British Journal of Pharmacology (2008) 153, 544–556

0007–1188/08 $30.00
www.brjpharmacol.org

RESEARCH PAPER

Cloning and pharmacological characterization of the guinea pig $P2X_7$ receptor orthologue

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Background and purpose: The human, rat, and mouse $P2X_7$ receptors have been previously characterized, and in this study we report the cloning and pharmacological properties of the guinea pig orthologue.
Experimental approach: A cDNA encoding for the guinea pig $P2X_7$ receptor was isolated from a guinea pig brain library. The receptor was expressed in U-2 OS cells using the BacMam viral expression system. A monoclonal antibody was used to confirm high levels of cell surface expression and the functional properties were determined in ethidium bromide accumulation studies.
Key results: The predicted guinea pig protein is one amino acid shorter than the human and rat orthologues and over 70% identical to the rat and human receptors. In contrast to human and rat $P2X_7$ receptors, 2′-&3′-O-(4benzoylbenzoyl)ATP (BzATP) was a partial agonist of the guinea pig $P2X_7$ receptor when compared to ATP and acted as an antagonist in some assays. However, as at other species orthologues, BzATP was more potent than ATP. The guinea pig $P2X_7$ receptor possessed higher affinity for 1-[N,O-bis(5-isoquinoline sulphonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine (KN62), suramin and Coomassie Brilliant Blue than human or rat $P2X_7$ receptors suggesting that it is pharmacologically different to other rodent or human $P2X_7$ receptors.
Conclusions and implications: The guinea pig recombinant $P2X_7$ receptor displays a number of unique properties that differentiate it from the human, rat and mouse orthologues and this structural and functional information should aid in our understanding of the interaction of agonists and antagonist with the $P2X_7$ receptor.
British Journal of Pharmacology (2008) **153,** 544–556; doi:10.1038/sj.bjp.0707596; published online 26 November 2007

Keywords: ion channel; BacMam; ATP; BzATP; U-2 OS

Abbreviations: BacMam, recombinant baculovirus in which the polyhedrin promoter has been replaced with a mammalian promoter; BzATP, 2′-&3′-*O*-(4benzoylbenzoyl) ATP; CBB, Coomassie brilliant blue; KN62, 1-[*N*, *O*-bis (5-isoquinolinesulphonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine; mAb, monoclonal antibody; PFU, plaque-forming units; PPADS, pyridoxal phosphate-6-azophenyl-2′, 4′-disulphonic acid; U-2 OS, human osteosarcoma cell line

Introduction

The $P2X_7$ receptor is a ligand-gated cation channel activated by extracellular ATP (for review see North, 2002; Burnstock and Knight, 2004; Liang and Schwiebert, 2005). The $P2X_7$ receptor exhibits a number of unusual properties. Following brief activation by low concentrations of agonist, the receptor acts as a nonselective cation channel permeable to calcium, potassium and sodium. However, repeated or prolonged activation by higher agonist concentrations creates a much larger aqueous pore permeable to molecules up to 900 kDa and eventually leads to cell lysis. Recent studies suggest that the large pore may reflect coupling to a pannexin hemichannel (Pelegrin and Surprenant, 2006). The $P2X_7$ receptors are expressed in immune cells and cells from immune origin (Surprenant *et al.*, 1996), and it has been reported recently in neurons (Anderson and Nedergaard, 2006). The localization of $P2X_7$ receptors on pro-inflammatory cells and its ability to modulate the release of the pro-inflammatory cytokine interleukin-1β (North, 2002) have suggested a role in inflammatory diseases. It has been

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Received 24 July 2007; revised 12 September 2007; accepted 30 October 2007; published online 26 November 2007

reported that extracellular ATP acting through the $P2X_7$ receptor may be an important modulator of the neuroinflammation in Alzheimer's disease (Rampe *et al*., 2004), and more recently it was demonstrated that $P2X_7$ knockout mice show reduced inflammatory and neuropathic pain (Chessell *et al*., 2005).

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). These three species orthologues possess different pharmacological properties with the rat and human $P2X_7$ receptors having higher affinity for the agonist 2′-&3′-*O*-(4benzoylbenzoyl) ATP (BzATP) than the mouse $P2X_7$ receptor (Chessell *et al*., 1998). The species orthologues also show very marked differences with respect to antagonist potency particularly between human and rodent receptors. Thus, 1-[*N*, *O*-bis(5-isoquinolinesulphonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine (KN62) possesses much higher affinity for human than rat or mouse receptors, whereas Coomassie brilliant blue (CBB) possesses higher affinity for rat and mouse than human receptors (Humphreys *et al*., 1998; Hibell *et al*., 2001). More recently, novel $P2X_7$ receptor antagonists such as AZ11645373 (Stokes *et al*., 2006) and even non-selective antagonists such as oxidized ATP, pyridoxal phosphate-6-azophenyl-2′, 4′-disulphonic acid (PPADS) and suramin (Hibell *et al*., 2001) have also been shown to possess higher affinity for human than for rodent receptors. These species differences in antagonist potency clearly complicate pre-clinical studies on $P2X_7$ receptor antagonists, which are currently under investigation for various therapeutic indications (Baraldi *et al*., 2004). In this regard, KN62 was found to block a guinea-pig putative $P2X_7$ receptor (Hu *et al*., 2001), which may indicate that the guinea-pig $P2X_7$ receptor is pharmacologically similar to the human receptor. To study this further, we have cloned and pharmacologically characterized this species orthologue.

In the present study, we report the isolation of the cDNA encoding the guinea-pig $P2X_7$ receptor from a brain library and its pharmacological characterization. For these studies, we used the BacMam (recombinant baculovirus in which the polyhedrin promoter has been replaced with a mammalian promoter) expression system (Kost *et al*., 2005) as an alternative to electroporation or stable cell line generation. This technology is safe, as the viruses are unable to replicate in mammalian cells, easy to use and amenable to use with a wide range of host cells. Furthermore, the BacMam technology has been used for the expression of recombinant proteins for cell-based functional assays, including G-protein-coupled receptors (Ames *et al*., 2004a), nuclear receptors (Boudjelal *et al*., 2005) and ion channels (Pfohl *et al*., 2002). In the present study, we evaluated this technology for functional expression of $P2X_7$ receptors. We used U-2 OS (human osteosarcoma cell line) cells as they have a null background for P2Y receptors (Ames *et al*., 2004b) and do not express $P2X_7$ receptors (Michel *et al*., 2007).

Pharmacological characterization of the guinea-pig $P2X_7$ receptor showed that the receptor does have some pharmacological similarities to human $P2X_7$ receptors but also that it has significant differences from human as well as rat and mouse $P2X_7$ receptor orthologues.

Methods

Cloning of the guinea-pig $P2X_7$ receptor

The 5′ and 3′ sequence ends of the guinea-pig $P2X_7$ gene were determined via rapid amplification of cDNA ends PCR and cloning from guinea-pig brain and spleen SMART cDNA (Clontech, Mountain View, CA, USA). Primers were designed based on an alignment between the rat (NM_019256), mouse (NM_011027) and human (NM_002562) sequences and rapid amplification of cDNA ends PCR was performed to amplify these ends. Once the 5′ and 3′ sequence ends were confirmed between the two different tissue sources, primers were then designed to amplify the full-length cDNA open reading frame. The full-length open reading frame was obtained by PCR from guinea-pig brain SMART cDNA. This sequence was confirmed by comparison with cDNA clones obtained from multiple tissue sources. The final clone was flanked with a 5′ CACC for directional cloning into the pcDNA3.1_V5-His-TOPO vector. The GenBank accession number for the guineapig $P2X_7$ receptor is EU275201.

Verification of sequence

To confirm the absence of amino acid at position 77 in the guinea-pig receptor compared with the human and rat orthologues (Figure 1), total RNA was isolated from guinea-pig brain using the RNeasy mini kit (Qiagen, Crawley, UK). cDNA synthesis and PCR were carried out using the Superscript One step reverse transcriptase-PCR with Platinum Taq kit (Invitrogen, Paisley, UK) and gene-specific primers (5′-CAAAGTCACCCGCATCCAGAGTAG-3′ and 5′-GTCCAGGT TGCAGTCCCAGTTGAT-3′) encompassing the amino acid of interest. A reaction containing no reverse transcriptase-PCR was used as a control. The 739 bp PCR product was agarose gel purified using Geneclean II (MP Biomedicals, Cambridge, Cambs, UK), cloned into pCR2.1 using the TOPO TA cloning kit (Invitrogen) and transformed into TOP10 cells. The resulting transformants were cultured overnight in Luria Bertani broth containing 100 $\mu g\,ml^{-1}$ ampicillin and plasmid DNA was then isolated using the Miniprep spin kit (Qiagen). The insert sequence was then confirmed by DNA sequencing in both directions, confirming the amino acids between positions 17 and 262.

