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RESEARCH PAPER

Identification of regions of the $P2X_7$ receptor that contribute to human and rat species differences in antagonist effects

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Background and purpose: Several $P2X_7$ receptor antagonists are allosteric inhibitors and exhibit species difference in potency. Furthermore, N^2-(3,4-difluorophenyl)-N^1-(2-methyl-5-(1-piperazinylmethyl)phenyl)glycinamide dihydrochloride (GW791343) exhibits negative allosteric effects at the human $P2X_7$ receptor but is a positive allosteric modulator of the rat $P2X_7$ receptor. In this study we have identified several regions of the $P2X_7$ receptor that contribute to the species differences in antagonist effects.
Experimental approach: Chimeric human-rat $P2X_7$ receptors were constructed with regions of the rat receptor being inserted into the human receptor. Antagonist effects at these receptors were measured in ethidium accumulation and radioligand binding studies.
Key results: Exchanging regions of the $P2X_7$ receptor close to transmembrane domain 1 modified the effects of KN62, 4-(4-fluorophenyl)-2-(4-methylsulphinylphenyl)-5-(4-pyridyl)1*H*-imidazole (SB203580) and GW791343. Further studies, in which single amino acids were exchanged, identified amino acid 95 as being primarily responsible for the differential allosteric effects of GW791343 and, to varying degrees, the species differences in potency of SB203580 and KN62. The species selectivity of pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid was affected by multiple regions of the receptor, with potency being particularly affected by the amino acid 126 but not by amino acid 95. A further region of the rat receptor (amino acids 154–183) was identified that, when inserted into the corresponding position in the human receptor, increased ATP potency 10-fold.
Conclusions: This study has identified several key residues responsible for the species differences in antagonist effects at the $P2X_7$ receptor and also identified a further region of the $P2X_7$ receptor that can significantly affect agonist potency at the $P2X_7$ receptor.
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Keywords: $P2X_7$ receptor; BzATP; KN62, SB203580; GW791343; PPADS

Abbreviations: BzATP, 2′- and 3′-*O*-(4benzoylbenzoyl) ATP; ECD, extracellular domain; GW791343, N^2-(3, 4-difluorophenyl)-N^1-(2-methyl-5-(1-piperazinylmethyl)phenyl)glycinamide dihydrochloride; LysoPC, palmitoyl L-α-lysophosphatidylcholine; PPADS, pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid; RT, room temperature; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulphinylphenyl)-5-(4-pyridyl)1*H*-imidazole; SNP, single-nucleotide polymorphism

Introduction

The $P2X_7$ receptor is a non-selective cation channel receptor gated by extracellular ATP (North, 2002). The receptor plays a key role in the release of the inflammatory cytokine, interleukin-1β (Ferrari *et al*., 2006), and in recent years there has been considerable interest in the development of selective $P2X_7$ receptor antagonists due to the potential utility of such compounds in the treatment of inflammatory disorders and pain (Donnelly-Roberts and Jarvis, 2007). This has led to the development of a new generation of $P2X_7$ receptor antagonists to complement the earlier tool compounds used to define this receptor such as oxidized ATP (Murgia *et al*., 1993), KN62 (Gargett and Wiley, 1997) and

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brilliant blue G (Jiang *et al*., 2000). The new generation of compounds includes compounds described in patents or publications from pharmaceutical companies and academic groups (Baraldi *et al*., 2004; Romagnoli *et al*., 2005; Donnelly-Roberts and Jarvis 2007) and more recently specific exemplars have been described including AZ11645373 (Stokes *et al*., 2006), A-740003 (Honore *et al*., 2006), compound-17 (Michel *et al*., 2007) and N^2-(3,4-difluorophenyl)-N^1-(2-methyl-5-(1-piperazinylmethyl)phenyl)glycinamide dihydrochloride (GW791343) (Michel *et al*., 2008).

The new generation of $P2X_7$ antagonists, similar to earlier antagonists such as KN62, often displays marked selectivity for human as opposed to rodent $P2X_7$ receptors, which clearly hampers preclinical studies to evaluate these compounds (Donnelly-Roberts and Jarvis, 2007). These compounds are also highly selective and specific $P2X_7$ receptor antagonists (Honore *et al*., 2006; Stokes *et al*., 2006). This would seem unexpected if the compounds were simple competitive antagonists of ATP given the ubiquity of ATP and nucleotide-binding proteins. However, recent studies have shown that several $P2X_7$ receptor antagonists are allosteric inhibitors, which may explain their specificity (Michel *et al*., 2007, 2008). One of these compounds, GW791343, exhibits quite unusual properties in that it inhibits ATP responses at the human receptor but increases ATP responses at the rat $P2X_7$ receptor (Michel *et al*., 2008).

