Quantitative analysis of the agonist and antagonist actions of some ATP analogues at P_{2X}-purinoceptors in the rabbit ear artery

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1 The agonist and antagonist effects of a series of β,γ -methylene dihalo- and 2-methylthio-substituted analogues of ATP at P_{2X}-purinoceptors have been analysed on the rabbit isolated ear artery preparation. Cumulative and sequential dosing experimental protocols were employed in the construction of agonist concentration-effect curves in order to address the possible influence of acute receptor desensitization on subsequent analyses.

2 Using the cumulative curve design the following results were obtained: D-AMP-PCBr₂P, 2-methylthio-D-AMP-PCCl₂P, L-AMP-PCF₂P, L-AMP-PCCl₂P and LAMP-PCBr₂P each behaved as partial agonists. D-AMP-CPP was used as a reference full agonist and these analogues were analysed by the comparative method of Barlow *et al.* (1967), to provide estimates of affinity and efficacy. 2-Methylthio-L-AMP-PCBr₂P was virtually silent as an agonist and was analysed as a competitive antagonist by Schild analysis.

3 Two agonists, L-AMP-PCCl₂P and L-AMP-PCBr₂P, were analysed by the sequential curve design, and the antagonist effects of one of the agonists, L-AMP-PCBr₂P were also analysed using this protocol. The resulting estimates of affinity and efficacy, while similar to those obtained with the cumulative design, indicated that acute desensitization may affect curve definition and estimation of these quantities.

4 The following structure-activity trends emerged: D-analogues tended to have higher efficacy but lower affinity than L-analogues; efficacy varied markedly and inversely with the atomic weight of the halogen while affinity was only minimally affected; 2-methylthio- substitution also reduced efficacy with minimal effect on affinity.

5 The results of this analysis are discussed in terms of the utility of affinity and efficacy information in the classification of purinoceptors and the design of chemical probes for them.

Keywords: P_{2X}-purinoceptors; ATP analogues; partial agonist; antagonist; affinity; efficacy; rabbit ear artery; receptor classification; medicinal chemistry

Introduction

The absence of selective and competitive antagonists for P₂-purinoceptors has meant that their pharmacological classification has depended primarily on the use of agonists (Burnstock & Kennedy, 1985; Kennedy, 1990). In turn, the use of agonists has been limited to the provision of potency order information. No attempt appears to have been made to analyse agonist action quantitatively, that is, to provide estimates of affinity and efficacy, despite the potential impact on and refinement to receptor classification that such information can provide. The purpose of the present study was to determine whether such data could be obtained for P_{2X}-purinoceptors.

Affinity and efficacy estimates can, in principle, be obtained for full agonists by the method of receptor inactivation (Furchgott, 1966), and for partial agonists by the comparative (Barlow *et al.*, 1967) or interaction (Stephenson, 1956) methods. The former method relies on the availability of an irreversible receptor antagonist. Unfortunately, in the case of P_{2X}-purinoceptors such an agent has not been discovered, meaning in the present study that full agonists could not be used to provide affinity and efficacy information. Such estimates would only be possible for partial agonists.

Few reports of partial agonism have appeared in the purinoceptor literature. However, in a study by Cusack and colleagues (1987), data were presented suggestive of such behaviour at P_{2X}-purinoceptors. Those workers investigated

the effects of a series of phosphonate ATP analogues in the guinea-pig taenia coli and urinary bladder. In the latter tissue, which was classified as a P_{2X}-purinoceptor system, some β,γ -methylene dihalo- and 2-methylthio-substituted compounds appeared to produce lower maximal effects than other analogues. This feature of the data was not remarked upon but served to stimulate us to reinvestigate this series of compounds.

We chose to use the rabbit isolated ear artery preparation for this study. This tissue has previously been shown to contain P_{2X}-purinoceptors and, unlike other smooth muscle preparations, to be amenable to precise agonist-concentration effect curve shape definition (O'Connor *et al.*, 1990), the latter being particularly important for affinity and efficacy analysis. The results of this study are discussed in terms of the confidence with which affinity and efficacy estimates for P_{2X}-purinoceptor agonists can be provided, and the utility of this information in the definition of P_{2X}-purinoceptors.

