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ATP, a partial agonist for the P2Z receptor of human lymphocytes

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1 Although extracellular adenosine 5′-triphosphate (ATP) is the natural ligand for the P2Z receptor of human lymphocytes it is less potent than 3′-*O*-(4-benzoylbenzoyl)-ATP (BzATP) in opening the associated ion channel, which conducts a range of permeants including Ba^{2+} and ethidium^{+}. We have quantified the influx of ethidium^{+} into lymphocytes produced by BzATP, ATP, 2-methylthio-ATP (2MeSATP) and ATPγS, studied competition between ATP and BzATP and investigated the effects of KN-62, a new and potent inhibitor of the P2Z receptor.

2 BzATP and ATP stimulated ethidium^{+} influx with EC_{50} values of 15.4 ± 1.4 μM ($n=5$) and 85.6 ± 8.8 μM ($n=5$), respectively. The maximal response to ATP was only 69.8 ± 1.9% of that for BzATP. Hill analysis gave n_H of 3.17 ± 0.24 ($n=3$) and 2.09 ± 0.45 ($n=4$) for BzATP and ATP, suggesting greater positive cooperativity for BzATP than for ATP in opening the P2Z receptor-operated ion channel.

3 A rank order of agonist potency of BzATP > ATP = 2MeSATP > ATPγS was observed for agonist-stimulated ethidium^{+} influx, while maximal influxes followed a rank order of BzATP > ATP > 2MeSATP > ATPγS.

4 Preincubation with 30–50 μM oxidized ATP (ox-ATP), an irreversible P2Z inhibitor, reduced the maximal response but did not change the steepness of the Ba^{2+} influx-response curve produced by BzATP (n_H 3.2 and 2.9 for 30 and 50 μM ox-ATP, respectively ($n=2$)).

5 ATP (300–1000 μM) added simultaneously with 30 μM BzATP (EC_{90}) inhibited both ethidium^{+} and Ba^{2+} fluxes to a maximum of 30–40% relative to the values observed with BzATP alone. Moreover, ATP (300 μM) shifted the concentration-response curve to the right for BzATP-stimulated Ba^{2+} influx, confirming competition between ATP and BzATP.

6. KN-62, a new and powerful inhibitor of the lymphocyte P2Z receptor, showed less potency in antagonizing BzATP-mediated fluxes than ATP-induced fluxes when maximal concentrations of both agonists (BzATP, 50 μM; ATP, 500 μM) were used.

7 These data suggest that the natural ligand, ATP, is a partial agonist for the P2Z receptor while BzATP is a more efficacious agonist. Moreover the competitive studies show that only a single class of P2-receptor (P2Z class) is expressed on human leukaemic lymphocytes.

Keywords: P2Z receptor; BzATP; lymphocytes: human leukaemic; KN-62; extracellular ATP receptor; ethidium^{+} influx; cation channel, lymphocyte; Ba^{2+} influx; partial agonist

Introduction

Extracellular adenosine 5′-triphosphate (ATP) mediates a wide range of effects by acting on P2-receptors expressed on many tissues throughout the body. Stimulation of P2-receptors rapidly elevates cytosolic Ca^{2+} concentration, either by influx from the extracellular medium via cation channels or by release of Ca^{2+} from internal stores subsequent to activation of the phospholipase C signalling cascade (Dubyak & El-Moatassim, 1993). Most functional effects mediated by extracellular ATP result from this rise in cytosolic Ca^{2+} concentration. The two different signalling mechanisms and the molecular structure of cloned P2-receptors form the basis of classifying these receptors into 2 major families (Burnstock & Kennedy, 1985; Fredholm *et al*., 1994). The P2X family of receptors have a putative structure of 2 transmembrane domains connected by a large extracellular loop (North, 1996) and are mainly expressed on neurones and smooth muscle cells. At these sites, ATP functions as a co-transmitter and spasmogen respectively (Burnstock, 1990; Dubyak & El-Moatassim, 1993). The P2Y family of receptors have the seven transmembrane spanning regions typical of G-protein coupled receptors and are found on many different tissue types, including endothelium and excitable tissues, where they exert multiple effects, including secretion and smooth muscle relaxation (Dubyak & El-Moatassim, 1993; Barnard *et al*., 1996). These two families of P2-receptors have been further divided into a number of subclasses ($P2X_1-P2X_6$ and $P2Y_1-P2Y_7$) based on pharmacological profiles of agonist responses and structures of the cloned receptors (Abbracchio & Burnstock, 1994; Burnstock, 1996). A possible third group of P2-receptor, originally termed P2Z, is a ligand-gated ion channel which forms a pore and shows selectivity for the ATP[4] species (Fredholm *et al*., 1994). Recently, a P2Z receptor was cloned from rat brain and the first 395 amino acids shown to have 35–40% homology with P2X receptors. This cloned receptor, designated $P2X_7$ on the basis of this homology, contains a long carboxyl terminal domain not found in other P2X subtypes, which confers the unique permeability of P2Z-channels to large cations such as fluorescent dyes (Surprenant *et al*., 1996). The P2Z pore-forming receptor, which is mainly expressed by cells of haemopoietic and immune origin (Wiley *et al*., 1990; 1992; Pizzo *et al*., 1991; Dubyak & El-Moatassim, 1993), may mediate apoptosis of rat thymocytes and murine lymphocytes and macrophages, since extracellular ATP causes release of lactic dehydrogenase and cytolysis (Di Virgilio, 1995). In human leukaemic lymphocytes, we have previously shown that extracellular ATP exerts multiple effects by occupancy of the P2Z receptor expressed on

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these cells. Thus extracellular ATP increases the permeability of lymphocytes to Ca^{2+}, Ba^{2+} and ethidium$^+$ (Wiley *et al*., 1990; 1993; 1994; 1996), stimulates phospholipase D activity (Gargett *et al*., 1996) and induces shedding of the surface adhesion molecule L-selectin (Jamieson *et al*., 1996).

