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Effects of antagonists at the human recombinant $P2X_7$ receptor

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1 We have used whole-cell patch clamping methods to examine the properties of the recombinant human $P2X_7$ (P2Z) receptor stably expressed in HEK-293 cells.

2 In an extracellular solution with lowered concentrations of divalent cations (zero Mg^{2+} and 0.5 mM Ca^{2+}), both ATP and the nucleotide analogue, 2′- and 3′-O-(4-benzoylbenzoyl)-adenosine 5′-triphosphate (Bz-ATP) evoked concentration-dependent whole-cell inward currents with maxima of 4658 ± 671 and 5385 ± 990 pA, respectively, at a holding potential of -90 mV. Current-voltage relationships determined using 100 μM Bz-ATP reversed at -2.7 ± 3.1 mV, and did not display significant rectification.

3 Repeated applications of 300 μM Bz-ATP produced inward currents with similar rise-times (approx. 450 ms, 5–95% current development) but with progressively slower 95–5% decay times, with the eighth application of this agonist yielding a decay time of 197% of the first application.

4 Concentration-effect curves to ATP and Bz-ATP produced estimated EC_{50} values of 780 and 52.4 μM, respectively. Consecutive concentration-effect curves to Bz-ATP produced curves with similar maxima and EC_{50} values.

5 The non-selective P2 antagonists, pyridoxal-phosphate-6-azophenyl-, 2′,4′-disulphonic acid (PPADS) and suramin, both produced concentration-dependent increases in maximal inward currents to Bz-ATP, with IC_{50} concentrations of approximately 1 μM and 70 μM, respectively. The profile of antagonism produced by PPADS was not that of a competitive antagonist.

6 The isoquinolene derivatives 1-(N,O-bis[5-isoquinolinesulphonyl]-N-methyl-L-tyrosyl)-4-phenylpiperazine (KN-62) and calmidazolium both produced antagonism which was not competitive, with IC_{50} concentrations of approximately 15 and 100 nM, respectively. HMA (5-(N,N-hexamethylene)- amiloride) was also an effective antagonist at a concentration of 10 μM. The group IIb metal, copper, also displayed antagonist properties at the human $P2X_7$ receptor, reducing the maximum response to Bz-ATP by about 50% at a concentration of 1 μM.

7 These data demonstrate that the human recombinant $P2X_7$ receptor displays functional behaviour which is similar to the recombinant rat $P2X_7$ receptor, but has a distinct pharmacological profile with respect to agonist and antagonist sensitivity.

Keywords: Human $P2X_7$ receptors; suramin; ATP; antagonist; pore; KN-62

Introduction

Adenosine 5′-triphosphate (ATP) can cause receptor-mediated permeabilization of membranes in certain human cell types, including lymphocytes, macrophages and mast cells (Blanchard *et al*., 1995; Gargett *et al*., 1997; Hickman *et al*., 1994). The receptor mediating this effect was originally termed P2Z, whose activation induces membrane permeabilization by formation of large pores, with consequent cytolysis upon persistent stimulation by agonists (Pizzo *et al*., 1992; Chiozzi *et al*., 1996). On the basis of its unusual transductional characteristics, this receptor was considered distinct from either of the other two major classes of ATP receptors, the P2X and P2Y purinoceptors. However, the isolation of a cDNA encoding the P2Z receptor from a rat cDNA library (Surprenant *et al*., 1996), identified this receptor as belonging to the growing family of ionotropic P2X receptors, and thus this receptor has been termed $P2X_7$. More recently, the cDNA encoding the human homologue of the $P2X_7$ receptor has been isolated (Rassendren *et al*., 1997).

A number of studies were directed toward the characterization of the human P2Z receptor (Blanchard *et al*., 1995; Wiley *et al*., 1992; 1994; Hickman *et al*., 1994) prior to its identification as the $P2X_7$ receptor. However, many of these studies have revealed differing pharmacology with respect to agonist and antagonist activity. This may be attributable to the wide variety of methods used to study this receptor, from radiolabelled ion uptake, to fluoroscopic measurements of intracellular dyes. In addition, many of these studies have been performed in cell types where other P2 receptor subtypes are known to exist (Wiley *et al*., 1992; Blanchard *et al*., 1995). Given the pharmacological discrepancies, and the knowledge that there is at least one case of heteropolymerization between differing P2X subunits (Lewis *et al*., 1995), it seems possible that the presence of additional P2 subunits may alter the functional properties observed for the $P2X_7$ receptor. At the recombinant human receptor itself, only suramin and pyridoxal-phosphate-6-azophenyl- 2′,4′-disulphonic acid have been tested for antagonist activity (Rassendren *et al*., 1997). Other studies have revealed a marked species variation in the properties of $P2X_7$ receptors, particularly between rodent and human homologues. As an example, 5-(N,N-hexamethylene)-amiloride (HMA) has been shown to be an effective antagonist at the human P2Z receptor (Wiley *et al*., 1990), whereas it was ineffective at the mouse $P2X_7$ receptor (Chessell *et al*., 1997).