Methods

Rabbit ear artery

Male New-Zealand White rabbits (2.5–3.5 kg) were killed by an overdose of pentobarbitone sodium (300 mg, i.v.). The ears were removed and following insertion of a polythene cannula (0.75 mm o.d.) the central artery ear was dissected free; 5–10 mm rings of tissue were mounted on fine tungsten wire hooks and suspended horizontally in 20 ml organ baths containing Krebs solution of the following composition

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(mM): NaCl 117.56, NaH₂PO₄ 0.89, NaHCO₃ 25.0, MgSO₄ 1.18, glucose 11.1, KCl 5.36 and CaCl₂ 2.55. The solution routinely contained the P₁-purinoceptor antagonist 8-sulphophenyltheophylline (8-SPT) (3×10^{-4} M) (Collis *et al.*, 1987) to exclude possible direct or indirect effects mediated through P₁-receptors, and indomethacin (2.8×10^{-6} M) to eliminate the influence of products of cyclo-oxygenase. The tissues were maintained at 37°C and gassed continually with 95% O₂/5% CO₂.

Each ring was subjected to an initial force of 1.0 g and allowed to equilibrate for 1 h before any further intervention. Changes in the contractile state of the tissues were recorded isometrically with Ormed force transducers and displayed on Advance Bryans chart recorders.

Experimental protocols

In all experiments tissues were initially contracted with KCl (80 mM) in order to establish viability. Once the response had reached steady state, acetylcholine (10^{-6} M) was added to confirm endothelium denudation. Following washing, tissues were allowed to equilibrate for 45 min then a cumulative agonist-concentration effect (E/[A]) curve was constructed with D- α,β -methylene ATP (D-AMP-CPP). Following washing, tissues were again allowed to settle for 70 min before being subjected to one of the following protocols.

Cumulative curve design In this protocol a second cumulative E/[A] curve was then constructed to D-AMP-CPP or a test agonist. Following subsequent washing, D-AMP-CPP (3×10^{-5} M) was applied and its contractile response allowed to fade (15 min). Cumulative applications of the test agonist were then repeated in the continued presence of D-AMP-CPP.

One compound, which was virtually inactive as an agonist was examined for antagonist properties. It replaced the test agonist in the protocol and was incubated for 45 min prior to the construction of a second E/[A] curve to D-AMP-CPP.

When histamine, phenylephrine or KCl were employed, they replaced D-AMP-CPP in the protocol.

The reproducibility of paired curves was analysed by comparing first and second D-AMP-CPP curves. The following mean differences in parameter estimates were computed: $\Delta p[A_{50}] = 0.01 \pm 0.01$; $\Delta \alpha = -3.18 \pm 2.10$; $\Delta p = 0.06 \pm 0.03$ ($n = 29$) (see Data analysis). These differences were not significant.

Sequential curve design In this protocol, a second E/[A] curve was obtained either to D-AMP-CPP or to a test agonist by making individual applications of ascending concentrations with a 40 min re-equilibration period between each application. Reproducibility of individual applications was assessed by repeated administration (6 times) of an $[A_{50}]$ and a maximal concentration of a single agonist. Variation, expressed as standard error on the measured effects across time, was in the order of only 1%. Reproducibility between the first cumulative and second sequential D-AMP-CPP curve was tested giving the following mean differences in parameter estimates: $\Delta p[A_{50}] = -0.06 \pm 0.02$; $\Delta \alpha = -5.22\% \pm 3.39\%$; $\Delta p = 0.22 \pm 0.06$ ($n = 18$) (see Data analysis). Although the differences in $p[A_{50}]$ and p were significant it was calculated that changes of this magnitude would have trivial effects on subsequent parameter estimation.

In experiments involving interactions between two agonists, application of the second agonist was made immediately after maximal response had been attained to the first.

In all experiments contractile effects of agonists were expressed as percentages of the maximum of the first curve to D-AMP-CPP obtained in the tissue.

Data analysis

General curve fitting In order to obtain objective estimates of E/[A] curve parameters, data sets were fitted using the logistic equation

$$E = \frac{\alpha [A]^p}{[A_{50}]^p + [A]^p} \quad (i)$$

in which α , $[A_{50}]$ and p are respectively the asymptote, location and slope parameters. Location parameter estimates are quoted in the text as $p[A_{50}]$ ($-\log_{10}[A_{50}]$) values.

Each individual curve, whether obtained cumulatively or sequentially, was fitted separately.

When the interaction between a partial agonist and D-AMP-CPP was studied the effects of the full agonist were measured from the response level produced by the partial agonist alone.

Paired Student's t test was used to compare parameter estimates for test agonists with those for D-AMP-CPP.