Construction of pFastBac-Mam-1 expression plasmids and BacMam expression viruses

Guinea-pig $P2X_7$ cDNA was subcloned as a 1860 bp *Hin*d3/*Not*1 fragment obtained from the plasmid pcDNA3.1/guinea pig $P2X_7$ into the *Hin*d3 and *Not*1 sites of pFastBac-Mam-1. The BacMam baculovirus transfer vector pFastBac-Mam-1 has been described previously (Condreay *et al*., 1999). The resulting plasmid, pFBMam-1/guinea-pig $P2X_7$, was then used to generate the BacMam baculovirus BacMam-guinea-pig $P2X_7$. The rat, mouse and human $P2X_7$ receptor sequences (Surprenant *et al*., 1996; Rassendren *et al*., 1997; Chessell *et al*., 1998) were excised from the plasmid pCDNA3 and then ligated into the pFastBac-Mam-1 vector using conventional techniques (Condreay *et al*., 1999). In these studies, the mouse sequence described by Young *et al*. (2007)

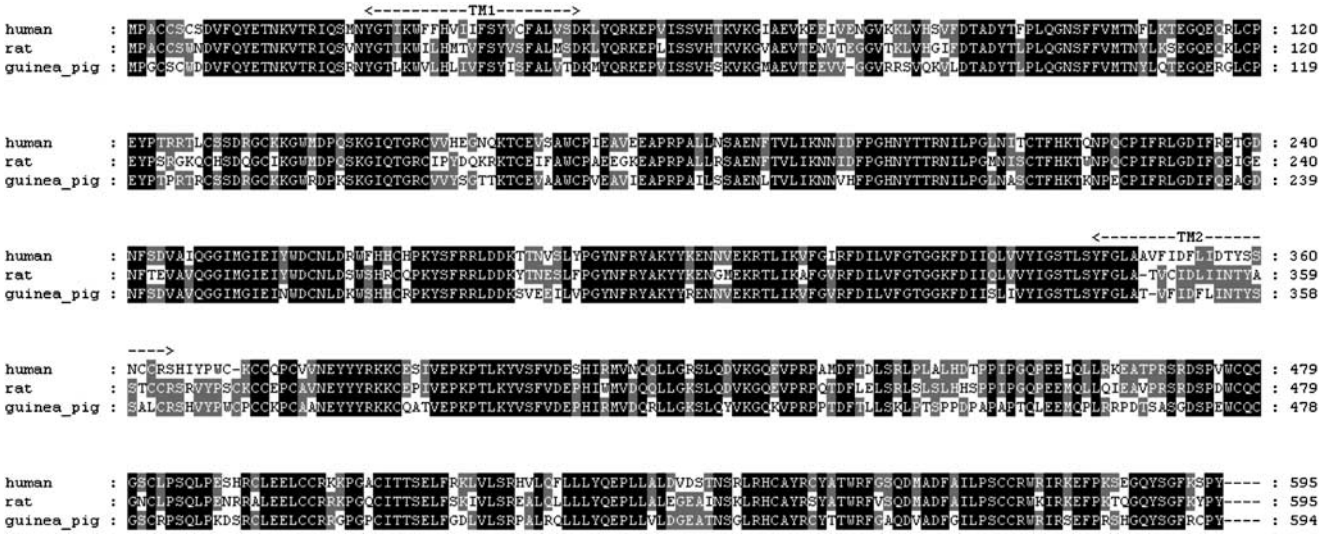

Figure 1 Pairwise alignment of primary amino-acid sequences of human, rat and guinea-pig P2X$_7$ proteins. The two predicted transmembrane-spanning regions are annotated as TM1 and TM2 with dashes and arrows. Completely conserved residues are coloured black, whereas positions that differ between all three sequences are coloured white. Amino-acid positions that display an intermediate level of conservation are shaded grey.

was utilized. This differs from the mouse P2X$_7$ receptor described by Chessell *et al*. (1998) at three amino acids, specifically, leucine instead of phenylalanine at amino acid 11, threonine instead of alanine at position 221 and threonine instead of methionine at position 283. Identical results were obtained using the mouse P2X$_7$ receptor described by Chessell *et al*. (1998) (data not shown). All BacMam baculoviruses were generated from their transfer plasmids in Sf9 insect cells using standard methods described previously (Clay *et al*., 2003).

Cell culture and viral transductions

Human osteosarcoma U-2 OS cells (obtained from ATCC, see Ames *et al*., 2004b) were maintained in adherent culture conditions in the presence of Dulbecco's modified Eagle's medium:nutrient mixture F-12 supplemented with Glutamax (DMEM:F12 + Glutamax, Invitrogen) and 10% foetal bovine serum (PAA laboratories GmBH, Pasching, Austria) at 37 °C, 5% CO_2. One day prior to assay, cells were harvested from the culture flasks using 0.05% trypsin/EDTA (Invitrogen), pelleted and resuspended at a concentration of $\sim 750 \times 10^3$ cells ml^{-1} in culture media in the presence of varying concentrations of the test BacMam viruses (1.25–20% BacMam virus in culture media v/v). Unless stated, human, rat and guinea-pig P2X$_7$ BacMam viruses were used at 10% (v/v), whereas the mouse P2X$_7$ receptor was used at 2.5% (v/v). For ethidium bromide and quantitative antibody binding fluorometry studies, cells (70–80 000) were plated into individual wells of poly-L-lysine pretreated 96-well plates (Costar, High Wycombe, UK) and the plates were incubated at 37 °C, 5% CO_2 overnight. Alternatively, for immunocytochemistry (ICC) studies, cells were plated in 22 mm diameter poly-D-lysine-treated BioCoat coverslips (BD Biosciences, Oxford, UK). For the experiments reported here, titres of the different viruses were as follows: 2×10^8 plaque-forming units (PFU) ml^{-1} for the human P2X$_7$-BacMam, 2.2×10^8 PFU ml^{-1} for the rat P2X$_7$-BacMam, 1.8×10^8 PFU ml^{-1} for the guinea-pig P2X$_7$-BacMam and 1.22×10^9 PFU ml^{-1} for the mouse P2X$_7$-BacMam.

ICC and quantitative antibody binding fluorimetry

ICC was performed as described elsewhere (Fonfria *et al*., 2002), using a monoclonal antibody specific for human P2X$_7$ receptor (P2X$_7$mAb) (Buell *et al*., 1998) diluted 1:500, and an Alexa-488-conjugated goat anti-mouse secondary antibody (Invitrogen), diluted 1:200. At the end of the procedure, cells were washed in phosphate-buffered saline (Invitrogen) and mounted onto glass slides using ProLong Gold antifade mounting media (Invitrogen). Fluorescent images were captured using a Leica DMR microscope (Leica Microsystems GmbH, Germany).

Quantitative antibody binding fluorimetry was performed using the same primary and secondary antibodies as above. The assay buffer comprised 2% BSA (Sigma-Aldrich, Poole, UK) in phosphate-buffered saline (Invitrogen). Briefly, adherent cells in 96-well plates were washed twice with 350 µl of assay buffer, and exposed to a mixture of increasing concentrations of primary antibody (0.1–3 µg ml^{-1}) and 10 µg ml^{-1} of secondary antibody in assay buffer. Plates were covered with aluminium foil and shaken at 10 r.p.m. min^{-1} in an orbital shaker (Stuart Scientific, Staffordshire, UK) for 3 h at room temperature. After the incubation period, cells were washed three times with assay buffer and 100 µl of fresh assay buffer was added to the cells. P2X$_7$ antibody binding was determined by measuring fluorescence (excitation wavelength of 485 nm and emission wavelength of 530 nm) from below the plate with a FlexStation (Molecular Devices, Wokingham, UK).

Ethidium bromide accumulation assays

For the ethidium bromide accumulation studies, the assay buffer comprised (in mM): HEPES 10, *N*-methyl-D-glucamine 5, KCl 5.6, D-glucose 10, $CaCl_2$ 0.5 (pH 7.4) and was supplemented with either 140 mM NaCl (NaCl buffer) or 280 mM sucrose (sucrose buffer). Studies were performed as described previously (Michel *et al*., 2006a). Cells grown in poly-L-lysine pretreated 96-well plates (see above) were washed with 350 µl of assay buffer and incubated for 30 min at room temperature (19–21 °C) in the presence or absence of the $P2X_7$ receptor antagonists before addition of agonist (ATP or BzATP, Sigma-Aldrich) and ethidium bromide (100 µM final assay concentration, supplied as 10 mM stock by Pierce, Cramlington, UK). Incubations were continued for the length of time indicated and were rapidly terminated by addition of 25 µl of 1.3 M sucrose assay buffer containing 5 mM of the $P2X_7$ receptor antagonist, Reactive black 5. Cellular accumulation of ethidium in the cell monolayer was determined by measuring fluorescence (excitation wavelength of 530 nm and emission wavelength of 620 nm) from below the plate with a FlexStation (Molecular Devices). For studies using BzATP as an antagonist, a 15 min pre-incubation period was used. The antagonists CBB, KN62, PPADS and decavanadate were obtained from Sigma-Aldrich. Decavanadate solutions were prepared as described previously (Michel *et al*., 2006b).

