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Apparent species differences in the kinetic properties of $P2X_7$ receptors

[1]A.D. Hibell, [1,2]E.J. Kidd, [1]I.P. Chessell, [1]P.P.A. Humphrey & *,[1]A.D. Michel

[1]Glaxo Institute of Applied Pharmacology, Department of Pharmacology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QJ

1 Apparent species differences in the responses of recombinant $P2X_7$ receptors to repeated application of 2′- and 3′-*O*-(4-benzoylbenzoyl)-ATP (BzATP) have been investigated.

2 Repeated application of 100 μM BzATP resulted in a progressive increase in current magnitude (current growth) at mouse and human, but not rat $P2X_7$ receptors.

3 Current growth was thought to reflect progressive dilation of the $P2X_7$ ion-channel to a pore permeable to large molecules (MW $<$ 900), suggesting that channel dilation was not occurring at the rat $P2X_7$ receptor. However, 100 μM BzATP produced a rapid influx of YO-PRO-1 (MW375) in cells expressing rat or human $P2X_7$ receptors.

4 There were, however, species differences in agonist potency such that 100 μM BzATP was a supra-maximal concentration at rat, but not human or mouse, $P2X_7$ receptors. Importantly, when sub-maximal concentrations of BzATP or ATP were examined, current growth occurred at rat $P2X_7$ receptors.

5 The rate of current growth and YO-PRO-1 accumulation increased with agonist concentration and appeared more rapid at rat and human, than at mouse $P2X_7$ receptors.

6 The potency of BzATP and ATP was 1.5–10 fold lower in naïve cells than in cells repeatedly exposed to ATP.

7 This study demonstrates that current growth occurs at mouse, rat and human $P2X_7$ receptors but only when using sub-maximal concentrations of agonist. Previously, current growth was thought to reflect the progressive increase in pore diameter of the $P2X_7$ receptor ion channel, however, the results of this study suggest a progressive increase in agonist potency may also contribute.
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Abbreviations: BzATP, 2′- and 3′-*O*-(4-benzoylbenzoyl)-ATP; CEC, concentration-effect curves; DMEM, Dulbecco's modified Eagle's medium; FBS, foetal bovine serum; G418, geneticin sulphate; YO-PRO-1, quinolinium,4-[(3-methyl-2 (3H) -benzoxazolylidene) methyl]-1[3-(triethylammonio) propyl]-, diiodide

Introduction

The $P2X_7$ receptor is a ligand-gated cation channel activated by extracellular ATP. It has been described as a bi-functional entity (Surprenant *et al*., 1996) since it is known that with brief agonist exposure the ion-channel is only permeable to small inorganic cations including calcium, potassium and sodium but, with repeated or prolonged exposure to agonist, the channel dilates and becomes permeable to molecules as large as 900 Da (Steinberg *et al*., 1987; Nuttle & Dubyak, 1994).

The rat, human and mouse $P2X_7$ receptors have been cloned and their pharmacological and functional properties determined (Surprenant *et al*., 1996; Rassendren *et al*., 1997; Chessell *et al*., 1998). The species orthologues possess different pharmacological properties with the rat and human $P2X_7$ receptors having much higher affinity for agonists than the mouse $P2X_7$ receptor (Chessell *et al*., 1998). There are also marked species differences in responses of $P2X_7$ receptors to repeated application of agonist. Thus, repeated agonist application leads to a progressive increase in current (current growth) in cells expressing the mouse (Chessell *et al*., 1998), but not the rat or human $P2X_7$ receptors (Rassendren *et al*., 1997; Surprenant *et al*., 1996). Furthermore, with repeated agonist application, the time taken for responses to decay to baseline after agonist removal (decay time) increases at the rat $P2X_7$ receptor (Surprenant *et al*., 1996), whereas changes in decay time are less pronounced at the human $P2X_7$ receptor (Rassendren *et al*., 1997) and not detected at the mouse $P2X_7$ receptor (Chessell *et al*., 1997). The changes in decay time and current growth with repeated agonist application are either irreversible or slowly reversible ($>$20 min) and have been suggested to be associated with the dilation of the $P2X_7$ channel (Chessell *et al*., 1997).

In the present study we have further examined the responses of rat, human and mouse $P2X_7$ receptors to repeated and sustained agonist application in order to determine the reasons for the inability to observe current growth at the rat $P2X_7$ receptor. Current growth has been studied using whole-cell patch-clamp electrophysiology while $P2X_7$ receptor-mediated pore formation has been studied by measuring the cellular accumulation of quinolinium,4-[(3-methyl-2 (3H) -benzoxazolylidene) methyl]-1[3-(triethylammonio) propyl]-, diiodide (YO-PRO-1) using confocal microscopy or a 96-well plate reader. YO-PRO-1 is a membrane-impermeant cation (629 Daltons for the di-iodide salt; 375 Daltons for free base) whose fluorescence increases upon binding nucleic acids and thus

*Author for correspondence;
E-mail: adm7393@glaxowellcome.co.uk
[2]Current address: Department of Pharmacology, Welsh School of Pharmacy, Cardiff University, Redwood Building, King Edward VII Avenue, Cardiff, CF1 3XF.

represents an excellent probe for detecting permeability changes in $P2X_7$ receptor channels (Hickman *et al*., 1994; Surprenant *et al*, 1996; Michel *et al*., 1999).

