British Journal of Pharmacology (1998) 123, 1425–1433

Validation of Furchgott's method to determine agonist-dependent A_1-adenosine receptor reserve in guinea-pig atrium

[1]Timothy E. Morey, [2,3]Luiz Belardinelli & [1,3,4]Donn M. Dennis

Departments of [1]Anesthesiology, [2]Medicine and [3]Pharmacology, College of Medicine, University of Florida, Gainesville, Florida, U.S.A.

1 The ubiquitous distribution of A_1-adenosine receptors (A_1AdoR) represents an impediment to achieve organ and/or response selectivity of A_1AdoR agonists. Differential receptor reserve may be exploited to overcome this problem. We hypothesize that A_1AdoR reserve is agonist-dependent and can be accurately estimated with Furchgott's method.

2 Concentration-response curves were constructed from measurement of the atrial monophasic action potential duration in guinea-pig, isolated hearts treated with **R**(−) N^6-(2-phenylisopropyl)adenosine (**R**-PIA) or 2-chloro-N^6-cyclopentyl-adenosine (CCPA) before and after treatment with the selective, irreversible A_1AdoR antagonist 8-cyclopentyl-3-[3-[[4-(fluorosulphonyl)benzoyl]oxy]propyl]-1-propyl-xanthine (FSCPX). Using Furchgott's method, we determined the equilibrium dissociation constant (K_A) of **R**-PIA and CCPA, and the fraction of non-inactivated A_1AdoRs remaining after FSCPX treatment ($q_{functional}$). Values of $q_{functional}$ were correlated to the fraction of specific binding sites after FSCPX treatment labelled by [^{3}H]-8-cyclopentyl-1,3-dipropylxanthine ([^{3}H]-CPX) derived from saturation binding normalized to control ($q_{binding}$).

3 Both **R**-PIA and CCPA are full A_1AdoR agonists, but have significantly different potencies (pD_2 [EC_{50}] = 6.84 ± 0.04 [145 nM] vs 7.36 ± 0.04 [44 nM], respectively), receptor affinities (pK_A [K_A] = 6.54 ± 0.10 [288 nM] vs 6.13 ± 0.03 [734 nM]), and pharmacological shift ratios defined as K_A/EC_{50} (2.2 ± 0.6 vs 15.9 ± 1.5). Values for $q_{functional}$ and $q_{binding}$ were highly correlated ($r^2 = 0.96$). The ratio between the intrinsic efficacies of CCPA and **R**-PIA derived from Furchgott's analysis was 5.9, a value similar to the ratio of 6.2–6.6 calculated from previously obtained binding data.

4 Radioligand binding studies validated the use of Furchgott's method to estimate A_1AdoR reserve. A_1AdoR reserve was agonist-dependent. CCPA was shown to be a high intrinsic efficacy, low affinity agonist, whereas **R**-PIA was found to be a low intrinsic efficacy, high affinity agonist.

Keywords: Adenosine; A_1-adenosine receptor; CCPA; Furchgott's method; receptor reserve; **R**-PIA

Introduction

Although Furchgott's method of irreversible receptor inactivation has been extensively used to determine receptor reserve, a phenomenon wherein submaximal receptor occupancy by an agonist causes a maximal response, for various G protein-coupled receptors (Harden *et al*., 1986; Meller *et al*., 1987; 1990; Adams *et al*., 1990), its validity to determine A_1-adenosine receptor (A_1AdoR) reserve has yet to be established. By validating Furchgott's method, it would be possible to determine adenosine receptor reserve in tissues (or cells) that are not amenable to radioligand binding studies (e.g., sinoatrial or atrioventricular node). If validated, this method would become a valuable pharmacological tool to study selective organ response(s) to A_1AdoR agonists, and differential regulation of tissue (or cellular) responsiveness to agonists during dynamic changes in receptor expression.

The A_1AdoR is ubiquitously distributed throughout the body and mediates a number of important physiological actions of adenosine (Collis & Hourani, 1993). This has been a major impediment to the development of drug therapies that selectively mimic or block specific actions of endogenous adenosine mediated by the A_1AdoR subtype. Knowledge of the receptor reserve for actions mediated by A_1AdoRs and of the relative dependence of an agonist on receptor affinity (K_A) and intrinsic efficacy (ε) for its potency, may form a theoretical framework whereby the problems of organ and response selectivity to agonists can be solved.

Using the method of Furchgott and Bursztyn (1967), we previously demonstrated the presence of an A_1AdoR reserve in guinea-pig atrioventricular node (Dennis *et al*., 1992), and recently showed a differential receptor reserve for A_1AdoR-mediated activation of the inwardly rectifying potassium current (I_{KAdo}) and inhibition of the isoprenaline-stimulated L-type calcium current (β-$I_{Ca,L}$) in atrial cardiomyocytes (Srinivas *et al*., 1997). Because receptor reserve has been found to be an agonist-dependent phenomenon in the dopamine (Meller *et al*., 1987), muscarinic acetylcholine (Harden *et al*., 1986), 5-hydroxytryptamine (5-HT; Meller *et al*., 1990), and μ opiate (Adams *et al*., 1990) receptor systems, it is reasonable to infer that receptor reserve for A_1AdoR-mediated responses, like those of other G-protein-coupled receptors, will also be agonist-dependent. Agonist-dependent differences in receptor reserve might be exploited to attain site- and event-specificity for the actions of A_1AdoR agonists. Thus, identification of A_1AdoR agonists with different magnitudes of receptor reserve for the various physiological effects of adenosine may have therapeutic implications.

In guinea-pig isolated perfused hearts we (1) investigated the phenomenon of receptor reserve in isolated hearts using two full A_1AdoR agonists, **R**(−)-N^6-(2-phenylisopropyl)adenosine (**R**-PIA) and 2-chloro-N^6-cyclopentyl-adenosine (CCPA), (2) determined the relative contribution of K_A and ε to the potency of **R**-PIA and CCPA, and (3) validated

[4]Author for correspondence at: Department of Anesthesiology, College of Medicine, University of Florida, P.O. 100254, Gainesville, FL 32610-0254, U.S.A..

Furchgott's model for A_1AdoR reserve by directly comparing its results against those of radioligand binding.

Methods

Isolated perfused hearts

Isolation and perfusion of hearts All protocols were reviewed and approved by the Animal Use Committee of the University of Florida Health Sciences Center. Hartley guinea-pigs of either sex weighing 300–400 g were anaesthetized with halothane (Halocarbon Laboratories, River Edge, NJ) and killed by cervical dislocation. The hearts were rapidly removed and rinsed in ice-cold Krebs-Henseleit (K-H) solution containing (mM): NaCl 117.9, KCl 4.8, $CaCl{\cdot}2H_2O$ 2.5, $MgSO_4{\cdot}7H_2O$ 1.18, KH_2PO_4 1.2, $Na_2EDTA{\cdot}2H_2O$ 0.5, ascorbic acid 0.14, glucose 5.5, pyruvic acid (sodium salt) 2.0 and $NaHCO_3$ 25. The ascending aorta was cannulated for perfusion of the coronary arteries at a constant flow of 8 ml min^{-1} with K-H solution gassed continuously with 95% O_2/5% CO_2. The oxygen tension, temperature and pH of the K-H solution were maintained at 500–600 mmHg. 36.0±0.5°C and 7.3–7.4, respectively.

