Pharmacological and biochemical analysis of FPL 67156, a novel, selective inhibitor of ecto-ATPase

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1 FPL 67156 (6-N,N-diethyl- β , γ -dibromomethylene-D-ATP), is a newly synthesized analogue of ATP.

2 In a rabbit isolated tracheal epithelium preparation, measuring P_{2U} -purinoceptor-dependent chloride secretion, FPL 67156 was discovered to potentiate the responses to UTP but not those to ATP- γ -S. UTP agonist-concentration effect ($E/[A]$) curves were shifted to the left by 5-fold in the presence of 100 μ M FPL 67156. The differential effect of FPL 67156 on UTP and ATP- γ -S was hypothesized to be due to the greater susceptibility of UTP to enzymatic dephosphorylation and the ability of FPL 67156 to inhibit this process.

3 FPL 67156 was tested as an ecto-ATPase inhibitor in a human blood cell assay, measuring [γ - 32 P]-ATP dephosphorylation. The compound inhibited [γ - 32 P]-ATP degradation with a pIC_{50} of 4.6.

4 FPL 67156 was then tested for its effects on ATP and α , β -methylene-ATP responses at P_{2X} -purinoceptors in the rabbit isolated ear artery. In the concentration range 30 μ M–1 mM, the compound potentiated the contractile effects of ATP but not those of α , β -methylene-ATP. At 1 mM, FPL 67156 produced a 34-fold leftward shift of ATP $E/[A]$ curves.

5 The effects of FPL 67156 on ATP $E/[A]$ curves in the rabbit ear artery were analysed using a theoretical model (Furchgott, 1972) describing the action of an enzyme inhibitor on the effects of a metabolically unstable agonist. This analysis provided an estimate of the pK_i for FPL 67156 as an ecto-ATPase inhibitor of 5.2.

6 Using appropriate assays, FPL 67156 was shown to have weak antagonist effects at P_{2X} - and P_{2T} -purinoceptors ($pA_2 \approx 3.3$ and 3.5 respectively), and weak agonist effects at P_{2U} -purinoceptors ($p[A_{50}] \approx 3.5$).

7 The degree of potentiation of ATP and UTP effects elicited by FPL 67156 confirms previous results concerning the influence that ecto-ATPase has on the position of $E/[A]$ curves for metabolically unstable agonists. The magnitude of this influence is predicted to have a major effect on the agonist potency orders currently used to designate purinoceptors.

8 This study indicates FPL 67156 to be a potentially valuable probe in studies on the action of nucleotides and in the classification of purinoceptors.

Keywords: FPL 67156; P_{2X} -purinoceptors; P_{2U} -purinoceptors; ATP; ecto-ATPase; enzyme inhibition; agonist potency orders; receptor classification

Introduction

A well-recognised problem in the pharmacological classification of receptors is the susceptibility of natural agonists and synthetic analogues to enzymatic degradation by the assay tissues used. Of particular concern is the effect that metabolic processes have on the measured potency of agonists. In quantitative terms, this can be a substantial influence. For example, the potency of acetylcholine in chick smooth muscle is reduced by some 1000-fold due to acetylcholinesterase activity (Green *et al.*, 1978). Uptake and metabolism of noradrenaline by the guinea-pig trachea decreases the potency of this agonist by 30-fold (Foster, 1967). In the same tissue, the potency of adenosine is reduced by 350-fold due to the presence of adenosine deaminase (Satchell, 1984). Clearly, such displacement of agonist-concentration effect ($E/[A]$) curves from their 'true' positions is of major concern when considering agonist potency orders. For example, in the guinea-pig trachea, under conditions when enzymatic degradation operates, salbutamol is some 20 fold more potent than noradrenaline whereas, when degradation is blocked, the two agonists have indistinguishable potency (Kenakin, 1980).

In the case of nucleotide agonists, mechanisms for their enzymatic dephosphorylation have been well-demonstrated and characterized (Welford *et al.*, 1986; 1987), although evidence for the influence of these on the pharmacological potency of agonists has been limited (Welford *et al.*, 1987). However, a recent study conducted in our laboratory (Crack *et al.*, 1994) indicated that the measured potency of ATP at P_{2X} -purinoceptors in the rabbit isolated ear artery is reduced by some 400-fold by the operation of ecto-ATPase in that tissue. Moreover, when the potency of ATP is adjusted to reflect its true value, its position, relative to metabolically stable agonists such as α , β -methylene-ATP, changes dramatically. This result led us to suggest that the agonist potency order currently accepted to define P_{2X} -purinoceptors may need to be reconsidered. Importantly, that study was conducted without the benefit of a selective ecto-ATPase inhibitor. This is unlike those quoted above in relation to other receptor systems where substances like physostigmine, cocaine and dipyrindamole were available to block the metabolic or transport processes concerned and quantify the associated changes in potency.