Affinity and efficacy estimation Analogues which behaved as partial agonists were analysed by the comparative method (Barlow *et al.*, 1967) using operational model-fitting procedures described elsewhere (Leff *et al.*, 1990). The comparative method involved the comparison of the partial agonist with a reference full agonist. D-AMP-CPP was adopted as the reference full agonist in the present study. This agonist has been shown elsewhere to have efficacy corresponding to full agonism in the ear artery (Wood *et al.*, 1992).

The operational model has the following algebraic form (Black & Leff, 1983):

$$E = \frac{E_m \tau^n [A]^n}{(K_A + [A])^n + \tau^n [A]^n} \quad (ii)$$

K_A is the equilibrium dissociation constant for the agonist and is a reciprocal measure of affinity. For convenience, pK_A values are quoted in the text. τ is the efficacy of the agonist. E_m is the maximum possible effect and n is the slope parameter in the receptor system.

For agonists of high efficacy, equation (ii) reduces to an equation of the logistic form

$$E = \frac{E_m [A]^n}{[A_{50}]^n + [A]^n} \quad (iii)$$

E_m and n are estimated by fitting this equation to the full agonist E/[A] curve data. Once these two parameters are known K_A and τ can be obtained by fitting the partial agonist data with equation (ii). In practice, both steps are carried out in a single computer fit.

Each piece of tissue provided a reference full agonist and a partial agonist E/[A] curve and, therefore, an estimate of pK_A and τ .

Analysis of antagonism Paired E/[A] curves to D-AMP-CPP obtained in the absence and presence of an antagonist were fitted to equation (i). Parallelism was tested by one-way analysis of variance on the paired differences in computed asymptote, α , and slope, p , values. Paired differences in $p[A_{50}]$ estimates were used to calculate dose-ratios (r) and analysed according to Arunlakshana & Schild (1959):

$$\log_{10}(r-1) = n \log_{10}[B] + pK_B \quad (iv)$$

in which n is the Schild plot slope and pK_B is the antagonist affinity constant. If n was found not to be significantly different from unity the pK_B was estimated with n constrained to that value.

Data on the antagonistic interaction between a test agonist and D-AMP-CPP were also analysed using this equation.

Data are presented as mean $\pm$ s.e.mean. On occasions when weighted averages were used, the reciprocal of the variance was used as the weighting factor. All fitting procedures were carried out with a VAX Mainframe computer employing the BMDP Statistical Software package.

Drugs and solutions

Drugs were obtained from the following sources: acetylcholine bromide, histamine dihydrochloride, D-adenosine 5'-(α,β -methylene)triphosphonate (D-AMP-CPP) dilithium salt, D-adenosine 5'-(β,γ -methylene)triphosphonate (D-AMP-PCP) disodium salt, phenylephrine hydrochloride and indomethacin, Sigma Chemical Co., Poole, U.K.; 8-sulphophenylthio-phylline (8-SPT), Research Biochemicals Inc., St Albans, U.K.; pentobarbitone sodium (Euthatal), RMB Animal Health Ltd., Dagenham, UK; (L-adenosine 5'-(β,γ -methylene)triphosphonate (L-AMP-PCP), L-adenosine 5'-(β,γ -difluoromethylene)triphosphonate (L-AMP-PCF₂P), L-adenosine 5'-(β,γ -dichloromethylene)triphosphonate (L-AMP-PCCl₂P), L-adenosine 5'-(β,γ -dibromomethylene)triphosphonate (L-AMP-PCBr₂P), D-adenosine 5'-(β,γ -difluoromethylene)triphosphonate (D-AMP-PCF₂P), D-adenosine 5'-(β,γ -dichloromethylene)triphosphonate (D-AMP-PCCl₂P), D-adenosine 5'-(β,γ -dibromomethylene)triphosphonate (D-AMP-PCBr₂P), 2-methylthioadenosine 5'-(β,γ -dichloromethylene)triphosphonate (2-methylthio-D-AMP-PCCl₂P) and (2-methylthio-L-adenosine 5'-(β,γ -dibromomethylene)triphosphonate (2-methylthio-L-AMP-PCBr₂P) [all tetrasodium salts] were synthesized by P.A. Cage, D. Cox and A.H. Ingall, Department of Medicinal Chemistry, Fisons R&D Labs, Loughborough, U.K.

Indomethacin was dissolved in 10% w/v Na₂CO₃ at 10 mg ml⁻¹ and subsequently diluted in Krebs buffer. Histamine was dissolved in distilled water at 0.2 M, the solution was neutralised with 10 M NaOH (10 μ l to 2 ml). All other drugs were dissolved in distilled water.