We have previously demonstrated that the whole-cell configuration of patch-clamp recording can be effectively used to give a direct readout of $P2X_7$ receptor activation. This technique has advantages over other techniques of studying the $P2X_7$ receptor, such as radiolabelled ion uptake, or fluorescent dye studies, as driving forces for ions can be more easily

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controlled by effective voltage clamp, agonists can be applied by concentration-clamp, and kinetics of receptor activation can be observed. In the present study, we have used this recording technique to examine the human recombinant $P2X_7$ receptor, stably expressed in HEK-293 cells. The aim of this study was to establish the pharmacological profile of various P2 antagonists at the homo-oligomeric form of this receptor, while avoiding many of the confounding factors present in other studies.

Methods

Cell line

Wild type Human Embryonic Kidney 293 (HEK-293; 1×10^6 cells) cells were transfected with human $P2X_7$ receptor plasmid (in pcDNA3; 5 μg; Rassendren *et al*., 1997) using an electroporator (EasyJect, FlowGen, U.K.). Cells were maintained in Dulbecco's modified Eagle medium (DMEM) nutrient mix supplemented with foetal calf serum (10%) and 0.5 mg ml^{-1} G418 (Gibco BRL, Paisley, U.K.), and selection-cloned using standard procedures. Stably transfected cells were incubated in a water-saturated atmosphere of 95% O_2/5% CO_2 and passaged when confluent by trypsinization (trypsin-EDTA 1 × solution). When required for study, cells were attached to glass coverslips (13 mm; Menzel-Glaser, Fisher, Leicester, U.K.) and used for electrophysiological investigations not less than 2 h after plating. Coverslips were used within 2 days.

Electrophysiological recording

For each experiment, coverslips were transferred to a perfused recording chamber (volume approximately 400 μl, flow rate 2 ml min^{-1}) mounted on the stage of an inverted microscope (Nikon Diaphot, Nikon U.K.). The whole cell configuration of the patch-clamp technique (Hamill *et al*., 1981) was used to record nucleotide-evoked ionic currents. Cells were perfused with a low-divalent cation containing solution, consisting of (in mM): NaCl 145, KCl 2, $CaCl_2$ 0.5, HEPES 10, D-glucose 10 (pH 7.3, osmolarity 300 mOsm). Patch electrodes were pulled from 1.2 mm borosilicate glass (GC120F-10, Clarke Electromedical Supplies, Pangbourne, U.K.), firepolished, and when filled with (in mM) Cs aspartate 145, EGTA 11, HEPES 5, NaCl 2 (pH 7.3, osmolarity 290 mOsm) had resistances of 2–5 MΩ.

Tight seal (>10 GΩ) whole-cell currents were recorded using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA, U.S.A.). Currents were filtered with a corner frequency of 1–5 kHz (8-pole Bessel filter), digitised at 2–10 kHz using a Digidata 1200A (Axon Instruments) interface, and stored on computer. Data was only recorded from cells with a residual series resistance of less than 18 MΩ, and compensation for series resistance was used (>75%). Cells were voltage-clamped at −90 mV, unless otherwise stated. For voltage-ramp experiments, reported voltages were corrected for the junction potential between the internal solution and the extracellular solution in which zero current was obtained before forming a seal. All experiments were performed at room temperature (22–24°C).

Experimental procedures

In all cases, agonists were applied using a computer-controlled fast-flow U-tube system (Fenwick *et al*., 1982), modified to include an extra solenoid valve, which allowed both rapid application and removal of applied drugs (onset and offset latencies of approximately 90 and 50 msec, respectively). In all cases where the effects of antagonists were investigated, these drugs were included at the appropriate concentrations in the U-tube application system.

Initially, concentration-effect curves to ATP, or 2′- and 3′-O-(4-benzoylbenzoyl)-adenosine 5′-triphosphate (Bz-ATP), were determined using a 2 s agonist application time with serially increasing agonist concentrations, and a wash period of 2 min between applications. Only one agonist was tested on each cell. In some cells it was noted that Bz-ATP caused successively longer deactivation times. In order to investigate these progressive changes in kinetics, Bz-ATP was repeatedly applied for 1 s to each naïve cell. This was repeated up to 10 times, with a 30 s wash period between successive applications. The effects of the nucleotides, adenosine 5′-diphosphate (ADP) and adenosine 5′-monophosphate (AMP) were tested on transfected cells at concentrations of 1 mM. Non-transfected wild-type HEK-293 cells were also tested for any endogenous P2X receptor subtypes by application of 1 mM ATP for 2 s.

For Bz-ATP, the effects of suramin, pyridoxal-phosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS), 1-(N,O-bis[5-isoquinolinesulphonyl]-N-methyl-L-tyrosyl)-4-phenylpiperazine (KN-62), calmidazolium, 5-(N,N-hexamethylene)-amiloride (HMA) and copper were investigated by continuous perfusion of various concentrations of antagonist for 10 min before and during determination of a second agonist concentration-effect curve. To investigate the kinetics of the antagonist actions of PPADS (Khakh *et al*., 1994), repeated applications of 100 μM Bz-ATP were made in the absence and presence of PPADS (1–10 μM) with increasing incubation times.