Methods

Cell culture

HEK293 cells stably expressing the rat (Surprenant *et al*., 1996), human (Rassendren *et al*., 1997) or mouse (Chessell *et al*., 1998) orthologues of the $P2X_7$ receptor were used in all experiments. 1×10^6 wild-type HEK293 cells, were transfected with 5 μg of the relevant $P2X_7$ cDNA plasmid construct using an electroporator (EasyJet, EquiBio, Kent, U.K.). Transfected cells were selected in a growth medium comprising Dulbecco's modified Eagle's medium F12 supplement mix (DMEM/F12) containing 10% foetal bovine serum (FBS) and 500 μg ml^{-1} geneticin sulphate (G418) to obtain stable expression of the relevant $P2X_7$ receptor. Cells were passaged when confluent by incubation in trypsin and dilution into fresh growth medium.

Electrophysiological recordings

Recordings from HEK293 cells stably transfected with recombinant $P2X_7$ receptors were made essentially as described (Chessell *et al*., 1997). Briefly, cells were perfused with extracellular solution containing a reduced divalent cation concentration, consisting of (in mM): NaCl 145, KCl 2, $CaCl_2$ 0.5, HEPES 10, D-glucose 10 (pH 7.3, osmolarity 300 mOsm). All experiments were performed at 22–24°C. Whole cell patch-clamp recordings (Hamill *et al*., 1981) were made with electrodes (resistance 2–5 MΩ) filled with (in mM): Cs aspartate 145, EGTA 11, HEPES 5, NaCl 2 (pH 7.3, osmolarity 290 mOsm). In some studies the NaCl of the extracellular solution was substituted with 145 mM Na-glutamate. Currents were filtered with a corner frequency of 1–5 kHz (8-pole Bessel filter), digitized at 2–10 kHz using a Digidata 1200A (Axon Instruments, Palo Alto, CA, U.S.A.) interface, and stored on computer. Data was only obtained from cells with a residual series resistance of less than 18 mΩ. Cells were voltage clamped at −90 mV.

Concentration-effect curves (CEC) for the ability of ATP and 2′- and 3′-*O*-(4-benzoylbenzoyl)-ATP (BzATP) to elicit inward currents were determined by application of agonists to naïve cells for either 0.5 s (for rat $P2X_7$) or 2 s (for human and mouse $P2X_7$) using a computer controlled fast-flow U-tube system (Fenwick *et al*., 1992). Current growth was studied by repeated application of agonist (for 2 s every 20–30 s), up to 30 times. CEC to BzATP were also determined on cells which had been repeatedly exposed to 1 mM ATP every 30 s until there was no further current growth.

Confocal microscopy

HEK293 cells stably transfected with rat, human or mouse $P2X_7$ receptors were maintained as described above. Cells were plated on poly-l-lysine coated (20 μg ml^{-1} poly-l-lysine) 22 mm diameter glass coverslips (Chance Proper, Agar Scientific Ltd., Essex, U.K.) at a density of 2×10^5 cells per coverslip. When required for study, coverslips were removed from the wells and placed in coverslip holders (Digitimer, Welwyn Garden City, Herts., U.K.). The cells were incubated in a buffer of similar composition to the extracellular recording solution used in the electrophysiological studies, but also containing YO-PRO-1 (1 μM). All experiments were performed at 22–24°C. The coverslips were visualized using a confocal laser scanning microscope (Zeiss LSM 510, Welwyn Garden City, Herts., U.K.) with an excitation wavelength of 488 nm and all emitted light above 505 nm was collected. Images were collected every second at a resolution of 512×512 pixels and BzATP (100 μM) was added after eight images had been scanned and the cells were imaged for a further 30 min. The intensity of YO-PRO-1 accumulation in the cells was measured using the region of interest analysis software provided with the LSM 510 microscope. An average of 10 cells were analysed on each coverslip and each experiment was repeated at least three times. Data for cells containing the mouse $P2X_7$ receptor in NaCl buffer are the average of four cells which were examined in one experiment.

YO-PRO-1 accumulation in cell suspensions

Studies on the cellular accumulation of YO-PRO-1 in cells suspensions were performed as described previously (Michel *et al*., 1999). Briefly, confluent HEK293 cells, stably expressing the rat, human or mouse recombinant $P2X_7$ receptors were harvested, washed in ice-cold phosphate buffered saline and centrifuged at $250 \times g$ for 5 min. The supernatant was discarded and the cells washed once in assay buffer consisting of (in mM): sucrose 280, HEPES 10, *N*-methyl-D-glucamine (NMDG) 5, KCl 5.6, D-glucose 10, $CaCl_2$ 1 (pH 7.4). YO-PRO-1 accumulation was studied at 37°C by addition of 100 μl of cell suspension to 50 μl of buffer containing YO-PRO-1 (final assay concentration 1.4 μM) and BzATP in a 96-well polystyrene 1/2 area flat bottom plate (Costar, Bucks, U.K.). Fluorescence was measured from below the plates in a plate reader (FluoroCount, Canberra-Packard, Berks, U.K.) using an excitation wavelength of 485 nm and emission wavelength of 530 nm.

Data analysis

All data are expressed as the mean ± s.e.mean. EC_{50} values were determined using a three or four parameter logistic equation (GraphPad Prism 3, CA, U.S.A.). In studies using repeated application of agonist, the data are presented graphically as a percentage of the response to the first application of agonist. The significance of any changes in inward current upon repeated agonist application were determined using the raw non-transformed values by use of a one way ANOVA followed by Tukey's test (GraphPad Prism 3).