Data analysis

Data are mean ± s.e.mean of three to four independent experiments. Curve fitting and statistical analysis were performed using GraphPad Prism 3 (GraphPad Software Inc., San Diego, CA, USA). For quantitative immunocytochemical studies, nonspecific binding of the $P2X_7$mAb to non-transduced U-2 OS cells was measured at each mAb concentration and subtracted from the binding signal measured in the U-2 OS cells transduced with the various $P2X_7$ receptors using the BacMam virus expression system in order to measure specific binding of the $P2X_7$mAb. For studies on HEK293 cells stably expressing human or rat $P2X_7$ receptors, nonspecific binding was measured in wild-type HEK293 cells and subtracted from total binding to determine specific binding of the $P2X_7$mAb. For statistical tests, one-way ANOVA followed by Dunnett's or Tukey's *post hoc* test was used.

Results

Cloning of the guinea-pig $P2X_7$ receptor

The sequence of the guinea-pig $P2X_7$ receptor was identical in cDNA clones generated from two different cDNA tissue sources, guinea-pig brain and guinea-pig spleen SMART cDNA. The predicted final protein sequence is 594 amino acids in length, one amino acid less than the human and rat orthologues, which are both 595 amino acids in length (Figure 1). The difference was due to the absence of the glutamic acid at position 77, which is present in the human, rat and mouse orthologues. The absence of the residue was confirmed by PCR amplification of this region of the receptor from mRNA obtained from guinea-pig brain. This residue is present across all P2X receptors (North, 2002). However, for the $P2X_1$-$P2X_6$ receptors, several residues between amino acids 72 and 86 ($P2X_7$ nomenclature) in exon 2 are missing or show a high degree of divergence (North, 2002).

Table 1 Pair distances using ClustalW method (settings = slow/accurate, Gonnet)

	Percentage identity		
	Guinea-pig	*Human*	*Rat*
Guinea-pig		77.2	74.2
Human	27.1		80.2
Rat	31.6	23.1	
	Percentage divergence		

Percent similarity is displayed in the upper triangle and percent divergence in lower triangle.

The homology of the generated guinea-pig $P2X_7$ receptor clone is 77% to the human, and 74% to the rat $P2X_7$ receptors (Table 1). Figure 1 shows the alignments of the guinea-pig clone compared to previously reported human and rat $P2X_7$ receptors (Surprenant *et al*., 1996; Rassendren *et al*., 1997). The absence of glutamine 77 in the guinea-pig receptor complicates comparison across species, so all comparisons have been made based on the sequence alignment in Figure 1 and using the human numbering. The guinea-pig $P2X_7$ receptor had the same 10 conserved cysteine residues present in the other P2X receptors at positions 119, 129, 135, 152, 162, 168, 216, 226, 260 and 269 (North, 2002). In addition, the guinea-pig receptor contained the putative N-glycosylation sites (Asparagine-Xaa-Serine/Threonine) at asparagine residues 187, 202, 213 and 241 present in rat, mouse and human receptors. The rat has two extra putative N-glycosylation sites at asparagine 74 and 284, this later site also being present in human and mouse receptors, but these residues were not present in the guinea-pig receptor (Figure 1 and Young *et al*., 2007).

Several single nucleotide polymorphisms of the human and mouse $P2X_7$ receptor have been identified. Histidine 155 to tyrosine is a reported gain of function polymorphism in human receptors (Cabrini *et al*., 2005), and in guinea-pig receptors, the corresponding residue was tyrosine as is in rat and mouse (Figure 1 and Young *et al*., 2007). Human polymorphisms arginine 307 to glutamine (Gu *et al*., 2004), threonine 357 to serine (Shemon *et al*., 2006), glutamate 496 to alanine (Gu *et al*., 2001), isoleucine 568 to asparagine (Wiley *et al*., 2003) and arginine 574 to histidine (Fernando *et al*., 2005) are reported to affect function of human $P2X_7$ receptors either by altering the trafficking to the plasma membrane or producing defective pore formation. In guinea-pig, rat and mouse, the corresponding residues are arginine at position 307, threonine at position 357, glutamine at position 496, isoleucine at position 568 and arginine at position 574 (human numbering, Figure 1 and Young *et al*., 2007).

The mouse $P2X_7$ receptor exhibits loss-of-function mutations in positions serine 342 to phenylalanine (Denlinger *et al*., 2003) and proline 451 to leucine (Adriouch *et al*.,

2002). These two residues were serine 342 and proline 451 in guinea-pig P2X$_7$ receptor and were conserved among the guinea-pig, rat and human orthologues (Figure 1 and Young *et al*., 2007).

Finally, the mouse and rat receptors possess differential sensitivity to ATP and BzATP and it is thought that the lysine at residue 127 in rat (alanine in mouse) and asparagine at residue 284 in rat (aspartate in mouse) are responsible for this difference (Young *et al*., 2007). In the guinea-pig receptor, the residues are threonine and glutamine, respectively, whereas the human orthologue has threonine and asparagine (Figure 1). Note that if the missing amino acid at position 77 in the guinea-pig P2X$_7$ receptor affects the relative location of the other residues then the corresponding amino acid at position 127 would be arginine.

ICC of P2X$_7$ receptors expressed in U-2 OS cells

Expression of P2X$_7$ receptor orthologues was studied in U-2 OS cells transduced with 10% of each individual BacMam virus (v/v) using ICC. The primary P2X$_7$mAb, which recognizes the human P2X$_7$ receptor (Buell *et al*., 1998) was used, with a secondary antibody conjugated to the fluorophore Alexa-488. Images in Figure 2 upper panels show a positive cell surface staining for human, rat and guinea-pig P2X$_7$ orthologues, suggesting that this P2X$_7$mAb also detects rat and guinea-pig P2X$_7$ receptors. Non-transduced U-2 OS cells showed negative staining (data not shown). All micrographs were captured under the same conditions in order to enable comparisons of the relative levels of labelling between the species orthologues.

The staining in cells transduced with the guinea-pig P2X$_7$ receptor was greater than observed in cells transduced with either the rat or human P2X$_7$ receptor. To investigate the reason for this, we used a quantitative method to analyse antibody binding to the different orthologues (Figure 2, lower panels). Binding of the antibody to each of the species orthologues increased in a concentration-dependent manner and showed signs of saturation at the highest concentration of 3 $\mu g\,ml^{-1}$ tested (Figure 2B). For the guinea-pig and rat orthologues, expression increased with increasing levels of virus used for the transduction (Figure 2C) with maximal expression at approximately 10% virus (one-way ANOVA $P>0.05$ when comparing the mAb binding with titres of 10, 15 or 20%). For the human orthologue, even 2.5% virus produced nearly maximal levels of binding (Figure 2C). In cells transduced with 15 and 20% BacMam virus, the cell density on the plates after the extensive wash procedures used to measure the mAb bound appeared less than in cells transduced with the lower virus titres. The loss of cells did not appear to differ greatly between cells transduced with the different species orthologues. In cells transduced with the highest virus titre of 20%, the pK_D values ($g\,ml^{-1}$) for the antibody were 5.53 ± 0.08, 5.68 ± 0.07 and 6.51 ± 0.09, respectively, for guinea-pig, rat and human P2X$_7$ receptors ($n=3$ independent experiments). Similar pK_D values were obtained at the other virus titres (data not shown). In cells transduced with 20% of the human, rat and guinea-pig receptor BacMam viruses, the maximal fluorescence was approximately 2-, 4.7- and 6.8-fold, respectively, of the background binding in non-transduced cells. When the data were expressed relative to the human P2X$_7$ receptor, the

