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Pharmacological analysis of ecto-ATPase inhibition: evidence for combined enzyme inhibition and receptor antagonism in P_{2X}-purinoceptor ligands

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1 Previous studies have shown that suramin and FPL 66301 are competitive antagonists at the P_{2X}-purinoceptor in the rabbit ear artery. Those studies employed α,β-methylene ATP, a poorly hydrolysable ATP analogue, as the agonist. In this study these compounds have been tested using ATP as the agonist.

2 Suramin, in the concentration range 30–1000 μM, *potentiated* the contractile effects of ATP, producing a 3-fold leftward shift of the ATP E/[A] curves. FPL 66301, in the concentration range 100–1000 μM, produced a significant but small (≈3-fold) rightward shift of the ATP curves. These results are in marked contrast with previous studies using α,β-methylene ATP in which 30-fold rightward shifts were achieved using the same concentration ranges of suramin and FPL 66301.

3 Suramin and FPL 66301 were tested as ecto-ATPase inhibitors in a human blood cell assay. Suramin inhibited the enzyme with a pIC_{50} of 4.3, FPL 66301 with a pIC_{50} of 3.3.

4 The pharmacological data were analysed using a theoretical model describing the action of a compound with dual enzyme inhibitory and receptor antagonistic properties on the effects of an agonist susceptible to enzymatic degradation. The model was found to fit the data well using the known pK_B estimates for suramin and FPL 66301 and similar relative (but not absolute) pK_I estimates to those obtained for the compounds in the enzyme assay.

5 From this analysis it was concluded that the limited shifts of ATP E/[A] curves produced by suramin and FPL 66301 were the result of 'self-cancellation' of the potentiating (enzyme inhibitory) and rightward-shifting (receptor antagonistic) properties.

6 The analysis also indicated that the presence of ecto-ATPase activity in the rabbit ear artery preparation has a marked effect on the apparent potency of ATP. The experimental $p[A_{50}]$ was 3.4, whereas the 'true' value, that is the value which would be obtained in the absence of ecto-ATPase activity, was 6.0, some 400-fold higher.

7 Two conclusions are drawn from this study. Firstly, caution must be exercised in the use of suramin and FPL 66301 as tools for receptor classification. Absence of overt antagonism by these compounds when metabolically unstable agonists are used could lead to erroneous claims for receptor subtypes. Secondly, the agonist potency order currently used to designate P_{2X}- purinoceptors may require modification.

Keywords: P_{2X}-purinoceptors; ATP; ecto-ATPase; suramin; FPL 66301; agonist potency orders; receptor classification

Introduction

A well-recognized issue in the classification of P_2-purinoceptors is the susceptibility of the natural agonists and some synthetic agonist analogues to enzymatic dephosphorylation in tissues used in pharmacological experiments. Previous studies have demonstrated the metabolic instability of agonists and determined their structure–activity dependence (Welford *et al.*, 1986; 1987). The potential pharmacological complications produced by such processes are generally 2-fold: to convert the applied agonist to other chemical species which may be active as agonists or antagonists at the receptor under study or at other receptors in the tissue preparation and to reduce the concentration of the agonist available to interact with the receptors under study so that the applied agonist concentration does not reflect the active one. Although there are a number of reports describing effects at purinoceptors of the former kind (Maguire & Satchell, 1981), evidence for the pharmacological impact of the latter is limited (Welford *et al.*, 1987). However, studies conducted in other receptor systems, for example adrenoceptors and muscarinic receptors, show that problems of this kind manifest as incorrect determination of agonist potency orders (Kenakin, 1980) and cause difficulties in the quantitative analysis of antagonist effects (Kenakin & Beek, 1985).

A particular problem that has received attention elsewhere is the pharmacological analysis of receptor antagonists which possess additional properties that confound the expression of simple competitive antagonism (Black *et al.*, 1986). When the additional property is inhibition of an enzyme which acts to degrade the experimental agonist, potentiation of agonist concentration effect (E/[A]) curves can occur (Kenakin & Beek, 1985). Combination of this property with receptor antagonism can result in 'self-cancellation' of E/[A] curve displacements in which neither the enzyme inhibitory nor receptor antagonistic components are fully expressed. In this study we address a problem of this kind affecting the pharmacological analysis of P_{2X}-purinoceptor antagonists.

In previous studies carried out in this laboratory, using the rabbit isolated ear artery preparation, suramin was shown to demonstrate simple competitive antagonism of P_{2X}-purinoceptors (Leff *et al.*, 1990). Significantly, those experiments were conducted using a α,β-methylene ATP and β,γ-methylene L-ATP, agonists which have been reported to be relatively stable to hydrolysis by ectonucleotidase (Welford *et al.*, 1986). A synthetic analogue of ATP, FPL 66301 (β,γ-methylenedibromo-2-methylthio-L-ATP) also demonstrates competitive antagonism under these conditions (Leff *et al.*, 1993). In the present paper we report further studies on these antagonists which demonstrate the presence of ecto-

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ATPase-inhibitory properties in each of them and which quantify the extent to which these are expressed pharmacologically. The results of this study are considered in relation to the impact of ecto-ATPase activity on the pharmacological analysis of purinoceptors and in relation to the utility of compounds such as suramin and FPL 66301 in purinoceptor classification.