Results

Figure 1 illustrates mean cumulative E/[A] curves for a series of D- and L- β,γ -dihalomethylene ATP analogues and two compounds with an additional 2-methylthio-substituent. Paired *t* tests on the computed asymptotes of the curves showed that five of the compounds produced maximal effects which were significantly lower than that of D-AMP-CPP. They were: D-AMP-PCBr₂P; 2-methylthio-D-AMP-PCCl₂P; L-AMP-PCF₂P; L-AMP-PCCl₂P; L-AMP-PCBr₂P. A sixth

Figure 1 E/[A] curves for a series of D- (a) and L- (b) substituted adenosine 5'-(β,γ -methylene)triphosphonates in isolated rings of rabbit ear artery. The points are the mean effects ($\pm$ s.e.mean, vertical bars, $n = 3-10$) calculated from the second curves obtained in each piece of tissue using the cumulative protocol: (a) (x) D-AMP-PCP; (●) D-AMP-PCF₂P; (▲) D-AMP-PCCl₂P (■) D-AMP-PCBr₂P; (Δ) 2-methylthio-D-AMP-PCCl₂P; (b) (x) L-AMP-PCP; (●) L-AMP-PCF₂P; (▲) L-AMP-PCCl₂P; (■) L-AMP-PCBr₂P; (□) 2-methylthio-L-AMP-PCBr₂P. See text for full names.

Table 1 E/[A] Curve shape parameters for D-AMP-CPP (the reference full agonist) and a series of D- and L-adenosine-5'-(β,γ -methylene) triphosphonates

Analogue	$p[A_{50}]$ ($-\log_{10} M$)	Parameter	
		α (%)	p
D-AMP-CPP	6.49 ± 0.03 (6.57 ± 0.05)	97.1 ± 2.1 (95.3 ± 3.5)	1.55 ± 0.04 (1.70 ± 0.06)
D-AMP-PCP	4.36 ± 0.12	105.9 ± 6.4	1.94 ± 0.20
D-AMP-PCF ₂ P	4.47 ± 0.07	99.7 ± 10.7	2.48 ± 0.23
D-AMP-PCCl ₂ P	4.32 ± 0.12	94.2 ± 3.5	2.62 ± 0.21
D-AMP-PCBr ₂ P	3.89 ± 0.07	$78.6 \pm 2.0^{****}$	2.63 ± 0.14
L-AMP-PCP	5.52 ± 0.04	100.2 ± 4.1	1.87 ± 0.09
L-AMP-PCF ₂ P	5.57 ± 0.10	$90.9 \pm 3.3^*$	1.76 ± 0.04
L-AMP-PCCl ₂ P	5.04 ± 0.05 (4.73 ± 0.09)	$49.7 \pm 3.4^{****}$ ($69.8 \pm 4.9^{***}$)	1.54 ± 0.07 (1.42 ± 0.22)
L-AMP-PCBr ₂ P	4.35 ± 0.11 (4.46 ± 0.13)	$15.4 \pm 2.7^{****}$ ($15.5 \pm 1.5^{****}$)	1.15 ± 0.17 (0.96 ± 0.10)
2-Methylthio-D-AMP-PCCl ₂ P	4.05 ± 0.06	$55.3 \pm 8.74^{**}$	1.62 ± 0.11

Values are means ($\pm$ s.e.mean) of parameter estimates made by fitting the logistic equation (equation (i)) to individual, second E/[A] curves, using the cumulative or sequential (values in parentheses) protocol.

Asterisks indicate significant differences between the maximal effect (α) of the agonist and that of D-AMP-CPP; * $P < 0.05$; ** $P < 0.02$; *** $P < 0.01$; **** $P < 0.001$ (paired Student's *t* test).

Figure 2 Analysis of partial agonism by the comparative method. Examples of typical experimental data are shown with computer-generated lines obtained by fitting equations (ii) and (iii). Each panel shows an E/[A] curve to the test compound and the corresponding curve to the reference full agonist, D-AMP-CPP (●). The data for each panel were obtained in a single piece of rabbit ear artery using the cumulative protocol. The resulting affinity (pK_A) and efficacy (τ) values from these individual analyses are given below: (a) (□) D-AMP-PCBr₂P, $pK_A = 2.94$, $\tau = 4.93$; (b) (Δ) 2-methylthio-D-AMP-PCCl₂P, $pK_A = 4.14$, $\tau = 0.78$; (c) (x) L-AMP-PCF₂P, $pK_A = 4.94$, $\tau = 4.51$; (d) (▲) L-AMP-PCCl₂P, $pK_A = 5.04$, $\tau = 1.00$; (e) (■) L-AMP-PCBr₂P, $pK_A = 4.27$, $\tau = 0.25$.