Pacing and electrophysiological measurements Hearts were paced by use of an interval generator (A310 Accupulser, World Precision Instruments, Sarasota, FL) which delivered stimuli via a stimulus isolation unit (A360R, World Precision Instruments) as square wave pulses of 3 ms duration and twice threshold intensity. The stimuli were delivered at a basic cycle length of 250 ms via a stainless steel, teflon coated, bipolar electrode placed on the epicardium of the right atrium.

Monophasic action potentials (MAP) were recorded with pressure contact silver-silver chloride electrodes (Langendorff Probe, EP Technologies, Inc. Sunnyvale, CA) placed on the epicardial surface of the left atrium as previously described (Raatikainen *et al*., 1996). The signals were amplified and filtered by an isolated biological amplifier (IsoDam, World Precision Instruments) and displayed in real time on a digital oscilloscope (2201, Tektronix Inc., Beaverton, OR). The amplitudes of the monophasic action potentials were determined from the diastolic baselines to the plateaux. Signals were considered adequate if they were stable over 10 min and their amplitudes exceeded 10 mV. Data were digitized at 2 kHz by a DT-2801A digitizing board (Data Translation, Marlboro, MA) and stored by use of the Snapshot data acquisition program (Snapshot Storage Scope, HEM Data Corp., Southfield, MI) for later analysis. The duration of the atrial monophasic action potential was measured at 90% repolarization ($MAPD_{90}$) by use of the Snapshot program. After completion of dissection and instrumentation the hearts were allowed to equilibrate for 20 min before experiments were commenced.

Experimental protocols After random assignment to receive either **R**-PIA or CCPA, concentration-responses curves for atrial $MAPD_{90}$ shortening were constructed for each heart. After the highest concentration of agonist was administered, the infusion of the A_1AdoR agonist was stopped. Fifteen minutes later, FSCPX (2 μM) was infused for 30–45 min followed by 30 min of washout. Concentration-response curves were then constructed again with the same agonist. At the conclusion of each experiment, the atria (right and left atrium) were excised and stored at −80°C for subsequent radioligand binding assays to determine the specific binding of [^{3}H]-CPX.

Analysis of concentration-response curves Concentration-response relationships were fit to a multiparameter logistic equation by use of a non-linear regression technique employing the Marquart-Levenberg algorithm (Prism 2.01, GraphPad Software, Inc., San Diego, CA):

$$\text{Response} = \frac{E_{max}[A]^{n_H}}{[A]^{n_H} + EC_{50}^{n_H}} \qquad \text{Equation 1}$$

where E_{max} is the maximal response ($MAPD_{90}$ shortening), A is the agonist concentration, EC_{50} is the concentration of an agonist causing half-maximal response, and n_H is the Hill coefficient.

Estimation of receptor reserve The method of Furchgott and Bursztyn (1967) was used to estimate the agonist equilibrium dissociation constant (K_A) of **R**-PIA and CCPA, and the fraction of non-inactivated receptors remaining after exposure of the heart to FSCPX ($q_{functional}$). Pairs of concentrations of an agonist that caused equal degrees of shortening of $MAPD_{90}$, before and after inactivation of a fraction of A_1AdoRs with FSCPX, were selected. The equieffective concentrations were determined at twenty levels of response between 20% and 100% of the maximum effect after FSCPX treatment by interpolation from concentration-response curves (see examples in Figure 2). To avoid heteroscedastic errors of linear regression (Kenakin, 1993), these twenty equieffective concentrations were fitted to the following untransformed equation by non-linear regression analysis (Prism 2.01) to yield values of K_A and $q_{functional}$ as previously described (Dennis *et al*., 1992):

$$[A] = \frac{[A']q_{functional}K_A}{K_A + (1 - q_{functional})[A']} \qquad \text{Equation 2}$$

where [A] and [A′] indicate the concentration of **R**-PIA or CCPA that causes a specific degree of atrial $MAPD_{90}$ shortening before and after treatment with FSCPX, respectively. Based on the law of mass action, the K_A values for **R**-PIA or CCPA to elicit A_1AdoR-mediated shortening of $MAPD_{90}$ were used to estimate the fractional A_1AdoR occupancy of either agonist as a function of agonist concentration:

$$\rho = \frac{[A]}{[A] + K_A} \qquad \text{Equation 3}$$

where [A] is the agonist concentration (**R**-PIA or CCPA), and ρ is the fractional receptor occupancy. From these data, a plot of the relationship between shortening of the MAP caused by **R**-PIA or CCPA and A_1AdoR occupancy was constructed.

The receptor reserve of these agonists was also determined by calculation of the pharmacological shift ratio (PSR) for both **R**-PIA and CCPA. PSR is an index of the extent of receptor reserve and is defined by the ratio between K_A and EC_{50} (i.e., K_A/EC_{50}). According to the null methodology of Furchgott's model of receptor theory, the differential potency of two full agonists is dependent only on the two factors unique to the drug-receptor interaction, that is ε and K_A. Hence, at any given magnitude of the same response (e.g., 50% maximal response) for two full agonists (i.e., **R**-PIA and CCPA), the following relationship is true (Furchgott, 1966).

$$\frac{\varepsilon_{CCPA}}{\varepsilon_{\mathbf{R}-PIA}} = \frac{\rho_{\mathbf{R}-PIA}}{\rho_{CCPA}} \qquad \text{Equation 4}$$

Substitution with Equation 3 where [A] is the concentration that causes 50% maximal response (i.e., EC_{50}) yields the

relative intrinsic efficacy of CCPA to **R**-PIA in terms of EC_{50} and K_A:

$$\frac{\varepsilon_{CCPA}}{\varepsilon_{\mathbf{R}-PIA}} = \frac{\dfrac{(EC_{50})_{\mathbf{R}-PIA}}{(EC_{50})_{\mathbf{R}-PIA} + (K_A)_{\mathbf{R}-PIA}}}{\dfrac{(EC_{50})_{CCPA}}{(EC_{50})_{CCPA} + (K_A)_{CCPA}}} \qquad \text{Equation 5}$$

Saturation radioligand binding

Membrane preparation Guinea-pig atrial membranes were prepared according to the method described by Lohse *et al.* (1985). In brief, guinea-pig atria harvested from the isolated perfused hearts were minced and then homogenized in ice-cold 50 mM Tris-HCl buffer, pH 7.4. Homogenates were filtered through cotton gauze and centrifuged at 48,000 × *g* for 15 min. The membrane pellets were washed twice by resuspension in fresh buffer and centrifugation. Final pellets were resuspended in 50 mM Tris-HCl buffer, pH 7.4, and frozen at −80°C until use.

Estimation of the fraction of non-inactivated A_1-adenosine receptors To complement the functional studies described above, the fraction of specific binding sites labelled by [^{3}H]-8-cyclopentyl-1,3-dipropylxanthine ([^{3}H]-CPX) after FSCPX treatment ($q_{binding}$) was determined by use of saturation binding studies and normalized to control B_{max}. Atrial membranes (0.2–0.7 mg), adenosine deaminase and [^{3}H]-CPX were incubated for 3 h in a 300 μl volume of 50 mM Tris-HCl buffer, pH 7.4. After the incubation period, bound and free radioligands were diluted by the addition of 5 ml of ice-cold Tris-HCl buffer and immediately separated through vacuum filtration of assay contents onto Whatman GF/C filters and washing of trapped membranes with 20 ml of Tris-HCl buffer. Filter disks containing membrane-bound radioactivity were placed in 4 ml of Scintiverse (Fisher Scientific, Pittsburgh, PA) and the radioactivity was quantified by the use of a liquid scintillation counter. Specific binding of [^{3}H]-CPX was defined as membrane binding displaced in the presence of 8-cyclopentyltheophylline (10 mM), and normalized to the specific binding of control atrial membranes that were performed in parallel experiments. Assays were carried out in triplicate at room temperature (22°C).

