RESEARCH PAPER

Pharmacological properties of the rhesus macaque monkey P2X7 receptor

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BACKGROUND AND PURPOSE

The human P2X7 (hP2X7) receptor exhibits striking pharmacological differences from its rodent counterparts, particularly in terms of its antagonist profile. Here, we characterized the functional and pharmacological properties of the rhesus macaque monkey P2X7 (rmP2X7) receptor in comparison with the hP2X7 receptor.

EXPERIMENTAL APPROACH

The rmP2X7 and hP2X7 receptors were heterologously expressed in HEK293 cells. The receptor surface and total expression levels were examined by biotin-labelling and Western blotting. The functional and pharmacological properties were characterized using patch-clamp recording and single-cell imaging.

KEY RESULTS

The rmP2X7 receptor showed strong cell surface expression. Both ATP and 2'(3')-O-(4-benzoylbenzoyl) adenosine-5'-triphosphate (BzATP) were full agonists in activating the rmP2X7 receptor; the EC₅₀ values were 802 μ M for ATP and 58 μ M for BzATP, respectively, in extracellular low divalent cation solution. Prolonged activation of the rmP2X7 receptors induced detectable but low level YO-PRO-1 uptake. KN-62, AZ11645373 and A-438079, three hP2X7 selective antagonists, all potently inhibited the rmP2X7 receptor-mediated currents; the IC₅₀ values were 86, 23 and 297 nM respectively.

CONCLUSION AND IMPLICATIONS

The rmP2X7 receptor exhibits similar pharmacological properties to the hP2X7 receptor. The rhesus macaque monkey thus may represent a valuable model species in elucidating the mechanisms and pharmacological interventions of hP2X7 receptor-related diseases.

Abbreviations

BzATP, 2'(3')-O-(4-benzoylbenzoyl) adenosine-5'-triphosphate

Introduction

ATP is one of the important extracellular signalling molecules, acting on P2 purinergic receptors at the cell surface (Ralevic and Burnstock, 1998; Neary and Zimmermann, 2009; Surprenant and North, 2009). The P2X7 receptor belongs to the trimeric P2X receptor family (Browne *et al.*, 2010; receptor nomenclature follows Alexander *et al.*, 2009). Each of the three subunits that form the P2X7 receptor is composed of intracellular N- and C-termini, and two transmembrane segments connected by a large extracellular domain (Surprenant *et al.*, 1996). Expression of P2X7 receptors is well documented in immune, glial, bone and epithelial cells, where the recep-

tor mediates the functions of extracellular ATP in immune responses, inflammation, neuron–glia interactions, nociception, bone remodelling and saliva secretion (Duan and Neary, 2006; Di Virgilio, 2007; Surprenant and North, 2009; Jarvis, 2010; Skaper *et al.*, 2010). Alterations in the P2X7 receptor expression and/or function appear to be causally associated with the pathogenesis and/or progress of an increasing number of conditions, including arthritis (Solle *et al.*, 2001; Labasi *et al.*, 2002), inflammatory and neuropathic pain (Chessell *et al.*, 2005), morphine tolerance (Zhou *et al.*, 2010), neurodegeneration and neuroinflammation (Parvathani *et al.*, 2003; Choi *et al.*, 2007; Matute *et al.*, 2007; Díaz-Hernández *et al.*, 2009; Sanz *et al.*, 2009) and epithelial

cancers (Gorodeski, 2010). These findings point to a great potential for the human P2X7 (hP2X7) receptor as a therapeutic target. Indeed, over the past few years there have been substantial efforts in developing novel and potent hP2X7 antagonists (Donnelly-Roberts and Jarvis, 2007; Romagnoli *et al.*, 2008; Guile *et al.*, 2009).