Figure 3 Analysis of antagonism by 2-methylthio-L-AMP-PCBr₂P. (a) E/[A] curves for D-AMP-CPP, in the rabbit ear artery, in the presence of zero (○), 0.1 (■), 0.3 (▲) and 1 mM (●) 2-methylthio-L-AMP-PCBr₂P. The points are the mean effects ($\pm$ s.e.mean, vertical bars, $n = 5$) calculated from second curves obtained in each piece of tissue using the cumulative protocol. (b) Corresponding Schild plot of the effect of 2-methylthio-L-AMP-PCBr₂P on E/[A] curves to D-AMP-CPP. Points show mean (dose ratio - 1) values $\pm$ s.e.mean.

Table 2 Affinity (pK_A) and efficacy (τ) estimates for partial agonists

Analogue	pK_A ($-\log_{10}$ M)	τ range	Parameter E_m (%)	n
D-AMP-PCBr ₂ P	3.49 ± 0.13	2.87 2.40–3.44	96.9 ± 1.49	1.75 ± 0.13
L-AMP-PCF ₂ P	5.05 ± 0.06	4.18 3.80–4.59	99.2 ± 0.75	1.77 ± 0.06
L-AMP-PCCl ₂ P	4.98 ± 0.02	0.99 0.98–1.00	100.6 ± 0.43	1.52 ± 0.03
	$(4.58 \pm 0.04^*)$	(1.26 1.23–1.29)	(99.3 ± 0.72)	(1.63 ± 0.05)
L-AMP-PCBr ₂ P	4.49 ± 0.09	0.26 0.25–0.28	101.2 ± 0.82	1.43 ± 0.04
	(4.63 ± 0.07)	(0.32 0.31–0.34)	(100.3 ± 0.50)	(1.46 ± 0.03)
2-Methylthio-D-AMP-PCCl ₂ P	3.93 ± 0.06	0.95 0.91–0.99	99.7 ± 0.98	1.56 ± 0.07

The values are the weighted averages ($\pm$ s.e.mean, $n = 4-10$) of the operational model parameters obtained using the comparative analysis. The main table shows estimates obtained from cumulative data. Values in parentheses show estimates obtained by the sequential protocol.

Asterisks indicate significant differences between the pK_A estimates obtained by the cumulative and sequential protocols: $*P < 0.01$ (Student's t test).

Figure 4 Comparison of $E/[A]$ curves obtained in the rabbit ear artery, using the cumulative (closed symbols) and sequential (open symbols) protocols: (● $n = 29$, ○ $n = 18$) D-AMP-CPP; (▲ $n = 10$, △ $n = 5$) L-AMP-PCCl₂P; (■ $n = 5$, □ $n = 5$) L-AMP-PCBr₂P. Points show mean $\pm$ s.e.mean (vertical bars).

compound, 2-methylthio-L-AMP-PCBr₂P, produced effects which were only barely measurable. The remaining three D- and one L- analogues produced maximal effects which were indistinguishable from that of D-AMP-CPP. Table 1 summarizes these findings. In all cases, responses were obliterated in the presence of 3×10^{-5} M D-AMP-CPP.

The five compounds which gave reduced but measurable maximal effects were then analysed by the comparative method (Barlow *et al.*, 1967) in order to estimate affinity and efficacy values. Figure 2 illustrates typical experimental data for the compounds, each obtained in a single piece of tissue. Each example shows the corresponding $E/[A]$ curve for D-AMP-CPP, the reference full agonist and computer-generated lines obtained by fitting equations (ii) and (iii). The resulting affinity (pK_A) and efficacy (τ) values from these individual analyses are given in the legend to Figure 2. Average estimates from replicate analyses are shown in Table 2.