Agonists for the P2Z receptor show a rank order of potency which clearly distinguishes these receptors from other P2-receptors. In murine macrophages (El-Moatassim & Dubyak, 1992; Nuttle *et al*., 1993), human lymphocytes (Wiley *et al*., 1994; 1996) and HEK cells expressing $P2X_7$ receptors (Surprenant *et al*., 1996) 3′-*O*-(4-benzoyl)benzoyl ATP (BzATP) was a far more potent agonist than ATP. Indeed, BzATP stimulated Ba^{2+} influx with an EC_{50} of 8 μM compared with 89 μM for ATP in lymphocytes suspended in divalent cation free KCl medium (Wiley *et al*., 1994). In addition to its greater potency relative to ATP, BzATP showed greater maximal stimulation of P2Z-mediated responses such as Ba^{2+} influx in human lymphocytes (Wiley *et al*., 1994), phospholipase D (PLD) activity in both human lymphocytes and murine macrophages (El-Moatassim & Dubyak, 1992; Gargett *et al*., 1996) and current amplitude in J744 macrophages and HEK cells transfected with $P2X_7$ receptors (Surprenant *et al*., 1996). Differences in maximal response elicited by agonists have also been demonstrated for both native and cloned $P2Y_1$ receptors, for which it was concluded that ATP acted as a partial agonist (Barnard *et al*., 1996). Partial agonism is a well recognised feature of many agonist-receptor interactions and is generally attributed to a diminished ability of the agonist to change receptor conformation, which results in lower measured responses, even at maximal receptor occupancy (Limbird, 1996).

Cells often co-express several P2-receptor subtypes and thus maximal responses to a variety of ATP analogues may be due to the additive responses of these co-expressed receptors (O'Connor *et al*., 1991). In the present study we have used four agonists to stimulate ethidium$^+$ influx through the P2Z receptor-operated ion channel. The results suggest that ATP, 2MeSATP and ATPγS are partial agonists for the P2Z receptor, while BzATP functions as a more efficacious agonist. Competitive inhibition by ATP of responses to BzATP with or without preincubation with ox-ATP, showed that a single class of P2-receptor is expressed on human leukaemic lymphocytes.

Methods

Source of lymphocytes

Peripheral blood lymphocytes were obtained from patients with B-cell chronic lymphocytic leukaemia whose cells showed permeability responses to ATP in our previous studies (Wiley *et al*., 1993; 1994; Gargett *et al*., 1996).

Lymphocyte preparation

Venous blood (20 ml) from patients was added to heparin anticoagulant and diluted with 2 vol of HEPES buffered saline (composition mM: HEPES 10, pH 7.4, NaCl 145, KCl 5, $CaCl_2$ 1, D-glucose 5 and bovine serum albumin (BSA) 1 g l^{-1}). Mononuclear cells were separated by density gradient centrifugation over Ficoll-Paque and washed twice in HEPES buffered saline. Contaminating monocytes were removed by incubation of the cells in plastic culture flasks for 60 min at 37°C. Cytocentrifuge preparations showed that >99% of cells were small mature lymphocytes, while 96% had B-cell phenotypes of $CD5^+$, $CD20^+$.

Ethidium$^+$ influx measurement by time resolved flow cytometry

Washed lymphocytes (10^8 ml^{-1}) suspended in HEPES buffered saline were diluted to 10^6 ml^{-1} in 1 ml of 150 mM KCl medium containing HEPES 10 mM, pH 7.4, BSA 1 g l^{-1} and D-glucose 5 mM. These samples were stirred and maintained at 37°C, the agonist added, followed 2 min later by addition of 25 μM ethidium$^+$. Cell associated fluorescence signals were analysed for 5 min by a Coulter Elite flow cytometer (Coulter, Hialeah, FL) with an argon laser excitation at 488 nm and fluorescent emission collected with a 590 nm long-pass filter. Data were collected at a rate of 500 cells s^{-1} and analysed by computer by use of Elite software, version 4.0 to produce histograms of fluorescence intensity (channel number) at consecutive 6 s intervals. The mean channel of fluorescence intensity was then calculated for each of the 6 s intervals and plotted against time.

Cytosolic Ba^{2+} measurements by fluorometry

Washed lymphocytes (10^7 ml^{-1}) suspended in HEPES buffered saline were loaded with 2 μM fura-2-acetoxymethyl ester by incubation at 37°C for 20 min in the dark. Cells were then washed twice with HEPES buffered saline and stored in the dark at 20°C. Lymphocytes (10^8 ml^{-1}) were diluted to 2.0×10^6 ml^{-1} in 3 ml of HEPES buffered 150 mM KCl medium and stimulated with ATP 1 min after additon of 3 μl $BaCl_2$ (final concentration, 0.25, 0.4 or 0.5 mM). Fluorescent signals were monitored at 37°C in a stirred cuvette by a Johnson Foundation Fluorometer with excitation at 340 nm and emission at 500 nm. Calibration of F_{max} and F_{min} was performed after each run by adding digitonin (25 μg ml^{-1}, final concentration) followed by EGTA (final concentration, 6 mM in 50 mM Tris, pH 8.5) (Grynkiewicz *et al*., 1985). Control experiments showed that addition of ATP did not release Ca^{2+} from the internal stores of lymphocytes suspended in medium containing EGTA.