Methods

Rabbit ear artery

Male New Zealand White rabbits weighing 2–3.5 kg were killed with an overdose of pentobarbitone sodium (Euthatal) via the marginal ear vein. The ears were removed and the central ear artery located at the base of the ears. A small section of the artery was cleared of connective tissue and an incision was made to allow the insertion of a nylon cannula (external diameter 0.75 mm). The cannula was lightly scored with a scalpel blade prior to insertion into the vessel, to remove the vessel endothelium. The artery, mounted on the cannula, was then dissected from the ear, cleared of connective tissue and cut into 5 mm rings. These were mounted horizontally over tungsten wire hooks (0.25 mm diameter) in 10 ml organ baths. The lower hook was attached to a stationary support in the organ bath and the upper hook was connected to an Ormed transducer for recording changes in isometric force. Tissues were bathed in Krebs' buffer (pH 7.4) of the following composition (mM): NaCl 117.56; $NaH_2PO_4.2H_2O$ 0.89; $NaHCO_3$ 25.0; $MgSO_4.7H_2O$ 1.18; glucose 11.1; KCl 5.36 and $CaCl_2$ 2.55. The tissues were maintained at 37°C and gassed continuously with 95% O_2/5% CO_2. The Krebs' solution also contained indomethacin (2.8 μM) to prevent the formation of cyclo-oxygenase products and 8-sulphophenyltheophylline (300 μM) to exclude possible influence of P_1-purinoceptor activation.

The tissues were subjected to an initial force of 1.0 g and then allowed to equilibrate for a minimum of 1 h, during which time the force was adjusted to achieve a final value of 0.5–1.0 g. The tissues were then washed by exchange of the bath solution.

Tissue viability was assessed by treatment with KCl (80 mM). Only those tissues producing an increase in force of 0.5 g or more were included in the study. The functional state of the endothelium was tested by subsequent addition of acetylcholine (1 μM). In these studies, the vasorelaxation to acetylcholine was invariably less than 15% of the KCl-induced contraction. Tissues were then washed and allowed to re-equilibrate for 45 min.

Experimental protocols for the rabbit ear artery

A paired curve design was adopted in which all tissues were initially treated with cumulative additions of ATP at 0.5 log unit intervals. Following washout and re-equilibration, FPL 66301 or suramin (0–1000 μM) was applied. Subsequently, a second cumulative curve was constructed using ATP. In the case of FPL 66301, all concentrations were incubated for 45 min between paired curves. In the case of suramin, incubation times were chosen based on previous kinetic studies (Leff *et al.*, 1990) as follows: 30 μM (220 min); 100 μM (86 min); 300 μM (31 min); 1 mM (10 min). The interval between paired ATP curves was 70 min in the case of FPL 66301 and 240 min in the case of suramin. Contractile effects were expressed as percentages of the first curve maximum.

Ecto-ATPase assay

Human blood was obtained by venepuncture from healthy volunteers, heparinized then centrifuged (2000 g, 10 min). Plasma was removed, care being taken not to disturb the buffy coat. The remaining cells were washed twice with buffer of composition (mM) Tris 20.0, glucose 5.0, NaCl 140.0, KCl 5.0, adjusted with HCl to pH 7.4, 20°C. The cell suspensions were then diluted 7-fold with buffer.

[γ^{32}P]ATP assays were carried out at 37°C in a shaking water bath. The total incubation volume was 200 μl, of which the added volume of cells was 75 μl. The incubation medium contained, in addition to buffer, NaN_3 (5.0 mM) and $CaCl_2$ (0.5 mM). The substrate concentration of ATP was 10 μM containing 80,000–90,000 d.p.m. [γ^{32}P]ATP. This concentration was near the K_M determined in previous studies (Beukers *et al.*, 1993). Suramin or FPL 66301 was added to achieve final concentrations in the range 0.3–1000 μM. Their inhibitory effects were measured as percentages of the degradation achieved in the presence of 10 μM ATP alone, over a period of 30 min.

Incubations were terminated by addition of activated charcoal (400 μl of 40 mg ml^{-1} charcoal in 0.1 N HCl), then centrifugation (6500 G, 2 min). Supernatant samples of 350 μl were homogenized with 4 ml of emulsifier (Emulsifier Safe, Packard) and radioactivity was counted in an LKB-Wallace 1214 Rackbeta Excel Spectrometer. Controls, without cells, were processed identically.