There are also species differences in agonist potency between the species orthologues, and recent studies have started to identify regions of the $P2X_7$ receptor that contribute to the differences in agonist potency. In particular, residues at positions 127 and 284 have been shown to greatly affect agonist potency at rat, human and mouse receptors (Thompson, 2001; Young *et al*., 2007). However, there is little structural information regarding the residues responsible for the species differences in antagonist potency. A previous study identified residues in the extracellular domain (ECD) of the $P2X_7$ receptor as being responsible for the species selectivity of KN62 (Humphreys *et al*., 1998), while we evaluated antagonists in chimeric human–rat receptors and found that undefined residues within the first 255 amino acids of the human $P2X_7$ receptor were responsible for the higher potency of KN62 (Thompson, 2001) and 4-(4-fluorophenyl)-2-(4-methylsulphinylphenyl)-5-(4-pyridyl)1*H*-imidazole (SB203580) (Michel *et al*., 2006a) at the human than at the rat $P2X_7$ receptor.

In this study, we have extended our earlier work to identify the regions of the human and rat $P2X_7$ receptor that contribute to the species differences in antagonist effects. For this study, we focused on identifying regions of the receptor that contributed to the selectivity of GW791343, as this compound produced such contrasting effects at human and rat receptors (Michel *et al*., 2008). We have constructed chimeric human–rat receptors comprising the human $P2X_7$ receptor into which specific residues or short domains of the rat sequence have been introduced. The domains were selected to contain regions of the species orthologues that exhibited the lowest levels of homology (Figure 1a). In addition, one of the domains contained amino acids 79–86 of the $P2X_7$ receptor, which are in the ECD and are not present in other members of the P2X receptor family (North, 2002).

From these studies, we have identified a key amino acid at position 95 that seems to be largely responsible for the species difference in effect of GW791343 and that also contributes to the species selectivity in antagonist effect of SB203580 and KN62. In addition, we have identified other regions of the $P2X_7$ receptor that affect agonist and antagonist potency.

Materials and methods

Plasmid construction, chimeric receptor generation and site-directed mutagensis

The nomenclature used for describing the $P2X_7$ receptor conforms to *Br J Pharmacol*'s Guide to Receptors and Channels (Alexander *et al*., 2008). The generation of the chimeric rat–human $P2X_7$ and human–rat $P2X_7$ receptors have been described previously (Michel *et al*., 2006a). BacMam reagents for expression of human and rat $P2X_7$ receptors have been described previously (Michel *et al*., 2007; Fonfria *et al*., 2008).

A human–rat chimeric receptor containing the entire ECD of the rat receptor (amino acids 48–332) was prepared in the background of an otherwise wild-type human receptor (rat ECD receptor, Figure 1). The DNA sequence of the rat ECD was based on the rat cDNA clone described previously (Fonfria *et al*., 2008) and was synthesized as a *Pml*1-to-*Xcm*1 fragment in pUC19 (Midland Certified Reagent Co. Inc., Midland, TX, USA, 79701). The rat ECD *Pml*1/*Xcm*1 fragment was then subcloned into the unique *Pml*1- and *Xcm*1-digested pFBM1-hum$P2X_7$ plasmid used for generating BacMam reagents (Fonfria *et al*., 2008).

The five mini-domain swap ECD mutants (Figure 1) were obtained using a modification of the mutagenesis method described previously (Geiser *et al*., 2001). For each of the domain swap mutants, a small region of the DNA sequence of the human receptor was replaced with that of the rat DNA sequence as indicated in Figure 1. The plasmid constructs were obtained using the following oligonucleotide pairs: (5′3′) Domain1 = AAGGTGAAGGGGATAGCAGAGGTGACA GAGAATGTCACGGAGGGC and AAAGAGTTCCCCTGCAAA GGGAGGGTGTAGTCGGCCGTGTC; Domain 2 = AGGGGA ACTCTTTCTTCGTGATGACAAATTATCTCAAGTCAGAAGGC and GCTCTGGGGGTCCATCCATCCCTTTATACAACCCTGG TCAGAATG; Domain 3 = ATTCAGACCGGAAGGTGTATACC TTACGACCAGAAGAGG and CACAGTGAAGTTTTCGGCGC TCCTCAAGAGTGCAGGCCGTGG; Domain 4 = ACCACGAG AAACATCCTGCCAGGTATGAACATCTCTTGTACC and CA TTATTCCGCCCTGAATTGCCACCTCTGTAAAGTTCTCTCC; and Domain 5 = TACTGGTACTGCAACCTAGACAGCTGGTC CCATCGCTGTCAAC, and GACTTTTATCAGAGTCCGTTTCT CCATGCCATTTTCCTTATAGTAC.