In the present paper we describe the discovery of FPL 67156, a novel ATP analogue synthesized at Fisons. Biochemical and pharmacological analyses of this compound

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are described which indicate it to be a selective inhibitor of ecto-ATPase suitable for use in resolving the kinds of agonist potency and receptor classification issues described above. The results of this study extend and confirm our previous paper (Crack *et al.*, 1994) as regards the influence of ecto-ATPase on agonist potency and, in turn, on 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) into 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 117.6, NaH_2PO_4 0.9, NaHCO_3 25.0, MgSO_4 1.2, glucose 11.1, KCl 5.4 and CaCl_2 2.6. 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 (8-SPT) (300 μM) to exclude the possible influence of P_1 -purinoceptor activation.

The tissues were subjected to an initial force of 1.0 g 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 response to acetylcholine was invariably less than 17% of the KCl-induced contraction. Tissues were then washed and allowed to re-equilibrate for 45 min.

Experimental protocols A paired curve design was adopted in which all tissues were initially treated with cumulative additions of ATP or α,β -methylene-ATP at 0.5 $\log_{10}$ unit intervals. Following washout and re-equilibration, FPL 67156 was applied for 45 min, then a second cumulative agonist-concentration effect ($\text{E}/[\text{A}]$) curve was constructed. Contractile effects were expressed as percentages of the first curve maximum.

Rabbit isolated epithelium

Male New Zealand White rabbits (2.5–4.0 kg) were killed with an overdose of pentobarbitone sodium (Euthatal) into the marginal ear vein. The thorax was opened and the trachea removed and placed in gassed Krebs solution of the following composition (mM): NaCl 117.6, KCl 5.4, CaCl_2 2.6, MgSO_4 1.2, NaHCO_3 24.9, NaH_2PO_4 1.2 and glucose 11.1. Dissections were carried out at room temperature. The trachea was divided transversely into two segments. Each segment was opened along the posterior membrane, pinned flat, and bathed in gassed Krebs during the dissection. The epithelial sheet was dissected away from the underlying cartilaginous tissue and mounted in a Ussing chamber with an

aperture of 0.64 cm^2 . Both surfaces of the epithelial sheet were bathed with 10 ml of Krebs buffer warmed to 37°C and gassed with 95% O_2 /5% CO_2 (pH 7.4) which also served to circulate the buffer using a gas-lift system.

Electrophysiological recordings The Ussing chamber was connected to a voltage clamp unit (DVC1000, World Precision Instruments Inc., Connecticut, U.S.A.) via 3 M KCl-4% agar bridges connected to silver-silver chloride electrodes. The trans-epithelial potential difference (V_t) was measured by electrodes sited close to the tissue surface and held at a value of 0 mV by passing current (the so called 'short circuit current' (I_{sc})) through the tissue via current electrodes. Under these conditions, where there is no concentration or electrical gradient for passive ion movement, only active ion transport will be measured. The contribution of fluid resistance to I_{sc} was corrected automatically.

Isolation of chloride transport pathway Preliminary experiments showed that the two major active transport processes in rabbit tracheal epithelium are sodium absorption and chloride secretion. In order to isolate the chloride secretion pathway, the Krebs bathing the apical surface of the tissue always contained amiloride (100 μM). To confirm that the residual I_{sc} was due largely to chloride secretion, bumetanide (100 μM) was added to the basolateral surface of the tissue to inhibit the cotransport process which accumulates chloride ions within the epithelial cells. This decreased I_{sc} from 48.5 ± 2.7 to $6.5 \pm 2.4 \mu\text{A cm}^{-2}$ (s.e., $n = 3$). Furthermore, when low chloride Krebs (Na isethionate substituting for NaCl) rather than normal Krebs was used to bathe both sides of amiloride treated tissues, I_{sc} fell from 52.7 ± 5.6 to $9.6 \pm 2.7 \mu\text{A cm}^{-2}$ (s.e., $n = 6$).