Chemicals

R(−)-N^6-(2-phenylisopropyl)adenosine (**R**-PIA) and 2-chloro-N^6-cyclopentyl-adenosine (CCPA) were purchased from Research Biochemicals Internationals (Natick, MA). Stock solutions of these chemicals were prepared in dimethyl sulphoxide (Sigma Chemical Company, St. Louis, MO). The stock solutions were further dissolved in perfusion medium immediately before experiments. [^{3}H]-8-cyclopentyl-1,3-dipropylxanthine ([^{3}H]-CPX) was purchased from Dupont New England Nuclear Research Products (Wilmington, DE). 8-Cyclopentyl-3-[3-[[4-(fluorosulphonyl)benzoyl]oxy]propyl]-1-propyl-xanthine (FSCPX), a specific, irreversible, selective antagonist of the A_1AdoR (Srinivas *et al.*, 1996), was synthesized and generously supplied by Dr Peter J. Scammels (Deakin University, Victoria, Australia). A stock solution of FSCPX (10 mM) was prepared in pure dimethyl sulphoxide and further dissolved in perfusion medium before each experiment. The concentration of dimethyl sulphoxide did not exceed 0.2% (vol/vol) in the perfusion medium.

Data analysis

All values are expressed as mean ± s.e.mean. Concentration-response and correlation data were analysed by use of GraphPad Prism 2.01 (San Diego, CA). Statistical significance of the difference between individual mean values in this study was determined by the unpaired *t* test. Multiple comparisons of individual mean values were made by one-way ANOVA followed by Student-Newman-Keuls testing. $P \leqslant 0.05$ was considered statistically significant.

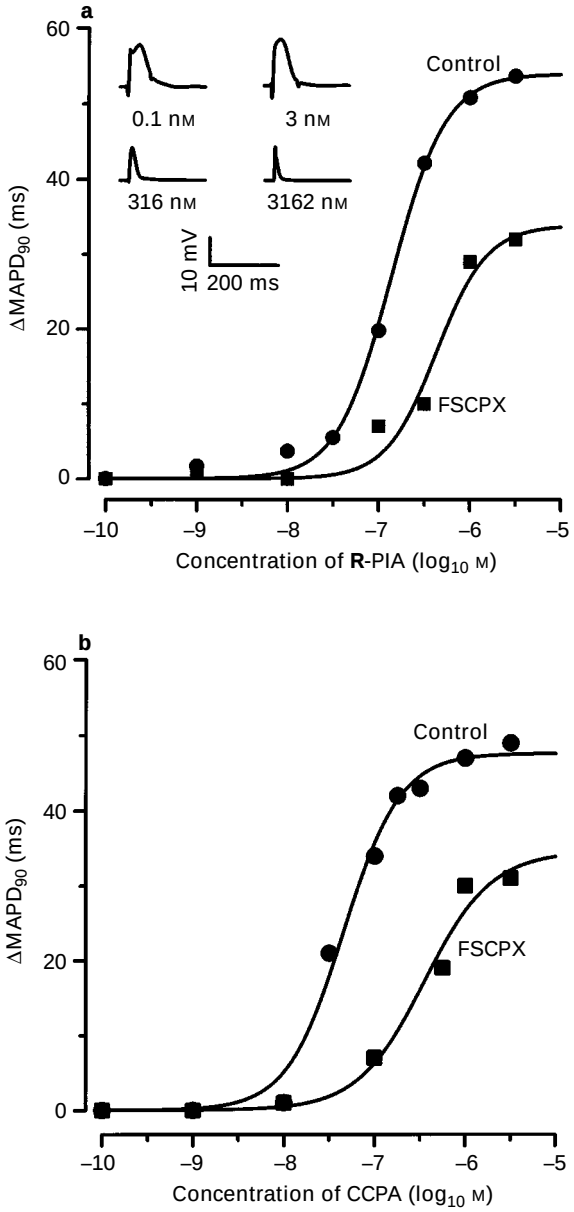

Figure 1 Representative concentration-response relationships for A_1-adenosine receptor agonists, (a) **R**(−)-N^6-(2-phenylisopropyl)-adenosine (**R**-PIA) and (b) 2-chloro-N^6-cyclopentyladenosine (CCPA), to shorten the atrial monophasic action potential at 90% repolarization ($\Delta MAPD_{90}$) before (control) and after treatment with the selective, irreversible, A_1-adenosine receptor antagonist, FSCPX in guinea-pig, isolated heart. (a) Concentration-response relationship for **R**-PIA to shorten atrial monophasic action potential (MAP) before and after treatment with FSCPX (2 μM). Inset: typical MAP traces recorded in the presence of various concentrations of **R**-PIA. (b) Concentration-response relationship for CCPA to shorten atrial MAP before and after treatment with FSCPX (2 μM).

Results

*Concentration-response relationships for shortening of the atrial MAP by **R**-PIA and CCPA*

The baseline atrial $MAPD_{90}$ values were 86.2 ± 5.9 and 81.8 ± 5.9 ms for **R**-PIA ($n=6$) and CCPA ($n=5$), respectively ($P=0.25$). Both A_1AdoR agonists **R**-PIA and CCPA shortened the atrial monophasic action potential (MAP) in a concentration-dependent manner. The concentration-response relationships of **R**-PIA and CCPA to shorten atrial MAP are shown in Figure 1. **R**-PIA and CCPA maximally shortened atrial MAP to 32.5 ± 7.2 and 29.8 ± 5.2 ms, respectively ($P=0.50$). However, CCPA was significantly more potent than **R**-PIA. The pD_2 (and EC_{50}) values for these agonists are summarized in Table 1.

Table 1 Pharmacological parameters of **R**-PIA and CCPA on the A_1-adenosine receptor to shorten atrial MAP in guinea-pig, isolated heart

	***R*-PIA**	***CCPA***	P
pD_2	6.84 ± 0.04 (6)	7.36 ± 0.04 (4)	<0.001
EC_{50} (nM)	145	44	
pK_A	6.54 ± 0.09 (4)	6.13 ± 0.03 (3)	0.02
K_A (nM)	288	734	
PSR	2.15 ± 0.63 (4)	15.9 ± 1.5 (3)	<0.001

Values are expressed as mean $\pm$ s.e.mean for number of experiments indicated in parentheses. Group means were analysed by the unpaired *t*-test. Abbreviations: EC_{50}, concentration of an agonist that causes half-maximal shortening of the atrial monophasic action potential; K_A, equilibrium dissociation constant; pD_2, $-\log_{10}$ (EC_{50}); pK_A, $-\log_{10}$ (K_A); PSR, pharmacological shift ratio ($=K_A/EC_{50}$); MAP, monophasic action potential.

