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Pharmacological classification of adenosine receptors in the sinoatrial and atrioventricular nodes of the guinea-pig

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1 The effects of seven agonist and three antagonist adenosine receptor ligands were compared on the guinea-pig sinoatrial (SA) node (isolated right atrium) and atrioventricular (AV) node (perfused whole heart). Single agonist concentration-effect curves were obtained to 5′-N-ethylcarboxamidoadenosine (NECA), **R**(−)-N^6-(2-phenylisopropyl)adenosine (**R**-PIA), N^6-cyclohexyladenosine (CHA), 2-chloroadenosine (CADO),),S(+)-N^6-(2-phenylisopropyl)adenosine (L-PIA), 2-phenylaminoadenosine (CV 1808) and N^6-aminoadenosine (MeAdo). Adenosine and/or NECA curves were obtained in the absence and presence of the antagonists 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), 9-chloro-2–(2-furanyl)-5,6-dihydro-1,2,4-triazolo[1,5-c]quinazolin-5-imine (CGS15943) and N^6-(endonorbornan-2-yl)-9-methyladenine (N-0861).

2 A formal comparison of the agonist and antagonist potency data was made by fitting the data to a straight line using a least squares procedure based on principal components analysis to account for the variance on both axes. The antagonist affinity estimates made on the two assays did not deviate significantly from the line of identity.

3 The agonist $p[A]_{50}$ data obtained on the two assays did not deviate from the line of identity, indicating that there were no significant differences in potencies between the two assays. The $p[A]_{50}$ ratio of **R**-PIA and **S**-PIA was 1.24 ± 0.09 in the SA node and 1.36 ± 0.11 in the AV node, indicating no difference in the stereoselectivity of the PIA isomers between the two tissues.

4 The agonist potency and antagonist affinity data obtained are consistent with previous findings showing that the AV and SA node data are pharmacologically indistinguishable and belong to the adenosine A_1-receptor class. No evidence for the reported A_3-receptor was found.

Keywords: Atrioventricular node; sinoatrial node; adenosine receptor

Introduction

We have found that dipyridamole is more potent at inhibiting adenosine uptake in the sinoatrial (SA) node than in the atrioventricular (AV) node as judged by the leftward displacement of adenosine concentration-effect curves in guinea-pig hearts (Meester *et al*., 1998). Application of a model of agonist uptake (Kenakin, 1981) gave significantly different pK_i estimates for dipyridamole, indicative of different adenosine transporters in the two tissues. However, in the same study, we also found that adenosine uptake in the AV node, but not in the SA node, appeared to potentiate the effects of adenosine receptor activation. We were able to develop a model which incorporated this feature. The model explains how different pK_i estimates can be obtained even if the adenosine transporters in the two tissues were identical. However, in the new model there was a critical assumption that the adenosine receptors in the two tissues were pharmacologically indistinguishable. There appears to be general agreement that the sinoatrial (SA) and atrioventricular (AV) nodes of the guinea-pig both contain adenosine A_1-receptors (Evans *et al*., 1982; Belardinelli *et al*., 1983; 1989; Clemo & Belardinelli, 1986; Collis *et al*., 1989). However, some doubt has been expressed by Ribeiro & Sebastiao (1986) who suggested that the A_1-receptor of the SA node was atypical; they proposed an 'A_3' subtype although this putative subtype is very different from the subsequently cloned A_3-receptor which is much less sensitive to established A_1-receptor antagonists (Zhou *et al*., 1992; Linden, 1994). Because of this and our need to be as sure as possible that the receptors could be considered to be homogeneous under the exact assay conditions employed in the studies involving dipyridamole, we have performed a pharmacological receptor classification exercise designed to allow the formal comparison of the data obtained on each assay.

We compared the potencies of three well-classified antagonists and seven agonists. Although competitive antagonists are usually considered to be the most reliable tools for pharmacological receptor classification (Colquhoun, 1986), in this case a comparison of agonist potency values offered a potential analytical advantage. The conclusion of A_1-receptor heterogeneity by Ribeiro & Sebastiao (1986) was based on agonist potency order discrepancy between receptors coupled to intracellular calcium in excitable tissue (e.g. cardiac and neural) and those coupled to inhibition of adenylate cyclase in smooth muscle. If these differences were due to a single receptor protein, displaying what would now be referred to as 'promiscuity' (Kenakin, 1995), then comparison of agonists, the potencies of which are governed by both affinity and efficacy, might expose heterogeneity which need not be evident from comparing antagonist potencies which are solely determined by their affinities. In addition, the study provided an opportunity to make the first comparison on an AV nodal assay of the potency of the **R**- and **S**-stereoisomers of N^6-(2-phenylisopropyl)adenosine (PIA) which clearly discriminate A_1- and A_2-receptors (see Fredholm *et al*., 1994). Finally, the formal comparison of the antagonist affinity and agonist

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potency data obtained on the two assays was made by fitting the data to a straight line by least squares. This entailed the application of a principal components analysis to account for the error on both x and y values. A detailed description of the analysis is provided because it has general applicability for the comparison of ligand potency values obtained on different assays and is more appropriate than correlation-based analyses usually used in the literature.