Experimental protocols When the baseline I_{sc} of each tissue was stable, ATP (30 μM) was added to the apical side of the tissue to establish viability. Once this response had reached a plateau, both the apical and basolateral sides of the system were drained and each side washed with 10 ml of Krebs. The basolateral side was then refilled with 10 ml of Krebs and the apical side with 10 ml of Krebs containing amiloride (100 μM). This washing and refilling procedure was used after each of the subsequent $\text{E}/[\text{A}]$ curves which were time matched and constructed by cumulative addition of agonist in 0.5 $\log_{10}$ unit increments. Two consecutive $\text{E}/[\text{A}]$ curves to ATP were obtained in each tissue. Then, following 5 min exposure of FPL 67156 (100 μM) or vehicle to the apical side, an $\text{E}/[\text{A}]$ curve to UTP or ATP- γ -S was constructed. Preliminary experiments had shown that consecutive curves to UTP were not sufficiently reproducible to permit a paired curve design, so treatment and control $\text{E}/[\text{A}]$ curves were obtained in different tissues in this preparation.

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, glucose 5, NaCl 140, KCl 5, adjusted with HCl to pH 7.4, 20°C. The cell suspensions were then diluted 7 fold with buffer.

$[\gamma^{32}\text{P}]\text{-ATP}$ 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. $[\gamma^{32}\text{P}]\text{-ATP}$. This concentration was near the K_M determined in previous studies (Beukers *et al.*, 1993). FPL 67156 was added to achieve final concentrations in the range 0.1–1000 μM . Inhibitory effects were measured as percentages of degradation of 10 μM ATP in the absence of drugs, 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 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

Acetylcholine bromide, adenosine 5'-triphosphate [disodium salt] (ATP), uridine 5'-triphosphate [disodium salt] (UTP), α,β -methylene ATP [dithidium salt], amiloride hydrochloride, bumetanide, dimethyl sulphoxide (DMSO) were obtained from the Sigma Chemical Co., Poole, U.K.; FPL 67156 (tetrasodium salt) was synthesized in the Medicinal Chemistry Department, Fisons by S.F. Hunt; 8(*p*-sulphophenyl)-theophylline (8-SPT), 2-methylthio-ATP [tetrasodium salt] were supplied by Research Biochemicals Inc., St. Albans, U.K.; adenosine-5'-O-(3-thiophosphate) (ATP- γ -S) [tetrasodium salt] was obtained from Boehringer Mannheim, Lewes, U.K.; activated charcoal, particle size 4–7 µm was supplied by Serva, Heidelberg, Germany; ATP (for ecto-ATPase studies) was obtained from Aldrich Chemie, Steinheim, Germany; [³²P]-ATP was from Amersham, U.K.

For rabbit ear artery experiments, ATP, FPL 67156 and 8-SPT were dissolved in distilled water. Indomethacin was prepared as a 28 mM solution in Na₂CO₃ (0.35 M) and subsequently diluted in Krebs solution.

For experiments in the rabbit epithelium, nucleotides were made up as stock solutions in normal Krebs solution. Amiloride hydrochloride was prepared as a 100 mM stock in DMSO and diluted in Krebs to give a final concentration of 100 µM. Bumetanide was prepared as a 100 mM stock in DMSO and 10 µl was added to the basolateral side of the chamber to give a final concentration of 100 µM.

Analysis of E/[A] curve data

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

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

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, $p[A_{50}]$.

Paired E/[A] curves obtained in the ear artery were fitted and the effects of FPL 67156 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 amongst treatment groups. Therefore, non-parametric tests were adopted: the Kruskal-Wallis test for analysing group differences; Mann-Whitney U-test for individual comparisons. Data obtained in the epithelium were not paired, and the effects of FPL 67156 on parameter estimates were analysed by Student's *t* test.

In experiments in the rabbit epithelium, FPL 67156 demonstrated a degree of agonism and it was necessary to take this into account when analysing its effects on the positions of E/[A] curves. To do this, the method of Pösch & Holzmann (1980) was employed. Briefly, this method involves the comparison of experimental E/[A] curves with those predicted to apply when two agonists interact in the same system. This allows detection and quantification of any potentiating or antagonistic properties that the ligand may have over and above their agonist properties. In practice it works by plotting the theoretical E/[A] curve for one agonist in the presence of the other assuming that the two agonists are purely additive in their interaction. Deviation of the experimental curve from this theoretical line indicates potentiation

(if the deviation is leftward) or antagonism (if the deviation is rightward).