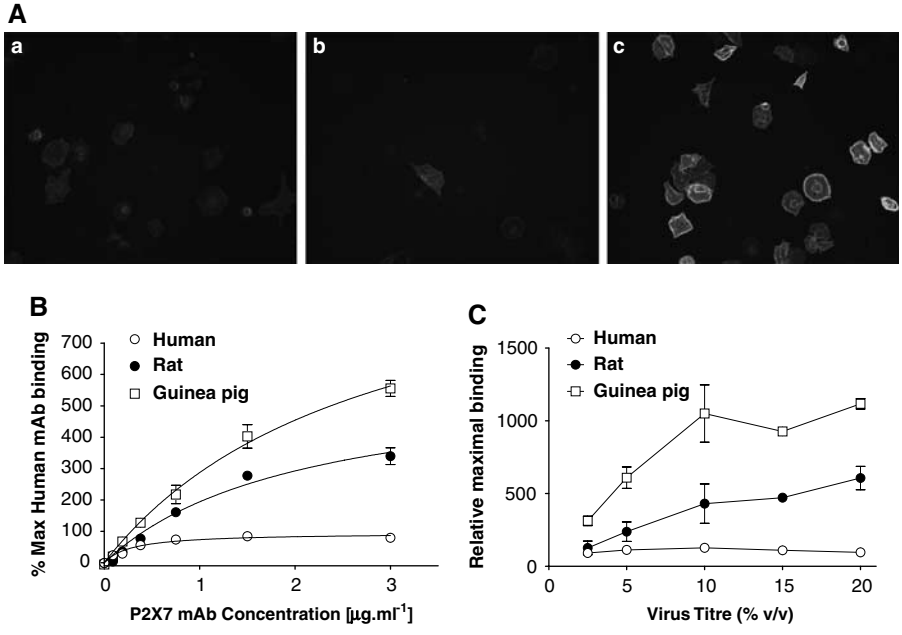

Figure 2 Characterization of P2X$_7$ receptor expression on BacMam-transduced U-2 OS cells. (**A**) Immunofluorescence images for U-2 OS cells transduced with the human (a), rat (b) and guinea-pig (c) recombinant P2X$_7$ receptors. Expression was detected using a primary anti-P2X$_7$ antibody (Buell *et al*., 1998) and an Alexa-488-conjugated secondary antibody. Images shown are representative of three micrographs taken from three independent experiments. (**B**) Specific binding of the P2X$_7$mAb to U-2 OS cells transduced with the human, rat or guinea-pig recombinant P2X$_7$ receptors. (**C**) Effect of virus titre on the maximal level of mAb binding to U-2 OS cells transduced with the human, rat or guinea-pig recombinant P2X$_7$ receptors. Data are shown as mean ± s.e.mean of three independent experiments run in duplicate.

estimated maximal levels of binding obtained from the curve fits to the data for the rat and guinea-pig receptor were 6.07 ± 0.8- and 11.2 ± 0.04-fold, respectively. Taken together, these findings suggest that the higher staining observed in the immunocytochemical studies reflects a higher level of expression of the guinea-pig receptor compared to the human and rat orthologues.

To further validate the methodology, stably transfected recombinant human and rat orthologues in HEK cells, previously shown to possess functional P2X$_7$ receptors (Surprenant *et al*., 1996; Rassendren *et al*., 1997), were also tested using this technique. A 17-fold and 4-fold increase in mAb binding over wild-type HEK293 cells was seen in cells expressing human and rat P2X$_7$ receptors, respectively. The pK_D of the antibody was 6.43 ± 0.07 for human and 5.78 ± 0.02 for rat P2X$_7$ receptors ($n = 3$ independent experiments), which is in close agreement with the respective pK_D values determined using the BacMam-transduced U-2 OS cells.

Agonist-induced ethidium bromide accumulation in recombinant P2X$_7$ U-2 OS cells

The functional properties of the guinea-pig P2X$_7$ receptor were evaluated using ethidium bromide accumulation assays. Initial experiments on functional expression were performed at a fixed transduction rate of 10% BacMam virus (v/v), as this condition showed optimal cell surface expression of the human, rat and guinea-pig P2X$_7$ orthologues (Figure 2). Figure 3 shows concentration-dependent ethidium bromide accumulation following ATP exposure for 2–64 min for the human receptor (panel A), rat receptor (panel C) and guinea-pig receptor (panel E). ATP potency was not affected by agonist exposure time (one-way ANOVA, $P > 0.05$), whereas the maximal effect increased over time and after 64 min agonist exposure resulted in a ~20-fold increase in fluorescence for each orthologue. The maximal rate of ethidium accumulation at the highest concentrations of ATP studied could be described by a single exponential (data not shown) and was characterized by a K_{obs} of 0.012,

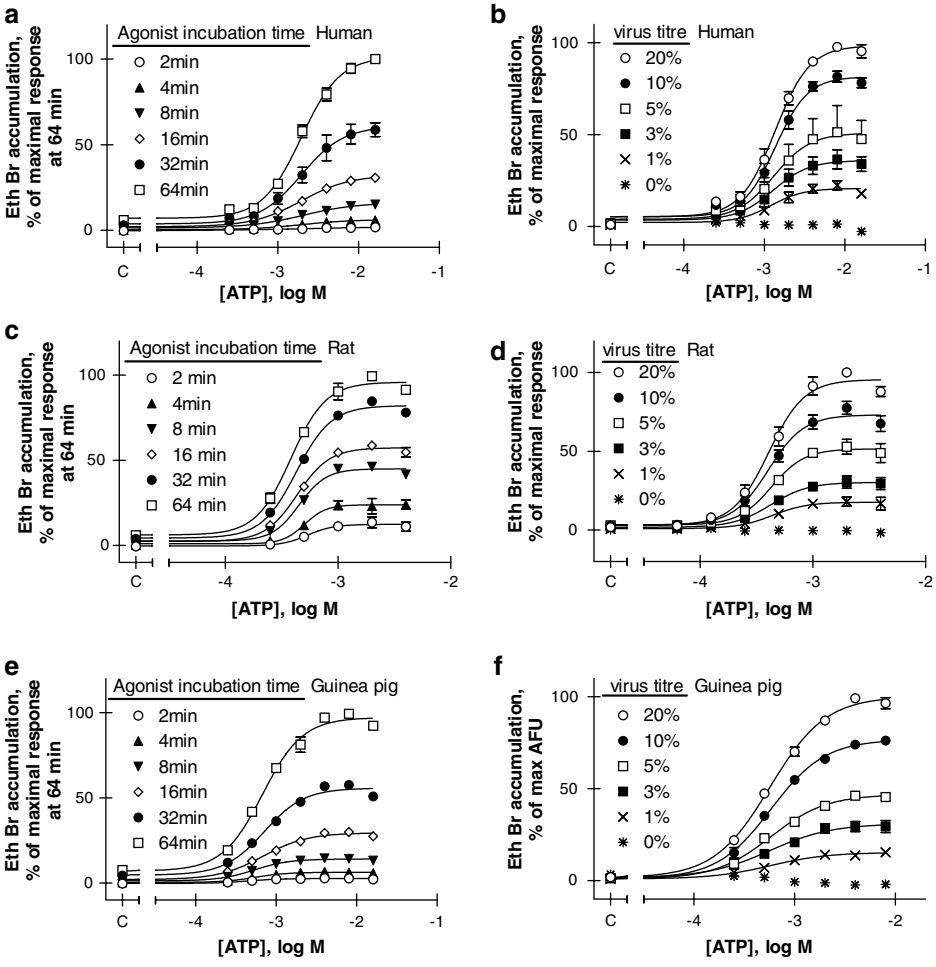

Figure 3 ATP-stimulated ethidium (Eth Br) bromide accumulation in U-2 OS cells transiently transduced with P2X$_7$ orthologues. (**a**, **c**, **e**) U-2 OS cells were transduced with BacMam virus at 10% (v/v) and ethidium bromide accumulation following ATP exposure (2–64 min) was measured in cells transduced with human (**a**), rat (**c**) or guinea-pig (**e**) recombinant P2X$_7$ receptors. (**b**, **d**, **f**) U-2 OS cells were transduced with various titres of BacMam virus (1.25–20%, v/v) and ethidium bromide accumulation following ATP exposure was measured in cells transduced with human (**b**), rat (**d**) or guinea-pig (**f**) recombinant P2X$_7$ receptors. ATP exposure was 16 min for human and guinea-pig orthologues, and 8 min for the rat orthologue. NaCl buffer was used in all studies. Control values obtained in the absence of ATP are shown on the abscissa (denoted as **c**). Data are mean ± s.e.mean of three experiments run in duplicate.

0.058 and 0.011 min^{-1} for cells expressing human, rat and guinea-pig P2X$_7$ receptors, respectively. The maximum ethidium accumulation measured after 64 min in the presence of the highest concentration of ATP was 215 338 ± 22 068, 216 467 ± 20 934 and 150 505 ± 9862 relative fluorescence units in cells expressing human, rat and guinea-pig P2X$_7$ receptors, respectively, and these values were not significantly different from each other ($P>0.05$, one-way ANOVA followed by Tukeys's *post hoc* test). To account for the differences in kinetics at the species orthologues, the predicted maximal ethidium accumulation for each orthologue was also calculated from the curve fits of the rate data. The values were 392 106 ± 6376, 217 865 ± 23 807 and 299 985 ± 53 812 relative fluorescence units in cells expressing human, rat and guinea-pig P2X$_7$ receptors, respectively. The predicted maximal accumulation in cells expressing the rat P2X$_7$ receptor was slightly, but significantly, lower than in the cells expressing human or guinea-pig P2X$_7$ receptors ($P<0.05$, one-way ANOVA followed by Tukeys's *post hoc* test).