In some cases, current-voltage relationships for Bz-ATP induced responses were determined by performing voltage ramps from −80 to +60 mV in a 400 msec sweep.

Data are expressed as mean (±s.e.mean) absolute current evoked by agonists, mean percentage of the current evoked by 1 mM ATP, or mean percentage of the maximal inward current observed in a particular experimental paradigm. Where appropriate, concentration-effect curves were fitted using a three parameter logistic equation (GraphPad Prism, San Diego, CA, U.S.A.). EC_{50} values are expressed as geometric means with 95% confidence intervals. Differences in mean values were tested using the Student's paired or unpaired *t*-test, as specified in the text.

Materials

Dulbecco's modified Eagle medium (DMEM), foetal calf serum, G418 and trypsin-EDTA solution were all obtained from GibcoBRL (Paisley, Scotland). Adenosine 5′-triphosphate (ATP), 2′- and 3′-O-(4-benzoylbenzoyl)-ATP, 5-(N,N-hexamethylene)-amiloride, calmidazolium (CMZ) and 1-(N,O-bis[5-isoquinolinesulphonyl]-N-methyl-L-tyrosyl)-4-phenylpiperazine were all obtained from Sigma (Poole, U.K.). Pyridoxal-phosphate-6-azophenyl-2′,4′-disulphonic acid was obtained from Cookson (Southampton, U.K.). Suramin was a kind gift from Bayer. All other chemicals were purchased from Sigma or BDH Laboratory Supplies (Leicester, U.K.). The human recombinant $P2X_7$ receptor cDNA was kindly provided by Dr G. Buell (GBRI, Geneva, Switzerland).

Results

Effects of agonists

In low divalent cation containing solution, Bz-ATP evoked whole-cell inward currents at a holding potential of −90 mV

in all hP2X$_7$ transfected HEK-293 cells tested, with Bz-ATP being more potent than ATP. Application of 1 mM ADP or AMP produced no significant inward currents in hP2X$_7$-transfected cells (currents <10 pA, $n=5$ for both agonists). In non-transfected HEK-293 cells, application of 1 mM ATP evoked inward currents of no greater than 10 pA in all cells tested (mean current of 6.4 ± 1.3 pA, $n=17$). In hP2X$_7$ expressing cells, at the highest concentrations of the agonists tested (300 μM for Bz-ATP and 3 mM for ATP), inward currents were 5385.6 ± 990.9 and 4658 ± 671.1 pA, respectively ($n=22$ and 14). Concentration-effect curves to these agonists did not reach a clearly defined maximum, and thus only estimated EC_{50} values of 52.4 [36.3–75.6] μM and 779.9 [372.9–1631.0] μM could be determined, respectively (Figure 1). No significant tachyphylaxis was observed for either agonist with up to five applications at the highest concentrations tested (see below). Current-voltage relationships generated using 100 μM Bz-ATP were approximately linear between -60 and $+50$ mV, reversed at -2.72 ± 3.1 mV ($n=4$), and showed no significant rectification (Figure 1b).

Repeated applications, for 1 s, of 300 μM Bz-ATP, produced inward currents of similar amplitude (Figure 2a), with no significant difference between the amplitude produced by the first application (3092 ± 448 pA) and the eighth (4235 ± 768 pA). The rise-times of inward currents also did not vary over the eight application protocol (5–95% rise-times 490.2 ± 29.0 and 493.2 ± 23.7 msec, first and eighth application, respectively). However, a successive increase in the time for receptor deactivation (i.e. the time required for the current to return from its peak value, to basal values, after removal of the agonist), consistent with previous reports for the recombinant P2X$_7$ receptor (Rassdendren *et al.*, 1997), was observed (Figure 2a), such that the mean 95–5% current decay time for the eighth application was 197 ± 37% of the first application decay (Figure 2b). A significant change in the deactivation time was reached after 6 applications ($P<0.05$, Student's paired *t*-test). Deactivation kinetics would not be accurately described by first or second order exponentials.

Effects of antagonists

There were no significant differences between the EC_{50} values, or the maxima observed, between two consecutive concentration-effect curves to Bz-ATP, with a 10 min intervening wash period between curves (EC_{50} values of 86.9 [58.8–128.5] and 43.9 [32.6–59.1] μM, maxima of 202.2 ± 31.5 and 217.1 ± 48.3% of 1 mM ATP responses, first and second concentration-effect curves, respectively, $n=6$).