Analysis of ecto-ATPase inhibition

Data for the inhibitory effects of FPL 67156 on ATP degradation were fitted to equation (i). 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 ecto-ATPase inhibition

A theoretical model proposed by Furchgott was employed (Furchgott, 1972). 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. The concentration of agonist within the biophase is defined as $[A_i]$, and its rate of entry is described by:

$$d[A_i]/dt = k_1([A_o] - [A_i]) \quad (ii)$$

in which k_1 is the diffusion constant for the agonist.

The degradation of agonist is assumed to be the consequence of enzyme activity within the biophase and to follow Michaelis-Menten kinetics:

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

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

$$k_1[A_i]^2 + (V_{max} + k_1K_M - k_1[A_o])[A_i] - k_1K_M[A_o] = 0 \quad (iv)$$

This is a quadratic equation in $[A_i]$. In theoretical simulations, values of k_1 , 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 (iv).

To generate theoretical E/[A] curves, values of $[A_i]$ were substituted into a simple hyperbolic function, equivalent to equation (i) 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 . Since the enzyme inhibitor used, FPL 67156, also showed some receptor antagonism at high concentrations, it was necessary to simulate this component. This was done by multiplying the $[A_{50}]$ by the factor $(1 + [X]/K_b)$, in which K_b is the receptor dissociation constant of X .

Results

Figure 1 shows the effect of FPL 67156 (100 µM) on E/[A] curves to UTP (Figure 1a) and ATP- γ -S (Figure 1b) in the rabbit epithelium. In the presence of FPL 67156, UTP E/[A] curves were displaced to the left: control $p[A_{50}] = 6.01 \pm 0.17$ (s.e., $n = 5$); treatment $p[A_{50}] = 6.69 \pm 0.15$ (s.e., $n = 5$). This difference was significant ($P < 0.02$, unpaired *t* test). In contrast, FPL 67156 did not potentiate ATP- γ -S; indeed the E/[A] curve was displaced to the right: control $p[A_{50}] = 6.13 \pm 0.15$ (s.e., $n = 5$); treatment $p[A_{50}] = 5.75 \pm 0.08$ (s.e., $n = 5$). FPL 67156 alone produced a change in I_{∞} indicated by the raised base lines of the curves in its presence. It was not possible to construct a complete E/[A] curve for FPL 67156 so its potency as an agonist could only be estimated ($p[A_{50}] \leq 3.5$). Thus, FPL 67156 is considerably weaker than UTP and ATP- γ -S, and also ATP which has a $p[A_{50}]$ of 5.36 in this tissue (data not shown). The influence of this agonist

Figure 1 Effects of FPL 67156 on UTP and ATP- γ -S E/[A] curves in the rabbit tracheal epithelium preparation. The figure shows average data ($n = 5$) for the effects of UTP (a) and ATP- γ -S (b) on short-circuit current (I_{sc}) obtained in the absence (O) and presence (Δ) of FPL 67156 (100 μ M). The dotted line depicts the theoretical dose-additive curve indicating the effects on UTP and ATP- γ -S E/[A] curves of FPL 67156 acting simply as an agonist. The leftward displacement of the experimental curve for UTP from the dose-additive line indicates synergy. The correspondence between the experimental curve for ATP- γ -S and the theoretical line indicates dose additivity.

effect on the location of the E/[A] curves to UTP and ATP- γ -S was analysed by the method of Pösch & Holzmann (1980). Thus, it was assumed that the agonist effects of FPL 67156, UTP and ATP- γ -S were subserved by the same receptor system and, therefore, that they were dose-additive. Theoretical lines were calculated indicating the position that the UTP and ATP- γ -S E/[A] curves should occupy in the presence of the experimentally found level of agonism produced by FPL 67156. These are depicted in Figure 1 as the dotted lines. Comparison of the experimental E/[A] curve for UTP in the presence of FPL 67156 with the theoretical additive curve indicated a clear leftward displacement (see Figure 1a). The $p[A_{50}]$ of the theoretical curve was 5.8, some 8-fold to the right of the value of 6.69 for the experimental curve. In contrast, a similar analysis of the effects of FPL 67156 on the effects of ATP- γ -S showed no deviation between the position of the experimental E/[A] curve and the theoretical additive curve (see Figure 1b). Since it was known that UTP is more susceptible to enzymatic hydrolysis than ATP- γ -S (Welford *et al.*, 1987) we considered that the

differential effect of FPL 67156 on the E/[A] curves for the two agonists may have been due to its ability to inhibit the breakdown of UTP and, therefore, to potentiate its effects.