A preliminary account of the data was presented to the British Pharmacological Society (Meester *et al*., 1994).

Methods

Guinea-pig isolated right atrium (SA node) assay

Chronotropic responses were measured in isolated, spontaneously-beating, right atria from male guinea-pigs (Dunkin-Hartley, 300–400 g), prepared according to previously described methods (Black *et al*., 1985a). In brief, the atria were suspended in 20 ml of Krebs-Henseleit (K-H) solution (composition, mM: Na^+ 143, K^+ 5.9, Ca^{2+} 2.5, Mg^{2+} 1.2, Cl^- 128, $H_2PO_4^-$ 2.2, HCO_3^- 24.9, SO_4^{2-} 1.2, dextrose 10) maintained at 37 ± 0.3°C and gassed with 95% O_2 and 5% CO_2. Tissues were loaded with an initial 0.5 g resting tension. Each isometric transducer output was amplified (Ormed 3559) and processed by a digital rate meter (Ormed 4461) which gave a direct readout of rate (beats min^{-1}) continuously displayed on a potentiometric chart recorder.

Guinea-pig isolated perfused heart (AV node) assay

Dromotropic effects were measured in isolated hearts of male guinea-pigs (Dunkin-Hartley, 200–300 g). The hearts were retrogradely perfused via the aorta according to Langendorff at a constant perfusion pressure of 74 cmH_2O (Döhring & Dehnert, 1986) with K-H solution, maintained at 35 ± 0.3°C and gassed with 95% O_2 : 5% CO_2. They were allowed to stabilize for 20–25 min. The sinoatrial region and most of the right atrium were excised. Removal of the SA node allowed the heart to be driven at a constant, high, rate of atrial pacing (3.5 Hz). Hearts were electrically stimulated (Grass S88) with square wave pulses (1 ms duration, twice threshold voltage) delivered via a stimulus-isolation unit (Grass SIU5) using a bipolar silver electrode placed on the left atrium. Removal of most of the right atrium allowed for the placement of two teflon-coated electrodes (Ag-5T), one on the intra-atrial septum and one on the left ventricle. The extracellular bipolar electrogram (EG) was then displayed on a digital oscilloscope (Nicolet 4094) at a sweep speed of 1 ms or 500 ms per point. The stimulus-to-R wave (SR) interval, calculated from the EG using Nicolet data analysis software (Mathpak 4094), was used as a measure of AV conduction (ms).

The temperature was reduced from 37 to 35°C as this was found to abolish spontaneous rhythms which occasionally arose at frequencies above the rate of external pacing. This step was taken in preference to increasing the rate of pacing which would have had the effect of reducing the experimental window in which agonist effects could be measured.

Experimental protocols

Guinea-pig isolated right atrium assay Six isolated right atrium preparations were used simultaneously and were allocated to control and treatment groups so that, as far as was practical, the design was balanced over days and organ baths. Control preparations were dosed with the maximal amount of vehicle used for any one treatment. None of the vehicles (see Compounds) had a significant effect on basal rate. Preparations were allowed to stabilize for 60 min during which time the organ bath fluid was replaced with pre-warmed K-H solution at 15 min intervals. Adenosine receptor activation slows the pacemaker frequency. Therefore, to increase the signal-to-noise ratio in the assay, histamine (3 μM, corresponding to ~90% of the histamine concentration-effect, E/[A], curve maximum) was added to the organ baths to increase the basal rate of ~200 to ~300 beats min^{-1}. Histamine responses are maintained in the SA node assay for at least the duration of the current experiments even when supramaximal concentrations are used (as long as the solutions of the dihydrogen chloride salt solution are back-neutralized, Black *et al*., 1981). Timolol (3 μM, ~3000 fold K_B at β_1-adrenoceptors; Robert Hull, personal communication) was added to annul possible β-adrenoceptor stimulation. A single agonist concentration-effect (E/[A]) curve was obtained on each preparation by cumulative dosing at half-log unit concentration increments. The total amount of vehicle added to the organ baths during an experiment did not exceed 7% of the original bath volume.

Guinea-pig isolated perfused heart assay Two isolated perfused heart preparations were used simultaneously and treatments, including vehicle controls, were allocated across the three replicate experiments performed daily so that, as far as was practical, the design was balanced over days and organ baths. None of the vehicle controls had a significant effect on the basal SR-interval. The adenosine uptake blocker, dipyridamole, and the adenosine A_1-receptor antagonists were added to the K-H reservoir and perfused for 25 min before a single E/[A] curve was obtained on each preparation. The agonists were administered into the perfusion lines at a fixed flow rate via a syringe pump. Preliminary experiments had shown that, at all concentrations of agonist, steady-state responses were always attained within 3 min for adenosine, 5 min for NECA and 10 min for the other agonists in the absence and presence of antagonists. The responses were measured from E.G. records displayed on an oscilloscope rather than from continuous chart records. Therefore, on the basis of the preliminary experiments, the responses to individual applications of agonist were recorded when a further 30 s had elapsed after the exposure times given above for each agonist.