*Effect of FSCPX on concentration-response relationships for **R**-PIA- and CCPA-mediated shortening of atrial MAP*

To determine if the 3.3 fold difference in potencies between **R**-PIA and CCPA is due to agonist-dependent differences in A_1AdoR reserve to shorten atrial MAP, we determined A_1AdoR reserve for each agonist by use of Furchgott's method of irreversible inactivation (see Methods). As illustrated in Figure 1, treatment of the hearts with FSCPX significantly reduced the maximal response for both agonists. After treatment with FSCPX, the maximal shortening of the atrial MAP caused by **R**-PIA and CCPA was 26.0 ± 5.1 and 31.7 ± 7.5 ms, respectively, and differed significantly from the maximal shortening caused by **R**-PIA (53.7 ± 2.7 ms) and CCPA (52.0 ± 4.7 ms) before FSCPX treatment. Likewise, FSCPX reduced the potency of **R**-PIA and CCPA to shorten the atrial MAP. The pD_2 (EC_{50}) values for **R**-PIA and CCPA

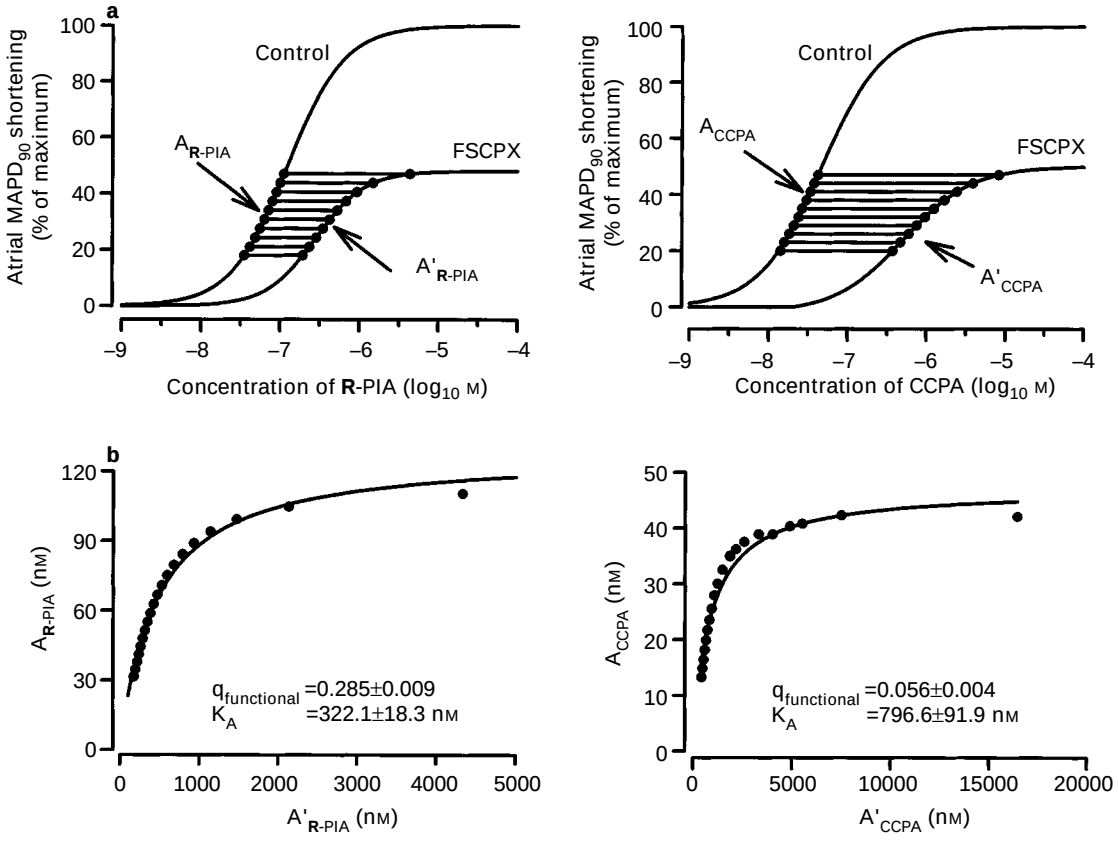

Figure 2 Analysis of the concentration-response relationship for **R**(−)-N^6-(2-phenylisopropyl)-adenosine (**R**-PIA) and 2-chloro-N^6-cyclopentyladenosine (CCPA) to shorten the atrial monophasic action potential at 90% repolarization ($\Delta MAPD_{90}$) by use of the method of Furchgott to calculate the equilibrium dissociation constant (K_A) and the fraction of non-inactivated A_1-adenosine receptors ($q_{functional}$) remaining after treatment with FSCPX in a guinea-pig, isolated heart. (a) Normalized data from a representative experiment. $A_{Agonist}$ and $A'_{Agonist}$ are the concentrations of **R**-PIA or CCPA that caused twenty equal levels (horizontal lines, 10 levels shown) of atrial MAP shortening before ($A_{Agonist}$) and after ($A'_{Agonist}$) treatment with FSCPX. $A_{Agonist}$ and $A'_{Agonist}$ values were used to estimate the K_A value of **R**-PIA and CCPA to bind to A_1-adenosine receptors. (b) Plots of concentrations of **R**-PIA and CCPA ($A_{Agonist}$, $A'_{Agonist}$) that caused equal magnitudes of shortening of the monophasic action potential before and after FSCPX treatment. $A_{Agonist}$ (control-untreated) and $A'_{Agonist}$ (FSCPX-treated) values were obtained from (a), and used to estimate the K_A value of each agonist to bind A_1-adenosine receptors and $q_{functional}$ for each heart.

after treatment with FSCPX were 6.55±0.09 (280 nM) and 6.53±0.17 (295 nM) for **R**-PIA and CCPA, respectively, and significantly differed from pD_2 (EC_{50}) values before FSCPX treatment (Table 1). In summary, after treatment with FSCPX both the maximal effect and potency of **R**-PIA and CCPA were significantly reduced.

To exclude any reductions of agonist potency to shorten the atrial MAP duration caused by the time-related deterioration or an agonist-induced desensitization of the isolated heart preparation, we constructed two concentration-response curves with **R**-PIA in the absence of FSCPX treatment in the same heart. This protocol was identical to the experiments described above except that FSCPX was not infused during the 75–90 min interval between the two concentration-response curves for **R**-PIA. The control $MAPD_{90}$ values before and after this interval were 81.0±3.0 and 83.0±1.0 ms, respectively ($P=0.59$, $n=2$). **R**-PIA administered before and after this interval maximally shortened the atrial $MAPD_{90}$ to 32.5±6.5 and 31.5±7.5 ms, respectively ($P=0.67$). The pD_2 (EC_{50}) values for **R**-PIA before and after this interval were 6.99±0.17 (103 nM) and 6.94±0.16 (115 nM), respectively ($P=0.67$).

*Estimation of the equilibrium dissociation constant (K_A), pharmacological shift ratio (PSR), and intrinsic efficacy (ε) of **R**-PIA and CCPA by use of Furchgott's analysis*

Concentration-response curves for **R**-PIA and CCPA to shorten atrial MAP before and after treatment of hearts with FSCPX (Figure 1) were used to estimate the agonist equilibrium dissociation constants (K_A) and pharmacological shift ratios (PSR) of **R**-PIA and CCPA (Figure 2). The results of these experiments are summarized in Table 1. Estimates of K_A revealed that, although **R**-PIA was less potent than CCPA, its affinity for the A_1AdoR was 2.6 fold greater than CCPA. In contrast, the intrinsic efficacy of CCPA was 5.9 fold greater than that of **R**-PIA (Equation 5, Methods).