Agonist exposure times for subsequent studies were chosen, so that uptake was still on the linear phase of the time–response relationship. This was 16 min for human and guinea-pig receptors, and 8 min for the rat receptor. Exposure of U-2 OS cells to increasing amounts of human, rat or guinea-pig P2X$_7$ receptor BacMam virus (1.25–20% v/v) resulted in a titre-dependent increase in maximal fluorescence for all three species orthologues (Figure 3b, d and f, for human, rat and guinea-pig, respectively). The effect of varying virus concentration on maximal rates of ethidium accumulation was different from that seen when measuring expression using the mAb (Figure 2C). Whereas maximal levels of expression were observed at 2.5–10% virus (Figure 2C), there was no clearly defined maximal response in the ethidium accumulation studies even at 20% virus. The pEC$_{50}$ for ATP did not vary with virus titre at the human, rat and guinea-pig receptors, respectively, suggesting that potency was not affected by the level of expression. For all subsequent experiments on human, rat and guinea-pig receptors functional responses were measured using 10% BacMam virus.

One of the features of P2X$_7$ receptors is the difference in sensitivity to BzATP and ATP among the species orthologues (Young *et al.*, 2007). Figure 4 shows a direct comparison of ATP and BzATP potency at the human, rat, mouse and guinea-pig P2X$_7$ receptors in NaCl buffer. BzATP and ATP were agonists of rat, human and mouse receptors, although there were differences in the relative potencies and intrinsic activities between the orthologues (Figure 4 and Table 2). Most notably, BzATP and ATP produced comparable effects at rat P2X$_7$ receptors, but BzATP was a partial agonist when compared with ATP at human and mouse P2X$_7$ receptors and produced no effect at the guinea-pig P2X$_7$ receptor. Furthermore, BzATP was an antagonist of ATP-mediated responses in NaCl buffer (Figures 5a and b), although it was not a competitive antagonist as it reduced the maximum response to ATP without greatly affecting ATP potency.

To explore if the lack of effect of BzATP was due to low efficacy in this assay, additional studies were undertaken in sucrose buffer, as in this buffer, agonist potency and the

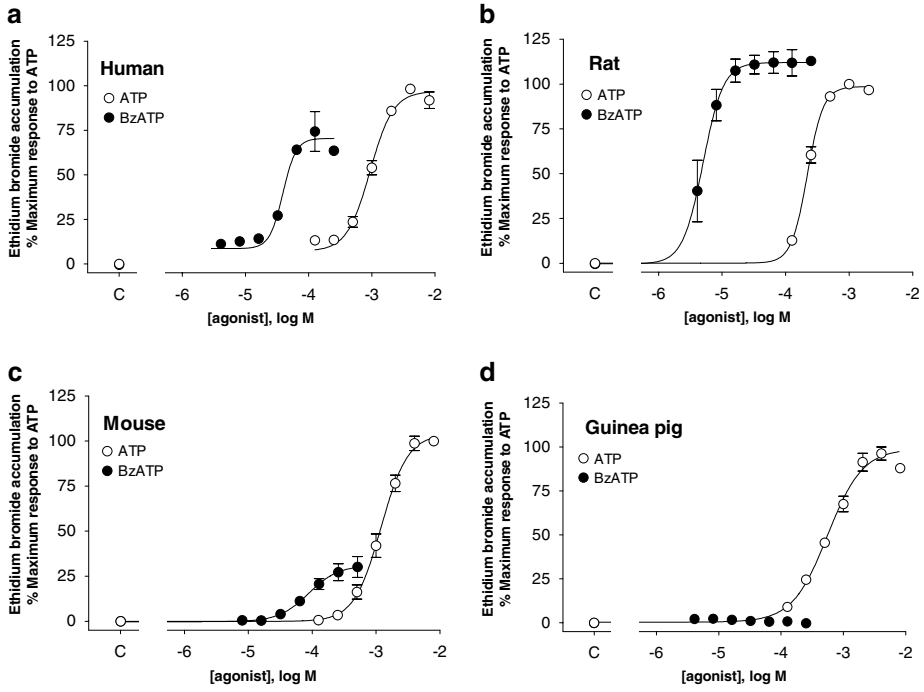

Figure 4 Comparison of BzATP- and ATP-stimulated ethidium bromide (Eth Br) accumulation in U-2 OS cells transiently transduced with P2X$_7$ orthologues. Ethidium bromide accumulation following agonist exposure was measured in cells transduced with human (**a**), rat (**b**), mouse (**c**) or guinea-pig (**d**) recombinant P2X$_7$ receptors. Agonist exposure was 16, 8, 8 and 32 min, respectively, for cells transduced with the human, rat, mouse or guinea-pig P2X$_7$ receptors. NaCl buffer was used in all studies. Control values obtained in the absence of ATP or BzATP are shown on the abscissa (denoted as **c**). Data are mean ± s.e.mean of three experiments run in duplicate. BzATP, 2′-&3′-*O*-(4benzoylbenzoyl) ATP.

ability of P2X$_7$ agonists to stimulate ethidium accumulation is more easily demonstrated (Michel *et al*., 1999, 2000). In sucrose buffer, ATP potency was increased and BzATP produced an agonist effect, although it was still a partial agonist (Figure 5c). Similarly, the maximal effect of BzATP, relative to ATP, at the human receptor was increased, and BzATP was a full agonist in sucrose buffer (Figure 5d).

P2X$_7$ antagonist effects at the guinea-pig P2X$_7$ receptor

Antagonist potency was determined in ethidium bromide accumulation assays performed on U-2 OS cells transduced with 10% guinea-pig P2X$_7$ receptor BacMam virus. Figure 6 shows data for antagonists that are more potent at the human receptor than at the rat or mouse orthologue (Anderson and Nedergaard, 2006). KN62 has a pIC$_{50}$ value of 6.5 at human P2X$_7$ receptors and <5 at rat receptors (Hibell *et al*., 2001); in this study, it was a potent antagonist (pIC$_{50}=6.88\pm0.09$, $n=4$) of 1 mM ATP-stimulated ethidium bromide accumulation in cells expressing guinea-pig P2X$_7$ receptors (Figures 6a and b). One-way ANOVA revealed no statistically significant differences in the antagonist potency with respect to the doses of ATP used ($P>0.05$). PPADS has a pIC$_{50}$ value of 6.7 and 5.9, respectively, at human and rat P2X$_7$ receptors (Hibell *et al*., 2001) and here it also inhibited the guinea-pig receptor (Figures 6c and d), with relatively high potency (pIC$_{50}=6.54$–6.82, $n=4$). One-way ANOVA followed by Dunnett's *post hoc* test revealed a small but statistically significant ($P<0.001$) difference in its potency against 250 μM ATP (pIC$_{50}=6.82\pm0.02$, $n=4$) compared to its potency against 2 mM ATP (pIC$_{50}=6.54\pm0.04$, $n=4$). Suramin possesses a pIC$_{50}$ of 4.9 and 4.8 at rat and human receptors, respectively (Hibell *et al*., 2001), but in this study was a much more potent antagonist (pIC$_{50}=6.11\pm0.02$, $n=3$, against 1 mM ATP) at the guinea-pig receptor (Figures 6e and f). One-way ANOVA revealed no statistically significant differences in antagonist potency with respect to the doses of ATP used ($P>0.05$). In this study, PPADS and suramin were non-competitive antagonists of the guinea-pig

Table 2 Relative potencies and intrinsic activities of ATP and BzATP at the rat, human, mouse and guinea-pig P2X$_7$ orthologues

P2X$_7$ orthologue	*pEC$_{50}$ BzATP*	*pEC$_{50}$ ATP*	*Intrinsic activity BzATP*[a]
Rat	5.30 ± 0.08	3.66 ± 0.02	$112\pm6\%$
Human	4.43 ± 0.05*	3.06 ± 0.04*	$74\pm11\%$*
Mouse	4.06 ± 0.03*,†	2.92 ± 0.05*	$30\pm6\%$*,†
Guinea-pig	—	3.22 ± 0.10*,‡	$2\pm0.3\%$*,†,‡

Studies were performed in NaCl buffer.
[a]Compared to full agonist ATP (100%). Data are mean ± s.e.mean, $n=3$ experiments. One-way ANOVA followed by Tukey's *post hoc* test revealed statistically significant differences vs rat (*$P<0.05$), human (†$P<0.05$) or mouse P2X$_7$ (‡$P<0.05$).