Data analysis

Agonist concentration-effect curves Responses from the right atrium assays and isolated perfused hearts were expressed as changes in rate (Δ basal rate : beats min^{-1}) and SR-interval (Δ SR-interval : ms), respectively. The E/log[A] curves obtained in the right atria and isolated hearts had a parabolic rather than a classical sigmoidal shape. This was presumably because the maximal responses in the SA node were limited by either an abrupt onset of SA nodal arrest or SA nodal exit block (Belardinelli *et al*., 1983) and in the AV node by second or third degree AV block. Accordingly, in the right atrium assay the response to the highest concentration of agonist that produced a stable rate change and, in the isolated heart assay, the value of the longest stable SR-interval immediately before block, were taken as the maximum response (α). The concentration of agonist required to produce 50% of this maximal inhibitory action ($[A]_{50}$) was estimated graphically for

each individual curve. On the assumption that these values are log-normally distributed, $\log[A]_{50}$ values were used for subsequent analysis. In practice, the agonist concentration which produced the highest response before the onset of SA or AV block varied between the individual preparations. Thus, strictly, meaningful average (±s.e.mean) values could only be calculated at agonist concentrations which produced responses in all preparations. In an attempt to illustrate more of the data, the mean value of the highest response obtained in each preparation, regardless of the concentration of agonist, was calculated and expressed as a function of the average log[A] which produced these responses.

Competitive antagonism In order to quantify the effects of competitive antagonists $\log[A]_{50}$ values obtained in the absence ($[A]_{50}$) and presence ($[A]_{50B}$) of the antagonist (B) were fitted to the following derivation of the Schild equation (see Black *et al*., 1985b):

$$\log[A]_{50B} = \log[A]_{50} + \log(1 + [B]^b/10^{\log K_B})$$

If the Schild plot slope parameter (b) was not significantly different from unity, it was constrained to unity and the data re-fitted to provide a pK_B estimate. The substitution of $10^{\log K_B}$ for K_B into the equation above was based on the assumption that K_B values were log-normally distributed (Hills *et al*., 1996). When the estimated value of b was significantly different from unity, a pA_2 value was calculated from the data obtained with the lowest concentration of antagonist which produced a significant dose-ratio with the Schild equation (log dose-ratio = $\log[A]_{50B}/\log[A]_{50} = \log[B] + pA_2$).

All data are presented as mean±s.e.mean. Differences between sets of curve parameters (α and $\log[A]_{50}$) were tested by one-way analysis of variance. Differences were considered significant when $P<0.05$.

Comparison of ligand potency values The comparisons of agonist potency ($p[A]_{50}$) and antagonist affinity (pK_B) values estimated on the SA and AV node assays were made by fitting straight line models (see below) to the data by least squares. In standard least-squares fitting, it is the sum of squared residuals in the y-direction that is minimized, with the implicit assumption that all the error is in the y-variable, while the x-variable is error free. That was not the case here so an alternative analysis was applied. In this instance, by inspection of the data obtained on the two assays (Table 1 and Table 2), it seemed reasonable to make the simplifying assumption that the variances of the affinity estimates on each assay system were equal. This implies that no direction is more important than any other, and so it was optimal to fit the line by minimizing the sum of the squares of the shortest distances of the points from the line, that is, the perpendicular distance from the line. This procedure is equivalent to performing a principal components analysis on the data which can be carried out using, for example, Genstat 5 computer software (Numerical Algorithms Group, Oxford, U.K.). In practice a simple basic computer programme was written and copies of this are available on request together with more details of the analysis.

The process involved fitting a nested set of straight line models to the data. First, the most general straight line was fitted. This has unconstrained slope and intercept (i.e. y = m.x + c). A test of non-linearity can be performed by comparing the residual sum of squares from this unconstrained line to an independent estimate of background variance. Secondly, the straight line with slope constrained to unity was fitted (i.e. y = x + c). This reflects the hypothesis that there is a constant difference in absolute values between the two assay systems, but that the difference between any two compounds is the same when measured on either system. Finally, a further constraint on the line – this time on the

Table 1 Schild plot slope (b) and affinity (pK_B) parameters for adenosine receptor antagonists obtained in guinea-pig SA and AV node assays

	SA node assay			*AV node assay*			
Antagonist	*Agonist*	pK_B	*b*	*Agonist*	pK_B	*b*	ΔpK_B (95% c.l)
DPCPX	adenosine	8.83±0.09	0.96±0.04	adenosine	8.36±0.07	0.99±0.07	0.47 (0.23)
DPCPX	NECA	8.49±0.11	0.91±0.05	NECA	8.77±0.09	N.A.[1]	−0.28 (0.28)
CGS15943	NECA	7.31±0.16	1.26±0.07*	NECA	7.76±0.07	1.00±0.07	−0.45 (0.35)
N-0861	adenosine	6.38±0.08	1.01±0.05	NECA	5.69±0.09	0.85±0.11	0.69 (0.24)

Data shown are means±s.e.mean. *Indicates that the Schild plot slope parameter was significantly different from unity ($P<0.05$) under which condition the number given in the pK_B column is a pA_2 value. See text for details of the analysis. [1]The slope value was not estimated as the pA_2 value was calculated from the dose-ratio obtained with a single concentration of DPCPX (0.3 μM). The final column shows the difference between the pK_B values obtained on the two assays and, in parentheses, the combined 95% confidence interval of the individual estimates.