Comparison of the fraction of non-inactivated receptors by use of Furchgott's analysis ($q_{functional}$) and specific binding of [^{3}H]-CPX ($q_{binding}$)

To validate the method of Furchgott to estimate receptor reserve, we directly compared the fraction of non-inactivated A_1AdoRs after FSCPX treatment ($q_{functional}$) determined by Furchgott's analysis (Figure 2) to the fraction of the specific binding of [^{3}H]-CPX to atrial membranes ($q_{binding}$), harvested

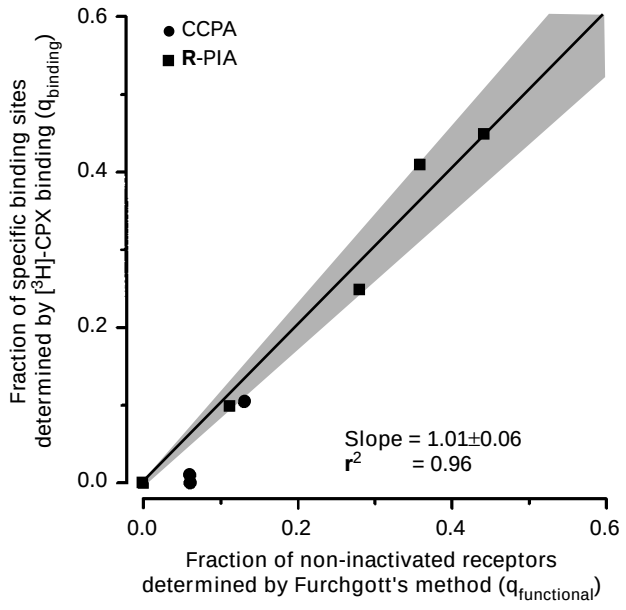

Figure 3 Correlation of the fraction of specific binding sites after FSCPX treatment labelled by [^{3}H]-CPX saturation binding normalized to control ($q_{binding}$) and the fraction of non-inactivated A_1-adenosine receptors remaining in guinea-pig, isolated atria after treatment with FSCPX determined by the method of Furchgott ($q_{functional}$, from Figure 2) with CCPA or **R**-PIA. The shaded area indicates the error in the fitted line by use of 95% confidence limits.

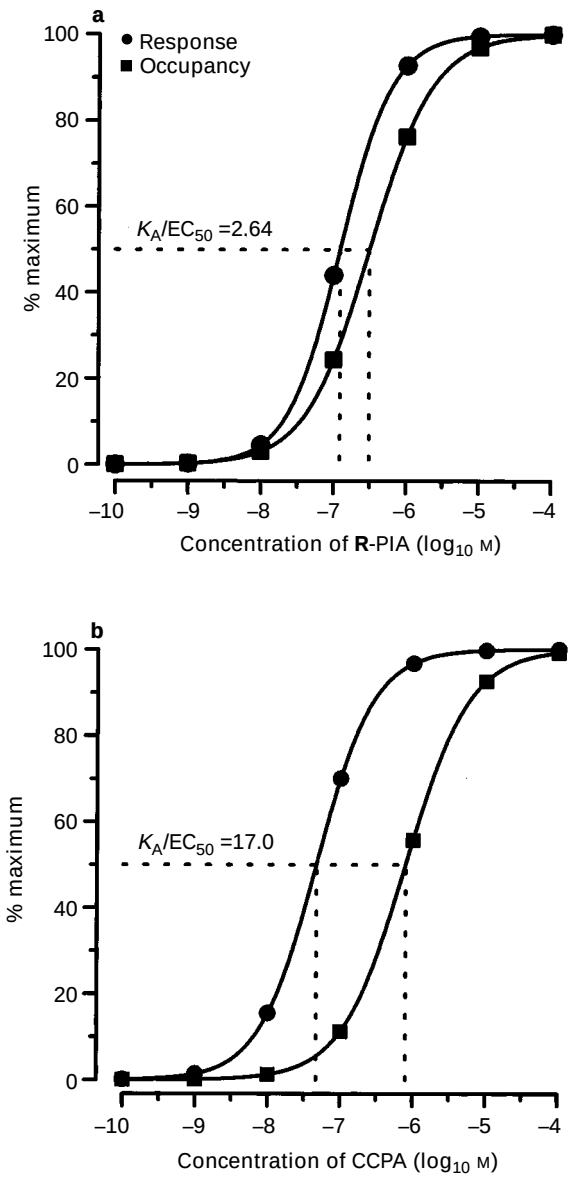

Figure 4 Examples of the dependencies of the A_1-adenosine receptor occupancy and response on the concentrations of **R**-PIA (a) and CCPA (b). The receptor occupancy was calculated from Equation 3 (Methods). Responses for **R**-PIA and CCPA are normalized data from representative experiments. The rightward position of the occupancy curve compared to the response curve indicates that amplification is present for both **R**-PIA and CCPA. The pharmacological shift ratio (PSR) equals K_A/EC_{50} and is an index of the magnitude of receptor reserve. K_A values for **R**-PIA and CCPA were 322 and 797 nM, respectively, whereas EC_{50} values were 122 and 47 nM, respectively. The dashed lines denote concentrations of **R**-PIA and CCPA required to cause half-maximal response and receptor occupancy.

from the isolated heart preparations as depicted in Figure 3. There was nearly a perfect 'one-to-one' correspondence ($r^2 = 0.96$) between $q_{functional}$ and $q_{binding}$. The best-fitted line of the regression analysis had a slope of 1.01 ± 0.06 and intersected the origin. Because Furchgott's analysis required a large fraction of receptors to be irreversibly inactivated, values of $q_{functional}$ exceeding 0.44 were not obtained. The single point at the origin in Figure 3 represents a heart in which, after FSCPX treatment, **R**-PIA at concentrations as high as 3.1 μM ($\sim$22 fold the EC_{50} value) did not shorten the atrial MAP. Consistent with this observation, no specific of [^{3}H]-CPX could be detected in atrial membranes from this heart.

*Occupancy-response relationships for **R**-PIA and CCPA to shorten atrial MAP*

The relationship between the concentrations of A_1AdoR agonist (**R**-PIA or CCPA) and fractional receptor occupancy to shorten atrial MAP was determined in two steps. First, atrial MAP shortening and fractional receptor occupancy were plotted against concentrations of CCPA and **R**-PIA concentrations (Figure 4). The concentration-response curves are normalized from data as shown in Figure 1. The A_1AdoR agonist concentration-fractional receptor occupancy data were calculated by use of values of K_A for CCPA and **R**-PIA, derived from the Furchgott's analysis (Figure 2) and the law of mass action (Equation 3, Methods). The concentration-response relationships for CCPA and **R**-PIA lay to the left of their respective occupancy curves (Figure 4). The ratios between K_A to EC_{50} (defined as the PSR) are measures of the extent of receptors reserve and were 2.6 and 17.0 for **R**-PIA and CCPA, respectively.