FPL 67156 was tested as an inhibitor of ecto-ATPase in the human blood cell assay. The compound inhibited degradation of [γ^{32} P]-ATP with a pIC_{50} of 4.62 ± 0.03 (s.e., $n = 3$). Average data are shown in Figure 2.

Figure 3a shows the effects of FPL 67156 (30–1000 μ M) on ATP E/[A] curves in the rabbit ear artery. Although a paired curve protocol was used, second curve data only are shown for clarity. Statistical analysis of the curve changes is given in Table 1. FPL 67156 produced substantial and highly significant leftward displacements of the ATP E/[A] curve. There were no significant effects on asymptote but there was a small (≈ 0.3 units compared with control) but significant decrease in slope in the presence of FPL 67156.

Assuming that FPL 67156 was behaving as an ecto-ATPase inhibitor in the rabbit ear artery, its effects were analysed using the model of Furchgott (1972) (see Methods). Figure 3b shows the resulting theoretical simulation. The values of the model parameters used to generate the simulation are recorded in Table 2. Figure 3 indicates a broad correspondence between the theoretical and experimental lines although there are some apparent inconsistencies. However, it is to be emphasised that Figure 3 shows second curve data for display purposes. Statistical analysis of the left-shifts of paired ATP E/[A] curves (Table 3) showed that the shifts predicted by modelling were consistent with the experimental values.

Of particular note are the following parameter estimates. The value of pK_i , the ecto-ATPase inhibitory potency, which needed to be chosen for FPL 67156 was higher than that obtained in the biochemical assay. The value was 5.2. Attempts to simulate the data with the lower values failed, in that the observed leftward shifts could not be generated at the concentrations of FPL 67156 used experimentally. Also, a $p[A_{50}]$ value of 6.0 was used in the simulations. This value represents the location of the ATP E/[A] curve that would be obtained in the absence of ecto-ATPase activity (or following complete inhibition of the enzyme). Again, this value was necessarily chosen in order to allow the magnitude of leftward shifts observed in the experimental data. In the absence of FPL 67156, the average experimental $p[A_{50}]$ value for ATP was 3.34 ± 0.19 (s.e., $n = 5$).

FPL 67156 (30–1000 μ M) was tested against α,β -methylene-ATP in the rabbit ear artery. The results are shown in Figure 4. Again, second curves only are illustrated. FPL 67156 had no significant effects on the slope or asymptote of

Figure 2 Effect of FPL 67156 on ATP dephosphorylation in human blood cells. The figure shows average data for the effect of FPL 67156 on [γ^{32} P]-ATP degradation. The estimated pIC_{50} was 4.62 ± 0.03 (s.e., $n = 3$).

Table 1 Statistical analysis of the effects of FPL 67156 on ATP E/[A] curves in the rabbit ear artery

[FPL 67156]	$\Delta\alpha$			Δm			$\Delta p[A_{50}]$		
	mean	s.e.	n	mean	s.e.	n	mean	s.e.	n
0 μM	5.6	3.2	5	0.09	0.08	5	0.19	0.15	5
30 μM	10.4	6.3	4	-0.34*	0.13	4	-0.48*	0.13	4
100 μM	21.2	4.5	5	-0.30*	0.08	5	-0.47	0.25	5
400 μM	23.8	6.0	5	-0.33*	0.10	5	-1.02*	0.13	5
1000 μM	12.9	7.0	5	-0.19*	0.09	5	-1.53*	0.16	5
Kruskal-Wallis test P-values:		0.13				0.034	0.001		

A paired curve design was adopted in which two ATP curves were constructed in each piece of tissue before and after exposures to FPL 67156 at the concentrations shown. Curve shape data are presented as mean paired differences in asymptote (α), slope (m) and location ($p[A_{50}]$) of curves, and n is the number of curve pairs for a particular treatment.

Mann-Whitney U-test: *indicates significant difference between treatment group and control.