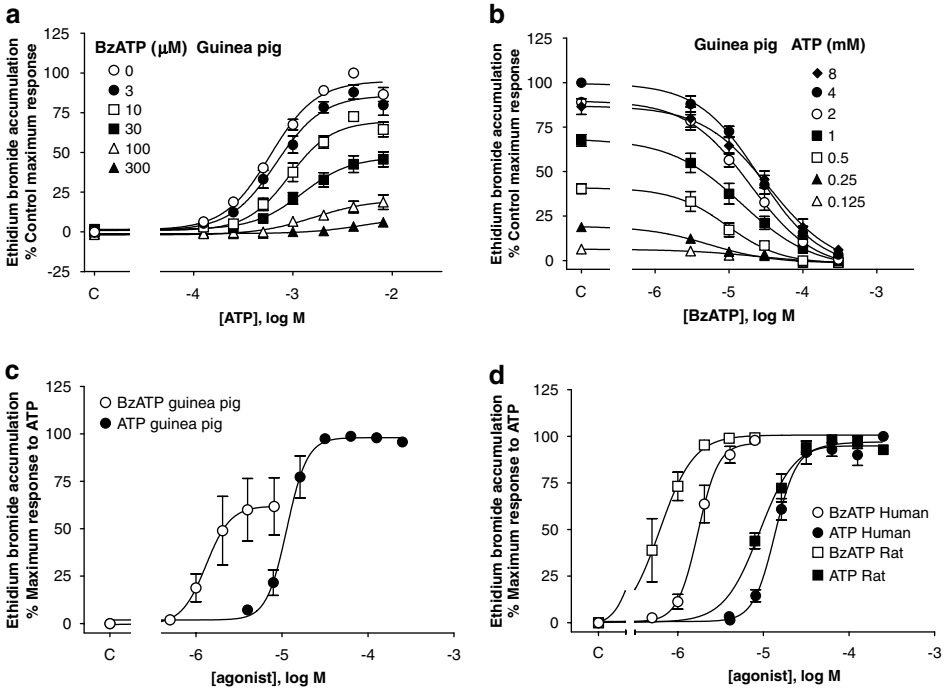

Figure 5 BzATP- and ATP-stimulated ethidium bromide (Eth Br) accumulation in U-2 OS cells transiently transduced with P2X$_7$ orthologues. (**a**, **b**) Cells were pretreated with the indicated concentrations of BzATP 15 min before the addition of ATP and ethidium. Ethidium bromide accumulation was measured after a further 32 min incubation in the presence of ATP and BzATP. These studies were performed in NaCl buffer: (**a**) effect of BzATP on ATP concentration–effect curve and (**b**) inhibition curves for BzATP at each ATP concentration employed in (**a**). (**c**, **d**) Concentration–effect curves for BzATP and ATP determined in sucrose buffer: (**c**) guinea-pig and (**d**) human and rat receptors. Agonist exposure was 2 min for human and rat and 8 min for the guinea-pig orthologue. Control values obtained in the absence of ATP or BzATP are shown on the abscissa (denoted as **c**). Data are mean ± s.e.mean of three experiments run in duplicate. BzATP, 2′-&3′-*O*-(4benzoylbenzoyl) ATP.

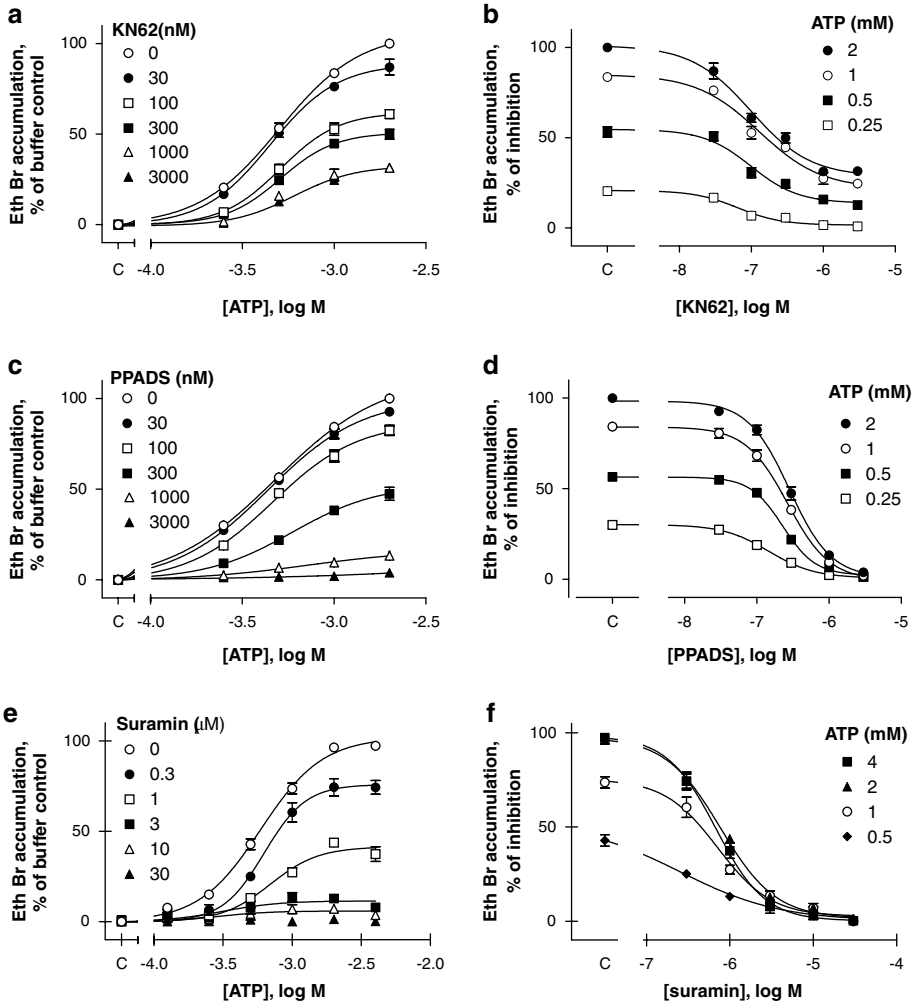

Figure 6 Antagonist effects on ATP-induced ethidium bromide (Eth Br) accumulation in U-2 OS cells transiently transduced with the guinea-pig P2X$_7$ receptor. Effects of KN62 are shown in (**a**, **b**), PPADS in (**c**, **d**) and suramin in (**e**, **f**). (**a**, **c**, **e**) The effects of the antagonists on the concentration–effect curve to ATP. In (**b**, **d**, **f**), the data from (**a**, **c**, **e**) are re-plotted to present antagonist inhibition curves at each of the indicated ATP concentrations. NaCl buffer was used in all studies. Control values obtained in the absence of the antagonists or ATP are shown on the abscissa (denoted as **c**). Results shown are mean ± s.e.mean of 3–4 independent experiments run in duplicate.

P2X$_7$ receptor, as they insurmountably and completely reduced the maximal response to ATP. KN62 was also a non-competitive antagonist but, unlike the other antagonists, the reduction in maximal response to ATP was saturable, the inhibition of ATP responses produced by 1 and 3 μM KN62 being identical (Figures 6a and b).

Figure 7 shows the effects of antagonists of the P2X$_7$ receptor, which are selective for the rat, compared to the human, orthologue, namely CBB (Hibell *et al*., 2001) or equipotent at both, namely decavanadate (Michel *et al*., 2006b). CBB was a potent non-competitive antagonist of the guinea-pig P2X$_7$ receptor ($pIC_{50} = 7.63 \pm 0.02$, $n = 3$, against 1 mM ATP), and one-way ANOVA revealed no statistically significant differences in the antagonist potency with respect to the doses of ATP used ($P > 0.05$). Decavanadate potency varied quite markedly with agonist concentration (Figures 7c and d) with its potency against 250 μM ATP ($pIC_{50} = 6.53 \pm 0.11$, $n = 4$) being significantly higher than against 8 mM ATP ($pIC_{50} = 5.61 \pm 0.02$, $n = 4$; $P < 0.001$, one-way ANOVA followed by Dunnett's *post hoc* test). Although decavanadate did not produce a clearly competitive antagonist effect, it was the only antagonist to significantly reduce ATP potency (one-way ANOVA followed by Dunnett's *post hoc* test). Thus, the control ATP potency (pEC_{50}) at the guinea-pig receptor was 3.24 ± 0.02 ($n = 3$), whereas in the presence of 1 μM decavanadate it was 2.85 ± 0.06 ($n = 3$, $P < 0.001$) and in the presence of 3 μM decavanadate it was 2.40 ± 0.03 ($n = 3$, $P < 0.001$).