Table 2 Agonist potency ($p[A]_{50}$) and maximum response (α) values obtained for adenosine receptor agonists in guinea-pig SA and AV node assays

	SA node assay		*AV node assay*	
	$p[A]_{50}$	α (beats min^{-1})	$p[A]_{50}$	α (ms)
NECA	7.21±0.08	158±23	6.82±0.10	58±8
R-PIA	6.86±0.08	190±22	6.86±0.05	48±3
CHA	6.83±0.02	192±30	6.92±0.08	67±7
CADO	6.62±0.10	195±28	6.49±0.07	57±6
S-PIA	5.62±0.05	188±12	5.50±0.10	50±5
CV 1808	4.57±0.10	168±23	4.41±0.07	70±10
MeAdo	4.35±0.04	87±16	4.60±0.05	65±5

Data shown are means±s.e.mean ($n=5/6$) — See text for details of the analysis.

intercept – was introduced by fitting the line of identity ($y = x$). This reflects the hypothesis that the values obtained for each compound on the two assays are indistinguishable. Differences in the residual sums of squares between the three models provide a formal test for unit slope and zero intercept in the usual way, though there was some question as to what to use for the estimate of the background variance (or 'experimental error'). One possibility was to use the standard errors of the individual affinity estimates, and create from them a pooled estimate of variance. This would mean that there was also a formal way of testing the fit of the unconstrained line ($y = m.x + c$), i.e. a test for non-linearity. However, in practice, the test for non-linearity of both the agonist and antagonist data sets returned statistically significant results, although the graphs, especially that of the agonist data (Figure 4), gave no visual evidence of systematic departure from the straight line. The likely reason for this is that such a pooled estimate of background variance reflects only within-experiment error and provides no information about how the results would differ if the experiments were repeated. Since this latter component of variation is often the dominant one, within-experiment estimates of error can be artificially small. When this is thought to be the case, there is the alternative of using the residual mean square from the model with the most parameters (i.e. the least constrained) to provide an estimate of baseline variance. This was the procedure followed here, the model with the most parameters being the unconstrained line itself.

A practical drawback of estimating the baseline variance by this method is a loss of degrees of freedom. The baseline variance appears in the denominator of the *F*-ratios and, if it has only a small number of degrees of freedom, those ratios will be rather unstable. This affects the formal properties of the associated significance tests: specifically there is a loss of statistical power which is particularly severe for the antagonists in this instance. It is still informative to look at the *F*-ratios: here they determine whether the mean squares for deviations from unit slope and zero intercept are big or not compared to the mean square for non-linearity. However, it should be borne in mind that statistical non-significance will not be a strong statement – any 'non-significances' are more due to a lack of evidence of differences, than to positive evidence of sameness.

Compounds

Adenosine, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), dipyridamole, and N^6-aminoadenosine (MeAdo), 5′-N–ethylcarboxamidoadenosine (NECA) and **R**(−)-N^6-(2-phenylisopropyl) adenosine (**R**-PIA) were purchased from Sigma Chemical Company Ltd. (U.K.) and 2-chloroadenosine (CADO), N^6-cyclohexyladenosine (CHA), 2-phenylaminoadenosine (CV 1808) and **S**(+)-N^6-(2-phenylisopropyl)adenosine (L-PIA) were obtained from RBI Research Biochemicals International (U.K.). N^6-(endo-2-norbornyl)-9-methyladenine (N-0861) and 9-chloro-2-(2-furanyl)-5,6-dihydro-1,2,4-triazolo[1,5-c]quinazolin-5-imine (CGS15943) were generously supplied as gifts from Discovery Therapeutics (U.S.A.) and Ciba-Geigy Corporation (U.S.A.), respectively. All test compounds, except GGS15943 which was dissolved at 2 mM, were prepared as 20 mM stock solutions in 0.05 M HCl for NECA, 0.1 M HCl for dipyridamole and N-0861, 50% ethanol for CADO and CHA, 100% dimethyl sulphoxide (DMSO) for CGS15943 and DPCPX, 50% DMSO for CV 1808, 33% DMSO for L-PIA, and distilled water for adenosine and MeAdo. Further serial dilutions were made in distilled water.

Results

Antagonist affinity determination

Three adenosine receptor antagonists were studied using either NECA or adenosine (in the presence of dipyridamole) as agonist. DPCPX was chosen as it has been used as an A_1-receptor selective antagonist (Martinson *et al*., 1987) in many other classification studies. N-0861 was included as it is reported to express particular selectivity for the A_1 over the A_2-receptor (Shyrock *et al*., 1992) and CGS15943 because of its A_2-receptor selectivity (Williams *et al*., 1987; Klotz *et al*., 1998). The initial aim was to perform a series of experiments in which each antagonist was investigated with the two agonists on both assays (12 experiments). In practice, the analysis of the results obtained when about half of the planned experiments had been performed made us doubt whether completion of the series was warranted because, although there were assay differences, no systematic evidence of receptor heterogeneity within and between assays had been found. Moreover, agonist potency estimates were those expected for A_1-receptors (see below). The order in which the experiments were performed was governed by laboratory expediency (e.g. compound availability) rather than design. This explains why, for example, N-0861 was studied using adenosine in the SA node assay and NECA in the AV node assay. However, because each analysis was internally controlled (performed according to a within-experimental block design), the results obtained with each agonist-antagonist pair could be treated as discrete information for the purpose of further statistical analysis.