$P2X_7$ receptors containing single amino-acid point mutations (rat amino acid introduced into wild-type human receptor) were obtained using a Quick Change II site-directed mutagensis kit following the manufacturer's protocol. The following single point mutants were generated using the plasmid pFBM1-hum$P2X_7$ previously described: lysine 72 to

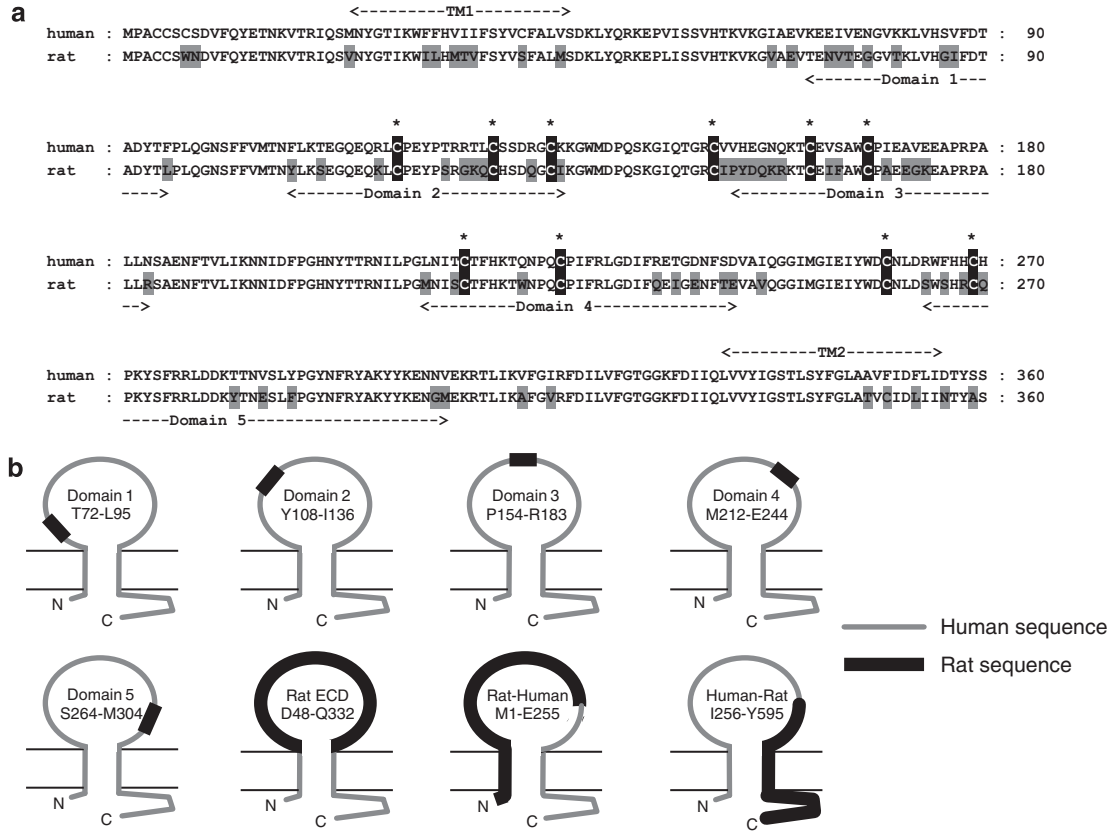

Figure 1 Comparison of rat and human P2X$_7$ receptors and illustration of receptor constructs used for these studies. (**a**) Sequence alignment of the first 360 amino acids of the human and rat P2X$_7$ receptors with residues that differ between the receptors shaded in grey. The location of the five domains of the receptor that were exchanged between rat and human receptors is shown. These domains were in the extracellular portion of the receptor between the two putative transmembrane domains (TM1 and TM2). The ten conserved cysteine residues of the P2X$_7$ receptor are shaded black and indicated by an asterix. (**b**) Representation of the P2X$_7$ receptor to illustrate the approximate location of the various constructs.

threonine (K72T), aspartate 74 to asparagine (E74N), asparagine 78 to glycine (N78G), lysine 81 to threonine (K81 T), serine 86 to glycine (S86G), phenylalanine 95 to leucine (F95L), arginine 126 to glycine (R126G), lysine 128 to glutamate (L128Q), arginine 133 to glutamate (R133Q). In addition, a histidine 155 to tyrosine human receptor mutant (H155Y) was generated, but this was in a human P2X$_7$ receptor containing a naturally occurring single-nucleotide polymorphism (SNP) at amino acid 270. This receptor contained arginine at amino acid 270 instead of histidine (H270R) as was present in the wild-type receptor used for the other studies. Each of the plasmids created above were used to generate BacMam baculoviruses as described previously (Clay *et al.*, 2003).