Materials

DMEM/F12, FBS and G418 were obtained from GibcoBRL (Paisley, U.K.). ATP and BzATP were obtained from Sigma (Poole, U.K.). YO-PRO-1 was obtained from Molecular Probes (Cambridge Bioscience, U.K.).

Results

Effects of repeated or prolonged application of 100 μM BzATP

Repeated application of 100 μM BzATP to HEK293 cells expressing the mouse $P2X_7$ receptor resulted in current growth (Figure 1) which became significant by the 4th application (139 ± 6.7% of the initial response; $P < 0.05$; one way ANOVA

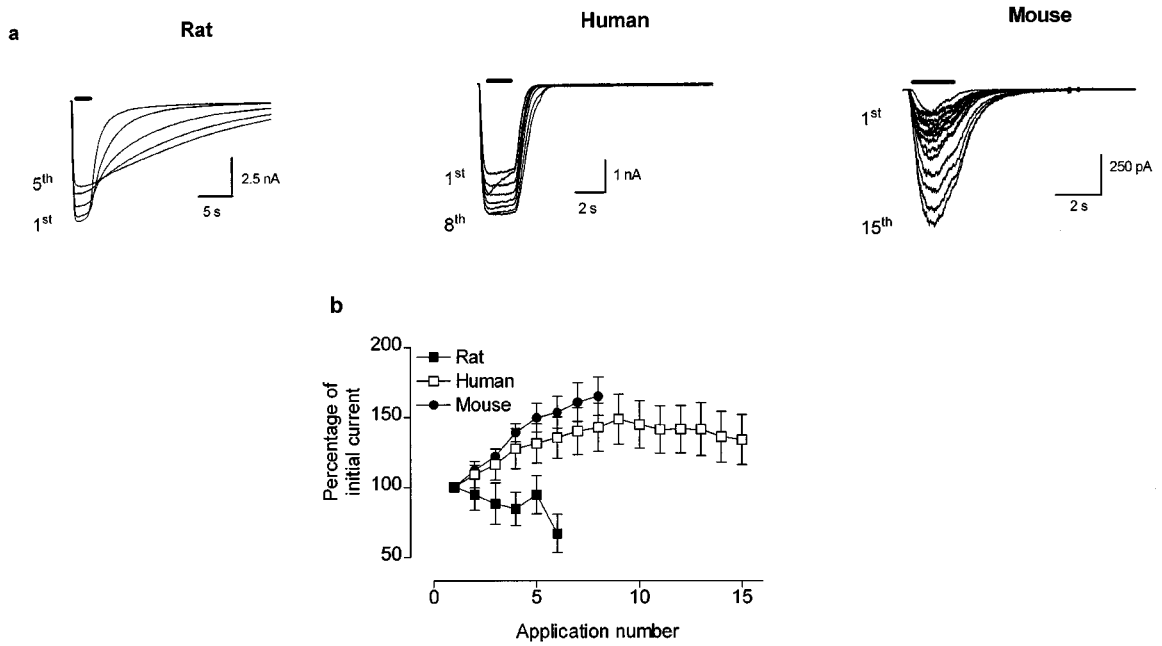

Figure 1 The effect of repeated application of BzATP (100 μM) applied for 2 s every 20–30 s to HEK293 cells expressing rat, human or mouse recombinant $P2X_7$ receptors. (a) Representative whole cell recordings from single cells. (b) Whole cell currents expressed as a percentage of the initial response to 100 μM BzATP. The data are the mean$\pm$s.e.mean.

followed by Tukey's test). Current growth was detectable by the 8th application of BzATP to cells expressing the human $P2X_7$ receptor (143$\pm$17.2% of the initial response; $P<0.05$; one way ANOVA followed by Tukey's test) but was not detected in those cells expressing the rat $P2X_7$ receptor (Figure 1).

Studies were also conducted with adherent cells using confocal microscopy to visualize BzATP-stimulated changes in permeability to YO-PRO-1 (Figure 2). In cells expressing the rat or human $P2X_7$ receptor, the BzATP-stimulated increase in YO-PRO-1 fluorescence (YO-PRO-1 accumulation) was rapid, being detectable within 10–30 s of agonist application (Figure 2). The initial rate of YO-PRO-1 accumulation appeared slower in cells expressing the human $P2X_7$ receptor than in those expressing the rat $P2X_7$ receptor (Figure 2), but there was little difference in the overall time taken for the maximal YO-PRO-1 accumulation to be achieved (approximately 20–30 mins; data not shown). In cells expressing the mouse $P2X_7$ receptor, YO-PRO-1 accumulation was only observed after a 20–30 min incubation period (data not shown).