Materials

Ficoll-Paque (density 1.077) was obtained from Pharmacia (Uppsala, Sweden). ATP, BzATP, 2′,3′-dialdehyde ATP (oxidized ATP), ethidium bromide, barium chloride and bovine serum albumin (BSA) were from Sigma Chemical Co. (St. Louis, MO). Fura-2-acetoxymethyl ester was from Molecular Probes (Eugene, OR). Adenosine 5′-[γ-thio]triphosphate and HEPES were from Boehringer Mannheim (W. Germany). 2-Methylthio-ATP and KN-62 (1-[*N*, *O*-bis(5-isoquinolinesulphonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine) were from Research Biochemicals, Inc. (Natick, MA). KN-04 (N-[1-[N-methyl-p-(5 isoquinolinesulphonyl)benzyl]-2-(4 phenylpiperazine)ethyl]-5-isoquinoline sulphonamide) was from Seikagaku, (Tokyo, Japan). ARL-67156 (6-*N*,*N*-diethyl-D-β,γ-dibromomethyleneATP) was a gift from Dr P. Leff (Astra Charnwood, Loughborough, U.K.).

Calculations of ATP^{4-} species concentration

Calculation of the relative concentrations of ATP^{4-}, $BaATP^{2-}$, and free Ba^{2+} for solutions containing varying concentrations of ATP and Ba^{2+} were calculated by use of an updated version (3.0) of a previously published programme, Bound and Determined (Brooks & Storey, 1992).

Data presentation and analysis

Concentration-response curves were obtained by non linear regression analysis by use of the programme Flexifit (Guardabasso *et al*., 1988). Hill plots were constructed from these curves from the Hill equation, and Hill coefficients calculated by linear regression analysis of slopes from individual experiments. Data are presented as mean ± s.e.mean (*n*), unless otherwise stated in figure legends.

Results

BzATP produced a greater maximal response than ATP

The influx of ethidium$^+$ through the lymphocyte P2Z receptor-operated ion channel was studied with concentrations of

BzATP and ATP known to give maximal Ba^{2+} influx (Wiley *et al.*, 1994). Addition of 500 μM ATP or 50 μM BzATP to lymphocytes suspended in Ca^{2+}-free KCl medium induced an uptake of ethidium$^+$, which was linear with time as measured by an increase in mean channel fluorescence over 5 min (Figure 1). Ethidium$^+$ influx was greater for BzATP compared to ATP. Concentration-response curves for BzATP and ATP were then analysed and uptake slopes gave EC_{50} values of 15.4 ± 1.4 μM (mean $\pm$ s.e.mean, $n = 5$ experiments from 4 patients) and 85.6 ± 8.8 μM ($n = 5$ experiments from 3 patients), respectively (Figure 2). The maximal response for ATP was only $69.8 \pm 1.9\%$ ($n = 5$) of that for BzATP. Hill analysis of these data (Figure 3) gave Hill coefficients (n_H) of 3.17 ± 0.24 ($n = 3$) and 2.09 ± 0.45 ($n = 4$) for BzATP and ATP, respectively. Similar Hill plots derived from Ba^{2+} uptake data (Figure 5b, Wiley *et al.*, 1994) gave n_H of 3.27 ± 0.43 ($n = 3$) and 2.36 ± 0.22 ($n = 5$) for BzATP and ATP, respectively. Our group has previously obtained Hill coefficients of 2.3 and 2.5 for ATP-stimulated fluxes of Ba^{2+} and $^{86}Rb^+$ (Wiley *et al.*, 1992; 1993).

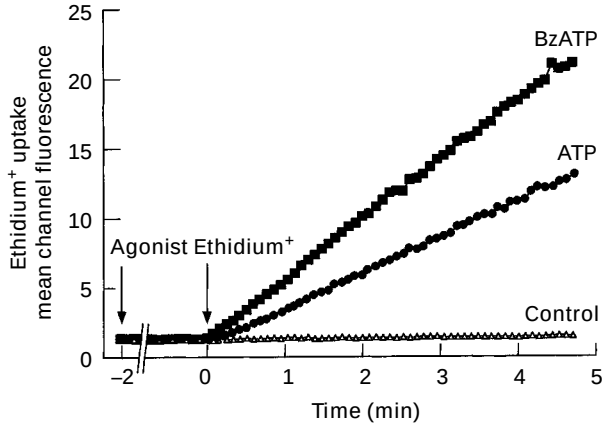

Figure 1 BzATP- and ATP-stimulated ethidium$^+$ influx in human lymphocytes. Cells (10^6 ml^{-1}) suspended in Ca^{2+}-free HEPES buffered KCl medium were pre-incubated for 2 min at 37°C with either 50 μM BzATP or 500 μM ATP (260 μM ATP^{4-}) before the addition of 25 μM ethidium$^+$. Control cells were incubated with 25 μM ethidium$^+$ alone. Mean channel of cell associated fluorescence was measured at 6 s intervals by flow cytometry. Data were collected at a rate of 500 cells s^{-1} for 5.5 min. Arrows indicate the sequential addition of agonist and ethidium$^+$. Results from a single experiment are shown, representative of 6.