In addition to these single-point mutations in the human receptor, we also generated a single-point mutation in the rat receptor using the same procedure described above. This was the leucine 95 to phenylalanine (L95F) mutant. This was produced in rat pcDNA3.1, and HEK293 cells were transfected with this plasmid and a stable cell line generated for use in functional studies.

Cell culture and viral transductions

Studies on the human, rat, human–rat, rat–human, rat L95F and domain 3 receptors were performed using HEK293 cells stably transfected with the recombinant receptor. The rat ECD, other chimeric domain swaps and single-point mutant receptors were transiently expressed in human osteosarcoma U-2 OS cells using the BacMam viral transduction method (Fonfria *et al.*, 2008). For the later studies, wild-type human and rat receptors were also expressed in U-2 OS cells for comparative purposes. The BacMam viral transductions for ethidium accumulation studies were conducted as described previously (Fonfria *et al.*, 2008). Briefly, U-2 OS cells were maintained in adherent culture conditions in the presence of Dulbecco's modified Eagle's medium: nutrient mixture F-12 supplemented with Glutamax and 10% foetal bovine serum at 37 °C, 5% CO_2. One day before assay, cells were harvested from the culture flasks using 0.05% trypsin/EDTA and suspended at a concentration of $\sim 750 \times 10^3$ cells mL^{-1} in culture media in the presence of the various BacMam viruses ($1–2 \times 10^8$ plaque-forming units mL^{-1} of the BacMam virus stock in culture media). This concentration of BacMam virus produced maximal responses for all of the recombinant receptor (AD Michel, unpublished data). Cells (70–80 000) were plated into individual wells of poly-L-lysine-pretreated 96-well plates and the plates were incubated at 37 °C, 5% CO_2 overnight. HEK293 cells stably expressing the human, rat, human–rat, rat–human, rat L95F or domain 3 recombinant

$P2X_7$ receptors were prepared in a similar manner except that the BacMam virus was omitted.

In studies to measure KN62 effects in radioligand-binding studies, U-2 OS cells were grown to confluence in T175 cm^2 flasks and the media were replaced with fresh growth media containing BacMam virus ($1–2 \times 10^8$ plaque-forming units mL^{-1}). The cells were incubated overnight, harvested using Versene and membranes were prepared as described previously (Michel *et al*., 2007). Similar studies to determine the effects of agonists in radioligand-binding studies were performed using membranes prepared from HEK293 cells stably expressing the human, rat or domain 3 chimeric recombinant receptors using the same methods as described above for the U-2 OS cells.

Cellular ethidium accumulation measurements

Studies were performed as described previously (Michel *et al*., 2006a; Fonfria *et al*., 2008) using assay buffers comprising (in mM): HEPES 10, *N*-methyl-D-glucamine 5, KCl 5.6, D-glucose 10, $CaCl_2$ 0.5 (pH 7.4) and supplemented with either 280 mM sucrose (sucrose buffer) or 140 mM NaCl (NaCl buffer). Before use, growth medium was completely removed from the cells and they were rinsed with 350 μL of the appropriate assay buffer, which was also removed before assay additions were performed. All solutions were aspirated using 25-gauge bevelled syringe needles to provide complete solution removal. In all studies, the final assay volume was 100 μL and studies were performed at a room temperature (RT) of 19–21 °C.

Cells were incubated with antagonist for 40 min before addition of a mixture containing the agonists, ATP or 2′- and 3′-*O*-(4benzoylbenzoyl) ATP (BzATP), and ethidium bromide (100 μM final assay concentration). After agonist addition, incubations were continued until approximately 10–30% of maximal agonist-stimulated dye accumulation occurred. Reactions were rapidly terminated by addition of 25 μL of 1.3 M sucrose assay buffer containing 5 mM reactive black 5, and cellular accumulation of ethidium was determined by immediately measuring fluorescence (excitation wavelength of 530 nm and emission wavelength of 620 nm) from below the plate with a 96-well plate fluorescence reader (FlexStation, Molecular Devices, Wokingham, UK).