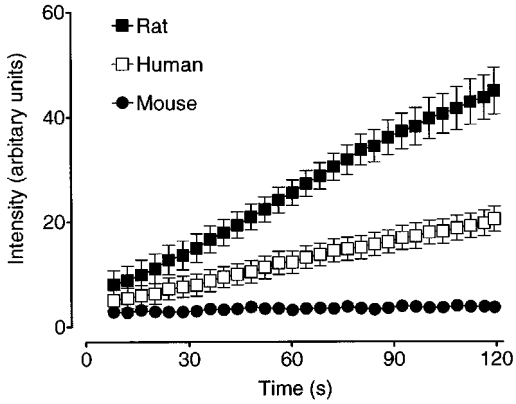

Figure 2 Kinetics of BzATP-stimulated YO-PRO-1 accumulation assessed using confocal microscopy. YO-PRO-1 accumulation was stimulated by addition of 100 μM BzATP to HEK293 cells expressing rat, human or mouse recombinant $P2X_7$ receptors. Data were obtained every second over a 110 s period after BzATP addition. For clarity some of the data points have been omitted. Experiments were performed at 22°C in a NaCl containing solution. Data from 10 cells were averaged and the data are the mean$\pm$s.e.mean of three experiments except for studies on the mouse $P2X_7$ receptor which represent data from four cells studied in one experiment.

Species differences in BzATP potency

The potency of BzATP to activate $P2X_7$ receptors differed markedly between the three species (Figure 3a). The pEC_{50} value for BzATP at the rat $P2X_7$ receptor in NaCl-buffer was 5.17$\pm$0.09 (EC_{50} 6.8 μM). The potency of BzATP at the human and mouse $P2X_7$ receptors was less than determined at the rat receptor, but a pEC_{50} value could not be determined since there was no clearly defined maximum at 300 μM BzATP (Figure 3a).

It was not possible to examine higher concentrations of BzATP at the mouse and human $P2X_7$ receptors due to a number of practical considerations including cost and availability. However, agonist potency at human $P2X_7$ receptors can be increased when extracellular NaCl is replaced by Na-glutamate (Michel *et al.*, 1999) and this approach was utilized to compare potency estimates between the three species. Thus, when experiments were performed in Na-glutamate buffer, the potency of BzATP at all three species increased and maximal responses to BzATP could be obtained at the rat and human $P2X_7$ receptors. The pEC_{50} values (EC_{50} value in parenthesis) for BzATP were 5.89$\pm$0.08 (1.3 μM) and 5.26$\pm$0.10 (5.5 μM) at the rat and human $P2X_7$ receptors, respectively (Figure 3b). A clearly defined maximum was not obtained at the mouse $P2X_7$ receptor but an estimated pEC_{50} of 4.08$\pm$0.08 ($EC_{50}=83$ μM) could be calculated by fitting the data to a logistic function.