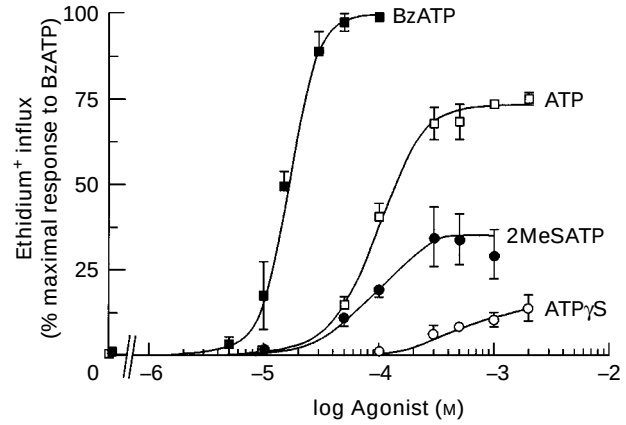

Figure 2 Concentration-response curves for ethidium$^+$ influx stimulated by ATP analogues. Lymphocytes (10^6 ml^{-1}) were suspended in Ca^{2+}-free HEPES buffered KCl medium and incubated with BzATP, ATP, 2MeSATP or ATPγS for 2 min at 37°C before the addition of 25 μM ethidium$^+$ and ethidium$^+$ influx measured by flow cytometry. Initial rates of agonist-stimulated ethidium$^+$ influx were calculated by linear regression analysis of uptake slopes and results expressed as a percentage of maximal response to BzATP, which was defined as 100% response. The curves shown were calculated by non linear regression analyses. Mean values $\pm$ s.e.mean from 3–6 experiments on 6 patients are shown.

Other ATP analogues were partial agonists of the P2Z receptor

Our previous work has shown that 2MeSATP and ATPγS are also agonists for the P2Z receptor, since both induce Ba^{2+} influx through the associated ion channel (Wiley *et al.*, 1994). Both 2MeSATP and ATPγS induced ethidium$^+$ influx into lymphocytes and analysis of the concentration-response curve for 2MeSATP gave an EC_{50} value of 77.5 ± 8.0 μM ($n = 3$). An EC_{50} was not determined for ATPγS, since in 2 out of 4 experiments the response to the highest concentration tested was not maximal, although it was greater than 300 μM. The maximal responses of 2MeSATP and ATPγS were $34.6 \pm 3.5\%$ ($n = 3$) and 13.1% ($n = 2$ of the 4 experiments shown) of that observed for BzATP (Figure 2). These values were compared to those found previously for the efficacy of four agonists in stimulating Ba^{2+} influx and phospholipase D (PLD) activity. All four agonists produced the same rank order of maximal P2Z responses, with similar relative values, whether measured by ethidium$^+$ uptake, Ba^{2+} influx or phospholipase D activation (Table 1).

BzATP concentration-response curves after partial receptor inactivation

To examine whether the steeper concentration-response curve to BzATP relative to ATP was due to the presence of

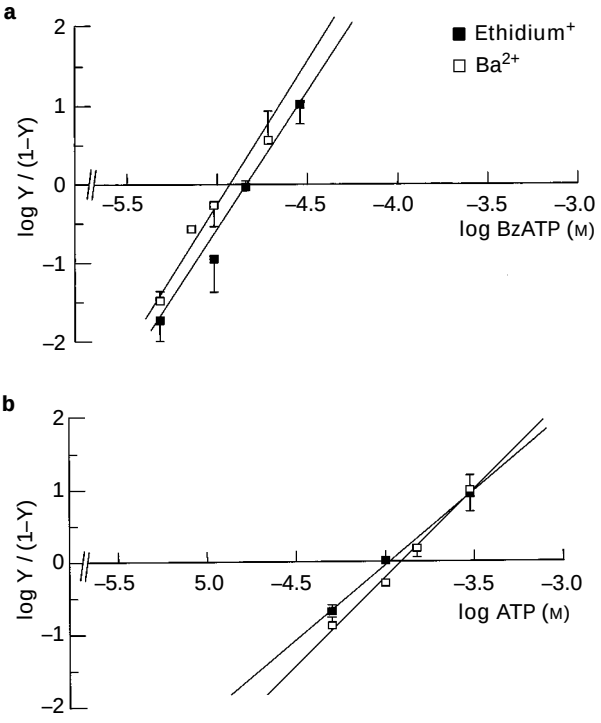

Figure 3 Hill plot analyses of BzATP- and ATP-stimulated cation fluxes. Data obtained from the concentration-response curves for (a) BzATP- and (b) ATP-stimulated ethidium$^+$ (Figure 2) and Ba^{2+} uptake (Figures 4 and 1b, Wiley *et al.*, 1994) were analysed by the Hill equation and plots for ethidium and Ba^{2+} constructed from the means of 3–5 experiments. Y is the fractional response, V/V_{max}, where V is the initial rate of ethidium$^+$ or Ba^{2+} influx for each agonist concentration and V_{max} is the rate at maximal agonist concentration. Hill coefficients were calculated by linear regression analysis of slopes from individual experiments and reported as means $\pm$ s.e.mean for n experiments.