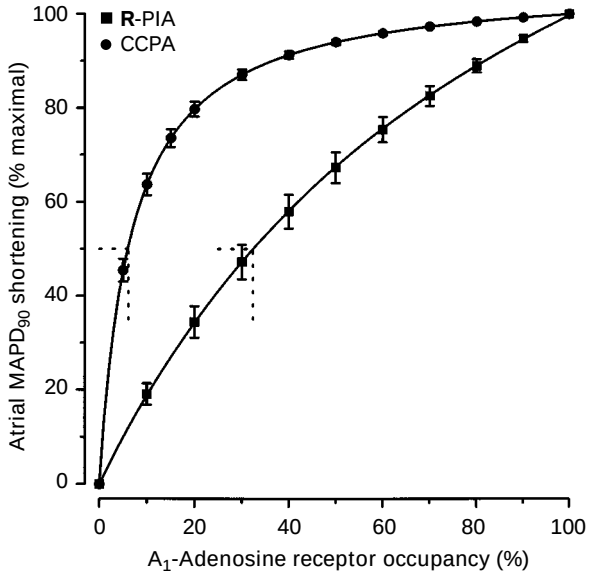

Figure 5 Relationships between A_1-adenosine receptor occupancy by **R**-PIA and CCPA and shortening of the atrial monophasic action potential at 90% repolarization ($MAPD_{90}$). Occupancy of A_1-adenosine receptors was calculated from Equation 3 (Methods) and estimates of the equilibrium dissociation constant (K_A, from Figure 2). The occupancy-response relationship for **R**-PIA is nearly linear and half-maximal response occurring at 32% receptor occupancy whereas this relationship for CCPA is curvilinear with half-maximal response occurring at 6% occupancy ($P = 0.0003$). Each point represents the mean and vertical lines show s.e.mean for four and three experiments with **R**-PIA and CCPA, respectively. The dashed lines denote the occupancy of A_1-adenosine receptors by **R**-PIA and CCPA required to cause half-maximal response.

To determine the magnitudes of receptor reserve for CCPA and **R**-PIA, the data shown in Figure 4 were used to calculate atrial MAP shortening as a function of receptor occupancy (Figure 5). This occupancy-response relationship of **R**-PIA was nearly linear indicating a low efficiency of occupancy-response coupling (low receptor reserve), whereas the occupancy-response relationship of CCPA was highly curvilinear indicating a high efficiency of occupancy-response coupling (high receptor reserve). The fractional A_1AdoR occupancies required for half-maximal shortening of the atrial MAP by **R**-PIA and CCPA were 32% and 6%, respectively, indicating 18% and 44% receptor reserves for the half-maximal response for **R**-PIA and CCPA, respectively. Likewise, the concentrations of **R**-PIA and CCPA required to cause maximal shortening of the atrial MAP, defined at the 90% maximal response level, were 82% and 37%, respectively, indicating 18% and 63% receptor reserves for this magnitude of response, respectively.

Discussion

This study is the first to validate systematically Furchgott's method of irreversible receptor inactivation as a means of estimating A_1AdoR reserve. In addition, we demonstrated important differences in the pharmacological basis of potency (receptor affinity vs intrinsic efficacy) for the two full A_1AdoR agonists **R**-PIA and CCPA. Evidence that CCPA is a high intrinsic efficacy, but a low affinity A_1AdoR agonist, whereas **R**-PIA is a low intrinsic efficacy, high affinity A_1AdoR agonist is supported by two independent but complementary methods of analysis, Furchgott's method and radioligand binding. By understanding the basis of agonist potency, similar studies in the future may provide insight into the design of highly selective A_1AdoR agonists. The evidence supporting these conclusions is discussed below.

Validation of Furchgott's method to estimate A_1AdoR receptor reserve

In this study, we provide compelling evidence that Furchgott's method of irreversible inactivation of receptors is a valid and an accurate method to measure the receptor reserve for A_1AdoR agonists in native tissue. Three separate lines of evidence support this conclusion. First, the fraction of non-inactivated A_1AdoRs remaining after FSCPX treatment ($q_{functional}$) derived from Furchgott's method and from saturation radioligand binding ($q_{binding}$) were in excellent agreement. Specifically, $q_{functional}$ and $q_{binding}$ were almost perfectly correlated ($r^2 = 0.96$), intersected the origin, and had a slope of unity. This finding is consistent with the results of Zernig *et al.* (1995), who used a variation of Furchgott's analysis to measure the receptor reserve for the antinociceptive effect of several μ opioid receptor agonists in mice. Like us, they found that $q_{functional}$ correlated with $q_{binding}$ ($r^2 = 0.92$), that the slope of the linear regression approximated unity and that the best-fitted line intersected the origin, i.e., $q_{functional} = 1.02(\pm 0.10)q_{binding} - 0.0507(\pm 0.062)$. In addition, others observed good agreement between $q_{functional}$ and $q_{binding}$ determined by functional or radioligand binding in the muscarinic cholinoceptor (Ehlert, 1987) and α_{2D}-adrenoceptor (Tian *et al.*, 1996) systems by use of [^{3}H]-N-methylscopolamine in homogenates of rabbit myocardium and [^{3}H]-rauwolscine binding in PC12 cell membranes, respectively. However, unlike the present and Zernig's study (Zernig *et al.*, 1995), these latter two studies used only single point comparisons between

$q_{functional}$ and $q_{binding}$. On the other hand, Minneman *et al.* (1984a,b), using phenoxybenzamine as an irreversible antagonist of α_1-adrenoceptors, found no relationship between $q_{functional}$ and $q_{binding}$ in rat vas deferens. The explanation for the lack of correlation of $q_{functional}$ and $q_{binding}$ was that the outer longitudinal muscles formed a barrier to diffusion of phenoxybenzamine and effectively shielded α_1-adrenoceptors located on the inner circular muscles from inactivation (Minneman *et al.*, 1984a). An alternative explanation was that sequestered α_1-adrenoceptors (i.e., intracellular, intramembrane) did not participate in signal transduction and were not inactivated by phenoxybenzamine, but were detectable with radioligand binding (Minneman *et al.*, 1984a). Regardless, in the studies in which a good agreement between $q_{functional}$ and $q_{binding}$ was observed, the irreversible antagonist was either administered intraperitoneally to whole animals (Zernig *et al.*, 1995) or intra-arterially to isolated perfused hearts (current study; Ehlert, 1987). Thus, we propose that it is preferable to administer the irreversible antagonist intra-arterially or intravenously to avoid potential diffusion barriers, to obtain a more homogeneous distribution of the irreversible ligand and to achieve uniform inactivation of receptors.

Second, the relative intrinsic efficacy of CCPA to **R**-PIA predicted by Furchgott's method (Equation 5) agreed well with the relative intrinsic efficacies calculated from historical competition radioligand binding values. Because the ratio between binding constants in the low (K_L) and high (K_H) affinity states in a two-site model is considered to be an index of intrinsic efficacy of an agonist (Kenakin, 1993), the relative intrinsic efficacies of CCPA to **R**-PIA can be calculated as $(K_L/K_H)_{CCPA}/(K_L/K_H)_{\mathbf{R}\text{-PIA}}$. The relative intrinsic efficacies determined from K_L and K_H data previously obtained (Bohm *et al.*, 1989; Musser *et al.*, 1993; Srinivas *et al.*, 1997) were 6.2 and 6.6 (Table 2). The ratio of the intrinsic efficacies calculated herein by use of Furchgott's analysis, 5.9, is in excellent agreement with the values obtained from results of radioligand binding studies.