2-Methylthio-L-AMP-PCBr₂P, which was virtually inactive as an agonist, was analysed as a potential competitive antagonist. Figure 3a shows the effects of this compound on $E/[A]$ curves to D-AMP-CPP. These data are the averages of the second curves obtained in each tissue and are shown for display purposes only. As explained in the Methods section,

Figure 5 Antagonist effects of L-AMP-PCBr₂P. $E/[A]$ curves were obtained in the rabbit ear artery to D-AMP-CPP, alone (●), and in the presence of L-AMP-PCBr₂P, (■) 100 μ M and (▲) 300 μ M. Points are the mean second curve data ($\pm$ s.e.mean, vertical bars, $n = 3$) obtained using the sequential protocol. The open symbols show the mean contraction induced by (□) 100 μ M and (△) 300 μ M L-AMP-PCBr₂P, before the addition of the full agonist ($\pm$ s.e.mean, $n = 15$ and 16 respectively).

tests for parallelism were based on paired comparisons. One way analyses of variance on computed asymptote (α) and slope (p) estimates revealed no significant differences ($P > 0.05$ in both cases). Figure 3b illustrates the corresponding Schild plot. The slope of the linear regression was 0.92 ± 0.08 (s.e.mean, 13 d.f.). Therefore, this analogue fulfilled the criteria for simple competition. The pK_B estimate, with n constrained to unity, was 4.35 ± 0.03 (s.e.mean, 14 d.f.). In addition, this compound, at 10^{-3} M, produced no displacement of $E/[A]$ curves constructed to phenylephrine, histamine and KCl (each $n = 4$) (data not shown).

The influence of experimental design was then examined, comparing analysis of $E/[A]$ curves obtained by the cumulative and sequential curve protocols. Figure 4 illustrates data for two agonists, L-AMP-PCCl₂P and L-AMP-PCBr₂P obtained by the sequential curve design. The cumulative curve for each agonist is included for comparison. Evidently, the two curves for the latter compound are superimposed, whereas for the former agonist the sequential curve is significantly larger and rightward-shifted compared to the corresponding cumulative curve (see Table 1 for parameter estimates). Also shown are data on the reference agonist, D-AMP-CPP, which, like the dibromo- analogue, exhibited no differences. The two halogenated compounds were analysed by the comparative method. Minimal differences in affinity and efficacy estimates were computed for the dibromo- analogue but significant differences were found for the dichloro- compound (see Table 2). It was notable from the chart tracings that the contractile effects of the L-AMP-PCCl₂P were subject to more pronounced fade than those of L-AMP-PCBr₂P and D-AMP-CPP although this was not quantified. Finally, an experiment was performed in which L-AMP-PCBr₂P was interacted with D-AMP-CPP. This involved the generation of D-AMP-CPP $E/[A]$ curves in the presence of different concentrations of the analogue. Preliminary experiments showed that cumulative curves to D-AMP-CPP constructed in the presence of partial agonists suffered a depression of their maxima. Since one of the aims of this experiment was to estimate the affinity of L-AMP-PCBr₂P as an antagonist of D-AMP-CPP and curve depression would compromise this, it was decided to try the sequential design. The results obtained with this protocol are shown in Figure 5. L-AMP-PCBr₂P produced rightward displacements of D-AMP-CPP curves which, from visual inspection, were consistent with the interaction between a partial and a full agonist. Analysis of the $[A_{50}]$ s of the curves using equation (iv) gave an affinity (pK_B) estimate of 4.18 ± 0.07 (s.e. mean, 5 d.f.). This value was significantly (although only 2 fold) below the estimate made by the comparative method (see Table 2).

Discussion

The general objective of this study was to determine whether affinity and efficacy information could be generated for P_{2x}-purinoceptor agonists. To this end we have shown that certain dihalomethylene- and 2-methylthio- substituted ATP analogues demonstrate apparently partial agonist activity in the rabbit ear artery preparation, in principle allowing affinity and efficacy estimates to be obtained by the comparative method (Barlow *et al.*, 1967). However, for the results of such an analysis to be accepted it must be shown that the agonists in question are genuinely partial, that their reduced maximal effects are not the consequence of other pharmacological properties and that their effects are mediated by the same receptor. A number of pieces of evidence support this. Firstly, the effects of all the agonists were obliterated in the presence of a maximum dose of D-AMP-CPP, confirming that they were mediated by the P_{2x}-purinoceptor (this procedure has been shown not to affect non-P_{2x}-contractile agents, O'Connor *et al.*, 1990). Secondly, no relaxation responses were observed in the presence or

absence of this 'occupancy/desensitization' manoeuvre which could have opposed the constrictor effects of the agonists. Indeed previous reports have shown that the rabbit ear artery lacks relaxant P_{2Y}-purinoceptors (Burnstock & Kennedy, 1985; O'Connor *et al.*, 1990) and the use of 8-SPT in the present study ruled out possible interactions with P₁-purinoceptors. Thirdly, the ability of L-AMP-PCBr₂P (an apparent partial agonist) and its 2-methylthio analogue (a 'silent' compound) to displace E/[A] curves to D-AMP-CPP was consistent with expected actions of a partial agonist and a competitive antagonist respectively, these data confirming mutual interactions of the three compounds with the same receptor and indicating that the two dihalo- analogues had genuinely low efficacy whilst retaining affinity.