Application of PPADS caused a concentration-dependent decrease in Bz-ATP induced inward currents. At a concentration of 10 μM PPADS, responses to the highest concentrations of Bz-ATP were abolished (response to 300 μM Bz-ATP in the presence of 10 μM PPADS 1.5 ± 0.4% of the first curve maximum, $P<0.05$). Responses to Bz-ATP were not antagonized in a competitive manner, and hence pK_B values could not be determined for this antagonist. However, at all concentrations of Bz-ATP, the IC_{50} of PPADS was approximately 1 μM (Figure 3a). Because PPADS has been previously described as a slow-acting antagonist, we investigated the kinetics of antagonism by repeated applications of 100 μM Bz-ATP in the presence of PPADS at increasing timepoints (Figure 3b). The antagonist reaction of PPADS was critically

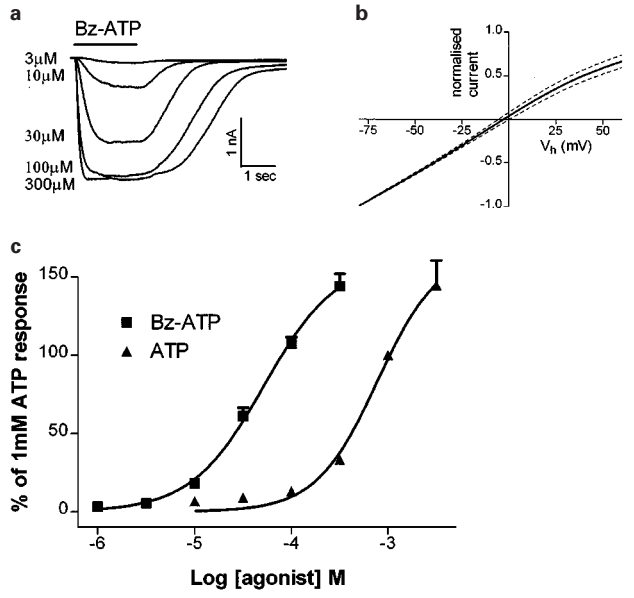

Figure 1 Characteristics of nucleotide-evoked responses in low divalent cation containing medium. (a) Steady-state, concentration-dependent inward currents to applications of Bz-ATP for 2 s (horizontal bar). (b) Current-voltage relationships for responses to 100 μM Bz-ATP, generated using a voltage ramp over a 400 msec period. For each cell, the mean of two control ramps was subtracted from the mean of two ramps generated in the presence of agonist ($n=4$). Dotted lines indicate s.e.mean. (c) Concentration-effect curves for Bz-ATP (■) and ATP (▲), $n=23$ and 15, respectively. Data points are the means ($\pm$s.e.mean) of n determinations.

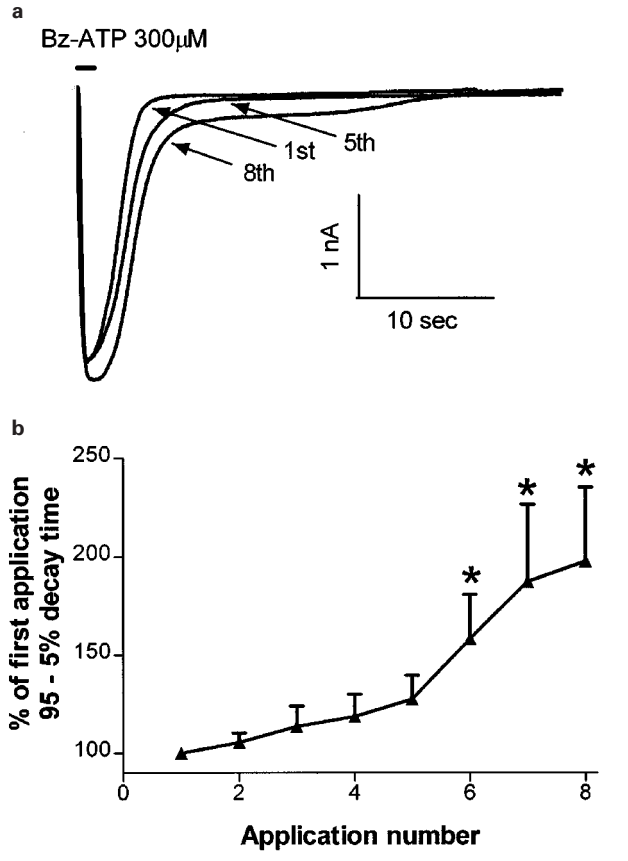

Figure 2 Effects of repeated applications of 300 μM Bz-ATP on hP2X$_7$ deactivation times. (a) Inward currents elicited by a 1 s application of Bz-ATP, showing responses to the first, fifth and eighth application. (b) Increase in time for current to decay from 95–5% of the peak current observed. *$P<0.05$, significantly different from 95–5% decay time of first application current, Student's paired *t*-test.

dependent on incubation time, with 50% reductions in responses to Bz-ATP for 1, 3 and 10 μM PPADS reached after approximately 8 min, 3 min and 70 s, respectively ($n=4$ for each concentration). The actions of PPADS were slowly reversible, with recovery of responses following a 15 min incubation with 1 μM PPADS to 67.2±12.6% of control after 30 min of washing.