All three antagonists produced a concentration-dependent rightward shift of the agonist E/log[A] curves in both assays. The data obtained from the interaction between adenosine plus dipyridamole and DPCPX are shown for illustrative purposes in Figure 1. Two of the antagonists had significant effects on the basal rate of the SA node assay following the addition of histamine (see Methods). CGS15943 reduced (maximum inhibition = 40 ± 13 beats min^{-1} at 10 μM) whereas N-0861 increased the basal rate. The increase in basal rate produced by N-0861 appeared to be concentration-dependent until a maximal increase was obtained at 30 μM (40 ± 14 beats min^{-1}). Thereafter, a higher concentration of N-0861 produced no further increase and the basal rate at 0.1 mM was not different from the control value. None of the antagonists had any effect on the basal conductance in the AV node assay. Although no formal test based on curve-fitting was made, the slopes of the agonist concentration-effect curves appeared to be consistent in the absence and presence of antagonists. Similarly, with the exception of the highest concentration of CGS15943 (3 μM) which produced a significant increase ($\Delta SR = 17 \pm 7$ ms) in the maximum response obtained with NECA in the AV node assay, there were no significant differences in α values.

Notwithstanding the effects of some of the antagonists on the basal activity of the SA node assay, the $\log[A]_{50}$ values obtained in the absence and presence of the antagonists were fitted to the derivation of the Schild equation (see Methods). The Schild plot slope parameter and pK_B values obtained are presented in Table 1. In the SA node the data from the interaction between CGS15943 and NECA produced a Schild plot slope parameter which was significantly greater than unity. In the other experiments the antagonists appeared to behave in a simple competitive manner with Schild plot slope parameter estimates which were not significantly different from unity.

To test the hypothesis that there was no overall difference between the antagonist affinity estimates made on the two

assays the values were fitted to a straight line by least squares (see Methods for details of the analysis). The analysis indicated that the data did not deviate significantly from the line of identity (Figure 2).

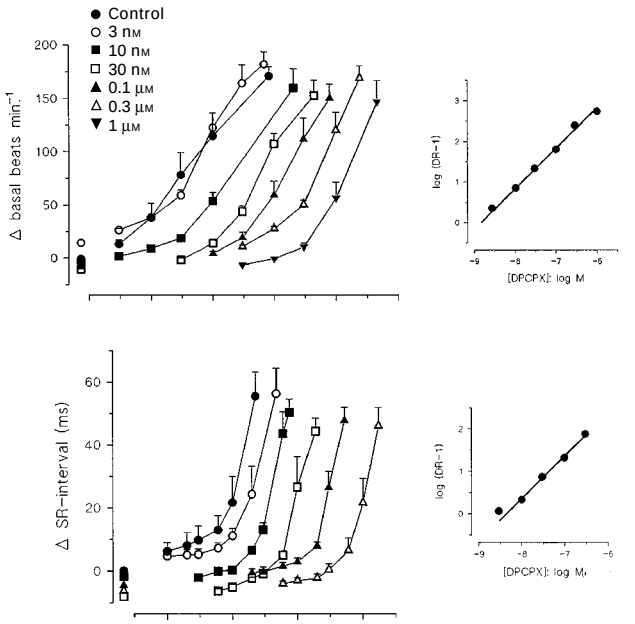

Figure 1 Adenosine (plus dipyridamole) concentration-effect curve data obtained in the (a) SA and (b) AV node assays in the absence (control) and presence of 3 nM, 10 nM, 30 nM, 0.1 μM, 0.3 μM and 1 μM DPCPX. Values represent mean of 5/7 preparations, vertical lines show s.e.mean. Inserts show the corresponding Schild plots (see Table 1 for details).