On this basis the analogues demonstrating reduced maximal effects were justifiably subjected to affinity and efficacy analysis. A factor which still remained, however, was the possible influence of experimental design. A common feature of all the agonists examined was the propensity of their contractile effects to fade with time. A possible consequence of the cumulative design is accumulation of error due to the progressive superimposition of transient response-time measurements as curves are constructed. This in turn could result in poor E/[A] curve definition and errors in affinity and efficacy estimation. Two partial agonists were studied using the sequential curve protocol which, in principle, eliminates this kind of error. In fact the results were different for the two compounds, the sequential and cumulative curves being superimposed for L-AMP-PCBr₂P but distinct for L-AMP-PCCl₂P. For D-AMP-CPP, the full agonist, the curves were superimposed. By inspection of the chart recordings, the discrepancy for the dichloro- analogue appeared to correlate with the more pronounced fade that this agonist exhibited compared to the others. However, a large number of compounds would need to be analysed in order to establish the generality or otherwise of such differences. The resulting affinity and efficacy estimates showed minimal changes in both for the dibromo- analogue but significant changes for the dichloro-compound. For example, the pK_A estimates showed an increase of 0.14 in one case and a decrease of 0.40 in the other. On the basis of these relatively small differences it would seem premature to discount values obtained by the cumulative design, but since the possibility remains that fade may affect response definition it would be cautious to recommend that the sequential protocol, although practically less convenient, is used at least to check results obtained by the cumulative design.

Finally, comparable affinity estimates were obtained for L-AMP-PCBr₂P when analysed as an agonist, by the comparative method, and as an antagonist, by Schild analysis. Although the estimates (4.49 and 4.18 respectively) were significantly different, the separation was only 2 fold. With some caution, therefore, we conclude that the estimates are mutually supportive.

Accepting the validity of the affinity and efficacy estimates shown in Table 2, a number of structure-activity features emerge. Comparing pairs of analogues between the D- and L-series it is evident that D-analogues are generally of higher efficacy than L-analogues. Only D-AMP-PCBr₂P showed overt signs of partial agonist behaviour in the absence of 2-methylthio- substitution, whereas all the L-dihalo-methylene- compounds exhibited low efficacy.

Efficacy clearly varied amongst the analogues. For the L-series the order was methylene > difluoromethylene > dichloromethylene > dibromomethylene (the un-halogenated compound being a full agonist). In the D-series, although the difluoro- and dichloro- analogues were full agonists, the dibromo- compound exhibited reduced efficacy. The effect of the 2-methylthio substituent in both series was a further fall in efficacy, converting the D-dichloro- analogue from a full into a partial agonist, and the L-dibromo- analogue from a partial agonist into a 'silent', competitive antagonist. Therefore, in both series of compounds halogenation and 2-

methylthio substitution conferred independent reductions in efficacy, in the former case the extent of reduction being proportional to the atomic weight of the halogen.

Regarding affinity, the effects of substituents were not so dramatic. For example, in the L-series, the difluoro- and dichloro- analogues exhibited indistinguishable affinities, and the dibromo- analogue had a 3 fold lower value. The effect of 2-methylthio incorporation was also marginal, causing less than a 2 fold drop in affinity between the unsubstituted agonist dibromo- analogue (pK_A = 4.49) and the substituted antagonist compound (pK_B = 4.35).