Drugs and materials

The following drugs were used: acetylcholine bromide, adenosine 5'-triphosphate, disodium salt (ATP) (Sigma Chemical Co., Poole, U.K.); FPL 66301, tetrasodium salt (synthesized by P.A. Cage, Medicinal Chemistry Department, Fisons); 8(*p*-sulphophenyl)theophylline (8-SPT) (Research Biochemicals, St. Albans, U.K.); suramin (a gift from Bayer, U.K.); indomethacin (Sigma); activated charcoal, particle size 4–7 μM (Serva, Heidelberg, Germany); ATP (for ecto-ATPase studies) (Aldrich Chemie, Steinheim, Germany); [γ^{32}P]ATP (Amersham, U.K.).

For rabbit ear artery experiments, ATP, FPL 66301 and 8-SPT were dissolved in distilled water, suramin in Kreb's solution. Indomethacin was prepared as a 28 mM solution in Na_2CO_3 (0.35 M) and subsequently diluted in Krebs' solution.

Analysis of E/[A] curve data

Agonist concentration effect (E/[A]) data were fitted to the logistic function:

$$E = \frac{\alpha[A]^m}{[A_{50}]^m + [A]^m} \quad (1)$$

in which α, $[A_{50}]$ and m are respectively the asymptote, location and slope parameters. $[A_{50}]$ values were assumed to be log-normally distributed and are presented as their negative logarithms, $p[A_{50}]$.

Each E/[A] curve in an experimental pair was fitted and the effects of suramin and FPL 66301 were tested by analysing paired changes in the three parameters, i.e. $\Delta\alpha$, $\Delta p[A_{50}]$ and Δm. Preliminary analysis showed that variances of Δ values were not consistent among treatment groups. Therefore, non-parametric tests were adopted: the Kruskal–Wallis test for analysing group differences and the Mann–Whitney *U*-test for individual comparisons.

Analysis of ecto-ATPase inhibition

Data for the inhibitory effects of suramin and FPL 66301 on ATP degradation were fitted to equation (1). The concentrations which produced 50% inhibition of degradation (IC_{50}) were read from the fitted lines and quoted as negative logarithms (pIC_{50}). Since the concentration of ATP used was near its K_M (Beukers *et al.*, 1993), IC_{50} values were assumed to approximate to K_I values for the purposes of theoretical modelling.

Theoretical analysis of combined ecto-ATPase inhibition and receptor antagonism

The theoretical model of Furchgott (1972) was employed. In this model, agonist is applied to the organ bath at concentration $[A_o]$. The access of the agonist to biophase containing the receptors is considered to be diffusion rate limited, and it is catabolized by enzymatic activity within the biophase. Hence the concentration of agonist presented to its receptor within the biophase, $[A_i]$, may be substantially lower than $[A_o]$. The relationship between $[A_o]$ and $[A_i]$ can be determined from the following equations. The rate of entry of agonist is described by:

$$d[A_i]/dt = k_l([A_o] - [A_i]) \quad (2)$$

in which k_l is the diffusion constant for the agonist.

The degradation of agonist is assumed to follow Michaelis–Menten kinetics:

$$d[A_i]/dt = V_{max}[A_i]/(K_M + [A_i]) \quad (3)$$

At steady-state the rates of agonist entry and degradation are balanced. Under this condition, the right-hand sides of equations (2) and (3) can be equated, and the following relationship can be derived:

$$k_l[A_i]^2 + (V_{max} + k_l K_M - k_l[A_o])[A_i] - k_l K_M[A_o] = 0 \quad (4)$$

This is a quadratic equation in $[A_i]$. In theoretical simulations, values of k_l, V_{max} and K_M are chosen, then $[A_i]$, the active, biophase agonist concentration can be calculated in terms of $[A_o]$, the applied concentration, by numerical solution of equation (4).

To generate theoretical E/[A] curves, values of $[A_i]$ were substituted into a simple rectangular hyperbolic function, equivalent to equation (1) with m set to unity, thereby producing curves plotted against the applied agonist concentration, $[A_o]$.

To simulate the effect of enzyme inhibition, K_m was multiplied by the factor $(1 + [X]/K_I)$, in which X is a competitive inhibitor with a dissociation constant K_I. To simulate receptor antagonism, $[A_{50}]$ was multiplied by the factor $(1 + [X]/K_B)$, where K_B is the receptor dissociation constant of X. To simulate the combined properties, both factors were varied simultaneously.

Results

Figure 1 shows the effect of suramin on ATP E/[A] curves in the rabbit ear artery. For ease of display, the average second curve data are shown. Statistical analysis was carried out using the paired curve data (Table 1). Kruskal–Wallis analysis indicated that, as a group, the $\Delta p[A_{50}]$ estimates obtained in the presence of suramin were significantly greater than control values ($p = 0.022$), that is suramin had potentiated the effects of ATP. The average control $p[A_{50}]$ was 3.39 ± 0.13 (SE, $n = 11$) and suramin produced a 3-fold leftward shift of the curves. The Mann–Whitney U-test indicated that the leftward shifts obtained at 100 μM and 1 mM suramin were individually significant ($0.01 < P < 0.05$). No significant effect of suramin on the values of α or m of the E/[A] curves was detected.