spare receptors, the irreversible P2Z antagonist, oxidized ATP (ox-ATP) (Murgia *et al*., 1993; Wiley *et al*., 1994), was used to inactivate a fraction of the P2Z receptors. Initial rates of BzATP-induced Ba^{2+} influx were measured fluorometrically, since Ba^{2+} and Ca^{2+} produce similar changes in the excitation and emission spectra of fura-2 (Schilling *et al*., 1989). Ba^{2+} was used as a permeant of the P2Z ion channel because the increase in fluorescent signal only reflects Ba^{2+} influx, since the Ba^{2+} taken up is neither pumped nor sequestered (Wiley *et al*., 1994). Fura-2 loaded cells were pretreated for 20 min at 37°C with 30 or 50 μM oxidized ATP. After washing, the cells were resuspended in Ca^{2+}-free KCl medium, 0.5 mM Ba^{2+} added and BzATP-induced Ba^{2+} influx measured. Figure 4 shows that the maximal response to BzATP in ox-ATP-treated cells was 66 and 50% (30 and 50 μM, respectively) of that for untreated cells ($n=2$). Hill analysis of the data gave n_H values for BzATP of 3.0, 3.2 and 2.9 for cells pretreated with 0, 30, and 50 μM ox-ATP, respectively ($n=2$), while EC_{50} values for BzATP were 16.1, 17.3 and 13.8 μM, respectively (Figure 4).

Table 1 Maximal P2Z-mediated responses induced by ATP analogues

	Maximal response		
Agonist	*Ethidium$^+$ influx*	*Ba^{2+} influx§*	*PLD activity†*
BzATP	100±3.5 (3)	100±6 (3)	100±18 (5)
ATP	69.8±1.9 (5)	52.9±2 (3)	72.6±7 (5)
2MeSATP	34.6±3.5 (3)	35.4±2 (3)	28.4±1 (3)
ATPγS	13.1 (2)	13.2±4 (3)	13.8±1 (3)

Maximal responses are expressed as a percentage relative to maximal responses for BzATP and are means±s.e.mean for (n) observations. Maximal rates for ethidium$^+$ influx were calculated by non-linear regression analysis of concentration-response plots by use of the programme Flexifit (Guardabasso *et al*. 1988). §Derived from data presented in Wiley *et al*. (1994) and †from Gargett *et al*. (1996).

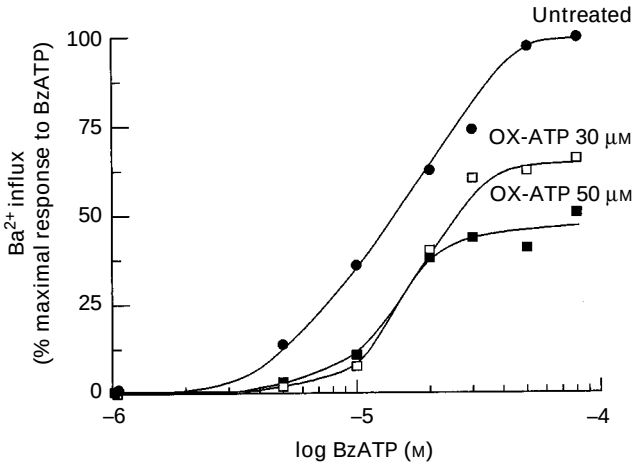

Figure 4 Effect of partial receptor inactivation on the BzATP concentration-response curve. Fura-2 loaded lymphocytes (2×10^6 ml^{-1}) suspended in HEPES buffered saline medium were either untreated or pretreated with ox-ATP for 20 min at 37°C, washed twice, resuspended (2×10^6 ml^{-1}) in Ca^{2+}-free HEPES buffered KCl medium at 37°C in a stirred fluorometer cuvette and Ba^{2+} (0.5 mM) was added 1 min before incubation with BzATP. Initial rates of Ba^{2+} influx were measured and results expressed as a percentage of maximal response to BzATP of untreated cells, which was defined as 100% response. Results from a single experiment are shown, representative of 2.

ATP, a competitive inhibitor of BzATP-mediated responses

To ascertain whether ATP binds to the same receptor as BzATP, classical full/partial agonist interaction studies were carried out. The putative partial agonist (ATP) was allowed to compete with the more efficacious agonist (BzATP), to find if the combination reduced the maximal response to BzATP. Cells were incubated with increasing concentrations of ATP (50–1000 μM) added simultaneously with a constant concentration of BzATP (30 μM; EC_{90}) and ethidium$^+$ fluxes measured. Concentrations of ATP less than 300 μM added concurrently with 30 μM BzATP did not affect the maximal response to BzATP (designated 100%) (Figure 5a). However, at concentrations of ATP above 300 μM, a progressive decrease in the rate of BzATP-stimulated ethidium$^+$ uptake was observed, such that the response at 1 mM ATP plus 30 μM BzATP matched that of 1 mM ATP alone (Figure 5a). A similar protocol was used to test ATP/BzATP competitive effects on Ba^{2+} influx. Fura-2 loaded cells, suspended in Ca^{2+}-free KCl medium containing 0.25 mM Ba^{2+}, were stimulated with ATP alone (50–1000 μM), or 30 μM BzATP added simultaneously with ATP (50–1000 μM). Again, low ATP concentrations did not alter the Ba^{2+} uptake stimulated by BzATP, but increasing the concentration of ATP to greater than 150 μM, gradually reduced the rate until it approximated that produced by ATP alone (Figure 5b). Another experiment was performed to show that ATP competes with BzATP for the same receptor. A fixed concentration of ATP (300 μM) was added together with BzATP (1–80 μM) in cells pretreated with ox-ATP (50 μM for 20 min at 37°C). After thorough washing, cells were incubated with BzATP with or without ATP and Ba^{2+} fluxes measured (Figure 6). In the presence of ATP (300 μM) plus low concentrations of BzATP (1–10 μM) the response was the same as ATP alone. However, at BzATP concentrations above 10 μM, an additive effect due to BzATP became apparent, although the concentration-response curve flattened and shifted to the right of the curve of BzATP alone (Figure 6).