Finally, the P2X$_7$mAb used to label cell surface P2X$_7$ receptors (Figure 3), which is an antagonist of the human receptor (Buell *et al*., 1998), was also tested as antagonist of the guinea-pig orthologue. Figure 8 shows that this mAb increased rather than inhibited ATP-induced ethidium bromide accumulation in guinea-pig. Thus, maximal ethidium accumulation was increased to $119 \pm 2.6\%$ ($n = 3$, $P < 0.001$) at 300 ng ml^{-1} and to $184 \pm 2.8\%$ ($n = 3$, $P < 0.001$) at 1 μg ml^{-1} when compared to maximal ethidium accumulation in the absence of the antibody ($97 \pm 1.6\%$, $n = 3$). At a concentration of 1 μg ml^{-1}, the antibody slightly decreased ATP potency (one-way ANOVA followed by

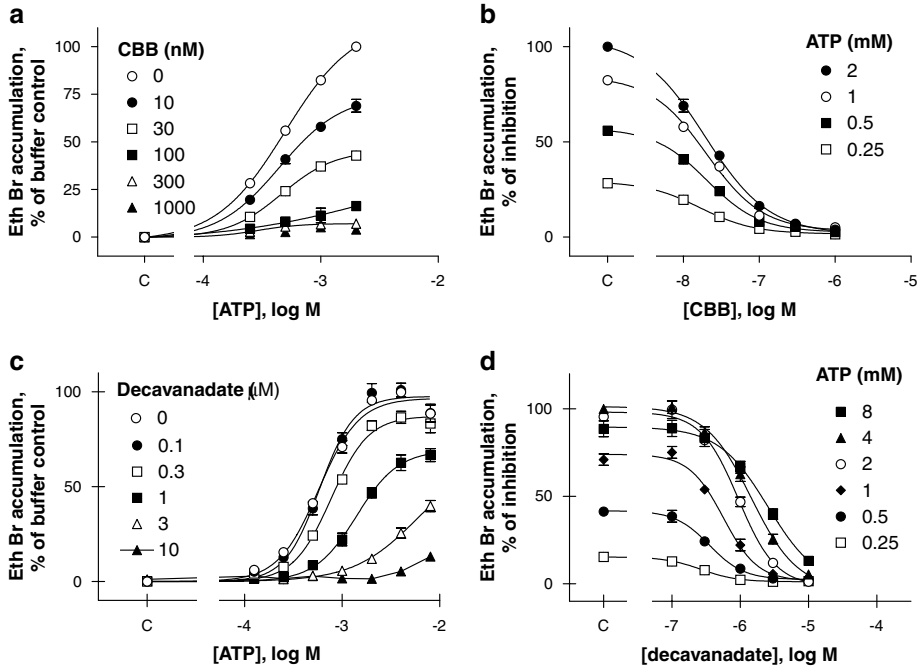

Figure 7 Antagonist effects on ATP-induced ethidium bromide (Eth Br) accumulation in U-2 OS cells transiently transduced with the guinea-pig P2X$_7$ receptor. Effects of CBB are shown in (**a**, **b**), and effects of decavanadate in (**c**, **d**). (**a**, **c**) The effects of the antagonists on the concentration–effect curve to ATP. In (**b**, **d**), the data from (**a**, **c**) are re-plotted to present antagonist inhibition curves at each of the indicated ATP concentrations. NaCl buffer was used in all studies. Control values obtained in the absence of the antagonists or ATP are shown on the abscissa (denoted as **c**). Results shown are mean ± s.e.mean of 3–4 independent experiments run in duplicate. CBB, Coomassie brilliant blue.

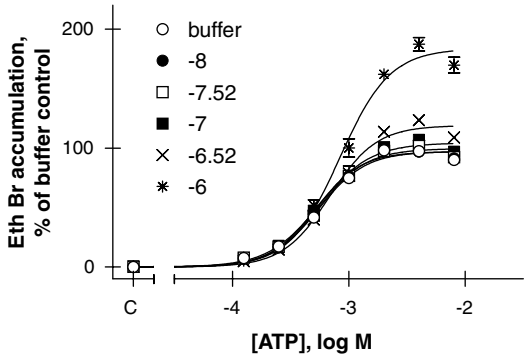

Figure 8 Effect of a range of concentrations of mAb against the P2X$_7$ receptor (Figure 3) on ATP-induced ethidium bromide (Eth Br) accumulation in U-2 OS cells transiently transduced with the guinea-pig P2X$_7$ orthologue. The different concentrations of antibody incubated with the cells are shown in the figure as $\log_{10}$(g antibody protein per ml); thus $-6.52 = 300\text{ng ml}^{-1}$. NaCl buffer was used and the control values obtained in the absence of ATP are shown on the abscissa (denoted as **c**). Results shown are mean ± s.e.mean of three independent experiments run in duplicate. mAb, monoclonal antibody.

Dunnett's *post hoc* test, $P<0.001$) from a pEC_{50} of 3.27 ± 0.03 ($n=3$) in the absence of the antibody to 3.08 ± 0.04 ($n=3$).

Discussion and conclusions

In this study, we have isolated a cDNA encoding the guinea-pig P2X$_7$ receptor from guinea-pig brain and functionally characterized this orthologue. This orthologue has some structural differences to the human and rodent receptors and distinctive pharmacological properties with both similarities and differences to the three previously characterized mammalian P2X$_7$ receptor orthologues.

The homology between the guinea-pig P2X$_7$ and the human, rat and mouse P2X$_7$ receptors was approximately 70%. The receptor was one amino acid shorter than the other species orthologues missing a glutamine at position 77 (human nomenclature). The guinea-pig receptor possessed the same conserved cysteines as present in all other P2X receptors, including the same conserved cysteine residue pairs, thought to be responsible for the tertiary structure of the receptor at positions 119–168, 129–152, 135–162, 216–226 and 260–269 (human nomenclature) (North, 2002).

P2X$_7$ receptors have up to six potential N-linked glycosylation sites on asparagine residues at positions 74, 187, 202, 213, 241 and 284. The guinea-pig receptor only possessed four potential N-linked glycosylation sites on asparagine residues corresponding to positions 187, 202, 213 and 241 (human nomenclature). The absence of asparagine at position 284 is of interest, as a recent study has implicated this region as being important to species differences in ATP and BzATP potency (Young *et al*., 2007). Despite having fewer potential N-linked glycosylation sites, the guinea-pig P2X$_7$ receptor expressed at high levels and was functional (see below). Several loss-of-function single nucleotide polymorphisms have been described in the literature for P2X$_7$ (Gu *et al*., 2001; Adriouch *et al*., 2002; Denlinger *et al*., 2003; Wiley *et al*., 2003; Gu *et al*., 2004; Fernando *et al*., 2005; Shemon *et al*., 2006), but the guinea-pig P2X$_7$ receptor did

not express any of these, which is consistent with it being functional.

We found that the guinea-pig $P2X_7$ receptor could be labelled using the $P2X_7$mAb previously used to label human $P2X_7$ receptors (Buell *et al*., 1998), although the $P2X_7$mAb possessed slightly lower affinity for guinea-pig than for human $P2X_7$ receptors. Immunocytochemical studies using this $P2X_7$mAb revealed even higher levels of cell surface receptor expression for guinea-pig $P2X_7$ receptors than for human or rat $P2X_7$ receptors. This may be due to a greater efficiency of transduction for the guinea-pig BacMam virus compared with the human or rat constructs, or represent a more efficient expression of the guinea-pig $P2X_7$ receptor, possibly due to the nature of the amino-acid residues in its intracellular C-terminus. Certainly, studies on $P2X_7$ receptors have identified multiple intracellular residues that affect cell surface expression of the $P2X_7$ receptor (Denlinger *et al*., 2003; Wiley *et al*., 2003; Fernando *et al*., 2005). Where positive labelling was obtained, it could be seen in the immunocytochemical micrographs that different cells had different degrees of staining, indicative of a non-homogenous expression of the transduced orthologues among the U-2 OS cell population. More quantitative saturation binding studies with the $P2X_7$mAb confirmed the high levels of cell surface expression of the guinea-pig $P2X_7$ receptor and showed that receptor expression could be increased with increasing titres of virus with evidence of maximal expression being achieved at the highest virus titres used. These data demonstrate that the BacMam viral expression system is suitable for producing a graded expression of $P2X_7$ receptors, although there are still cell-to-cell variations and gradation of expression is only apparent when dealing with populations of cells.