Rodents and their derived tissues and cells are useful in understanding the physiological functions and disease mechanisms of human proteins, as shown by studies of P2X7 receptors over the past decade (Surprenant and North, 2009; Jarvis, 2010; Skaper *et al.*, 2010), and are essential for development of new drugs. However, the hP2X7 receptor exhibits striking differences in its functional and pharmacological properties from its corresponding receptors in rodent species, the most prominent of which is the antagonist profile (Rassendren *et al.*, 1997; Chessell *et al.*, 1998; Hibell *et al.*, 2000; Jiang *et al.*, 2000; Stokes *et al.*, 2006; Fonfria *et al.*, 2008; Donnelly-Roberts *et al.*, 2009; Roger *et al.*, 2010a; Bradley *et al.*, 2011). Such differences between species could complicate or compromise the relevance of rodent animal models in elucidating hP2X7 receptor-related disease mechanisms and preclinical testing of drugs targeting the hP2X7 receptor, as have been discussed for other channels (Chen and Kym, 2009). The structure-activity relationships underlying the species specificity of the P2X7 antagonists are largely unknown. A recent study using chimeras and site-directed mutagenesis has identified the residue at position 95 to be involved in determining the differential sensitivity of hP2X7 (Phe⁹⁵) and rat (r) P2X7 receptors (Leu⁹⁵) to the antagonists SB203580 and KN-62 (Michel *et al.*, 2009). Understanding of the molecular basis governing the species-specific ligand–receptor interactions is important for screening *in silico* and *de novo* design of novel hP2X7 antagonists for therapeutic interventions.

Genome sequencing efforts have identified the genes encoding the P2X7 receptor in an increasing number of species. Functional characterization and comparison of P2X7 receptors from different species have been useful in delineating the molecular basis for species-dependent pharmacological properties (Young *et al.*, 2007; Michel *et al.*, 2009). The P2X7 receptor from non-human primates has not been characterized. In this study, we determined the functional and pharmacological properties of the rhesus macaque monkey P2X7 (rmP2X7) receptor after heterologous expression in HEK293 cells. We also carried out a direct comparison of the pharmacological properties between rhesus macaque monkey and human P2X7 receptors. Our results show that the rmP2X7 receptor exhibits very similar pharmacological properties to the hP2X7 receptor, demonstrating that the rhesus macaque monkey may be a valuable model species in elucidating the mechanisms of hP2X7 receptor-related diseases and developing pharmacological interventions targeting the hP2X7 receptor.

Methods

Biotin-labelling and Western blotting

The experiments were carried out by using the protocols described in our previous studies (Mei *et al.*, 2006; Xia *et al.*, 2008; Bradley *et al.*, 2010). In brief, HEK293 cells,

co-transfected with the plasmid encoding a P2X7 receptor or an empty pcDNA3.1 vector, and the plasmid encoding the enhanced green fluorescent protein (GFP) were labelled with sulpho-NHS-LC-biotin (Pierce, Rockford, IL, USA) for 30 min at 4°C. Total protein concentrations were determined using the bicinchoninic acid protein assay (Thermo Scientific, Northumberland, UK). The biotinylated proteins were purified from total protein (300 µg) by incubating the lysate with EZ view red streptavidin affinity beads (Sigma, St Louis, MI, USA) overnight at 4°C, and were eluted in 50 µL standard protein electrophoresis buffer (6% SDS, 10% glycerol, 50 mM Tris-HCl pH 6.8, 2 mM EDTA, 0.05% bromophenol blue and 10% β-mercaptoethanol). Whole-cell lysate (10 µg) or biotin-labelled samples (20 µL) were separated on 12% SDS-PAGE gels. Proteins were detected using primary rabbit anti-EE antibody (a dilution of 1:5000; Bethyl, Montgomery, TX, USA) or mouse anti-GFP antibody (1:2000; Santa Cruz, CA, USA) and secondary horseradish peroxidase-conjugated anti-rabbit (1:5000) or anti-mouse IgG (1:2000) antibodies (Santa Cruz) and visualized using supersignal west femto maximum sensitivity substrate (Thermo Scientific).