Figure 2 shows the effect of FPL 66301 on ATP E/[A] curves. Again average second curves are displayed. Kruskal–Wallis analysis of the paired data indicated an overall significant rightward shift ($P = 0.031$; Table 1). Mann–Whitney-U analysis indicated that the effects of 100 μM and 300 μM FPL 66301 were individually significant. No significant effects on curve asymptotes or slopes were detected.

Figure 3 shows the effects of suramin and FPL 66301 on [γ^{32}P]ATP degradation by human blood cells. Clear concentration-dependent inhibition of degradation was obtained in both cases, with suramin evidently being more potent ($pIC_{50} = 4.33 \pm 0.03$, SE, $n = 3$) than FPL 66301 ($pIC_{50} = 3.33 \pm 0.06$, SE, $n = 3$).

Figure 4 illustrates theoretical analysis of the effects of suramin on ATP E/[A] curves using the model for combined receptor antagonism and ecto-ATPase inhibition. For clarity the effects of only the highest concentration of suramin (1 mM) have been simulated in the figure. Figure 4a shows the experimental data obtained using ATP as agonist, Figure 4b the computer simulation and Figure 4c experimental data previously obtained using α,β-methylene ATP as agonist (replotted from Leff *et al.*, 1990). The pK_B value (4.8) describing the receptor antagonism component used in the simulation was that found experimentally when α,β-methylene ATP was used (Leff *et al.*, 1990). This is indicated in Figure 4 by the quantitative correspondence between the rightward shift of α,β-methylene ATP curves obtained experimentally (Figure 4c) and the simulated rightward shift corresponding to the

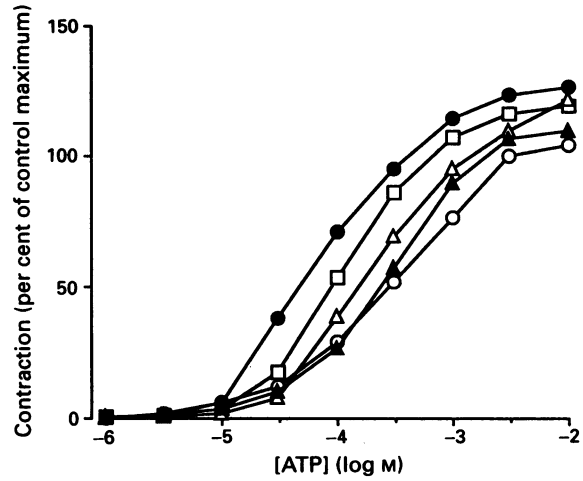

Figure 1 Effect of suramin on ATP E/[A] curves in the rabbit ear artery. Cumulative E/[A] curves to ATP were constructed in the presence of the following concentrations of suramin (μM): zero (○); 30 (▲); 100 (●); 300 (△); 1000 (□). A paired curve protocol was used (see Methods) but, for ease of display, the graph shows the average second-curve data, measured as percentages of the first-curve maximum ($n = 4$ or 5). The complete data together with statistical analyses are shown in Table 1.

Table 1 Statistical analysis of the effects of suramin and FPL 66301 on ATP E/[A] curves. Analysis of paired differences in asymptote (α), slope (m) and location ($p[A_{50}]$) of curves obtained in the absence and presence of the two compounds

	Δα		Δm		Δp[A_{50}]		
Antagonist	*Mean*	*SE*	*Mean*	*SE*	*Mean*	*SE*	n
Suramin (μM)							
0	2.9	12.0	0.08	0.05	−0.04	0.15	5
30	6.3	4.6	0.19	0.18	−0.21	0.05	5
100	17.8	11.6	0.28	0.10	−0.67*	0.13	4
300	22.7	10.5	0.21	0.36	−0.43	0.28	4
1000	11.6	8.5	0.45	0.18	−0.45*	0.11	5
FPL 66301 (μM)							
0	−2.1	4.2	−0.01	0.05	−0.04	0.07	6
100	−1.5	5.9	0.01	0.04	0.41*	0.10	5
300	3.6	7.2	0.03	0.15	0.38*	0.10	5
1000	3.3	7.1	−0.15	0.13	0.37	0.42	5

*Indicates difference between treatment group and control, ($P < 0.05$) by Mann–Whitney U-test.