Third, the K_A values determined by Furchgott's analysis approximated the values determined by competition radioligand binding studies previously (Bohm *et al.*, 1989; Musser *et al.*, 1993; Srinivas *et al.*, 1997). The K_A value (734 nM) derived from Furchgott's analysis for CCPA approximates the K_i value (530 ± 120 nM) obtained from guinea-pig atrial membranes in the presence of 100 μM GTP (Srinivas *et al.*, 1997). Likewise, the K_A value (288 nM) for **R**-PIA estimated by Furchgott's analysis is in excellent agreement with the K_i value (286 nM) determined in human atrial membranes in the presence of guanylylimododiphosphate (Gpp(NH)p) (Bohm *et al.*, 1989), but was greater than the K_i value (18 ± 5 nM) obtained in porcine atrial membranes treated with Gpp(NH)p (Musser *et al.*, 1993). However, the K_i value determined in porcine atrial membranes is likely to be an underestimate of K_A because not all A_1AdoRs were converted to a low-affinity state, as evidenced by a statistically superior fit of the data to a two-site binding model even after addition of Gpp(NH)p (Musser *et al.*, 1993). The good agreement between K_A and K_i for two distinct A_1AdoR agonists adds to the growing number of G-protein-coupled receptor systems, wherein K_A obtained by functional analysis corresponds well to the affinity equilibrium constant measured in the presence of guanine nucleotides by use of radioligand binding (Minneman & Abel, 1984b; May *et al.*, 1985; Nasseri & Minneman, 1987). In summary, Furchgott's method of irreversible receptor inactivation accurately estimated (1) the fraction of specific binding sites after FSCPX treatment labelled by [^{3}H]-CPX saturation binding ($q_{binding}$), (2) the relative intrinsic efficacies of CCPA to **R**-PIA determined by radioligand binding, and (3) the dissociation constant of CCPA and **R**-PIA in the presence of guanine nucleotides. Taken together, these results provide compelling and mutually supportive evidence that Furchgott's method is a valid technique to assess A_1AdoR agonist receptor reserve in guinea-pig atrium.

Table 2 Competition radioligand binding parameters and relative intrinsic efficacies of CCPA and **R**-PIA for the A_1-adenosine receptor in atrial membranes

	R-PIA		*CCPA*
K_L (nM)	38 ± 15	90 (47–168)	229 ± 21
K_H (nM)	0.30 ± 0.11	0.75 (0.3–2.0)	0.29 ± 0.03
K_L/K_H	128	120	790
K_i (nM)	18 ± 5	286 (146–557)	530 ± 120
$\varepsilon_{CCPA}/\varepsilon_{\mathbf{R}\text{-PIA}}$	6.2	6.6	–
Species	Pig	Human	Guinea-pig

All data are mean $\pm$ s.e.mean except where 95% confidence intervals are noted in parentheses. The ratio of intrinsic efficacy of CCPA to **R**-PIA was calculated as $(K_L/K_H)_{CCPA}/(K_L/K_H)_{\mathbf{R}\text{-PIA}}$. Abbreviations: ε, intrinsic efficacy of an agonist; K_H, high affinity state of receptor; K_L, low affinity state of receptor; K_i, affinity of receptors in the presence of guanine nucleotides. Data are from Musser *et al.* (1993); Bohm *et al.* (1989) and Srinivas *et al.* (1996).

Pharmacological basis of potency of A_1AdoR agonists

According to Furchgott's model of receptor theory, different agonists acting at the same receptor mediating a response in a given tissue may have different receptor reserves. Although this concept has been demonstrated for a number of G-protein-dependent responses such as dopamine (Meller *et al.*, 1987), muscarinic cholinoceptors (Harden *et al.*, 1986), 5-HT (Meller *et al.*, 1990), and μ opiate (Adams *et al.*, 1990) receptor systems, agonist-dependent differences in A_1AdoR reserve have not been previously studied. In addition, the model predicts that differences in receptor reserve must be attributable to factors unique only to the drug-receptor interaction, i.e. ε and K_A (Kenakin, 1993). Knowledge of the relative contribution of each parameter to the potency of an agonist is of major importance, not only for receptor classification, but also for the rational design of new agonists (Kenakin, 1984). Based on the results of the present study, **R**-PIA is a low intrinsic efficacy, relatively high affinity A_1AdoR agonist whereas CCPA is a high intrinsic efficacy, relatively low affinity A_1AdoR agonist.

The two drug-receptor related parameters, ε and K_A, are unique only to the drug-receptor interaction and should transcend differences in tissue and response (Kenakin, 1993). An understanding of whether an agonist relies predominantly on its affinity to bind to receptors or on its intrinsic efficacy to activate G-proteins for its physiological effects has significant therapeutic implications. For example, in tissue desensitized due to chronic therapy, an agonist with high intrinsic efficacy may still elicit the maximal response (i.e. no change in efficacy), albeit the concentration-response curve may be shifted rightward (Kenakin & Ferris, 1983). In contrast, an agonist with low intrinsic efficacy may not only fail to elicit a response, but may also act as a competitive antagonist and may cause a response opposite to the intended effect (Kenakin & Ferris, 1983). Although these phenomena have not been studied for the A_1AdoR system, the interaction of tolerance and μ opioid agonists have been described. Accordingly, cancer patients tolerant to morphine (>250 mg day^{-1}) noted little or no postoperative analgesia from epidural morphine, a known low

intrinsic efficacy μ opioid agonist, but experienced significant analgesia from an equieffective dose (in opiate naïve patients) of epidural sufentanil, a known high intrinsic efficacy μ opioid agonist (Mjanger & Yaksh, 1991; De Leon-Casasola & Lema, 1994). In keeping with this finding, the degree of tolerance that develops to equieffective doses of μ opioid agonists is inversely related to the intrinsic efficacy of the agonist (Paronis & Holtzman, 1992; Duttaroy & Yoburn, 1995). Thus, the determinations of ε and K_A may be at least, if not more, important than potency of an agonist. Lastly, knowledge of the dependence of the potency of an agonist on ε and K_A may provide a useful framework to classify receptors and agonists, and to guide the search for solutions to problems of drug and response selectivity (May *et al*., 1985).

Implications and future directions

Although the results are only directly applicable to A_1AdoRs in guinea-pig atrium, there is no *a priori* reason to suspect that the results presented here will not be equally applicable to native A_1AdoRs located in other tissues or linked to different responses. Previously, we employed Furchgott's method to demonstrate the presence of 20% and 54% spare A_1AdoRs at the half-maximal and maximal negative dromotropic response, respectively, caused by N^6-cyclopentyladenosine in guinea-pig, atrioventricular node (Dennis *et al*., 1992). Likewise, Lohse *et al*. (1986) used irreversible agonist photoaffinity labelling and adenosine 3′:5′-cyclic monophosphate measurements to show >40% spare A_1AdoRs at the IC_{50} level in rat, isolated adipocytes. Recently, we obtained a 24 fold higher PSR for adenosine to inhibit β-$I_{Ca,L}$ compared to the activation of $I_{K,Ado}$ in guinea-pig, atrial myocytes (Srinivas, 1997). These findings of A_1-AdoR agonist-dependent receptor reserve raise the possibility of exploiting differences in receptor reserve as a mechanism whereby A_1AdoR-mediated responses can be selectively elicited.

The development of new clinical therapies based on adenosine-related compounds cannot be successful if efforts are only based on the identification of new adenosine receptor subtypes and the synthesis of subtype selective ligands (agonists and antagonists). Equally important is the investigation of whether organ and response selectivity for A_1AdoR agonists can be achieved based on differences in receptor-effector coupling efficiency (i.e., differences in receptor reserve). Although the importance of adenosine in modulating cellular function(s) and its therapeutic utility as an antiarrhythmic agent are well established, an understanding of receptor reserve in a given organ, for distinct functions and agonists is required before one can fully appreciate the importance of this nucleoside and exploit the therapeutic potential of adenosine-related substances.

This work was supported by National Institutes of Health Grant HL 56785 and the I. Heermann Anesthesia Foundation, Inc. We thank Jackie Ruble for her assistance with the radioligand binding assays.