The response to ATP at the human, rat and guinea-pig orthologues in ethidium accumulation studies also increased with increasing virus titre and ATP pEC_{50} remained constant regardless of virus titre or agonist exposure time. This observation suggests that agonist potency was independent of receptor density at the expression levels examined in this study. There were some discrepancies between ethidium accumulation and expression studies, as expression appeared to saturate at the higher virus titres tested, whereas receptor function did not clearly saturate. This may be due to binding of the mAb being underestimated in immunocytochemical studies when using the higher virus titres. Certainly, there was a loss of cells from the 96-well plates in the immunocytochemical studies, but not the ethidium accumulation studies, when using the higher virus titres. This may reflect toxic effects of either the virus or high levels of $P2X_7$ receptor expression on cell adhesion leading to cell loss, which was only evident in the immunocytochemical studies due to the greater number of cell washing steps in those studies. Nevertheless, both techniques confirmed that a graded expression of the receptor is possible with the BacMam virus expression system.

The immunocytochemical studies suggested that rat and guinea-pig $P2X_7$ receptors were expressed at 6- to 11-fold higher levels than the human $P2X_7$ receptor. However, there was at most a twofold difference in maximal ethidium accumulation between the species orthologues. This mismatch may not be unexpected, as cellular ethidium accumulation is likely to be limited by total cellular DNA or RNA levels, which should be similar in all cases. Furthermore, recent studies have suggested that cellular entry of dyes such as ethidium occurs independently of the $P2X_7$ receptor (Jiang *et al*., 2005) and may occur via the pannexin hemichannel (Pelegrin and Surprenant, 2006). In such a situation, the maximal ethidium accumulation may reflect coupling of the $P2X_7$ receptor to endogenous pannexin channels, which should be present at similar levels in all cases. The rate of ethidium accumulation was faster in cells transduced with the rat than with the human or guinea-pig orthologues. This may reflect differences in the intracellular C-termini of the species orthologues and their ability to couple to pathways responsible for cellular entry of ethidium.

There were differences in agonist effect at the guinea-pig $P2X_7$ receptor compared with the other species orthologues. Thus, in studies on the rat, human and mouse $P2X_7$ receptors, ATP is a partial agonist compared to BzATP (Wiley *et al*., 1998; Young *et al*., 2007). However, in NaCl buffer, BzATP had no agonist activity at the guinea-pig $P2X_7$ receptor and instead was able to block responses to ATP. Interestingly, BzATP was also a partial agonist at the human and mouse $P2X_7$ receptors when studied in NaCl buffer, although this contrasts with previous studies (Wiley *et al*., 1998; Young *et al*., 2007). The reasons for the discrepancies in intrinsic activity between assay formats are not known, but these studies suggest that BzATP intrinsic activity differs at the species orthologues and is higher at rat and human $P2X_7$ receptors than at mouse or guinea-pig $P2X_7$ receptors. We have previously observed that $P2X_7$ receptor-simulated ethidium accumulation is more readily demonstrated in sucrose buffer than in NaCl buffer (Michel *et al*., 1999) and in sucrose buffer BzATP was able to evoke a response, although its intrinsic activity was still less than ATP.

The reasons for the lower intrinsic activity of BzATP at the guinea-pig receptor are not known but Surprenant and co-workers (Young *et al*., 2007) reported that amino-acid residues at positions 127 and 284 in rat and mouse $P2X_7$ orthologues affect agonist potency and also affected the intrinsic activity of BzATP. However, any comparison of the residues present at these positions with those in the guinea-pig receptor is complicated by the missing amino acid at position 77 in the guinea-pig $P2X_7$ receptor. Consequently, the guinea-pig amino acid corresponding to position 127 in the other orthologues may be arginine or threonine depending upon how the missing amino acid affects receptor structure. At position 127, arginine would differ from that present in all other orthologues, whereas threonine is present in the human receptor. Similarly, at position 284, the corresponding guinea-pig residues could be either glutamate 283 or glutamate 284, different from those in the other species orthologues. As amino acids at positions 130, 134 and 136 were also implicated in BzATP potency (Young *et al*., 2007), it seems likely that multiple residues affect agonist potency and efficacy. Consequently, determining the reasons for the low intrinsic activity of BzATP at guinea-pig $P2X_7$ receptors will be difficult.

Several antagonists that discriminate between the rat, mouse and human orthologues (Anderson and Nedergaard, 2006) were examined as antagonists of the guinea-pig $P2X_7$ receptor. KN62 and PPADS, which display some selectivity for the human receptor (Hibell *et al*., 2001), were also potent antagonists of the guinea-pig receptor. However, the effect of KN62 was clearly not competitive, as it produced only a small and apparently saturating shift in the ATP concentration–effect curve and produced incomplete inhibition of responses to ATP, which complicated analysis of its effects. We have observed similar behaviour with KN62 at human $P2X_7$ receptors (Michel *et al*., 2000) and believe this is due to it being an allosteric inhibitor of the $P2X_7$ receptor (Michel *et al*., 2007). Nevertheless, KN62 was a potent inhibitor of guinea-pig $P2X_7$ receptors with a pIC_{50} of 6.8 that was similar to that obtained at the human $P2X_7$ receptor (pIC_{50} of 6.5; Hibell *et al*., 2001). In the case of PPADS, the pIC_{50} value of 6.5 was also similar to its pIC_{50} of 6.7 at the human receptor (Hibell *et al*., 2001). Note that as antagonist affinities differ considerably between techniques, and even with buffer composition (Hibell *et al*., 2001), we have compared potencies only to those obtained with the same technique and in NaCl buffer.

The $P2X_7$mAb is an antagonist of the human $P2X_7$ receptor (Buell *et al*., 1998) but, surprisingly, it potentiated rather than inhibited ATP-induced responses at the guinea-pig $P2X_7$ receptor. This potentiation may represent positive cooperativity between the antibody and ATP binding sites. Previously, we have shown that the $P2X_7$mAb produces non-competitive blockade of response in functional studies (Buell *et al*., 1998), and in radioligand binding studies it partially inhibited radioligand binding to the human $P2X_7$ receptor, suggesting that it may be an allosteric regulator of the $P2X_7$ receptor (Michel *et al*., 2007). Presumably, structural differences in the human and guinea-pig receptor result in positive rather than negative cooperativity at the guinea-pig receptor.

CBB is selective for the rat over human $P2X_7$ receptors and its pIC_{50} of 7.5 at guinea-pig $P2X_7$ receptors was even higher than that observed at rat receptors ($pIC_{50} = 6.8$; Hibell *et al*., 2001). Decavanadate is a competitive antagonist equipotent at the rat and human receptors (Michel *et al*., 2006b). This compound possessed similar potency at guinea-pig receptors ($pIC_{50} = 6.5$–5.6) as at human or rat $P2X_7$ receptors ($pIC_{50} = 6.2$–6.5), although it did not display such a clearly defined competitive interaction against ATP responses as it did of BzATP-mediated responses at human and rat $P2X_7$ receptors (Michel *et al*., 2006b).

Suramin is a relatively non-selective antagonist of human, rat and mouse $P2X_7$ receptors but possessed 10- to 20-fold higher affinity at the guinea-pig $P2X_7$ receptor. Interestingly, the guinea-pig $P2X_7$ receptor has one less amino acid than the other species orthologues and this deletion is in a region of the receptor (glutamine 77, human sequence) that can affect suramin potency at P2X receptors. Thus, the equivalent residue in the $P2X_4$ receptor (amino acid 78) has been shown to affect sensitivity to suramin (Garcia-Guzman *et al*., 1997), as replacing glutamine in the rat $P2X_4$ receptor with lysine increased suramin potency. Clearly, the differences in amino-acid composition between $P2X_4$ and $P2X_7$ receptors make such comparisons complex, but it seems plausible that the absence of the glutamine at position 77 in the guinea-pig $P2X_7$ receptor could contribute to the higher potency of suramin at the $P2X_7$ receptor.

Several of the compounds tested as antagonists in this study produced an insurmountable antagonist effect. The reasons for this behaviour are not known but may reflect a limitation of the methodology with respect to agonist and antagonist equilibration. Certainly, BzATP would not be expected to be a non-competitive antagonist, as it presumably binds at the ATP binding site. In the case of PPADS, the non-competitive effect is very likely a reflection of its slow dissociation kinetics, as other studies have shown that it binds at the ATP binding site (Michel *et al*., 2006b, 2007).

In conclusion, here we report the cloning of the guinea-pig $P2X_7$ orthologue. The pharmacological characterization of this new orthologue revealed similar sensitivity to ATP as at the human, rat and mouse receptors but a greatly reduced efficacy of BzATP and a different sensitivity to $P2X_7$ receptor antagonists than observed at the human, rat and mouse orthologues. The receptor displays a number of unique properties that differentiate it from the human, rat and mouse orthologues and the structural information may aid in our understanding of the interaction of agonists and antagonist with the $P2X_7$ receptor.

Conflict of interest

The authors are employed by GlaxoSmithKline R&D Ltd.

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