The non-selective P2 antagonist, suramin, caused a concentration-dependent rightward-shift of the agonist concentration-effect curves to Bz-ATP (Figure 3c). When concentration-effect curves determined in the presence of suramin were fitted with maxima constrained to 100% of the control concentration-effect curves, the Hill slope estimates of these curves were not significantly different from control (1.1±0.2, 1.3±0.2 and 1.2±0.1 for curves determined in the presence of 1, 10 and 100 μM suramin, respectively, *cf* control curves 1.1±0.1). On the basis of this, the calculated agonist concentration ratio at a suramin concentration of 100 μM provided an antagonist pK_B estimate of 4.88±0.08, using the Gaddum-Schild equation, and assuming competitive antagonism (see Jenkinson *et al*., 1995). Following a 20 min washout period after incubation with 100 μM suramin, responses to 300 μM Bz-ATP partially returned, to 68.5±2.4% of the control curve maximum.

In a similar manner to PPADS, the isoquinoline derivative, KN-62, displayed non-competitive antagonism at the $hP2X_7$ receptor. This antagonist was considerably more potent than PPADS, with an IC_{50} value of approximately 25 nM at all concentrations of Bz-ATP (Figure 4a). At the highest concentration of KN-62 tested (100 nM), responses to 300 μM

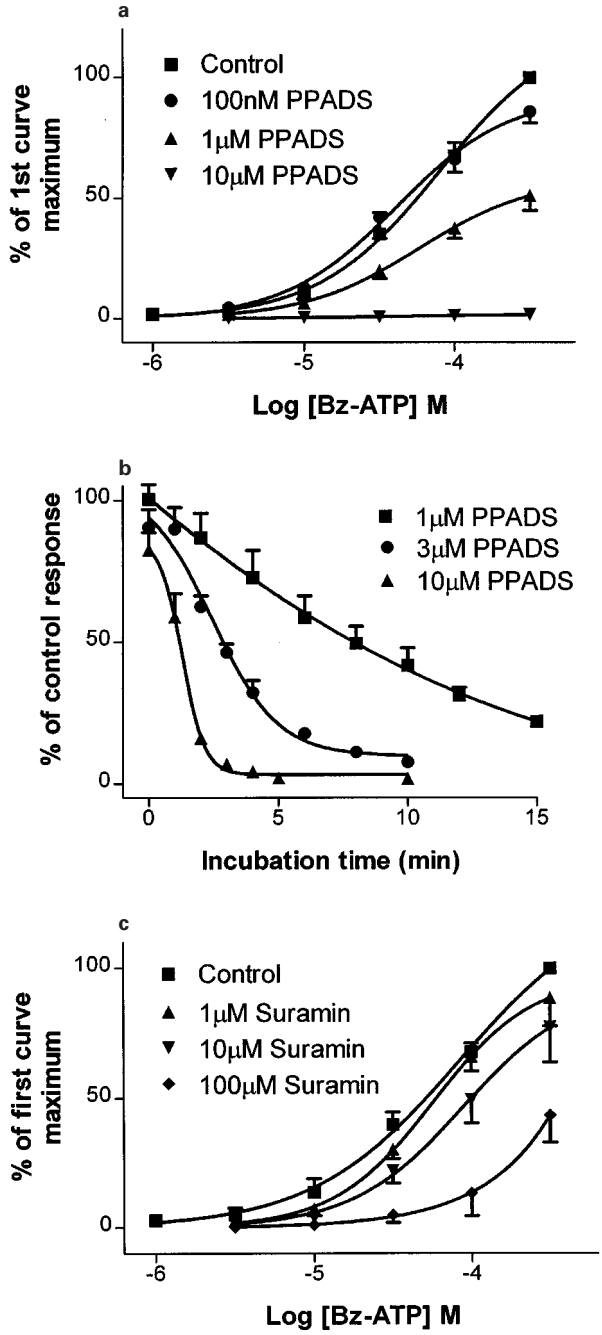

Figure 3 Effects of PPADS (a and b) and suramin (c) on Bz-ATP-induced inward currents. For (a) and (c), first concentration-effect curve determinations (■) were followed by determination of a second concentration-effect curve in the continued presence of various concentrations of PPADS or suramin, respectively. Incubation times with antagonists between curves were 10 min. One concentration of antagonist was evaluated on each cell, with at least 4 determinations made for each antagonist concentration. For (b), repeated applications of 100 μM Bz-ATP were made in the presence of 1 (■), 3 (●) or 10 (▲) μM PPADS, respectively, at the indicated time points. At least 4 determinations were made for each data point. Data points are expressed as the mean (±s.e.mean) percentage of the first curve maximum.