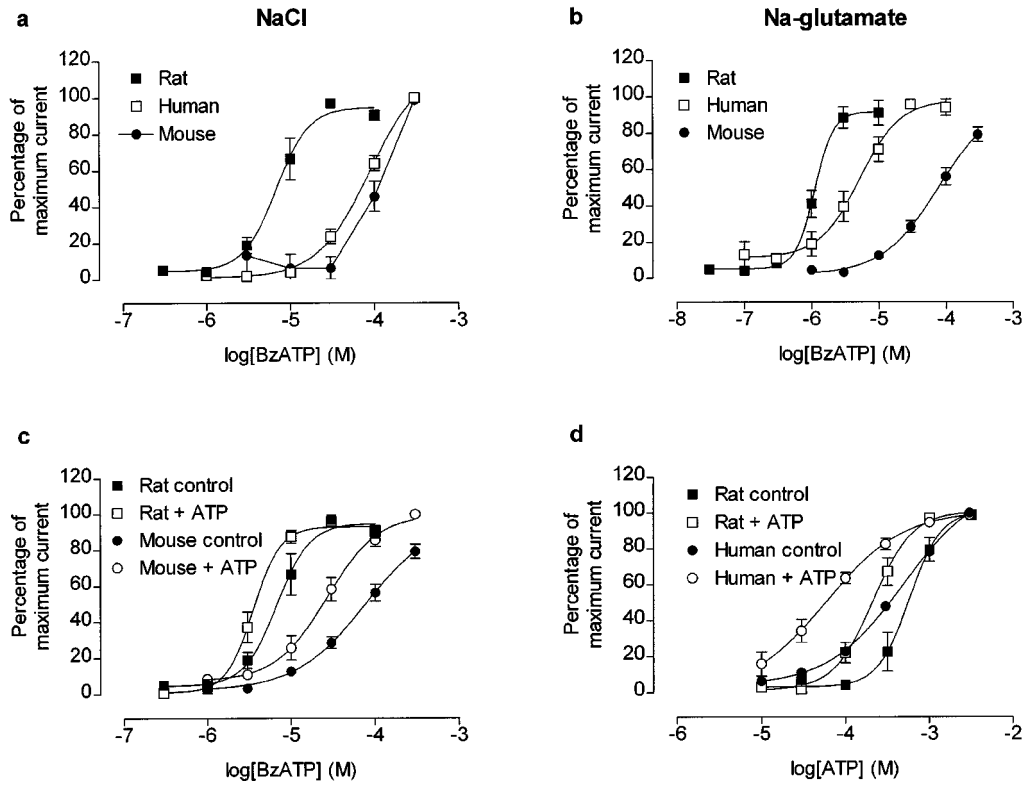

Figure 3 Species differences in agonist potency and the effects of repeated exposure to ATP on the potency of BzATP and ATP. Concentration-effect curves (CEC) to BzATP at the rat, human or mouse recombinant $P2X_7$ receptors, were constructed in an extracellular solution containing (a) 145 mM NaCl or (b) 145 mM Na-glutamate in place of NaCl. (c) CEC for BzATP were determined in cells expressing rat or mouse recombinant $P2X_7$ receptors. Studies were performed in either naïve cells (control) or in cells repeatedly exposed to 1 mM ATP as described in the Methods (+ATP). In studies on the rat $P2X_7$ receptor the extracellular solution contained 145 mM NaCl while in studies on the mouse $P2X_7$ receptor the extracellular solution contained 145 mM Na-glutamate in place of 145 mM NaCl. (d) CEC for ATP were determined in cells expressing rat or human recombinant $P2X_7$ receptors. Studies were performed in either naïve cells (control) or in cells repeatedly exposed to 1 mM ATP as described in the Methods (+ATP). In studies on the rat $P2X_7$ receptor the extracellular solution contained 145 mM NaCl while in studies on the human $P2X_7$ receptor the extracellular solution contained 145 mM Na-glutamate in place of 145 mM NaCl. In all figures data are expressed as a percentage of the maximum current obtained for each cell type and are the mean ± s.e.mean of 6–13 experiments.

Effect of repeated application of ATP on agonist potency at $P2X_7$ receptors

In addition to the species differences in potency described above, the potency of BzATP and ATP for the rat, human and mouse $P2X_7$ receptors increased after repeatedly exposing cells to ATP.

In studies on the rat $P2X_7$ receptor, the potency of BzATP determined in a NaCl containing buffer was slightly, but significantly ($P<0.05$; Student's *t*-test), lower in naïve cells (pEC_{50} 5.17 ± 0.09; $EC_{50}=6.8$ μM; $n_H=2.2\pm0.3$) than in cells pre-exposed repeatedly to ATP (pEC_{50} 5.39 ± 0.04; EC_{50} 4.1 μM: $n_H=2.7\pm0.4$; Figure 3c). Similarly, pEC_{50} values for ATP were 3.39 ± 0.1 ($EC_{50}=407$ μM; $n_H=2.3\pm0.2$) and 3.73 ± 0.07 ($EC_{50}=186$ μM; $n_H=1.8\pm0.3$), respectively in naïve and ATP-treated cells (Figure 3d).

In studies on the human $P2X_7$ receptor performed in NaCl buffer, the pEC_{50} values for BzATP were 4.09 ± 0.06 ($EC_{50}=81$ μM; $n_H=1.4\pm0.2$) and 4.42 ± 0.12 ($EC_{50}=38$ μM; $n_H=1.2\pm0.2$) in naïve and ATP-pretreated cells, respectively (data not shown). Complete CEC to ATP could only be obtained in Na-glutamate buffer and, in this buffer, pEC_{50} values for ATP, were 3.29 ± 0.07 ($EC_{50}=512$ μM; $n_H=1.0\pm0.2$) and 4.33 ± 0.19 ($EC_{50}=47$ μM; $n_H=0.9\pm0.1$), respectively in naïve and ATP-treated cells (Figure 3d).

Finally, the only convincing potency estimates that could be obtained at the mouse $P2X_7$ receptor were obtained in a Na-glutamate buffer. In this buffer (Figure 3c), the potency of BzATP was significantly ($P<0.05$; Student's *t*-test) lower in naïve cells ($pEC_{50}=4.08\pm0.08$; $EC_{50}=83$ μM; $n_H=1.0\pm0.2$) than in cells pre-exposed repeatedly to ATP ($pEC_{50}=4.58\pm0.09$; $EC_{50}=26$ μM; $n_H=1.4\pm0.2$). It should be noted that in these studies a clearly defined maximal response was only obtained in the ATP-pretreated cells and so pEC_{50} values in the naïve cells were calculated assuming the maximum response obtained using 300 μM BzATP as the maximum for the concentration-effect curve.

Concentration-dependence of current growth

In the initial studies performed in NaCl buffer, 100 μM BzATP was a supra-maximal concentration at the rat $P2X_7$ receptor, a sub-maximal concentration at the human $P2X_7$ receptor and threshold at the mouse $P2X_7$ receptor. To determine if this could affect the ability to detect current growth, studies on the rat $P2X_7$ receptor were performed using lower concentrations of BzATP.

In NaCl containing buffer, current growth was evident when lower concentrations of BzATP (1, 3 and 10 μM) were repeatedly added to cells expressing the rat $P2X_7$ receptor (Figure 4a). The rate of current growth appeared to increase with agonist concentration since a significant increase in current was detected after the 14th, 5th and 4th applications of BzATP, when using BzATP concentrations of 1, 3 and 10 μM, respectively.

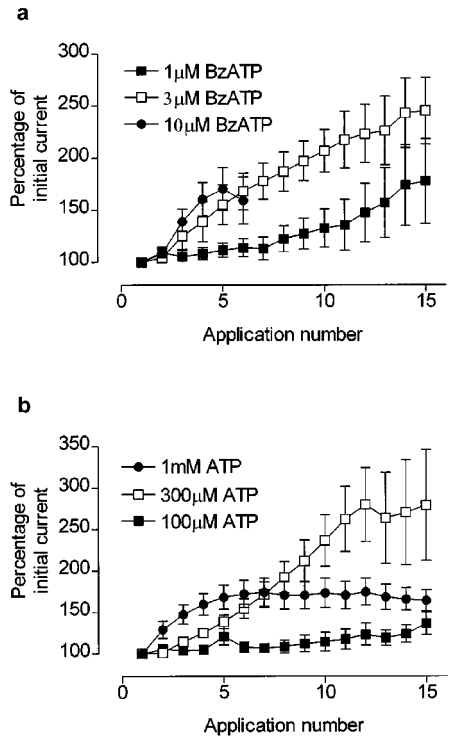

Figure 4 The effect of repeated application of BzATP or ATP to cells expressing the rat recombinant $P2X_7$ receptor. (a) The effect of repeated application of BzATP at 1, 3 or 10 μM BzATP. The data are presented as a percentage of the response to the 1st application of BzATP. (b) The effect of repeated applications of 100 μM, 300 μM or 1 mM ATP expressed as a percentage of the response to the 1st application of ATP. The data are the mean$\pm$s.e.mean of 6–9 experiments.

