor blocker 8-SPT antagonized the relaxations induced by adenosine and the various analogues in a competitive manner and with similar potency, suggesting that the relaxations are mediated by a homogeneous population of adenosine receptors. Moreover, the pA_2 values calculated for antagonism of adenosine and the various analogues by 8-SPT

were statistically not different and similar to the pK_D value of 8-SPT at the A_2 binding site, suggesting that the responses are mediated by adenosine A_2 receptors.

In conclusion, the data presented in this study suggest that the porcine coronary artery possesses only adenosine A_2

receptors which mediate dilatation and seem to be similar to the adenosine A_2 binding site in pig striatum, whereas no evidence was obtained for the presence of adenosine A_1 receptors.

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