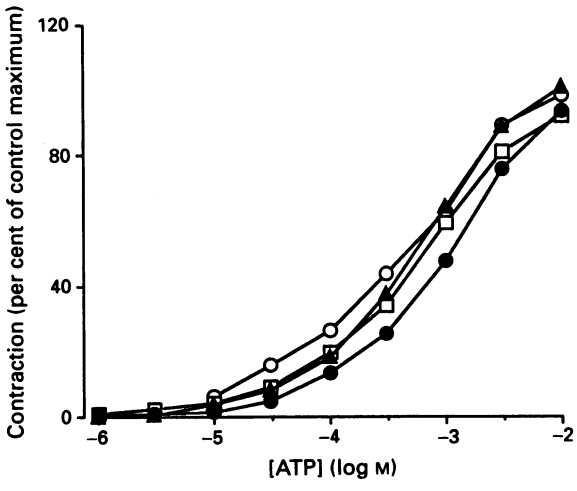

Figure 2 Effect of FPL 66301 on ATP E/[A] curves in the rabbit ear artery. Cumulative E/[A] curves to ATP were constructed in the presence of the following concentrations of FPL 66301 (μM): zero (○); 100 (□); 300 (●); 1000 (▲). A paired curve protocol was used (see Methods) but, for ease of display, the graph shows average second-curve data, measured as percentages of the first-curve maximum ($n = 5$ or 6). The full data together with statistical analyses are shown in Table 1.

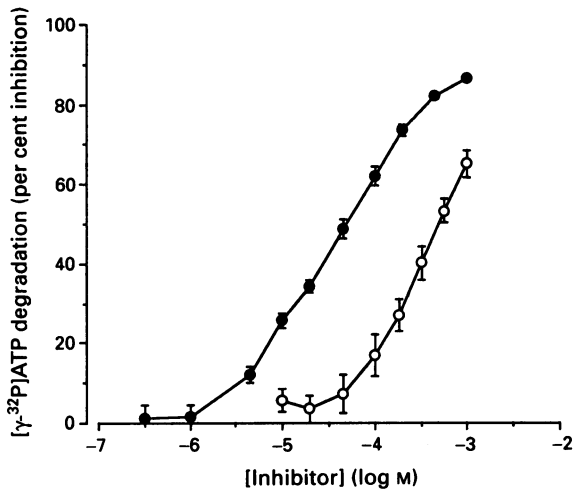

Figure 3 Effect of suramin and FPL 66301 on ATP degradation in human blood cells. Inhibition curves are shown for suramin (●) and FPL 66301 (○) (both $n = 3$) on [γ^{32}P]ATP dephosphorylation by ecto-ATPase in human blood cells. The estimated pIC_{50} values were 4.33 for suramin and 3.33 for FPL 66301.

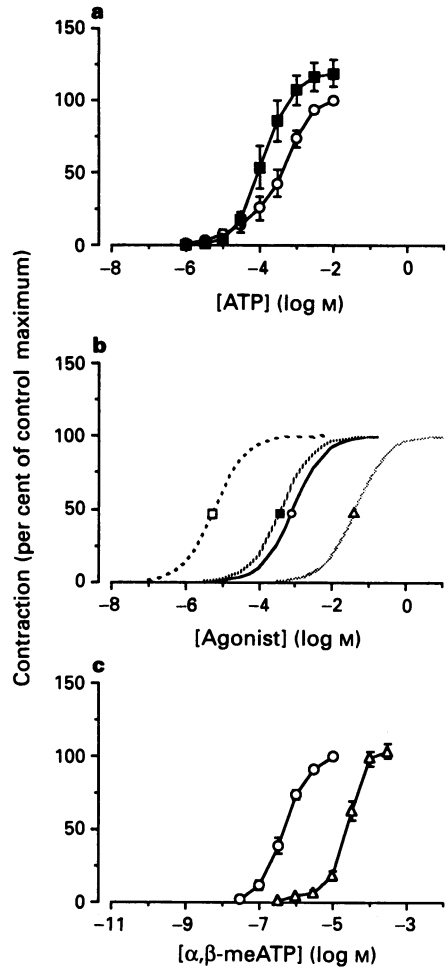

Figure 4 Theoretical analysis of combined ecto-ATPase inhibition and P_{2X}-purinoceptor antagonism by suramin. (a) ATP E/[A] curves obtained in the rabbit ear artery in the absence (○) and presence (■) of suramin (1 mM), redrawn from Figure 1. (b) A simulation of a theoretical model describing the effect of a compound with dual enzyme and receptor inhibitory properties on an agonist susceptible to enzymic degradation (see Methods section for details). The dual inhibitor was assumed to have a pK_B (receptor antagonism) of 4.8 and a pK_I (enzyme inhibition) of 5.2 and the simulation shows its predicted effects at 1 mM. Theoretical curves were generated under the conditions: control (○); receptor antagonism alone (△); enzyme inhibition alone (□); combined effects (■). (c) α,β-Methylene ATP curves obtained in the rabbit ear artery in the absence (○) and presence (△) of suramin (1 mM) (redrawn from Leff *et al.*, 1990).

receptor antagonism by suramin Figure 4b. Values of the other model parameters, including the pK_I value, were chosen to obtain reasonable correspondence between the theoretical curve simulating the combined effects of ATPase inhibition and receptor antagonism and the expermental data in Figure 4a.