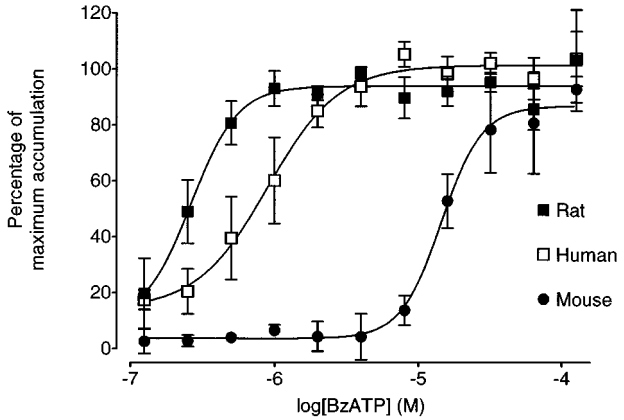

Figure 5 Concentration-effect curves for the ability of BzATP to increase YO-PRO-1 fluorescence in HEK293 cells expressing rat, human or mouse recombinant $P2X_7$ receptors. The data are expressed as a percentage of the maximum response to BzATP obtained in each cell type. Responses at the rat, human or mouse $P2X_7$ receptors were measured after 5, 10 or 40 min, respectively. The experiments were performed at 37°C in a NaCl-free, sucrose-containing, buffer and are the mean$\pm$s.e.mean of three determinations.

Furthermore, the number of applications required for a maximal increase in current to be achieved was less when using higher concentrations of agonist. Current growth was also detected after repeated application of ATP with significant increases in current observed after the 8th and 2nd applications of ATP at concentrations of 300 μM and 1 mM, respectively (Figure 4b).

In cells expressing the human and mouse $P2X_7$ receptor it was more difficult to study the concentration-dependence of current growth due to the inability to examine supra-maximal concentrations of BzATP. However, current growth was observed when using a lower concentration of 30 μM BzATP and was almost identical to that obtained in Figure 1 using 100 μM BzATP (data not shown). Furthermore, current growth at the human $P2X_7$ receptor was also observed when using ATP with significant ($P<0.05$; one way ANOVA followed by Tukey's test) increases in current measured after the 14th (131 ± 14% of initial response) or 13th application (271 ± 83% of initial response) when using ATP concentrations of 300 μM and 1 mM, respectively.

Concentration-dependence of YO-PRO-1 accumulation in cell suspensions

To study directly the concentration-dependence of channel dilation, additional studies were undertaken to measure YO-PRO-1 accumulation. These studies were performed on cells in suspension since mono-layers of HEK293 cells in 96-well plates would not withstand the multiple wash procedures required for quantitative measurement of YO-PRO-1 accumulation. Furthermore, studies were performed in a buffer in which sucrose was substituted for NaCl since full concentration-effect curves for BzATP could be obtained for all three species in this buffer (Figure 5). In agreement with the patch clamp studies, BzATP was most potent at the rat $P2X_7$ receptor and least potent at the mouse $P2X_7$ receptor with pEC_{50} values (EC_{50} in parenthesis) of 6.60 ± 0.03 (0.25 μM), 6.03 ± 0.31 (0.9 μM) and 4.93 ± 0.07 (11.7 μM) for the rat, human and mouse $P2X_7$ receptors, respectively. The maximal rate of YO-PRO-1 accumulation was faster in cells expressing rat and human $P2X_7$ receptors, than in cells expressing mouse $P2X_7$ receptors. Thus, the times taken for 50% of maximal BzATP-stimulated YO-PRO-1 accumulation to occur were 12 ± 1, 25 ± 5 and 109 ± 5 min for HEK293 cells expressing rat, human and mouse $P2X_7$ receptors, respectively.

Discussion

The aim of this study was to determine the reasons for the apparent species differences in current growth observed at the $P2X_7$ receptor following repeated agonist application. We found that current growth could in fact be detected at all three species orthologues and that the species differences in current growth reported previously can be attributed to the strong concentration-dependence of the phenomenon which results in it only being readily detected when using sub-maximal concentrations of agonist.

In agreement with our previous studies (Chessell *et al.*, 1998), we found that repeated application of 100 μM BzATP did not result in current growth in cells expressing the rat $P2X_7$ receptor while current growth was detected at the mouse and human $P2X_7$ receptor. The exact mechanism underlying current growth has not been determined, although it was thought to be associated with dilation of the $P2X_7$ channel (Chessell *et al.*, 1997; 1998). Assuming this hypothesis to be true then the failure to detect current growth in cells expressing the rat $P2X_7$ receptor with repeated applications of 100 μM BzATP suggested that channel dilation was not occurring in these cells. However,

BzATP evoked a marked cellular accumulation of YO-PRO-1 within 10–30 s of application to adherent cells expressing the rat or human $P2X_7$ receptors but only caused a small increase in YO-PRO-1 accumulation after 20–30 min in cells expressing the mouse $P2X_7$ receptor.