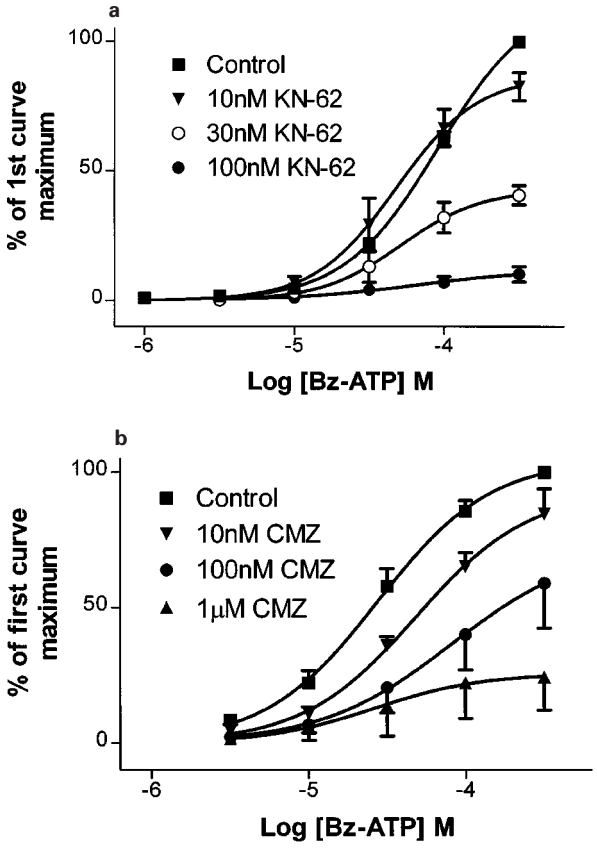

Figure 4 Effects of KN-62 (a) and calmidazolium (CMZ; b) on Bz-ATP-induced inward currents. In both cases, first concentration-effect curve determinations (■) were followed by determination of a second concentration-effect curve in the continued presence of various concentrations of KN-62 or calmidazolium. Antagonists were added to the perfusion buffer 10 min before, and during determination of the second curve. One concentration of antagonist was evaluated on each cell, with at least 4 determinations made for each antagonist concentration. Data points are expressed as the mean (±s.e.mean) percentage of the first curve maximum.

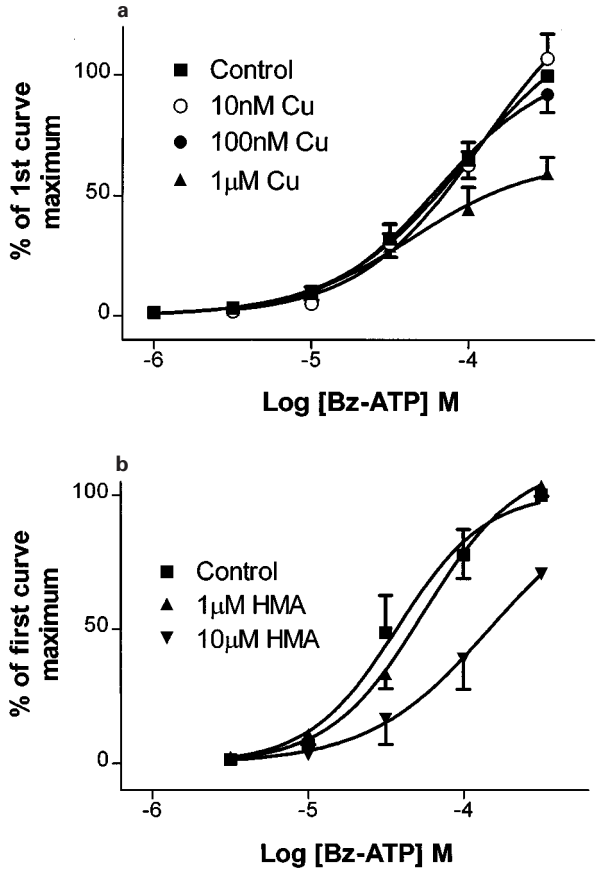

Figure 5 Effects of copper (a) and HMA (b) on Bz-ATP-induced inward currents. In both cases, first concentration-effect curve determinations (■) were followed by determination of a second concentration-effect curve in the continued presence of various concentrations of copper or HMA. Incubation times with antagonists between curves were 10 min. One concentration of antagonist was evaluated on each cell, with at least 4 determinations made for each antagonist concentration. Data points are expressed as the mean (±s.e.mean) percentage of the first curve maximum.

Bz-ATP were 10.3±2.9% of the first curve maximum. The effects of KN-62 were not reversed after a 20 min washout period (data not shown). Calmidazolium produced similar effects as KN-62, but was less potent, with an approximate IC_{50} of 100 nM (Figure 4b). At the highest concentration of calmidazolium tested (1 μM), responses to 300 μM Bz-ATP were 24.4±12.0% of the first curve maximum. Responses were not restored after 20 min of washing.

Incubation of cells with increasing concentrations of the group IIb metal, copper, failed to produce significant antagonism of Bz-ATP induced responses (Figure 5a), except at the highest concentration of copper tested (1 μM). There was a significant decrease in the response to 300 μM Bz-ATP in the presence of 1 μM copper, to 59.4±6.8% of the first curve maximum, but no significant differences in the amplitude of Bz-ATP induced responses were observed at any other Bz-ATP, or copper, concentration.