Figure 5 shows a similar theoretical analysis of the effects of FPL 66301 on ATP. Again, the pK_B value (4.4) used in the simulation was that previously obtained for FPL 66301 as antagonist of α,β-methylene ATP (Leff *et al.*, 1993). The pK_I value required to simulate the experimental data was lower than in the case of suramin, but the remaining parameter values were set to the same values as in Figure 4.

Table 2 summarizes the parameter values used to generate the computer simulations shown in Figures 4 and 5. Of note is the $p[A_{50}]$ value for ATP used in the simulations. This value is the theoretical position of the ATP E/[A] curve that would be obtained in the absence of ecto-ATPase activity. It contrasts markedly with the experimental control $p[A_{50}]$ (3.39). Also note that the values of pK_I are higher than those found in the blood cell assay, though they maintain a similar ratio of K_I values.

Discussion

The present study has investigated the effects of suramin and FPL 66301 on ATP E/[A] curves in the rabbit isolated ear artery. The results obtained are in marked contrast with those previously reported from this laboratory when the two compounds were tested, in the same preparation and under the same experimental conditions, but using α,β-methylene ATP or β,γ-methylene L-ATP as agonists (Leff *et al.*, 1990; 1993). In those studies, both suramin and FPL 66301 were shown to behave as competitive antagonists of the P_{2X}-purinoceptors in this preparation, producing parallel rightward shifts of E/[A] curves which accorded with a Schild plot slope of unity. In the case of suramin a pK_B estimate of 4.8

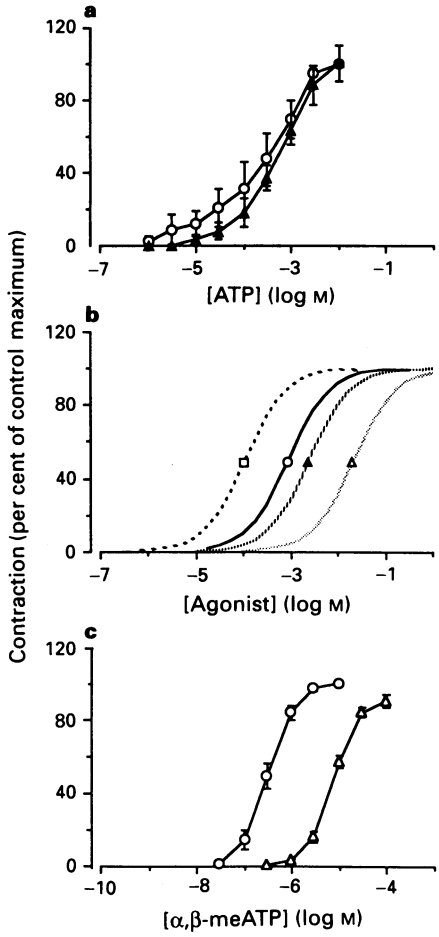

Figure 5 Theoretical analysis of combined ecto-ATPase inhibition and P_{2X}-purinoceptor antagonism by FPL 66301. (a) ATP E/[A] curves obtained in the rabbit ear artery in the absence (○) and presence (▲) of FPL 66301 (1 mM), redrawn from Figure 2. (b) A simulation of a theoretical model describing the effect of a compound with dual enzyme and receptor inhibitory properties on an agonist susceptible to enzymic degradation (see Methods section for details). The dual inhibitor was assumed to have a pK_B (receptor antagonism) of 4.4 and a pK_I (enzyme inhibition) of 3.9 and the simulation shows its predicted effects at 1 mM. Theoretical curves were generated under the conditions: control (○); receptor antagonism alone (△); enzyme inhibition alone (□); combined effects (▲). (c) α,β-Methylene ATP curves obtained in the rabbit ear artery in the absence (○) and presence (△) of suramin (1 mM) (redrawn from Leff *et al.*, 1993).

was reported and in the case of FPL 66301 an estimate of 4.4. In the present study, neither compound exhibited effects consistent with their P_{2X}-purinoceptor antagonist properties: suramin potentiated the effects of ATP rather than antagonized them; FPL 66301 produced only limited (no more than 3-fold) rightward shifts. In both cases the concentration range employed had produced rightward shifts of 30-fold in the previous study.