Patch-clamp recording

The whole-cell currents were recorded at room temperature using a HEKA EPC10 amplifier at a holding potential of –60 mV (Liu *et al.*, 2008). Normal extracellular solution contained (concentrations in mM): 147 NaCl, 2 KCl, 1 MgCl₂, 2 CaCl₂, 10 HEPES and 13 glucose, pH 7.3. The currents induced by the highest concentrations of agonists used [10 mM ATP or 1 mM 2'(3')-O-(4-benzoylbenzoyl) adenosine-5'-triphosphate (BzATP)] in cells expressing the hP2X7 or rmP2X7 receptors reached maximum in extracellular low divalent cation solution containing no MgCl₂ and 0.3 mM CaCl₂, but not in normal extracellular solution (Roger *et al.*, 2010a; Bradley *et al.*, 2011). The extracellular low divalent cation solution was therefore used for subsequent experiments. Intracellular solutions contained (in mM): 145 NaCl, 10 EDTA and 10 HEPES, pH 7.3. The agonist or antagonist concentration–current response curves shown in the Figures were constructed after full receptor facilitation (Roger *et al.*, 2010b). The inhibition by KN-62 was largely irreversible, and the inhibition by AZ11645373 was slowly and partially reversible after a 10–20 min wash. However, the inhibition by A-438079 was rapidly reversible, even during the 4 s application of ATP and was completely reversed after a 5 min wash (Figures 3 and S1). Thus, ATP alone was used to induce currents following exposure to each indicated concentration of KN-62 and AZ11645373 for 4 min (Figure 3A and B), but was co-applied with A-438079 after exposure to each indicated concentrations of A-438079 for 1 min (Figures 3C and S1). To further examine the antagonist actions, ATP concentration–current responses curves were constructed before, and after 4 min incubation with indicated concentrations of antagonists, which was present thereafter throughout the experiments. Stock solutions of KN-62, AZ11645373 and A-438079 (10–100 mM) were prepared in DMSO and, at the highest concentration used (0.03% v/v), DMSO had no effect on agonist-induced responses (data not shown). Agonist and antagonist were delivered using an RSC160 system (Bio-Logic Science Instruments, Claix, France).

Dye uptake assay

The YO-PRO-1 uptake experiments were carried out at room temperature using a Nikon confocal microscope (excitation 488 nm; $\times 20$ objective). In the extracellular low divalent cation solution containing YO-PRO-1 (1 μ M), cellular fluorescence was measured during a 5 min application of the indicated agonist. For each agonist concentration, 9–37 isolated cells were imaged and the fluorescence signal averaged for the time course, or the slope taken for the rate of YO-PRO-1 influx. In the absence of agonist, the YO-PRO-1 uptake was negligible for both hP2X7 and rmP2X7 expressing cells.

Structural modelling

The hP2X7 receptor structure was modelled based on the crystal structure of the zebrafish P2X4 receptor (PDB accession 3H9V) (Kawate *et al.*, 2009) in our recent study (Roger *et al.*, 2010a). The image in Figure 1B, showing the hP2X7 receptor with the location of the three extracellular residues that are different from the rmP2X7 receptor, was generated using UCSF Chimera version 1.4 (Pettersen *et al.*, 2004).

Data analysis

All results, where appropriate, are presented as means $\pm$ SEM. Agonist EC₅₀ values were estimated by fitting the data from individual cells (ionic currents; Figures 2 and 4) or the mean data (dye uptake; Figure 5) to the Hill equation: $R = R_{\max}/[1 + (EC_{50}/[A])^n]$, where $R_{\max}$ is the maximal responses (ionic currents or dye uptake) for each case, R is the response induced by given agonist concentrations ($[A]$) and n is the Hill coefficient. Antagonist IC₅₀ values were derived by fitting the data from individual cells to the Hill equation using two parameters: $I = 100/[1 + ([B]/IC_{50})^n]$, where I is the agonist-induced currents after exposure to given concentrations of antagonist ($[B]$) expressed as percentage of the control current before antagonist application, and n is the Hill coefficient (Figure 3). KN-62 concentration–current response curve was also fitted to the Hill equation with three parameters: $I = (100 - C)/[1 + ([B]/IC_{50})^n] + C$, where C represents the small residual currents, but the results using two or three parameters were not significantly different (Table 2). The smooth lines in figures represent the best fit to the mean data of all experiments. Curve fitting was done using the least squares method. Statistical analysis was performed using Student's *t*-test and $P < 0.05$ was considered to be significant.

Materials

All chemicals used in this study were purchased from Sigma except when indicated otherwise. AZ11645373 and A-438079 were from Tocris Bioscience. HEK293 cells (ATCC) were used to transiently express P2X7 receptors. The cDNA encoding the hP2X7 receptor protein with a C-terminal EYMPME (EE) epitope was subcloned in the mammalian expression pcDNA3.1 vector (Invitrogen, Paisley, UK). The nucleotide sequence encoding the rmP2X7 receptor, based on the gene located on chromosome 11 (gene LOC722096 with transcript ID ENSMUT00000045479), was synthesized *in vitro* (Epoch Biolabs, Missouri, TX, USA). The rmP2X7 receptor sequence, carrying the mammalian Kozak sequence (gcctgtcacc) upstream of the start codon and a sequence encoding a C-terminal EE epitope, was inserted between KpnI and NotI

sites in the pcDNA3.1 vector. Cell preparation and transfection with plasmids were performed as described previously (Bradley *et al.*, 2010; 2011).