In order to understand these paradoxical results we examined the concentration-dependence of the process of current growth in the three species. This was particularly important since the potency of BzATP was found to vary considerably between the three species with BzATP possessing much higher affinity for the rat than the mouse $P2X_7$ receptor. As a result of the species differences in BzATP potency, the concentration of BzATP (100 μM) employed in previous studies, and in our preliminary studies, would be supra-maximal at the rat $P2X_7$ receptor and sub-maximal at the human and mouse $P2X_7$ receptors. Importantly, when lower, sub-maximal, concentrations of BzATP were examined at the rat $P2X_7$ receptor, current growth was detected suggesting that current growth can be observed at all three species when using sub-maximal concentrations of agonist. This suggests that the failure to observe current-growth at the rat $P2X_7$ receptor in previous studies was due to the use of supra-maximal concentrations of agonist at the rat $P2X_7$ receptor.

Previously, current growth was thought to be due to dilation of the $P2X_7$ ion-channel (Chessell *et al*., 1997) and it has been suggested that the dilation of the $P2X_7$ channel to a pore permeable to YO-PRO-1 occurs more rapidly at the rat than the human $P2X_7$ receptor (Rassendren *et al*., 1997). Furthermore, in studies with cells expressing endogenous $P2X_7$ receptors, changes in ethidium permeability occurred within seconds in rat mast cells (Tatham & Lindau, 1990) but required 10–60 s in human lymphocytes (Wiley *et al*., 1998) and mouse macrophages (Humphreys *et al*., 1998). We could not demonstrate any marked differences in current growth between the three species, although this comparison was complicated by the concentration-dependence of the phenomenon. However, the maximal rate of YO-PRO-1 accumulation was fastest in suspensions of cells expressing the rat $P2X_7$ receptor and slower in cells expressing the human or mouse $P2X_7$ receptors. It should be noted that the maximum rate of YO-PRO-1 accumulation should be proportional to the channel density and so for any direct comparison cells should express the same density of channels. While, it was not possible to measure directly channel density for the $P2X_7$ receptor orthologues, maximal currents, which should be proportional to receptor/channel density, were not dissimilar for the three species. Thus, when measured in a Na-glutamate buffer the maximum currents elicited by BzATP were approximately 2.7, 3.7 and 1.8 nA for rat, human and mouse $P2X_7$ receptors, respectively (A.D. Hibell, unpublished observation).

A further finding of this study was that agonist potency appeared to be increased in cells repeatedly exposed to agonist compared to that measured in naïve cells. This has previously been shown for the native $P2X_7$ receptor in NTW8 cells (Chessell *et al*., 1997) and at the human recombinant $P2X_7$ receptor (Chessell *et al*., 1999). In the present study repeated exposure to ATP resulted in an increased potency of BzATP and ATP at the rat and human $P2X_7$ receptors and of BzATP at the mouse $P2X_7$ receptor.

We have previously speculated that current growth reflects a progressive dilation of the $P2X_7$ receptor channel (Chessell *et al*., 1997; 1999). Thus, it was assumed that a progressive pore dilation would result in an increase in current as a result of an increased permeability to the charge carrying ions. However, the present results suggest that changes in agonist affinity could also contribute to current growth. Thus, if agonist affinity were to increase with repeated agonist application, then for a fixed concentration of agonist, receptor occupancy would progressively increase thereby leading either to a greater number of channels open and able to pass current, or to an increase in open channel probability. It was not possible to determine the relative extent to which changes in affinity and channel dilation contributed to current growth. However, in the case of studies on the mouse $P2X_7$ receptor YO-PRO-1 accumulation required many minutes indicating that channel dilation only occurred slowly. Since current growth was observed within four 2 s applications of agonist, this would suggest that current growth primarily reflects changes in agonist affinity rather than changes in pore diameter, although further studies would be required to confirm this.

The mechanism for the changes in agonist affinity following repeated exposure to agonist are not known although it is of interest that both calmidazolium (Virginio *et al*., 1997) and a monoclonal antibody to the human $P2X_7$ receptor (Buell *et al*., 1998; Chessell *et al*., 1999) have been shown to exhibit differential blockade of $P2X_7$ receptor function depending upon the method of study. Thus, both agents potently inhibit responses to brief applications of BzATP in naïve cells, but have no, or less effect, in cells repeatedly or continually exposed to agonist. These observations suggest that the affinity of the $P2X_7$ receptor for both agonists and antagonists can change after agonist exposure. This change in affinity could be due to a direct agonist-mediated conformational change in the $P2X_7$ receptor or may reflect modifications to the receptor as a consequence of activation of intra-cellular signalling pathways.