Since most of the compounds in the D- series behaved as full agonists it was not possible to compare affinity estimates amongst different halogenated analogues. However, some general comparisons between the D- and the L- series can still be made. It is evident from the computed p[A₅₀] values that compounds in the D- series exhibited lower potency than equivalent members of the L- series. For example, D-AMP-PCCl₂P was a full agonist with a p[A₅₀] of 4.32, whereas L-AMP-PCCl₂P was a partial agonist with a p[A₅₀] of 5.04. Moreover, the pK_A (affinity) estimate for the latter compound was 4.98, a value which is clearly to the more potent side of the p[A₅₀] for the D- analogue. In general, it is theoretically impossible for the E/[A] curve for a full agonist to lie well to the right (on the log₁₀[A] scale) of the pK_A. This can be shown by the operational model equation which defines the relationship between [A₅₀] and K_A (Black & Leff, 1983; Leff, 1987). Conceptually, this situation is unreasonable since it would allow an agonist to produce a threshold effect after saturating the receptors and for two indistinguishable levels of occupancy to produce different pharmacological effects. Therefore, for compounds such as D-AMP-PCP, D-AMP-PCF₂P and D-AMP-PCCl₂P, which behaved as full agonists, it can be deduced that their pK_A values are substantially lower than their computed p[A₅₀] values, meaning that they are likely to be less than 4.0 on the log₁₀ scale. These are all an order of magnitude lower than the estimates for the corresponding L-analogues. In fact, in the one case where it was possible to compare pK_A estimates between the two series, D-AMP-PCBr₂P (3.49) with L-AMP-PCBr₂P (4.49), the difference was consistent with this reasoning.

It is important to recognise that the provision of reliable affinity information, as with potency measurements, presumes that the possible influence of ectonucleotidase activity is absent. It has been reported that the dihalomethylene analogues used in the present study (with the exception of the dibromo compounds) are resistant to metabolism, showing no degradation over a period during which ATP was completely dephosphorylated (Cusack *et al.*, 1987). The biochemical experiments which provided those results do not strictly represent the events occurring in the biophase where the receptors and nucleotidase enzymes may be located. Nor were they performed in the tissue which was the subject of the present study. However, with these limitations, the stability data suggest that if errors can occur they are likely to be minimal with these analogues. Definitive experiments must await the availability of a nucleotidase inhibitor for routine pharmacological use.

On the basis of these results it would appear that, predominantly, D-analogues owe their potency to efficacy whereas the L-analogues owe their potency to affinity. For example, D-AMP-PCP and L-AMP-PCBr₂P had virtually identical potencies (p[A₅₀]s of 4.36 and 4.35, respectively), the first resulting from a combination of low affinity and high efficacy, the latter from a combination of high affinity and low efficacy. This serves to emphasise one of the important reasons for estimating affinity and efficacy for agonists, rather than relying on potency measurements alone. Using the potency scale, compounds of the same [pA₅₀] would be regarded as having the same activity despite, as the example shows, the possibility that the compounds are different in the relative contributions of the two underlying properties which determine potency. Since, in addition, these two properties

can depend quite differently on chemical structure, the provision of affinity and efficacy information is obviously fundamental to medicinal chemistry on agonists. For example, in the rational design of molecules to probe P_{2x} -purinoceptors, the present data would suggest that D-analogues, having higher efficacies, are better leads to agonists, whereas L-analogues, having lower efficacies, are better leads to antagonists.

In the light of the present analysis it is interesting to re-evaluate the data produced by Cusack *et al.* (1987) which, as mentioned in the Introduction, contained evidence for partial agonist action using the guinea-pig urinary bladder preparation. In particular, L-AMP- $PCCl_2P$ and 2-methylthio-D-AMP- $PCCl_2P$ exhibited lower maximal effects than the other agonists studied (unfortunately, those authors did not report data on dibromo-substituted compounds). The present study shows both these analogues to be partial agonists in the rabbit ear artery. Although from inspection there appear to be some problems of $E/[A]$ curve shape definition in the bladder preparation, meaning that detailed analysis of the data is not possible, the qualitative consistency in the behaviour of the two agonists between the tissues is noteworthy and points to the way in which affinity and efficacy

estimation may be of value in the classification of P_{2x} -purinoceptors. Comparison of 'fingerprints' of affinity and efficacy information for series of agonists has been useful in the classification of receptors in other areas, establishing the similarity or difference between receptors in different tissues (Leff & Dougall, 1992). The power of this approach as compared with the use of potency orders is that each agonist provides two pieces of information for the purposes of receptor classification rather than only one. Also, as implied by preceding arguments, the fact that potency depends on both affinity and efficacy means that the same potency order could emerge from different combinations of affinity and efficacy, and receptors which are in fact different could be wrongly classified as the same. Estimation of affinities and efficacies avoids such potential pitfalls. In the present case, for example, the same 'fingerprint' of affinity and efficacy estimates in the ear rabbit ear artery and guinea-pig bladder would provide strong quantitative support to the belief that these preparations contain the same receptor type. While selective antagonists remain scarce in this field of receptor research, quantitative data on agonist action could make an important contribution to receptor classification.

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