Results

Amino acid sequence comparison of P2X7 receptors

The rmP2X7 receptor is predicted to have 595 amino acid residues and shares a sequence identity of 96% with the hP2X7 receptor (Rassendren *et al.*, 1997). There are 18 different amino acid residues between the two receptors that are dispersed along the receptor protein (Figure 1A), three of which are in the extracellular domain, as highlighted in the structural model of the hP2X7 receptor based on the crystal structure of the zebrafish P2X4 receptor (Kawate *et al.*, 2009; Roger *et al.*, 2010a) (Figure 1B). Pair-wise comparison indicates that the rmP2X7 receptor shares 78–85% sequence identity with the other previously characterized mammalian P2X7 receptors (rat: Surprenant *et al.*, 1996; mouse: Chessell *et al.*, 1998; guinea pig: Fonfria *et al.*, 2008; dog: Roman *et al.*, 2009) (Table 1) and 50–56% with the P2X7 receptors of non-mammalian species (frog: Paukert *et al.*, 2002; zebrafish: Kucenas *et al.*, 2003). The rmP2X7 receptor contains all the residues in the extracellular domain (K64, K66, N187, F188, T189, N292, F293, R294 and K311; Figure 1A) that are proposed to be the constituents of an extracellular inter-subunit ATP binding site (Browne *et al.*, 2010; Evans, 2010).

Protein and functional expression

Both the rmP2X7 and hP2X7 receptors were expressed with an EE-epitope tag in the C-terminus, and thus we carried out biotin-labelling and Western blotting using an anti-EE antibody to examine the surface and total receptor protein expression. As shown in Figure 1C, the results show substantial expression of the rmP2X7 receptor at the cell surface, which was similar to that of the hP2X7 receptor. The protein corresponding to the P2X7 receptor was not detected in cells transfected with empty vector (Bradley *et al.*, 2010; 2011).

We performed patch-clamp recordings to measure the whole-cell current responses to brief (4 s) applications of agonists, ATP and BzATP. In HEK293 cells expressing the rmP2X7 receptor, both ATP and BzATP evoked robust currents in a concentration-dependent manner (Figure 2A). The maximal currents evoked by ATP (160 ± 22 pA·pF⁻¹, $n = 11$) were not significantly different from those evoked by BzATP (220 ± 27 pA·pF⁻¹, $n = 4$; $P > 0.05$), indicating that ATP and BzATP are almost equi-effective in activating the rmP2X7 receptor. Data fit to the Hill equation yielded EC₅₀ values of 802 ± 87 μ M ($n = 11$) and 58 ± 4 μ M ($n = 6$) for ATP and BzATP, respectively, and thus the sensitivity of the rmP2X7 receptor to BzATP is more than 10-fold greater than that to ATP. Under the same experimental conditions, the hP2X7 receptor exhibited EC₅₀ values of 314 ± 24 μ M ($n = 12$) and 30 ± 2 μ M ($n = 5$) for ATP and BzATP, respectively, suggesting that the rmP2X7 receptor is slightly less sensitive to these agonists than the hP2X7 receptor (Figure 2B and C). While there was no difference in the maximal BzATP-

Figure 1

Protein expression of rhesus macaque and human P2X7 receptors. (A) Amino acid sequence alignment of the rhesus macaque (rm) and human (h) P2X7 receptors. Transmembrane domains (TM1 and TM2) are indicated. The asterisks beneath indicate the amino acid residue identity, and the conserved residues that are crucial in forming the ATP binding sites are in bold. Phe⁹⁵, highlighted in the rectangle, is involved in the sensitivity to inhibition by KN-62. (B) Structural model of the extracellular part of the trimeric hP2X7 receptor, viewed along the axis of symmetry, with each subunit shown in a different colour. The three different residues in the extracellular domain are highlighted in green in one subunit. The residues in brackets are from the rmP2X7 receptor. (C) Surface and total protein expression of rmP2X7 and hP2X7 receptors assessed by biotin-labelling and Western blotting.

induced currents carried by the rmP2X7 and hP2X7 receptors (hP2X7: 310 ± 36 pA·pF⁻¹, $n = 5$; $P > 0.05$), the maximal ATP-evoked currents mediated by the rmP2X7 receptor were significantly smaller than those by the hP2X7 receptor (330 ± 22 pA·pF⁻¹, $n = 12$; $P < 0.001$).