KN-62 was a less potent inhibitor of BzATP than of ATP-mediated responses

Recently we described the isoquinolinesulphonamides (KN-62 and KN-04) as potent inhibitors of the lymphocyte P2Z receptor, which are active in the low nanomolar range (Gargett & Wiley, 1997). Cells were preincubated with the more potent of these two inhibitors, KN-62 (5–2000 nM) for 15 min at 37°C before the measurement of ethidium$^+$ or Ba^{2+} fluxes both in the presence and absence of ATP or BzATP. With maximally effective concentrations of these agonists, KN-62 produced much greater inhibition of responses to ATP than to BzATP (Figure 7a, b). Similar results were obtained with KN-04 (results not shown). However, when BzATP (18 μM) was used at a concentration equiactive with a maximally effective ATP concentration, KN-62 showed the same inhibitory potency (Figure 7a, b).

Effect of an ecto-ATPase inhibitor on P2Z-mediated responses

Since B-cell lymphocytes (Segel *et al*., 1985; Barankiewicz *et al*., 1988) express an ecto-ATPase on the cell surface, it is possible that the lower potency of ATP may be due to its more rapid hydrolysis compared to BzATP. ARL-67156 (300 μM), a recently described ecto-ATPase inhibitor (Crack *et al*., 1995), was preincubated with fura-2 loaded cells for 5 min at 37°C, which were then stimulated with ATP or BzATP in the presence of 0.25 mM Ba^{2+}. ATP- and BzATP-induced Ba^{2+} fluxes were not potentiated by ARL-67156 but rather inhibited by about half (results not shown), indicating that the ecto-ATPase inhibitor has other pharmacological effects. Ecto-ATPase is both Ca^{2+}- and Mg^{2+}-dependent

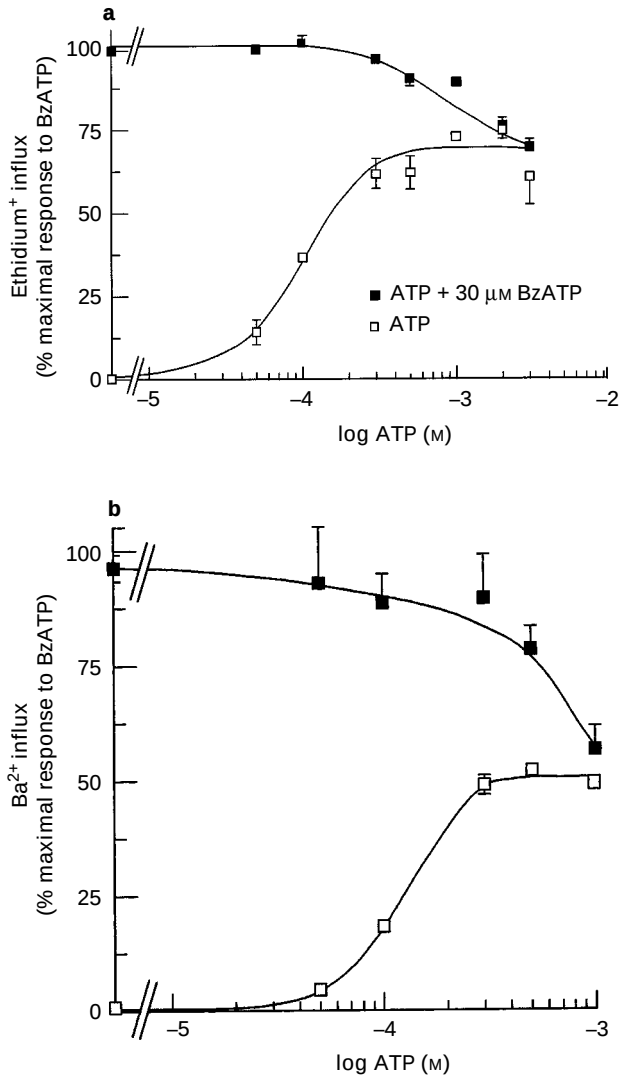

Figure 5 Partial inhibition of BzATP-stimulated cation fluxes by ATP. (a) Lymphocytes (10^6 ml^{-1}) were suspended in Ca^{2+}-free HEPES buffered KCl medium and incubated with either ATP alone or 30 μM BzATP added simultaneously with ATP for 2 min at 37°C before the addition of 25 μM ethidium$^+$. (b) Fura-2 loaded lymphocytes (2×10^6 ml^{-1}) were suspended in Ca^{2+}-free HEPES buffered KCl medium at 37°C in a stirred fluorimeter cuvette, 0.25 mM Ba^{2+} was added 1 min before the incubation with either ATP alone or 30 μM BzATP added simultaneously with ATP. Initial rates of ethidium$^+$ or Ba^{2+} influx were measured and results expressed as a percentage of response to 30 μM BzATP alone, which was defined as 100% response. The concentration-response curves were calculated by non linear regression analyses. Mean values, with vertical lines showing s.e.mean, from 3 experiments on 3 patients are presented.