HMA, which has been reported to inhibit responses at some P2Z purinoceptors (Nuttle & Dubyak, 1994) was also effective at blocking inward currents at the $hP2X_7$ receptor (Figure 5b). However, it should be noted that at concentrations higher than 10 μM, HMA compromised the quality of the seal, and thus precluded determination of the effects of higher concentrations of this antagonist. At a concentration of 10 μM, HMA reduced the maximal response to Bz-ATP to 70.5±2.1% of the first curve maximum. As with the other antagonists, the effects of HMA, where noted, appeared to be irreversible over the time scale of the experiment (20 min washing).

Discussion

In this study, we have used whole-cell patch-clamping methods to characterise the properties of the recombinant human $P2X_7$ receptor expressed in HEK-293 cells. This is the first time that a number of the antagonists used in this study have been studied at this recombinant receptor expressed in isolation. In extracellular solution containing 0.5 mM Ca^{2+} and no exogenously added Mg^{2+}, Bz-ATP was a more potent agonist than ATP, while ADP and AMP were ineffective. Repeated applications of the highest concentration of Bz-ATP (300 μM) produced inward currents of similar size, with similar risetimes. However the time for deactivation of the receptor (i.e. the time taken for the currents to return to basal values after removal of the agonist) increased progressively with serial agonist applications. This operational profile is very similar to that described previously for the recombinant $hP2X_7$ receptor expressed in HEK-293 cells (Rassendren *et al*., 1997). Of the compounds tested for antagonism at this receptor in the present study, the isoquinoline derivative, KN-62, was the most potent, followed by calmidazolium, PPADS, and copper. Suramin and HMA were the least potent antagonists tested. All of these compounds inhibited inward currents in a concentration-dependent manner, but only suramin displayed competitive antagonism. Quantitative differences in the profiles of these antagonists were found between the human $P2X_7$ receptor, and those observed for rodent and human $P2X_7$ receptors in previous studies, and these are discussed below.

A significant difference was noted between the operational profile of the $hP2X_7$ receptor, and that described for the mouse $P2X_7$ receptor found in microglial cells (Chessell *et al*., 1997) with respect to the changes observed in current amplitude and deactivation kinetics. For the mouse $P2X_7$ receptor, repeated applications of agonist produced successively larger inward currents, which reached a plateau after eight applications, whereas for the $hP2X_7$ receptor, inward currents elicited by the eighth application of agonist were not significantly different from those elicited by the first application. In addition, the rate of deactivation of the mouse $P2X_7$ receptor remained constant with successive agonist applications, whereas the recombinant $hP2X_7$ receptor displays a phenotype which is similar to that described for the $rP2X_7$ receptor (Surprenant *et al*., 1996). The underlying basis for these phenotypic differences remains to be fully explored, and the possibility that further P2X receptor subunits remain to be cloned cannot be excluded, but one possibility is that in cells expressing a $P2X_7$ phenotype endogenously, such as in the NTW8 microglial cells, a heteropolymeric combination of various P2X subunits exists. It seems unlikely that any endogenous receptor, capable of forming hetermultimeric combinations with the transfected receptors, exists in HEK-293 cells, as in our experiments, application of a high concentration of ATP failed to produce significant currents in the wild-type HEK-293 cells. The precise relationship between the observations of current augmentation, increases in deactivation time, and the formation of the large pore, characteristic of the $P2X_7$ receptor, also remains to be established. However, it is apparent that both extension of deactivation time and augmentation of inward current, for the human and rat recombinant $P2X_7$ and the mouse microglial $P2X_7$ receptors respectively, are associated with the formation

of the large pore, as evidenced by uptake studies of YO-PRO, a propridium dye with a molecular weight of approximately 380 (Chessell *et al*., 1997; Rassendren *et al*., 1997).

Two inhibitors of calmodulin-dependent enzymes, KN-62 and calmidazolium, were potent inhibitors of Bz-ATP induced inward currents in the present study. The potent antagonist action of KN-62 is consistent with previous reports of inhibition of the human P2Z receptor in lymphocytes (Gargett & Wiley, 1997) and human macrophages (Blanchard *et al*., 1995), where ethidium bromide uptake, ion flux, and cell lysis were used as indices of receptor activation. It seems unlikely that the effects of KN-62 can be attributed to its known action on CaM kinase II, since the IC_{50} value for enzyme inhibition is approximately 1 μM, and in the present study, marked abrogation of inward currents induced by Bz-ATP was observed at concentrations as low as 30 – 100 nM. These values are similar to those observed in experiments where KN-62 blocked uptake of YO-PRO-1, in cells expressing the hP2X$_7$ receptor (Michel *et al*., 1998). It is interesting to note that KN-62 did not block ATP-mediated membrane depolarization in human macrophages, but did prevent cell lysis (Blanchard *et al*., 1995), which may suggest that KN-62, like calmidazolium (Virginio *et al*., 1997), can differentiate between 'channel' and 'pore' forms of the receptor in some types of experiments. Calmidazolium has also been reported to block the P2Z receptor present in human macrophages (Blanchard *et al*., 1995), with an IC_{50} value of approximately 2 μM. This is considerably higher than the IC_{50} derived in the present study, of approximately 100 nM, and again may reflect differences in the methods used or the presence of additional P2X subunits. The use of these two antagonists also reveals species differences between the human and rat P2X$_7$ receptor, as shown by recent findings that calmidazolium is a very potent antagonist at the rat recombinant P2X$_7$ receptor, with an IC_{50} value of 13 nM (Virginio *et al*., 1997), while KN-62 is without significant antagonist activity in blocking YO-PRO-1 uptake at this P2X$_7$ homologue (A.D. Michel, unpublished observations).