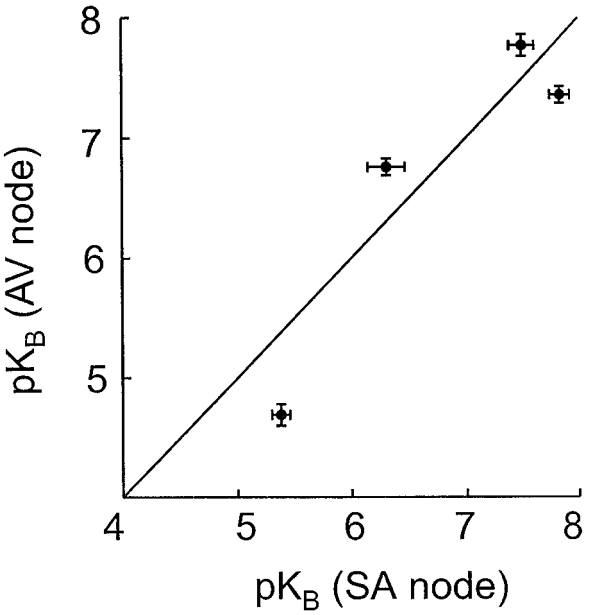

Figure 2 Comparison of pA_2/pK_B values ($\pm$s.e.) obtained for three adenosine receptor antagonists in the SA and AV node assays (see Table 1). The comparison of the data was made by fitting a nested set of straight line models to the data by least squares. The results of the analysis, which indicate that the data did not deviate significantly from the line of identity (i.e. y = x), are shown below. See text for details of the analysis. *F*-test for linearity: (y = m.x + c), $F(2,152) = 18.839$, $P<0.01$; *F*-test for unit slope: (y = x + c), $F(1,2) = 0.529$, $P>0.05$; *F*-test for zero intercept: (y = x), $F(1,2) = 0.126$, $P>0.05$.

Agonist potency determination

The seven agonists investigated all produced concentration-dependent negative chronotropic and dromotropic responses in the SA and AV node assays (Figure 3). Due to the abrupt onset of SA or AV node block, fully-defined agonist concentration-effect curves could not be obtained in either assay. Log concentration-effect curves obtained on the two assays were convex to the log[A] axis with no upper asymptote as though they were the lower regions of a sigmoid function (Figure 3a). Locating such curves by the negative logarithm of the agonist concentration required for half-maximal effect ($p[A]_{50}$) does not have the same meaning as a $p[A]_{50}$ value calculated for a full sigmoid function because the asymptote is not defined. However, comparison of $p[A]_{50}$ values within an assay tissue appeared to be valid because the curve shape and maximum response (α) appeared to be similar for each agonist. The consistency of measurements within each assay was judged to be the basis for comparing the relative potencies between assays.

Figure 3 shows the E/log[A] curves obtained with the seven agonists in the SA and AV node assays and the corresponding $p[A]_{50}$ and α values are shown in Table 2. With the exception of the least potent agonist, CV1808, which produced a low value on the SA node, there were no significant differences in the values of α between the agonists as judged by ANOVA. The comparison of the $p[A]_{50}$ estimates made on the two assays was made by fitting the values to a straight line by least squares (see Methods). This analysis indicated that the data did not deviate significantly from the line of identity ($F(1,70) = 0.693$), that is,

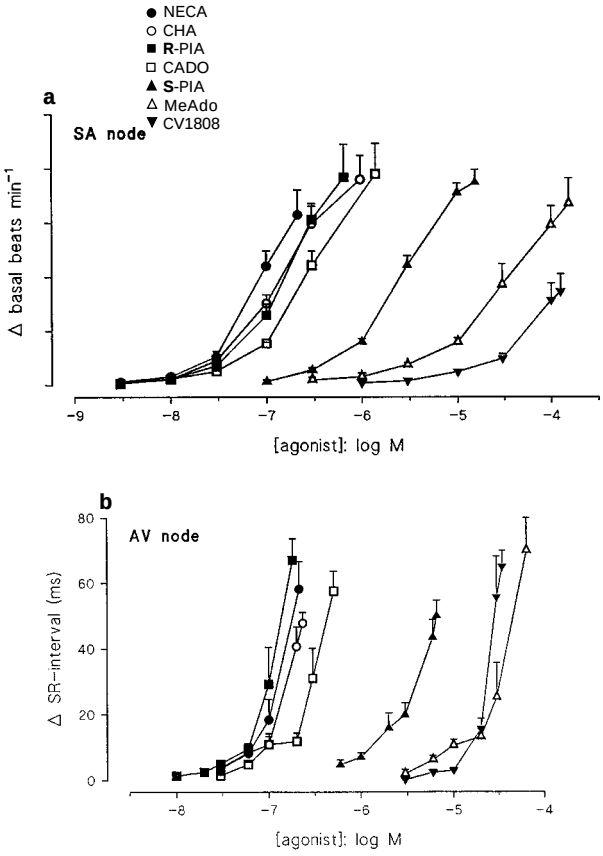

Figure 3 Concentration-effect curve data obtained with NECA, CHA, **R**-PIA, CADO, **S**-PIA, MeADO and CV 1808 in the (a) SA and (b) AV node assays. Values represent mean of 5/6 preparations; vertical lines show s.e.mean.

there were no significant differences in absolute potencies between the two assays. (Figure 4).

Discussion

The aim of this study was to determine within a single species of the adenosine receptors mediating chronotropic and dromotropic effects in the SA and AV node, respectively, could be considered to be homogeneous. This receptor classification exercise was performed as a prelude to a larger study of the role of adenosine in SA and AV nodal function (see Meester *et al*., 1998).

The experiments performed complement those previously reported by Belardinelli *et al*. (1983, 1989), Clemo & Belardinelli (1986), Collis *et al*. (1989) and Evans *et al*. (1982). SA nodal function was studied using the isolated spontaneously-beating right atrium preparation because it produces high quality data and has become a standard assay in our laboratory for several receptor classes (Black *et al*., 1985a). The Langendorff perfused assay (Jenkins & Belardinelli, 1988) was preferred over a superfused heart assay (Hoffman *et al*., 1959), because we found that this assay showed a lesser tendency to become hypoxic, as indicated by the basal AV conduction time (55 ± 5 ms, data not shown) which was equivalent to the physiological norm for the guinea-pig (56 ± 4 ms; Wagner & Manning, 1976).