α,β -methylene-ATP E/[A] curves. The paired control α,β -methylene-ATP E/[A] curves were virtually superimposable ($\Delta p[A_{50}] = -0.07 \pm 0.03$ (s.e., $n = 4$)) and the lower concentrations of FPL 67156 produced no measurable curve displacement. At the higher concentrations (300 μM and 1 mM) the compound caused small but significant rightward shifts. From the displacement of the α,β -methylene-ATP E/[A] curve at 1 mM an estimate of the pA_2 for FPL 67156 at P_{2X} -purinoceptors was made of 3.31 ± 0.06 (s.e., $n = 7$). This estimate was employed in the theoretical simulations of the

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}	0.1 (M min^{-1})
Location of ATP E/[A] curve; $p[A_{50}]$	6.0 ($-\log \text{M}$)
Dissociation constant for ecto-ATPase inhibition by FPL 67156; pK_i	5.2 ($-\log \text{M}$)
Dissociation constant for P_{2X} -purinoceptor antagonism by FPL 67156; pK_B	3.3 ($-\log \text{M}$)

Table 3 Comparison of experimental and theoretical E/[A] curve shifts analysing paired changes in $p[A_{50}]$ at each concentration of FPL 67156

[FPL 67156] (μM)	$\Delta p[A_{50}]$		
	Experimental	Theoretical	t test
30	0.67 ± 0.20	0.73	$P > 0.7$ (7 d.f.)
100	0.66 ± 0.29	1.13	$P > 0.1$ (8 d.f.)
400	1.21 ± 0.20	1.48	$P > 0.2$ (8 d.f.)
1000	1.72 ± 0.22	1.58	$P > 0.5$ (8 d.f.)

Experimental $\Delta p[A_{50}]$ values (and s.e.) were estimated from Table 1, and adjusted by 0.19 to account for the shift between the control ATP E/[A] curves. This analysis showed that there were no significant differences between the data and the predicted shifts.

Figure 3 Effect of FPL 67156 on ATP E/[A] curves in the rabbit ear artery preparation: (a) shows average second-curve data ($n = 4,5$) obtained in the absence of FPL 67156 (●) and in its presence at the following concentrations (μM): 30 (□); 100 (■); 400 (Δ); 1000 (▲). (b) Shows theoretical simulations of the data using the model of Furchgott (1972). A pK_i value of 5.2 for FPL 67156 as an ecto-ATPase inhibitor was required to fit the data. Also required was an estimate of 6.0 for the $p[A_{50}]$ of ATP in the absence of ecto-ATPase activity. The other parameter values used in the simulation are recorded in Table 2.

Figure 4 Effect of FPL 67156 on α,β -methylene ATP E/[A] curves in the rabbit ear artery preparation. The figure shows second curve data obtained in the absence of FPL 67156 (●) and in its presence at the following concentrations (μM): 30 (□); 100 (■); 400 (Δ); 1000 (▲) ($n = 3-7$). Paired analysis of $\Delta p[A_{50}]$ changes indicated significant rightward displacement (≈ 3 -fold) at 1000 μM , from which a pA_2 estimate of 3.31 ± 0.06 (s.e., $n = 7$) was made.

effect of FPL 67156 on ATP. FPL 67156 alone produced some weak agonist responses at the higher concentrations ($\approx 15\%$ at 1 mM).

FPL 67156 was tested for activity at P_{2T} -purinoceptors using an established assay (Humphries *et al.*, 1994). The compound behaved as a low affinity antagonist ($pA_2 \approx 3.5$, $n = 4$).

Discussion

The first indication that FPL 67156 may be an ecto-ATPase inhibitor was obtained in the rabbit tracheal epithelium preparation where it potentiated the effects of UTP, but not those of ATP- γ -S. Important in the analysis of these data was the ability to eliminate, as a complicating factor, the agonist effect of FPL 67156 itself. This was possible by use of the method of Pösch & Holzmann (1980). By plotting theoretical dose-additive curves it was possible to define the effect that FPL 67156 would have had, acting solely as an agonist, on the position of UTP and ATP- γ -S $E/[A]$ curves. The clear separation of the experimental curve for UTP obtained in the presence of FPL 67156 from the theoretical additive curve indicated that FPL 67156 had acted synergistically with UTP. This was unlike the interaction between FPL 67156 and ATP- γ -S, which was shown to be simply additive. Although this analysis did not prove the involvement of ecto-ATPase inhibition, this was suggested by the fact that UTP had previously been found to be more susceptible than ATP- γ -S to enzymic dephosphorylation by tissues (Welford *et al.*, 1987). This encouraged us to seek direct evidence for ecto-ATPase inhibition by FPL 67156. Such evidence was obtained using the human blood cell assay where the compound inhibited $[\gamma^{32}P]$ -ATP degradation in concentration-dependent manner with a pIC_{50} of 4.62.