(Ziganshin *et al*., 1994) and since neither of these cations were present in our experiments, it is unlikely that ecto-ATPase significantly affected the concentration-response curves of nucleotide agonists.

Discussion

ATP was a partial agonist for the lymphocyte P2Z receptor

This study showed that extracellular ATP, the natural ligand for the lymphocyte P2Z receptor, is a partial agonist, while BzATP is a more efficacious agonist for this receptor. Several lines of evidence support this conclusion. Firstly, maximal responses to ATP were less than BzATP for three different

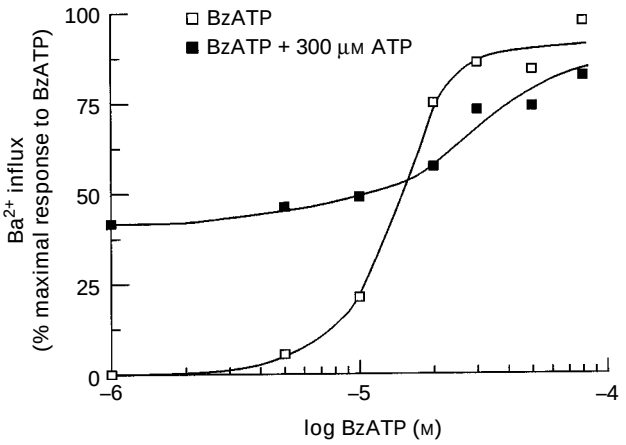

Figure 6 ATP inhibition of BzATP-stimulated Ba^{2+} influx. Fura-2 loaded lymphocytes (2×10^6 ml^{-1}) suspended in HEPES buffered saline medium were pretreated with 50 μM ox-ATP for 20 min at 37°C, washed twice and resuspended (2×10^6 ml^{-1}) in Ca^{2+}-free HEPES buffered KCl medium at 37°C in a stirred fluorimeter cuvette. Ba^{2+} (0.5 mM) was added 1 min before incubation with either BzATP alone or 300 μM ATP added simultaneously with BzATP. Initial rates of Ba^{2+} influx were measured and results expressed as a percentage of maximal response to BzATP alone of ox-ATP pretreated lymphocytes, which was defined as 100% response. Means of duplicates from a single experiment are shown (values showed less than 12% variation from respective means).

P2Z-stimulated responses: ethidium$^+$ uptake, Ba^{2+} fluxes and activation of phospholipase D (Table 1). Secondly, high concentrations of ATP competitively inhibited BzATP-stimulated cation fluxes in cells with and without partial receptor inactivation (Figures 5 and 6). These results also suggest that ATP, BzATP and the irreversible antagonist, ox-ATP all bind to the same P2Z receptor.

ATP is not only a partial agonist for the P2Z receptor, but also for the chick brain $P2Y_1$ receptor, giving 70% of the response elicited by 2MeSATP (Barnard *et al*., 1996), while for the P2Y receptor of rat brain microvascular endothelial cells, ATP produced a response that was only 55% of that for the full agonist, ADP (Feolde *et al*., 1995). However, ATP is generally a full agonist for cloned and native $P2X_{1-6}$ receptors, while various synthetic ATP analogues such as 2MeSATP, ATPγS and BzATP are partial agonists (Surprenant, 1996). 2MeSATP and ATPγS, like ATP also appeared to be partial agonists for the lymphocyte P2Z receptor (Figure 2, Table 1, Wiley *et al*., 1994).

Steep concentration-response curves for the effects of ATP have been documented for permeability responses mediated by both P2X and P2Z receptors, with n_H values of approximately 3 and 2, respectively (Tatham & Lindau, 1990; Wiley *et al*., 1990; 1992; 1993; Pizzo *et al*., 1991; Bean, 1992). These steep responses have been attributed to allosteric effects mediated by ATP on putative multisubunit ion channels (Bean, 1992; North, 1996). In this study, greater n_H values derived for BzATP (3.3 and 3.2; Figure 3a) compared with ATP (2.1 and 2.4; Figure 3b) suggest that in the lymphocyte P2Z system the more efficacious agonist exerts a greater positive co-operative effect than a partial agonist. The steepness of the BzATP concentration-response curve cannot be explained by the presence of spare receptors, since reduction of available receptors by the irreversible antagonist, ox-ATP, had little effect on either the location parameter (EC_{50}) or the steepness of the curve (n_H values ≈ 3.0) (Figure 4).