It was hypothesized that these differences were due to the susceptibility of ATP but not α,β-methylene ATP or β,γ-methylene L-ATP to metabolic degradation by ecto-ATPase in the tissue and that suramin and FPL 66301 could act as inhibitors of this enzyme as well as receptor antagonists. This combination of properties could, in theory, lead to a 'self-cancellation' of ATP E/[A] curve displacements in which the effects of receptor antagonism are offset by the potentiating influence of inhibiting the degradation of ATP. In contrast, when a metabolically stable agonist is used, only the receptor antagonism component would be apparent. In support of this hypothesis, *in vitro* studies on the metabolic degradation of

Table 2 Model parameter values used in theoretical simulations

Parameter	*Value*
Diffusion constant for ATP, k_1	0.1 min^{-1}
Ecto-ATPase, K_M	3×10^{-3} M
Ecto-ATPase, V_{max}	0.3 M min^{-1}
Location of ATP E/[A] curve, $p[A_{50}]$	6.0 (−log M)
Dissociation constant for ecto-ATPase inhibition, pK_I	
Suramin	5.2 (−log M)
FPL 66301	3.9 (−log M)
Dissociation constant for P_{2X}-purinoceptor antagonism*, pK_B	
Suramin	4.8 (−log M)
FPL 66301	4.4 (−log M)

*Values obtained experimentally with α,β-methylene ATP as agonist (Leff *et al.*, 1990; 1993).

nucleotides have shown triphosphates such as ATP to be susceptible to rapid hydrolysis. In contrast, phosphonates containing α,β- or β,γ-methylene substituents have been shown to be relatively stable. Thus, in a guinea pig taenia coli preparation ATP was dephosphorylated with a half-life of about 20 min, whereas α,β-methylene ATP degradation was less than 10% in 20 min and β,γ-methylene L-ATP appeared completely resistant to hydrolysis over this period (Welford *et al.*, 1986). Suramin has been reported to be an inhibitor, albeit at high concentrations, of ATP degradation in the guinea pig bladder (Hourani & Chown, 1989). Furthermore, in the present study, using the human blood assay, suramin and FPL 66301 have been clearly demonstrated to possess ecto-ATPase inhibitory properties, characterized by IC_{50} values in the concentration range relevant to the pharmacological studies.

However, as explained in the Introduction, another way in which the metabolism of agonists by tissues can lead to complications in pharmacological analysis is the production of metabolites which are active as agonists in their own right. In the present system, it was necessary to consider the possible conversion of ATP to ADP, AMP and adenosine. In particular, accumulation of adenosine may have resulted in the activation of P_1-purinoceptors mediating vasorelaxation, which would have opposed the P_{2X}-purinoceptor-mediated contractile effects of ATP. Inhibition of adenosine formation could have then led to potentiation of ATP effects. This explanation could be ruled out in this study because 8-SPT was present in all experiments at a concentration 30 to 100-fold higher than its dissociation constant for P_1-purinoceptors. Another potential complication may have been the local generation of ADP from ATP and its effects at P_{2X}-purinoceptors in the tissue. However, for this factor to explain the potentiation of ATP effects by inhibition of ecto-ATPase, ADP would had to have been acting as an antagonist of ATP under control conditions. In fact, ADP is an agonist at the receptors concerned, so we do not consider this to be a viable explanation. The possible effects of metabolites of ATP active at P_{2Y}-purinoceptors could also be excluded since these receptors are absent in this preparation (O'Connor *et al.*, 1990).

On the basis that the original hypothesis was plausible it was then necessary to assess whether it could account quantitatively for the E/[A] curve data. To do this a theoretical model proposed by Furchgott (1972) was applied. This model was originally developed to analyse the pharmacological influence of catecholamine metabolism and its inhibition on β-adrenoceptor agonist effects. Its application here showed it to be a valid description of the data, giving a quantitatively accurate fit to the E/[A] curves for ATP and α,β-methylene ATP obtained using both suramin and FPL 66301, as shown in Figures 4 and 5. It was considered, therefore, to be a

reasonable framework for assessing the influence of ecto-ATPase on the position of ATP E/[A] curves and for quantifying the effects of suramin and FPL 66301.

The choice of parameter values in the simulations requires some discussion. Firstly, the K_M value used was higher than that reported elsewhere for ATP dephosphorylation in human blood (Beukers *et al.*, 1993). This was considered necessary for the following reason. The E/[A] curves for ATP obtained in the rabbit ear artery in the presence and absence of suramin and FPL 66301 were evidently parallel. This indicated that, over the range of concentrations of ATP employed, the dephosphorylation mechanism was operating in a pseudo first-order manner, meaning in turn that the ATP concentrations used were less than the K_M. Had this not been the case, changes in slope of the E/[A] curves would have been expected (Furchgott, 1972). A value of K_M was chosen to ensure that this condition was fulfilled.

A key feature of the theoretical analysis was that it employed the known pK_B estimates for suramin and FPL 66301 as agonists of the P_{2X}-purinoceptors in the rabbit ear artery. These estimates fixed the degree of underlying shift associated with the receptor antagonism component of each compound when ATP was used as the agonist. On the basis that the experimental curves obtained in the presence of suramin and FPL 66301 represented the 'self-cancelled' position, the extent of underlying potentiation could be estimated. The magnitude of the potentiation determined the values of k_I and V_{max} used in the simulations because these are the model parameters which govern the difference between the ATP concentrations in the organ bath ($[A_o]$) and in the biophase ($[A_i]$) and, therefore, the impact of ecto-ATPase on the position of the ATP E/[A] curve. In the simulations, $[A_i]$ was some 1000-fold lower than $[A_o]$.