In conclusion then, we have shown that the phenomenon of current growth can be observed at rat, human and mouse $P2X_7$ receptors and is produced by both BzATP and ATP. Current growth and channel dilation appear more rapid at the rat and human $P2X_7$ receptors, than at the mouse $P2X_7$ receptor. Current growth either reflects the progressive increase in the size of the $P2X_7$ channel, or, more probably, an increase in agonist affinity. These long lasting changes in channel properties may be of physiological/pathophysiological importance. Thus, the *in vivo* relevance of the $P2X_7$ receptor can be questioned given that it is only activated by millimolar concentrations of ATP under physiological conditions. However, macrophages and other immune cells that express the $P2X_7$ receptor can be exposed repeatedly to pulses of ATP released from dying or damaged cells in conditions of inflammation or injury (Lutz & Kabler, 1997; Braun *et al*., 1998). The changes in agonist potency and channel conductance that result from this exposure may combine to make the $P2X_7$ receptor progressively more relevant in patho-physiological conditions.

References

BRAUN, N., ZHU, Y., KRIEGLSTEIN, J., CULMSEE, C. & ZIMMERMANN, H. (1998). Upregulation of the enzyme chain hydrolysing extracellular ATP after transient forebrain ischaemia in the rat. *J. Neurosci.,* **18,** 4891 – 4900.

BUELL, G., CHESSELL, I.P., MICHEL, A.D., COLLO, G., SALAZZO, M., HERREN, S., GRETENER, D., GRAHAMES, C., KAUR, R., KOSCO-VILBOIS, M.H. & HUMPHREY, P.P.A. (1998). Blockade of human $P2X_7$ receptor function with a monoclonal antibody. *Blood,* **92,** 3521 – 3528.

CHESSELL, I.P., MICHEL, A.D. & HUMPHREY, P.P.A. (1997). Properties of the pore-forming $P2X_7$ receptor in mouse NTW8 microglial cells. *Br. J. Pharmacol.,* **121,** 1429 – 1437.

CHESSELL, I.P., MICHEL, A.D. & HUMPHREY, P.P.A. (1999). Determinants of human $P2X_7$ large pore formation. *Br. J. Pharmacol.,* **126,** 19P.

CHESSELL, I.P., SIMON, J., HIBELL, A.D., MICHEL, A.D., BARNARD, E.A. & HUMPHREY, P.P.A. (1998). Cloning and functional characterisation of the mouse $P2X_7$ receptor. *FEBS Letts.,* **439,** 26 – 30.

FENWICK, E.M., MARTY, A. & NEHER, E. (1982). A patch-clamp study of bovine chromaffin cells and of their sensitivity to acetylcholine. *J. Physiol.,* **331,** 577 – 597.

HAMILL, O.P., MARTY, A., NEHER, E., SAKMANN, B. & SIGWORTH, F.J. (1981). Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflugers Arch.,* **391,** 85 – 100.

HICKMAN, S.E., EL KHOURY, J., GREENBERG, S., SCHIEREN, I. & SILVERSTEIN, S.C. (1994). P2Z adenosine triphosphate receptor activity in cultured human monocyte-derived macrophages. *Blood,* **84,** 2452 – 2456.

HUMPHREYS, B.D., VIRGINIO, C., SURPRENANT, A., RICE, J. & DUBYAK, G.R. (1998). Isoquinolines as antagonists of the P2X7 nucleotide receptor: High selectivity for the human versus rat receptor homologues. *Mol. Pharmacol.,* **54,** 22 – 32.

LUTZ, P.L. & KABLER, S. (1997). Release of adenosine and ATP in the brain of freshwater turtle (Trachemys scripta) during long term anoxia. *Brain Res.,* **769,** 281 – 286.

MICHEL, A.D., CHESSELL, I.P. & HUMPHREY, P.P.A. (1999). Ionic effects on human recombinant $P2X_7$ receptor function. *Naunyn-Schmiedeberg's Arch. Pharmacol.,* **359,** 102 – 109.

NUTTLE, L.C. & DUBYAK, G.R. (1994). Differential activation of cation channels and non-selective pores by macrophage P_{2Z} purinergic receptors expressed in Xenopus Oocytes. *J. Biol. Chem.,* **269,** 13988 – 13996.

RASSENDREN, F., BUELL, G., VIRGINIO, C., COLLO, G., NORHT, R.A. & SURPRENANT, A. (1997). The permeabilizing ATP receptor, $P2X_7$: cloning and expression of a human cDNA. *J. Biol. Chem.,* **272,** 5482 – 5486.

STEINBERG, T.H., NEWMAN, A.S., SWANSON, J.A. & SILVERSTEIN, S.C. (1987). ATP^{4-} permeabilizes the plasma membrane of mouse macrophages to fluorescent dyes. *J. Biol. Chem.,* **262,** 8884 – 8888.

SURPRENANT, A., RASSENDREN, F., KAWASHIMA, E., NORTH, R.A. & BUELL, G. (1996). The cytolytic P2Z receptor for extracellular ATP identified as a P2X receptor ($P2X_7$). *Science,* **272,** 735 – 738.

TATHAM, P.E.R. & LINDAU, M. (1990). ATP-induced pore formation in the plasma membrane of rat peritoneal mast cells. *J. Gen. Physiol.,* **95,** 459 – 476.

VIRGINIO, C., CHURCH, D., NORTH, R.A. & SURPRENANT, A. (1997). Effects of divalent cations, protons and calmidazolium at the rat $P2X_7$ receptor. *Neuropharmacology,* **36,** 1285 – 1294.

WILEY, J.S., GARGETT, C.E., ZHANG, W., SNOOK, M.B. & JAMIESON, G.P. (1998). Partial agonists and antagonists reveal a second state of human lymphocyte P2Z/$P2X_7$ channel. *Am. J. Physiol.,* **275,** C1224 – C1231.

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