Inhibition of ATP-induced currents by hP2X7 selective antagonists

We asked whether the rmP2X7 receptor exhibits the same antagonist sensitivity as the hP2X7 receptor, and here focused on three currently available and potent hP2X7 recep-

tor antagonists, KN-62 (Humphreys *et al.*, 1998), AZ11645373 (Stokes *et al.*, 2006) and A-438079 (Donnelly-Roberts *et al.*, 2009). As illustrated in Figure 3, these antagonists strongly inhibited ATP-induced currents mediated by the rmP2X7 receptor in a concentration-dependent manner. Upon washing in extracellular solution, the inhibition by KN-62 was largely irreversible (Figure 3A) and the inhibition by AZ11645373 was partially reversed (Figure 3B), whereas the inhibition by A-438079 showed rapid and complete recovery (Figures 3C and S1). The IC₅₀ values were 86 ± 19 nM (or 54 ± 8 nM using three parameters), 23 ± 3 nM and 297 ± 24 nM

Figure 2

Activation of rhesus macaque and human P2X7 receptors. (A) Left, representative currents evoked by indicated concentrations of ATP or BzATP. Right, agonist concentration–current response curves. $n = 8–11$ for ATP and $n = 4–6$ for BzATP. The smooth curves represent the fit of the mean data to the Hill equation. (B–C) Comparison of the agonist concentration–current curves between rmP2X7 and hP2X7 receptors. The data for the rmP2X7 are from A. $n = 12$ for ATP (B) and $n = 5$ for BzATP (C) for the hP2X7 receptor.

Table 1

Pair-wise amino acid identities among mammalian P2X7 receptors

	Human	Dog	Rat	Mouse	Guinea pig
Rhesus macaque	96	85	80	80	78
Human		85	80	80	77
Dog			76	76	73
Rat				84	74
Mouse					75

The sequence accession numbers are as follows: human, Q99572; rat, Q64663; mouse, Q9Z1M0; guinea pig, NP_001166578; dog, NP_001106927.

for KN-62, AZ11645373 and A-438079, respectively, and were not significantly different from those at the hP2X7 receptor (Figure 3D and Table 2).

To further study the actions of these antagonists on the rmP2X7 receptor, we determined the ATP concentration–current responses before and after treatment with 100 nM KN-62 or 30 nM AZ11645373 (Figure 4). The major effect of KN-62 and AZ11645373 was to suppress the maximal current responses with little or no effect on the ATP sensitivity (Table 3), suggesting dominant non-competitive antagonism.

Dye uptake pore formation

Finally, we assessed the ability of the rmP2X7 receptor to induce the formation of large pores in response to prolonged

activation, by measuring accumulative uptake of YO-PRO-1 fluorescent dye. Over a 5 min stimulation period by the maximal concentrations of agonists (5 mM ATP or 300 μM BzATP; left panels in Figure 5), there was a detectable level of dye uptake in HEK293 cells expressing the rmP2X7 receptor as compared with that in cells transfected with empty vector ($P < 0.05$), but the dye uptake level was markedly lower than that in cells expressing the hP2X7 receptor ($P < 0.001$). While it was difficult to determine the EC_{50} value for ATP at the rmP2X7 receptor due to weak concentration dependence, the data fit to the Hill equation yield an EC_{50} value of 770 μM for ATP at the hP2X7 receptor (right panel in Figure 5A). The EC_{50} values for BzATP were 160 and 50 μM at the rmP2X7 and hP2X7 receptors respectively (right panel in Figure 5B).

Figure 3

Inhibition of rhesus macaque and human P2X7 receptors by hP2X7 antagonists. (A–C) Representative currents in the absence (control) or presence of indicated concentrations of hP2X7 antagonists. The currents were evoked by 4 s exposure to ATP (3 mM for the rmP2X7 and 1 mM for the hP2X7). The antagonists were applied for 4 min between ATP applications in the case of KN-62 and AZ11645373, and 1 min for A-438079. A-438079 was co-applied with ATP during receptor activation. The washout time was 20 min for KN-62 (A), 15 min for AZ11645373 (B) and 5 min for A-438079 (C). (D) Comparison of the mean antagonist concentration–current response curves between the rmP2X7 and hP2X7 receptors. The number of cells in each case are as follows: KN-62: $n = 3$ for rmP2X7 and $n = 3$ for hP2X7; AZ11645373: $n = 3$ for rmP2X7 and $n = 3$ for hP2X7; A-438079: $n = 6$ for rmP2X7 and $n = 6$ for hP2X7. The dotted line shows the fit of the data for KN-62 at the rmP2X7 receptor to the Hill equation with three parameters.