References

ADAMS, J.U., PARONIS, C.A. & HOLTZMAN, S.G. (1990). Assessment of relative intrinsic activity of Mu-opioid analgesics *in vitro* by using β-funaltrexamine. *J. Pharmacol. Exp. Ther.,* **225,** 1027–1032.

BOHM, M., PIESKE, B., UNGERER, M. & ERDMANN, E. (1989). Characterization of A_1 adenosine receptors in atrial and ventricular myocardium from diseased human hearts. *Circ. Res.,* **65,** 1201–1211.

COLLIS, M.G. & HOURANI, S.M. (1993). Adenosine receptor subtypes. *Trends Pharmacol. Sci.,* **14,** 360–366.

DE LEON-CASASOLA, O.A. & LEMA, M.J. (1994). Epidural bupivacaine/sufentanil therapy for postoperative pain control in patients tolerant to opioid and unresponsive to epidural bupivacaine/morphine. *Anesthesiology,* **80,** 303–309.

DENNIS, D., JACOBSON, K. & BELARDINELLI, L. (1992). Evidence of spare A_1-adenosine receptors in guinea pig atrioventricular node. *Am. J. Physiol.,* **262,** H661–671.

DUTTAROY, A. & YOBURN, B.C. (1995). The effect of intrinsic efficacy on opioid tolerance. *Anesthesiology,* **82,** 1226–1236.

EHLERT, F.J. (1987). Coupling of the muscarinic receptors to adenylate cyclase in the rabbit myocardium: effects of receptor inactivation. *J. Pharmacol. Exp. Ther.,* **240,** 23–30.

FURCHGOTT, R.F. (1966). The use of β-haloalkylamines in the differentiation of receptors and in the determination of dissociation constants of receptor-agonist complexes. In *Advances in Drug Research*. Vol. 3, ed Harper, N.J. & Simmonds, A.B. pp. 21–55. New York: Academic Press.

FURCHGOTT, R.F. & BURSZTYN, P. (1967). Comparison of dissociation constants and of relative efficacies of selected agonists acting on parasympathetic receptors. *Ann. New York Acad. Sci.,* **144,** 882–899.

HARDEN, T.K., HENG, M. & BROWN, J.H. (1986). Receptor reserve in the calcium-dependent cyclic AMP response of astrocytoma cells to muscarinic receptor stimulation: Demonstration by agonist-induced desensitization, receptor inactivation, and phorbol ester treatment. *Mol. Pharmacol.,* **30,** 200–206.

KENAKIN, T.P. & FERRIS, R.M. (1983). Effects on in vivo β-adrenoceptor down-regulation on cardiac responses to prenalterol and pirbuterol. *J. Cardiovasc. Pharmacol.,* **5,** 90–97.

KENAKIN, T. (1993). *Pharmacological Analysis of Drug-Receptor Interaction*. New York: Raven Press.

KENAKIN, T.P. (1984). The Classification of drugs and drug receptors in isolated tissues. *Pharmacol. Rev.,* **36,** 165–222.

LOHSE, M.J., KLOTZ, K.-N. & SCHWABE, U. (1986). Agonist photoaffinity labeling of A_1 adenosine receptors: persistent activation reveal spare receptors. *Mol. Pharmacol.,* **30,** 403–409.

LOHSE, M.J., UKENA, D. & SCHWABE, U. (1985). Demonstration of Ri-type adenosine receptors in bovine myocardium by radioligand binding. *Naunyn-Schmeideberg's Arch. Pharmacol.,* **328,** 310–316.

MAY, J.M., ABEL, P.W. & MINNEMAN, K.P. (1985). Binding of agonists and antagonists to β-adrenoceptors in rat vas deferens: relationship to functional response. *Naunyn-Schmiedeberg's Arch. Pharmacol.,* **331,** 324–333.

MELLER, E., BOHMAKER, K., NAMBA, Y., FRIEDHOFF, A.J. & GOLDSTEIN, M. (1987). Relationship between receptor occupancy and response at striatal dopamine autoreceptors. *Mol. Pharmacol.,* **31,** 592–598.

MELLER, E., GOLDSTEIN, M. & BOHMAKER, K. (1990). Receptor reserve for 5-hydroxytryptamine$_{1A}$-mediated inhibition of serotonin synthesis: Possible relationship to anxiolytic properties of 5-hydroxytryptamine$_{1A}$ agonists. *Mol. Pharmacol.,* **37,** 231–237.

MINNEMAN, K.P. & ABEL, P.W. (1984a). 'Spare' Alpha$_1$-adrenergic receptors and the potency of agonist in rat vas deferens. *Mol. Pharmacol.,* **25,** 56–63.

MINNEMAN, K.P. & ABEL, P.W. (1984b). Relationship between α_1-adrenoceptor density and functional response of rat vas deferens. *Naunyn-Schmiedeberg's Arch. Pharmacol.,* **327,** 238–246.

MJANGER, E. & YAKSH, T.L. (1991). Characteristics of dose-dependent antagonism by B-funaltrexamine of the antinociceptive effect of intrathecal mu agonists. *J. Pharmacol. Exp. Ther.,* **258,** 544–550.

MUSSER, B., MORGAN, M.E., LEID, M., MURRAY, T.F., LINDEN, J. & VESTRAL, R.E. (1993). Species comparison of adenosine and β-adrenoceptors in mammalian atrial and ventricular myocardium. *Eur. J. Pharmacol.,* **246,** 105–111.

NASSERI, A. & MINNEMAN, K.P. (1987). Relationship between α_2-adrenergic receptor binding sites and the functional receptors inhibiting norepinephrine release in rat cerebral cortex. *J. Pharmacol. Exp. Ther.,* **32,** 655–662.

PARONIS, C.A. & HOLTZMAN, S.G. (1992). Development of tolerance to the analgesic activity of mu agonists after continuous infusion of morphine, meperidine or fentanyl in rats. *J. Pharmacol. Exp. Ther.,* **262,** 1–9.

RAATIKAINEN, M.J.P., NAPOLITANO, C.A., DRUZGALA, P. & DENNIS, D.M. (1996). Electrophysiological effects of a novel, short-acting and potent ester derivative of amiodarone, ATI-2001, in guinea pig isolated heart. *J. Pharmacol. Exp. Ther.,* **277,** 1454–1463.

SRINIVAS, M., SHRYOCK, J.C., DENNIS, D.M., BAKER, S.P. & BELLARDINELLI, L. (1997). Differential A_1-adenosine receptor reserve for two actions of adenosine on guinea pig atrial myocytes. *Mol. Pharmacol.,* **52,** 683–691.

SRINIVAS, M., SHRYOCK, J.C., SCAMMELLS, P.J., RUBLE, J., BAKER, S.P. & BELARDINELLI, L. (1996). A novel irreversible antagonist of the A_1-adenosine receptor. *Mol. Pharmacol.,* **50,** 196–205.

TIAN, W.N., DUZIC, E. & DETH, R.C. (1996). Evaluation of agonist efficacy and receptor reserve for α_{2D}-adrenergic receptor regulation G protein activation in PC12 cell membranes. *Pharmacology,* **52,** 252–262.

ZERNIG, G., ISSAEVITCH, T., BROADBEAR, J.H., BURKE, T.F., LEWIS, J.W., BRINE, G.A. & WOODS, J.H. (1995). Receptor reserve and affinity of mu opioid agonists in mouse antinociception: Correlation with receptor binding. *Life Sci.,* **57,** 2113–2125.

(Received October 29, 1997
Revised December 22, 1997
Accepted January 2, 1998)