Although the assays and physiological responses of the SA and AV nodes are different, the use of ratios of agonist concentrations in both the analysis of agonist potency and antagonist affinity allows the post-receptor, physiological, events to be cancelled out. It is generally accepted that antagonist pK_B value estimation is the most reliable method for pharmacological receptor classification (Colquhoun, 1986). However, inspection of the antagonist data (Table 1) showed that there were small differences between the assays for individual compounds. For example, the difference in values for N-0861 (0.69) was greater than the 0.5 half log unit difference which Furchgott (1966) proposed as a 'rule of thumb' indicator of receptor heterogeneity. In addition, in three out of four cases, the difference in pK_B value between assays was greater than the combined 95% confidence intervals associated with each estimate. Therefore, the data obtained raised the issue of how to make a formal comparison of pK_B and, for similar reasons, agonist potency values obtained on different assays. The null hypothesis, that the receptors are homogeneous, predicates that the data obtained on the two assays will lie on the line of identity. However, the formal test of linearity failed. The most likely reason for this result is that the pooled estimate of the background variance obtained from the variance of the individual estimates only reflects within-experiment error and not the variation which would be exposed if the experiments were repeated (see Methods).

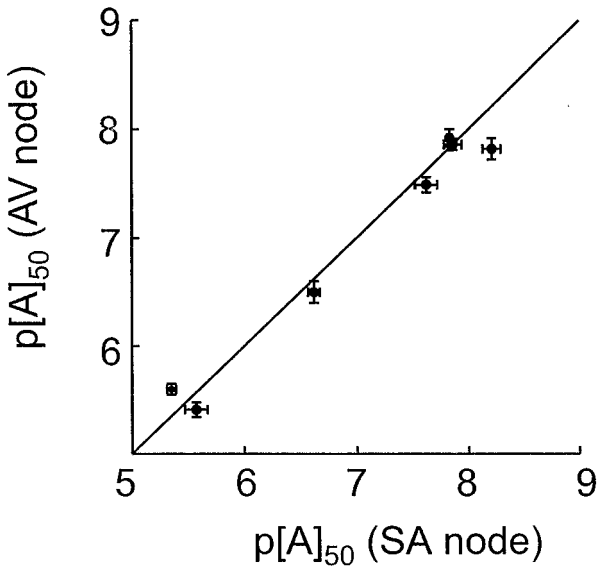

Figure 4 Comparison of $p[A]_{50}$ values ($\pm$s.e.mean) obtained with adenosine receptor agonists in the SA and AV node assays (see Table 2). The comparison of the data was made by fitting a nested set of straight line models to the data by least squares. The results of the analysis, which indicate that the data did not deviate significantly from the line of identity (i.e. $y = x$), are shown below. See text for details of the analysis. *F*-test for linearity: ($y = m.x + c$), $F(5,70) = 3.889$, $P < 0.01$; *F*-test for unit slope: ($y = x + c$), $F(1,5) = 0.718$, $P > 0.05$; *F*-test for zero intercept: ($y = x$), $F(1,5) = 0.693$, $P > 0.05$.

The analysis based on the simultaneous fit of all the antagonist data to the line of identity (see Methods) indicated that overall the values were indistinguishable (Figure 2). The absence of any basal effect of DPCPX in this assay indicates that any endogenous, extracellular, adenosine was only present at subthreshold concentrations. Therefore, we can conclude that the action of N-0861 to increase basal rate was not due to antagonism of endogenous adenosine. In the case of CGS15943, we have no idea of the mechanism involved in the effect on basal rate. However, previously both Ghia *et al*. (1987) and Prentice (1990) have reported steep Schild plots with this compound.

Traditionally, in the adenosine receptor field, classification has been based on agonist potency ratios because, until recently, there has been a relative paucity of antagonists with sufficient discriminatory power. In principle, the reliable estimation and comparison of agonist potency values requires that the concentration-effect curves are fully defined. In this study, in both assays, this was not possible due to the abrupt onset of SA and AV nodeblock. In practice, agonist potency ($p[A]_{50}$) values were estimated as the concentration of agonist required to produce 50% of the maximum response which was defined as the highest stable decrease in rate and the longest stable SR-interval before block in the SA and AV nodal assays, respectively. This method could lead to errors if the underlying curve shape and maxima (α) were not conserved for all the agonists within each assay. Within a single receptor class assay, agonist curve shape is expected to be conserved for high efficacy agonists (Black *et al*., 1985c). With the exception of MeAdo, which produced a low value on the SA node, there were no significant differences in the values of α between the agonists which suggests that these compounds were not behaving as partial agonists. Moreover, inspection of the experimental data shown in Figure 3 does not reveal any obvious differences in the shape of the curves between agonists within an assay. However, the lower maximum of MeAdo in the SA node, but not the AV node, may be a consequence of the differences in the shape of the curves between the two assays. Thus, inspection of the data in Figure 3 reveals that the SA node assay curves span approximately twice the concentration range (2 log units) of those obtained in the AV node assay. Therefore, although the absolute potency values were judged to be indistinguishable, higher concentrations (greater than 0.1 mM) of the least potent agonist, MeAdo, were required to define its curve maxima in the SA node than in the AV node (60 μM, see Figure 3). Perhaps, at these higher concentrations MeAdo was expressing an additional action to cause abrupt SA nodal arrest. Previously, it has been shown