Discussion

In this study we have characterized the pharmacological properties of the first non-human primate P2X7 receptor, the

rmP2X7 receptor, and have shown that the rmP2X7 receptor has very similar pharmacological properties to the hP2X7 receptor.

P2X7 receptors are well known to manifest an agonist profile that is strikingly distinct from all other P2X receptors,

Figure 4

Non-competitive inhibition of rhesus macaque and human P2X7 receptors by KN-62 and AZ11645373. (A) Left, representative currents in response to indicated concentrations of ATP before (control) or after 4 min exposure to 100 nM KN-62. Right, ATP concentration–current response curves. $n = 3$ for rmP2X7 and $n = 6$ for hP2X7. (B) Left, representative currents in response to indicated concentrations of ATP before (control) or after 4 min exposure to 30 nM AZ11645373. Right, ATP concentration–current response curves. $n = 3$ for rmP2X7 and $n = 3$ for hP2X7.

that is, submillimolar to millimolar sensitivity to its physiological agonist ATP and a greater potency of BzATP than ATP (North and Surprenant, 2000). This is clearly true for the rmP2X7 receptor because our results show that both ATP and BzATP are full agonists in activating the rmP2X7 receptor and that BzATP is >10-fold more potent than ATP (Figure 2). Extensive site-directed mutagenesis studies have defined a small subset of conserved residues to be critical for the formation of the inter-subunit ATP-binding sites in P2X receptors (Wilkinson *et al.*, 2006; Browne *et al.*, 2010; Evans, 2010) and these residues are found in the rmP2X7 receptor

(Figure 1A). Overall, the rmP2X7 receptor has an agonist profile that is very similar to the hP2X7 receptor. This suggests that the three non-conserved amino acid residues in the extracellular domain (Figure 1B) may contribute to the subtle difference in the sensitivity to both ATP and BzATP between the rmP2X7 and hP2X7 receptors (Figure 2B and C) but have no major role in determining the agonist profile of the P2X7 receptor.

KN-62 and AZ11645373 are two potent and selective hP2X7 antagonists that >1000-fold less potently inhibit the rP2X7 receptor (Humphreys *et al.*, 1998; Stokes *et al.*, 2006).

Figure 5

Uptake of YO-PRO-1 by cells expressing rhesus macaque and human P2X7 receptors. (A) Left, accumulative time course and rate of YO-PRO-1 dye uptake by cells expressing rmP2X7 and hP2X7 receptors, or cells transfected with empty vector, in response to 5 mM ATP. Right, ATP concentration-dye uptake response curves. The smooth curve represents the fit of the mean data to the Hill equation. The number of cells examined for each case is indicated. (B) Left, accumulative time course and rate of YOPRO-1 dye uptake by cells expressing rmP2X7 and hP2X7 receptors, or cells transfected with empty vector, in response to 300 μM BzATP. Right, BzATP concentration-dye uptake response curves. The smooth curves represent the fit of the mean data to the Hill equation. The number of cells examined for each case is indicated.

A-438079 is a relatively new P2X7 selective antagonist and is reported to be effective in inhibiting the hP2X7 receptor as well as the rodent P2X7 receptors (McGaraughty *et al.*, 2007; Donnelly-Roberts *et al.*, 2009). Here we showed that all three antagonists strongly inhibited the rmP2X7 receptor-mediated currents with IC₅₀ values of submicromolar concentrations, suggesting that they are potent antagonists at the rmP2X7 receptor (Figure 3D). In addition, we observed no significant difference in the sensitivity to these antagonists between the rmP2X7 and hP2X7 receptors (Table 2). Of note, the IC₅₀ values for KN-62 and AZ11645373 for the hP2X7 receptor were somewhat greater than those previously reported (Humphreys *et al.*, 1998; Stokes *et al.*, 2006), which may relate to the experimental conditions used in this study, including extracellular low divalent cation solutions and receptor facilitation. Our results revealed clear antagonist-specific differences in terms of the reversibility of the antagonistic effects; upon washing, the inhibition by KN-62 was largely irreversible and the inhibition by AZ11645373 was partially reversed, whereas the inhibition by A-438079 showed rapid and complete reversibility (Figures 3A–C and S1). Furthermore, we demonstrated that both KN-62 and AZ11645373 inhibit the rmP2X7 and hP2X7 receptors predominantly with non-competitive mechanisms (Figure 4 and Table 3), similar to previous reports for the hP2X7 receptor (Stokes *et al.*, 2006; Fonfria *et al.*, 2008). In summary, the rmP2X7 receptor exhibits the same sensitivity to these three potent antagonists as