Accepting that FPL 67156 could be classified as an ecto-ATPase inhibitor, its pharmacological effects were then studied using the rabbit ear artery preparation. This system has previously been shown to permit quantitative evaluation of the effects of agonists and antagonists acting at P_{2X} -purinoceptors (O'Connor *et al.*, 1990; Leff *et al.*, 1990; 1993). Recently, we used this preparation to analyse the ecto-ATPase inhibitory properties of ligands which also act as receptor antagonists (Crack *et al.*, 1994). We considered this system to be appropriate for providing a pharmacological estimate of the potency of FPL 67156 as an ecto-ATPase inhibitor.

As shown in Figure 3, FPL 67156 elicited substantial leftward shifts of ATP $E/[A]$ curves whereas, in the concentration range 30–100 μ M, FPL 67156 had no effect on the location of $E/[A]$ curves to α, β -methylene-ATP, a metabolically stable agonist (Welford *et al.*, 1986). At the highest concentration used (1 mM), FPL 67156 potentiated ATP responses by 34-fold. In fact, at that concentration and at 400 μ M, the compound also showed some P_{2X} -purinoceptor antagonism against the effects of α, β -methylene ATP, corresponding respectively to rightward shifts of 3-fold and 1.5-fold. This property would have opposed the leftward shift of ATP $E/[A]$ curves produced by ecto-ATPase inhibition. So the degree to which ATP responses can be potentiated, considerable as it stands, may have been underestimated. This is an important consideration when quantifying the impact of ecto-ATPase on the position of the ATP $E/[A]$ curve in this preparation.

In principle, the potentiating effect of FPL 67156 could have come about in two general ways, both related to ecto-ATPase inhibition. Desphosphorylation of the applied agonist may have produced other chemical species which oppose its pharmacological effects. Alternatively, as assumed here, the effects of metabolism may simply be to reduce the concentration of the agonist in the vicinity of the receptors. Inhibition of enzymatic degradation in both cases could lead to potentiation of the agonist responses. The former explana-

tion was rejected in the present study for the following reasons. In the rabbit ear artery experiments the issue would have been 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 a vasorelaxation response which would have opposed the P_{2X} -purinoceptor-mediated contractile effects of ATP. Inhibition of adenosine formation could then have 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 30–100 fold higher than its dissociation constant for P_1 -purinoceptors. Similarly, the possible complicating effects of metabolites active at P_{2Y} -purinoceptors could be excluded since these receptors are absent in this preparation (O'Connor *et al.*, 1990). Therefore, it was assumed that the potentiating effects of FPL 67156 could be attributed simply to the enhancement of the active concentration of ATP.

The model of Furchgott (1972) provides a formal way of analysing such data and it was used here to make a pharmacological estimate of the affinity of FPL 67156 as an ecto-ATPase inhibitor in the rabbit ear artery preparation. Evidently from Figure 3, it was possible to represent the experimental data quite accurately by the model. The parameters used to produce the simulations predicted parallel leftward displacements. In fact, there was some evidence for a flattening of the ATP $E/[A]$ curves in the presence of FPL 67156. According to Furchgott's model, such shape changes can occur. However, the magnitude of the change in slope observed in the present data was only ≈ 0.3 units and we did not consider it necessary to accommodate such a small change during the modelling process. The important requirement of the theoretical analysis was to account for the magnitude of the leftward displacements observed and, as explained in the Results section, the choice of parameter values required to do this was not arbitrary. In particular, the estimates pK_i and $p[A_{50}]$ merit some attention.

The pK_i estimate of 5.2 was needed in order to simulate potentiation of ATP effects in the appropriate range of FPL 67156 concentrations. This pharmacological pK_i estimate is higher than the one made in the human blood cell assay (4.6). Why this is so is not known, but similar discrepancies were found in the preceding analysis of ecto-ATPase inhibition (Crack *et al.*, 1994). In that study, suramin and FPL 66301, an analogue of ATP, were tested in the same two preparations and the pK_i estimates were, respectively, 8-fold and 4-fold higher in the rabbit ear artery than in human blood cells. From the pharmacological point of view, we consider the estimate made in the rabbit ear artery to have the greater relevance because this is the value which indicates the operational concentration-range for the potentiating effects of FPL 67156.

A criticism of this interpretation could be that the difference in pK_i estimates arose from uncharacterized properties of FPL 67156 expressed in the pharmacological preparation but not in the biochemical assay. Such properties may have led to potentiation of the effects of ATP by mechanisms other than ecto-ATPase inhibition, resulting in an overestimation of the potency of FPL 67156 as an enzyme inhibitor. We consider this unlikely because potentiating mechanisms other than inhibition of agonist metabolism would have to operate at the receptor or post-receptor level and affect metabolically stable agonists as well as unstable ones. The fact that α, β -methylene-ATP $E/[A]$ curves were completely unaffected by FPL 67156 at concentrations which produced substantial potentiation of those to ATP argues against the expression of additional potentiating properties.