Regarding the pK_I values for suramin and FPL 66301 used in the simulations, these needed to be higher than the estimates made in the human blood assay. Bearing in mind the fact that the pharmacological and biochemical data were obtained in different tissues and under different conditions, this result is perhaps not surprising. Ideally, an ecto-ATPase assay using cells from the rabbit ear artery would have been employed but, to our knowledge, such an assay is not available. Another consideration here is that in the theoretical analysis suramin and FPL 66301 are treated as competitive enzyme inhibitors. This assumption may be questioned in the case of suramin (Hourani & Chown, 1989). However, as explained above, dephosphorylation of ATP in the ear artery appeared to operate in a pseudo first-order manner. Under this condition, the relationship between $[A_i]$ and $[A_o]$ is given by $[A_i] = (V_{max}K_M/k_I)[A_o]$, so that multiplying K_M by the factor $(1 + [X]/K_I)$, as for a competitive interaction, is technically identical to dividing V_{max} by the same factor, as for a non-competitive interaction. In this sense, the theoretical simulations and their ability to account for the experimental data are not dependent on the assumption that the inhibition of ecto-ATPase by either compound is truly competitive.

The pK_I estimates required to model the E/[A] curve displacements have similar relative values to those obtained in the ecto-ATPase assay. So there is at least qualitative accordance between the pharmacological and biochemical analyses. Moreover, from the point of view of pharmacological analysis, we would consider the relevant estimates to be the ones obtained in the tissue study since these reflect the extent of potentiation that the compounds are able to produce. In these respects therefore, the model parameter values used in simulating the data were deduced rather than chosen.

An important conclusion from this analysis is that, in order to allow for the extent of potentiation that was required to offset the antagonist effects, it was necessary to assume that the location of the ATP curve, in the absence of ecto-ATPase activity, would have been some 400-fold to the left of its experimental position. This is signified in the theoretical analysis by the $p[A_{50}]$ value of 6.0 (see Table 2), contrasted with the experimental value of 3.39. Attention has been drawn to the potential influence of ecto-ATPase on the location of E/[A] curves for ATP (and other metabolically unstable agonists) obtained in tissue preparations (for example, Burnstock & Kennedy, 1985) but, to our knowledge, the present study is the first to attempt to quantify this influence. This finding has some important implications for P_2-purinoceptor classification. The degree to which ecto-ATPase affects the position of the ATP E/[A] curve in this preparation means that the true potency of ATP is underestimated by over two orders of magnitude. This is particularly relevant in the context of agonist potency orders. In previous studies (O'Connor *et al.*, 1990) the following agonist potency order ($p[A_{50}]$ values in parentheses) was obtained: α,β-methylene ATP (6.47) > β,γ-methylene L-ATP (5.52) > β,γ-methylene ATP (4.37) ≥ 2-MeS-ATP (4.15) > ATP (3.14). This potency order was characteristic of that designated for P_{2X}-purinoceptors (Burnstock & Kennedy, 1985). However, if the true $p[A_{50}]$ value for ATP is taken as 6.0 then its position in the potency order changes markedly relative to the metabolically stable α,β- and β,γ-methylene-substituted compounds. It is also reasonable to suggest that the position of 2-MeS-ATP should change since it has been shown to be as susceptible as ATP to dephosphorylation by ectonucleotidases (Welford *et al.*, 1986). If its true potency is calculated on the same basis as that of ATP then there would be little difference between α,β-methylene ATP and 2-MeS-ATP. This implies that the agonist potency order currently held to characterize P_{2X}-purinoceptors may need to be re-evaluated.

It may be relevant to consider these conclusions in relation to radioligand binding studies on P_{2X}-purinoceptors. A notable feature of the results from such studies is the high apparent affinity values obtained for agonists compared with the potency values obtained in isolated tissues (e.g. Michel & Humphrey, 1993). It is possible that this difference may partly be explained by the absence of ecto-ATPase activity in the binding assays. However, it is to be noted that the difference also applies to poorly hydrolysable agonists such as α,β-methylene ATP and so additional explanations need to be sought.

Regarding suramin and FPL 66301, the present study indicates that caution should be adopted when using these compounds in receptor classification studies. The propensity for their receptor antagonistic and ecto-ATPase inhibitory properties to 'self-cancel' when metabolically unstable agonists are used is a basis for misinterpretation. For example, in the present system, the ability of each compound to antagonize the effects of α,β-methylene ATP in an apparently simple competitive manner, but their failure to displace ATP E/[A] curves could have led to the conclusion that ATP acted at a different receptor from the other agonists. This interpretation would clearly have been quite misleading and unhelpful in purinoceptor classification.

Obviously, experimental resolution of these issues would be greatly aided by the provision of a selective ecto-ATPase inhibitor. A compound of this kind has been discovered in our laboratory and studies are currently under way in which it is being used to elucidate problems of the kind described here.

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