the hP2X7 receptor (Table 2). This finding implies no role of the non-conserved residues in the extracellular domain (Figure 1A and B) in interacting with these antagonists. It is perhaps worth mentioning that Phe⁹⁵ is preserved in the rmP2X7 receptor (Figure 1A). This residue is located in the deep part of the ATP binding site (Browne *et al.*, 2010), and as mentioned above it is involved in determining the differential sensitivity to KN-62 of the hP2X7 receptor (Phe⁹⁵) and rP2X7 receptor (replaced with Leu⁹⁵) (Michel *et al.*, 2009). Increasing knowledge of the pharmacological properties from different species should help to decipher the domains or subsets of residues governing the ligand–receptor interactions, including those that are species-specific, which in turn are important for screening *in silico* and *de novo* design of novel hP2X7 antagonists for therapeutic interventions.

Interestingly, the agonist-induced dye uptake in cells expressing the rmP2X7 receptor was markedly lower than that in cells expressing the hP2X7 receptor (Figure 5), despite comparable agonist-induced currents mediated by the rmP2X7 and hP2X7 receptors and particularly those induced by BzATP. These results are reminiscent of an early finding that BzATP induced strikingly lower dye uptake in HEK293 cells expressing the hP2X7 receptor than that in cells expressing rP2X7 receptors, with no significant difference in the BzATP-induced currents (Rassendren *et al.*, 1997). A recent study has shown that ATP can induce robust dye uptake but BzATP completely fails to do so in human osteosarcoma cells

Table 2

Summary of rhesus macaque and human P2X7 receptor pharmacology

	rmP2X7	hP2X7
ATP	(n = 11)	(n = 12)
EC ₅₀ (mM)	0.8 ± 0.09***†††	0.3 ± 0.02***
Hill coefficient	1.2 ± 0.06***†††	1.9 ± 0.06
BzATP	(n = 6)	(n = 5)
EC ₅₀ (μM)	58 ± 4†††	30 ± 2
Hill coefficient	1.9 ± 0.1	2.2 ± 0.2
KN-62	(n = 3)	(n = 3)
IC ₅₀ (nM)	86 ± 19 (54 ± 8 ^a)	130 ± 40
Hill coefficient	1.2 ± 0.4 (1.9 ± 0.2 ^a)	1.6 ± 0.2
AZ11645373	(n = 3)	(n = 3)
IC ₅₀ (nM)	23 ± 3	31 ± 3
Hill coefficient	1.2 ± 0.1	1.8 ± 0.5
A-438079	(n = 6)	(n = 6)
IC ₅₀ (nM)	297 ± 24	493 ± 94
Hill coefficient	0.8 ± 0.05††	1.1 ± 0.1

****P* < 0.001, compared between ATP and BzATP within the same species;

††*P* < 0.01 and †††*P* < 0.001 compared between rmP2X7 and hP2X7 receptors.

^aResults from fitting the data to the Hill equation with three parameters.

heterologously expressing the guinea pig P2X7 receptor, and unfortunately the study did not determine whether BzATP was an agonist activating the guinea pig P2X7 receptor channel (Fonfria *et al.*, 2008). Currently, the mechanisms underlying the dye uptake pore formation largely remain a subject of further investigation. On one hand, proteins other than the P2X7 receptor, such as pannexin-1 (Pelegri and Surprenant, 2006), have been proposed to form the dye uptake pore. On the other hand, studies have shown a critical role of the conserved residue Gly³⁴⁵ in the ion permeating pathway (Monif *et al.*, 2009), and particularly the intracellular C-terminus in large pore formation (Surprenant *et al.*, 1996; Rassendren *et al.*, 1997; Jiang *et al.*, 2005), supporting the fundamentally different mechanism by which the small cation-permeable channel dilates into the large pore (North, 2002). The rmP2X7 and hP2X7 receptors are most divergent in the C-terminus. This raised the possibility that the different residues in this part (Figure 1A) may contribute to the difference in the dye uptake. Other factors, such as species-dependent receptor facilitation (Roger *et al.*, 2010b) and surface and functional expression (Bradley *et al.*, 2011) may also influence the dye uptake pore formation. Our biotin-labelling results nonetheless show similar membrane protein expression of the rmP2X7 and hP2X7 receptors (Figure 1C). More studies are clearly required to understand better the formation of dye uptake pores, following prolonged P2X7 receptor activation.