Differential inhibitory potency of KN-62 for ATP- and BzATP-mediated responses

The isoquinolinesulphonamide derivative, KN-62, was found to be a potent inhibitor of ATP-stimulated Ca^{2+}, Ba^{2+} and

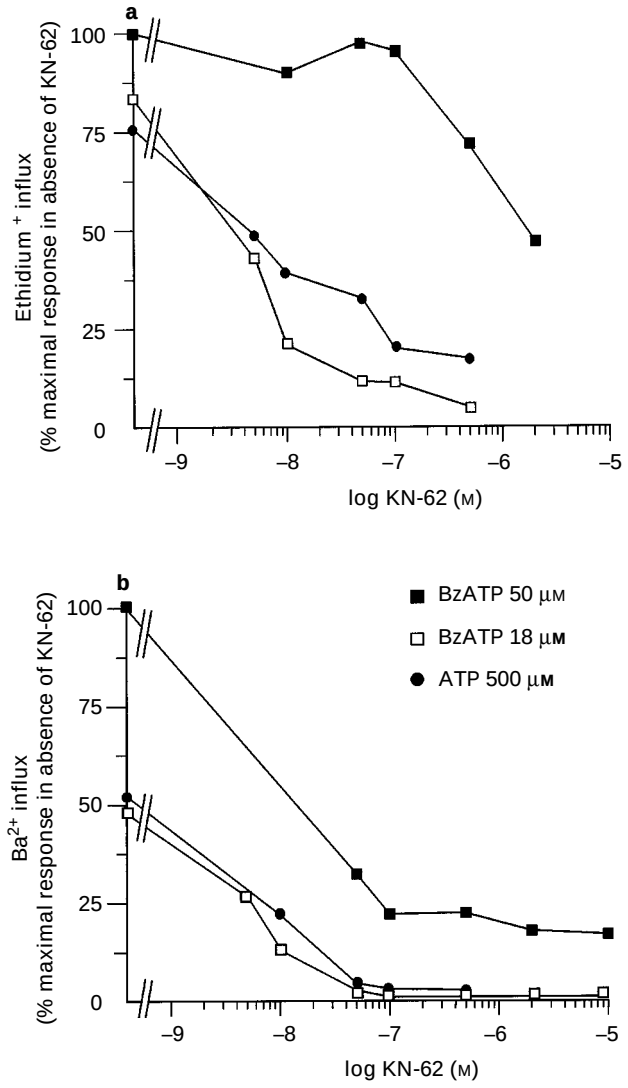

Figure 7 Inhibition of ATP- and BzATP-stimulated cation fluxes by KN-62. (a) Lymphocytes (10^6 ml^{-1}) suspended in Ca^{2+}-free HEPES buffered KCl medium, were preincubated with KN-62 for 15 min at 37°C followed by addition of BzATP or ATP and 25 μM ethidium$^+$. (b) Fura-2 loaded lymphocytes (2×10^6 ml^{-1}) were suspended in Ca^{2+}-free HEPES buffered KCl medium, preincubated with KN-62 for 15 min at 37°C in a stirred fluorometer cuvette, and 0.4 mM Ba^{2+} was added 1 min before the incubation with BzATP or ATP. Initial rates of ethidium$^+$ or Ba^{2+} influx were measured and results expressed as a percentage of maximal response to 50 μM BzATP in the absence of KN-62, which was defined as 100% response. Results from a single experiment are shown, representative of 2.

ethidium$^+$ fluxes (IC_{50} of 13 nM), ATP-stimulated PLD activity (IC_{50} 6 nM) and ATP-induced shedding of L-selectin, which are all P2Z-mediated responses of the human lymphocyte (Gargett & Wiley, 1997). The specificity of KN-62 has been shown by its lack of effect on the neutrophil $P2Y_2$ receptor (Gargett & Wiley, 1997) or the $P2X_1$ receptor of guinea-pig urinary bladder (Cocks, T. Gargett C.E. and Wiley, J.S., unpublished observation). In this study, KN-62 was an incomplete and less potent inhibitor of cation fluxes mediated by maximal BzATP concentrations (Figure 7). Furthermore, high concentrations of BzATP overcame the inhibitory effect of KN-62 after a delay of approximately 1 min (results not shown). Our previous data suggest that KN-62 may bind to one of the multiple agonist binding sites, since n_H values of ≈ 1.0 were obtained for the KN-62 inhibitory effect on P2Z-mediated responses (Gargett & Wiley, 1997). It has been suggested that the isoquinoline sulphonamides are competitive antagonists for the ATP-binding site of protein kinases, since they occupy the cleft that binds ATP (Xu *et al*., 1996), but the locus of the KN-62 effect on the P2Z receptor has not been defined.

Human leukaemic lymphocytes only express the P2Z receptor subtype

The presence of multiple P2-receptor subtypes complicates any analysis of relative potencies and maximal activities of an agonist series. For example, mast cells co-express $P2Y_1$ and P2Z receptors (Cockcroft & Gomperts, 1979; Osipchuck & Cahalan, 1992) and macrophages both $P2Y_2$ and P2Z receptors (Nuttle *et al*., 1993). In the above cells, any observed nucleotide or antagonist potency order will depend on the proportions of different types of P2-receptors present (O'Connor *et al*., 1991). Competitive experiments between different agonists distinguish true partial agonism from interaction of agonists with different classes of P2-receptors. For example, the failure of 2MeSATP, the putative partial agonist, to inhibit responses of piglet aortic endothelium to the full agonists ATP, UTP or ATPγS, aided the discovery of coexisting $P2Y_1$ and $P2Y_2$ receptors on endothelial cells (Needham *et al*., 1987; O'Connor *et al*., 1991). In contrast, a single class of P2-receptors was shown on the B10 clone of rat brain microvascular endothelial cells by competitive inhibition of ADP responses by the partial agonist ATP (Feolde *et al*., 1995). Likewise, our data (Figures 5 and 6) show that only one class of P2-receptor (P2Z class) is expressed on human leukaemic lymphocytes.

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