The non-selective P2 antagonists, suramin and PPADS both caused concentration-dependent decreases in the maximum responses obtained to Bz-ATP. Both of these antagonists were more effective at blocking the human P2X$_7$ receptor than the rat and mouse homologues (Surprenant *et al*., 1996; Chessell *et al*., 1997). In the one previous study describing the effects of these antagonists at the human recombinant receptor (Rassendren *et al*., 1997), both PPADS and suramin did antagonise Bz-ATP induced responses, with IC_{50} values of 62 and 92 μM, respectively. In the present study, PPADS appeared to be more potent, with an IC_{50} value of approximately 1 μM, respectively, while suramin was of similar antagonist potency (IC_{50} value of approximately 70 μM). The reason for the discrepancy with PPADS is not clear, but it may be related to the longer incubation times required to observe the antagonist effects of PPADS (Khakh *et al*., 1994). Indeed, in the present study, we have shown that the effects of PPADS are critically dependent on incubation time, and little antagonism was apparent when the agonist and antagonist were co-applied, without any prior pre-incubation. PPADS was also effective at inhibiting Bz-ATP induced YO-PRO influx in cells expressing the recombinant human P2X$_7$ receptor (Michel *et al*., 1998), where the pIC_{50} values obtained in that study were similar to those observed in the present study. Suramin, a weak antagonist at the human receptor, was nevertheless more potent at this receptor than at the rodent P2X$_7$ receptor homologues; at both the rat (Surprenant *et al*., 1996) and mouse (Chessell *et al*., 1997) P2X$_7$ receptors, the IC_{50} values of suramin were in excess of 100 μM, although it was not possible to determine exact values in those studies.

The amiloride derivative, HMA, has been reported to block P2Z receptors, for both murine (Nuttle & Dubyak, 1994) and human species variants (Wiley *et al*., 1992), although the degree of antagonism was dependent on ionic concentrations, methods of study, and temperature. In addition, one study proposed that HMA had effects only on a subpopulation of P2Z receptors, and rapidly gated Bz-ATP-triggered inward currents were not affected (Nuttle & Dubyak, 1994). In the present study, HMA did have a small but significant effect on Bz-ATP-induced inward currents at a concentration of 10 μM, which is a lower effective concentration than that described in other studies, but the effects of HMA were difficult to fully explore, since higher concentrations caused a loss of seal. Nevertheless, the results obtained in the present study are in keeping with those described for HMA in human lymphocytes, where, at concentrations of 40 μM or more, ATP-induced Na^+ ion flux was largely blocked (Wiley *et al*., 1990). It is intriguing that opposing effects of HMA were observed in studies using NTW8 mouse microglial cells, where this compound caused augmentation of inward currents (Chessell *et al*., 1997). The reasons for these differences are not clear, and may be related to species variation.

At the recombinant hP2X$_7$ receptor, copper caused inhibition of maximum responses to Bz-ATP, with an IC_{50} of approximately 1 μM. Similar effects were observed at the rat recombinant P2X$_7$ receptor (Virginio *et al*., 1997), P2X$_7$ receptors endogenously expressed in CHO-K1 cells (Michel *et al*., 1997), and NTW8 mouse microglial cells (I. Chessell, unpublished observations). Given that the group IIb metals often potentiate responses at other P2X receptors (Brake *et al*., 1994; Soto *et al*., 1998; Michel *et al*., 1997), these data suggest that these metal ions can be used to differentiate between some of the identified P2X receptor types and may prove to be useful in characterizing endogenous P2X receptors.

In summary, using electrophysiological techniques, we have identified several antagonists of the recombinant human P2X$_7$ receptor. There are currently very few antagonists that are selective for any single P2X receptor type, or even that distinguish between P2X and P2Y receptor classes. Further investigations are required to determine the activity of some of the compounds described in the present study not only at other P2X receptor types, but also at heteropolymeric combinations of the P2X$_7$ receptor with other P2X subunits. Nonetheless, there is already sufficient data to suggest that these compounds should prove to be useful as tools to investigate the properties of the human P2X$_7$ receptor.

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