that adenosine decreases primary and secondary pacemaker rates with different potencies (Belardinelli *et al*., 1983). This may provide an explanation for the observation that the slope of the E/[A] curves in the SA node was consistently lower than that obtained in the AV node. Thus, the low slope would be due to a progressive shift to lower frequency pacemaker activity with concomitant decrease in potency of the agonists. The comparison of the agonist potency values obtained on the two assays (Figure 4) indicates that the data can be described by the line of identity. According to classical receptor theory, this indicates that not only were the receptors in the two assays pharmacologically indistinguishable in terms of agonist affinity but also, remarkably, that the efficacy of the agonists was identical. The latter implies that the net effect of the tissue-dependent factors governing agonist potency, such as total receptor concentration and receptor-effector coupling efficiency were equal in the two assays. The rank order of agonist potency (CHA = **R**-PIA = NECA $\geqslant$ CADO > **S**-PIA > CV 1808 = MeAdo) was consistent with previous findings indicating that the adenosine receptors mediating negative chronotropic and dromotropic effects are of the A_1 subtype (Kennedy *et al*., 1992). As expected for adenosine A_1-receptors, the difference between the **R**-PIA and **S**-PIA $p[A]_{50}$ values was almost identical in the SA (1.24 ± 0.09) and AV (1.36 ± 0.11) nodal assays.

Notwithstanding data showing that there are species differences in radioligand binding studies for adenosine agonists and xanthine-based antagonists (Ukena *et al*., 1986), we found a remarkable comparison between our agonist potency data and binding values obtained by Bruns *et al*. (1986) for the same compounds on rat brain membranes. The model-fitting approach (see Methods) indicated that the data did not deviate significantly from a straight line with unit slope ($F(1,42) = 0.080$; Figure 5). The y-intercept (the affinity in the binding assays) obtained (1.74 ± 0.13) indicates that the affinity of the agonists in the binding assay was consistently ~ 55 fold higher than the $[A]_{50}$ values in the AV and SA nodal assays. Presumably, this constant discrepancy could be due to the expression of high affinity binding by agonists which is well-recognized at G protein-coupled receptors (e.g. De Lean *et al*., 1980).

In conclusion, the analyses of agonist and antagonist activity indicate that the adenosine receptors mediating chronotropic effects in the SA node and dromotropic effects in the AV node in the guinea-pig are pharmacologically indistinguishable and are of the A_1-receptor class. No evidence for so-called 'A_3'-receptors was found.

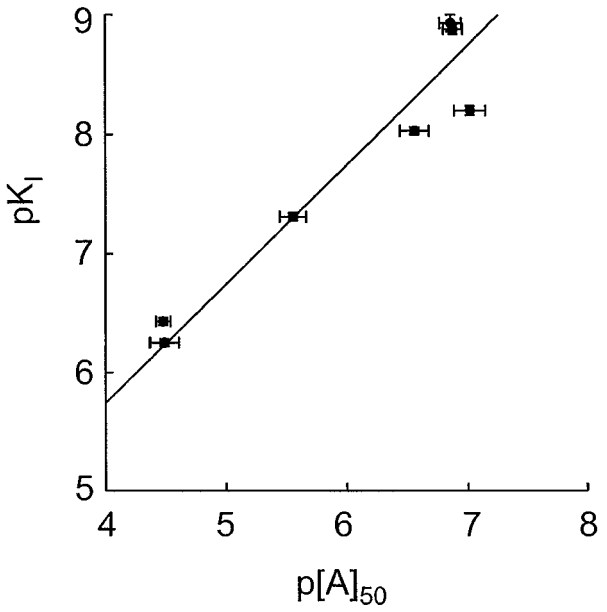

Figure 5 Comparison of the mean $p[A]_{50}$ values ($\pm$s.e.mean) obtained with adenosine receptor agonists in the SA and AV node assays in this study (see Table 2) with the apparent pK_i values obtained from an analysis of rat brain adenosine A_1-receptor radioligand binding assay data (Bruns *et al*., 1986). The comparison of the data was made by fitting a nested set of straight line models to the data by least squares. The line superimposed on the data shows the best fit obtained by least squares model-fitting to a straight line with unit slope and variable y-intercept (i.e. $y = x + c$). The y-intercept (c) estimated was 1.74 ± 0.13. See text for details of the analysis. *F*-test for linearity: ($y = m.x + c$), $F(5,42) = 9.558$, $P < 0.01$; *F*-test for unit slope: ($y = x + c$), $F(1,5) = 0.080$, $P > 0.05$; *F*-test for line of identity: ($y = x$), $F(1,5) = 177.2$, $P < 0.01$.

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