An alternative explanation for the difference in the pK_i estimates made in the two systems is, of course, that two different assay tissues were involved. Ideally, the pharmacological and biochemical analyses would have been conducted in the same tissue and in this regard the ear artery would have been the system of choice. However, to our

knowledge, no assay of nucleotide metabolism has been published for this tissue and we felt it necessary to employ an established and available method, as exemplified by the blood cell assay (Beukers *et al.*, 1993). This leaves open the criticism that FPL 67156 was inhibiting two quite distinct enzymes in the two systems. Against this is the correlation between the pharmacological and biochemical pK_i estimates for FPL 67156, suramin and FPL 66301, namely 5.2, 5.2, 3.9 and 4.6, 4.3, 3.3 respectively (present study and Crack *et al.*, 1994). The similarity of the relative values suggests that the same enzyme is involved, and the systematic difference between the two sets of numbers (5-fold on average) would not be unexpected for the same enzyme operating under different conditions.

The $p[A_{50}]$ value of 6.0 used in the model corresponds to the potency of ATP in the absence of ecto-ATPase, or under conditions of complete block of the enzyme. In other words, it represents an estimate of the 'true' potency of ATP in the tissue. This value was necessary in the simulations to allow for the magnitude and concentration-dependence of the potentiations produced experimentally by FPL 67156. Evidently this estimate of ATP's potency is considerably higher than the experimental $p[A_{50}]$ obtained in the absence of FPL 67156, which was 3.34. A difference of this magnitude (approximately 400-fold) was concluded to apply in our previous study (Crack *et al.*, 1994). In those experiments, the effects of suramin and FPL 66301 on ATP responses in the rabbit ear artery preparation were analysed. In order to explain the magnitude of leftward shifts of $E/[A]$ curves produced by each of these compounds an estimate of 6.0 for the 'true' $p[A_{50}]$ of ATP was again necessary.

In our previous paper (Crack *et al.*, 1994) we drew attention to the impact of ecto-ATPase on the experimental potency of ATP and, by implication, other metabolically unstable agonists. In the present study, the use of FPL 67156 has confirmed the degree to which the potency of ATP, at least, is underestimated in the rabbit ear artery preparation.

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- It also emphasises our previous arguments regarding the need to re-evaluate the agonist potency order currently held to designate P_{2X} -purinoceptors (Burnstock & Kennedy, 1985). If the results of these analyses are accepted, then dramatic changes will occur in the positions that ATP and, by implication, other metabolically susceptible agonists such as 2-MeSATP, occupy in the potency order. According to such calculations, the potency order used to designate P_{2X} -purinoceptors would read: α,β -methylene-ATP = 2-MeSATP > ATP > β,γ -methylene-L-ATP > β,γ -methylene-D-ATP. This is in marked contrast with the reported order (O'Connor *et al.*, 1990): α,β -methylene-ATP > β,γ -methylene-L-ATP > β,γ -methylene-D-ATP > 2-MeSATP > ATP.
- Similar arguments apply to the potency of UTP in the rabbit tracheal epithelium. Unfortunately, the agonist effects of FPL 67156 limited the concentration-range over which it could be used in that preparation. However, at 100 μ M it produced a degree of potentiation of UTP that was very similar to its effects on the action of ATP in the rabbit ear artery, and the potency of UTP, relative to ATP- γ -S, clearly changed. We presume that the receptors in the rabbit tracheal epithelium belong to the P_{2U} -class. However, further experiments, conducted in the presence of FPL 67156, are required before a meaningful agonist potency order can be reported for this system.
- Regarding the utility of FPL 67156 in purinoceptor classification, its relatively modest potency as an ecto-ATPase inhibitor is recognised. However, the compound was only weakly active at P_{2X} -, P_{2U} -, and P_{2T} -purinoceptors, demonstrating pA_2 or $p[A_{50}]$ values of 3.5 or less. Accepting the theoretical pK_i estimate (5.2), this means FPL 67156 has some 50-fold selectivity as an ecto-ATPase inhibitor. As such, it is hoped that this compound should have sufficient selectivity to approach the kinds of problems in pharmacological analysis described here and in our previous paper (Crack *et al.*, 1994).
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