As mentioned in the Introduction, the hP2X7 receptor displays strikingly different pharmacological properties to the P2X7 receptor of rodent species and particularly in terms of the antagonist profile. Such species differences could

Table 3

Effects of KN-62 and AZ11645373 on ATP-induced currents mediated by rhesus macaque and human P2X7 receptors

	rmP2X7	hP2X7
Maximal currents in pA/pF	(n = 3)	(n = 6)
Control	192 ± 58	345 ± 43
+100 nM KN-62	36 ± 9*	121 ± 18***
Inhibition (%)	81 ± 1.2	65 ± 2.8
ATP EC ₅₀ in μM	(n = 3)	(n = 6)
Control	676 ± 131 (1.1 ± 0.02 ^a)	284 ± 32 (1.8 ± 0.1 ^a)
+100 nM KN-62	457 ± 162* (1.3 ± 0.2 ^a)	252 ± 44 (1.4 ± 0.2 ^a)
Maximal currents in pA/pF	(n = 3)	(n = 3)
Control	214 ± 8.6	329 ± 32
+30 nM AZ11645373	56 ± 19**	148 ± 52**
Inhibition (%)	74 ± 8.1	57 ± 12
ATP EC ₅₀ in μM	(n = 3)	(n = 3)
Control	696 ± 110 (1.3 ± 0.1 ^a)	291 ± 47 (2.1 ± 0.1 ^a)
+30 nM AZ11645373	1667 ± 1304 (1.6 ± 0.4 ^a)	360 ± 92 (2.1 ± 0.1 ^a)

P* < 0.05, *P* < 0.01, ****P* < 0.001 compared between control and after exposure to the indicated antagonist within the same species.

^aHill coefficient.

compromise or complicate our efforts to understand the physiology of important human proteins, such as the hP2X7 receptor, and associated disease mechanisms, and perform preclinical testing of new drugs using rodent animals and their derived cells (Chen and Kym, 2009). Non-human primates show phylogenetic, immunological, physiological and behavioural similarities to humans and thus are increasingly recognized as invaluable and most relevant models for biomedical research and therapeutic development (Sasaki *et al.*, 2009; Schatten and Mitalipov, 2009). In particular, the Old World primate rhesus macaque monkey represents one of the more attractive non-human primate model species used to study human disease mechanisms (Yang *et al.*, 2008; Han *et al.*, 2009). In this study, we have characterized the pharmacology of the P2X7 receptor from this species (rmpP2X7), and have made direct comparison with the hP2X7 receptor. Our results show that the rmpP2X7 receptor exhibits very similar pharmacological properties to the hP2X7 receptor based on the receptor channel function (Table 2). Our findings demonstrate clearly that the rhesus macaque monkey is a valuable and more relevant model species than the widely used rodent animals in elucidating the mechanisms underlying the roles of the hP2X7 receptor in neurodegenerative, inflammatory and mood depressive diseases, and assessing clinical benefits of new hP2X7 antagonists as therapeutic drugs.

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Conflicts of interest

None.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Rapid and complete reversibility of the inhibition by A-438079. A. Left, representative current recordings showing rapid reversal of the inhibition of ATP-induced currents at the rmP2X7 (top) and hP2X7 receptors (bottom) by A-438079 during 4 s application of ATP, and complete recovery after a 5 min wash. Right, summary of the mean currents ($n = 3$) before treatment with A-438079 (control), at the end of 4 s ATP application after 1 min treatment with A-438079, and 5 min after wash, from experiments as shown on the left.

B. Summary of the mean currents ($n = 3$) before treatment with A-438079 (control), and 1 min after treatment A-438079 (and co-applied with ATP), and 5 min after wash from experiments as shown in Figure 3C.

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