Radioligand-binding studies

The radioligand-binding studies using [^{3}H]-compound-17 were performed as described previously (Michel *et al*., 2007). Briefly, studies with KN62 were performed using membranes prepared from U-2 OS cells transduced with human, rat or chimeric recombinant $P2X_7$ receptors. Studies with agonists were performed using membranes prepared from HEK293 cells stably expressing human, rat or domain 3 chimeric recombinant $P2X_7$ receptors. The radioligand, [^{3}H]-compound-17, was used at a concentration of 2–3 nM. Incubations were carried out for 60 min at RT in a final assay volume of 200 μL of 50 mM Tris-HCl buffer containing 0.01% BSA (pH 7.4 at RT) and were terminated by vacuum filtration. Non-specific binding was defined using 10 μM compound-17.

Materials

The Quick Change II site-directed mutagensis kit was obtained from StrataGene (La Jolla, CA, USA); αβ-methylene-ATP, adenosine-5′-*O*-(3-thiotriphosphate) (ATPγS), 2-methylthio-ATP, ATP, BzATP, ethidium bromide, KN62, pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS) and reactive black 5 were obtained from Sigma (Poole, UK). SB203580 and GW791343 were synthesized in the Chemistry Department of GSK (Harlow, UK). All culture media, 0.05% trypsin/EDTA and Versene were obtained from Invitrogen (Paisley, Scotland), whereas other reagents were obtained from VWR (Loughborough, UK); the poly-L-lysine-pretreated 96-well plates were obtained from Costar (High Wycombe, UK); foetal bovine serum was obtained from PAA laboratories GmBH (Pasching, Austria). [^{3}H]-compound-17 was from Tritec (Basel, Switzerland; specific activity was 2.1TBq $mmol^{-1}$ and purity was >99% by HPLC). KN62 (1-(*N*,*O*-bis(5-isoquinolinesulphonyl)-*N*-methyl-1-tyrosyl)-4-phenylpiperazine), SB203580, compound-17 and GW791343 were dissolved in DMSO as a 10 mM solution and were stored as frozen aliquots at −20 °C until required.

Data analysis

Individual concentration–effect or inhibition curves from each experiment were fitted to a four-parameter logistic function to determine the maximum and minimum responses and to calculate the EC_{50} or IC_{50} values and the Hill slope. For graphical purposes, most concentration–effect and inhibition curves are presented as a percentage of the maximal response obtained in the control group.

As the compounds produced non-competitive antagonist effects in the Schild studies (for example, see Figure 3), the data from the Schild studies were also analysed to calculate antagonist pIC_{50} values at each agonist concentration, as this provided some quantitative estimate of antagonist potency. To represent the effect of agonist concentration on antagonist pIC_{50} graphically, the agonist concentration was expressed relative to its EC_{50} at the various receptors (logarithm [Agonist Concentration/Agonist EC_{50}]). This enabled a simpler comparison of antagonist pIC_{50} values between the species orthologues and chimeric receptors. In addition, for the single-point mutants, the relationship between pIC_{50} and (logarithm [agonist concentration/agonist EC_{50}]) was linear (Figure 3e). To provide a statistical comparison of antagonist potency between these mutants, the pIC_{50} data for each experiment were analysed by linear regression and the antagonist pIC_{50} at the agonist EC_{50} was calculated from the fit. In graphical terms, this corresponded to the pIC_{50} at 0 on the *x* axis (Figure 3e), and this value is referred to as the normalized pIC_{50}. This approach was only followed for analyses where the slope of the best fit from the regression analysis did not differ between receptors being compared.

Statistical comparisons were made using Student's *t*-test or one-way ANOVA followed by Tukey's *post hoc* test. Differences were assessed as significant when $P<0.05$. In these studies, the data are the mean ± s.e.mean of 3–7 experiments. All curve fitting and statistical analysis was performed using GraphPad Prism 3 (San Diego, CA, USA).

Drug/molecular target nomenclature
Drug/molecular target nomenclature conforms with *Br J Pharmacol*'s Guide to Receptors and Channels (Alexander *et al*., 2008).

Results

Effect of GW791343 at chimeric receptors
GW791343 inhibits P2X$_7$ receptor-mediated responses in cells expressing human P2X$_7$ receptors, but in cells expressing the rat P2X$_7$ receptor, the predominant effect of GW791343 is to increase P2X$_7$ receptor-mediated responses (Michel *et al*., 2008). To identify the regions of the P2X$_7$ receptor contributing to the species differences in effects of GW791343, we constructed chimeric receptors in which regions of the rat P2X$_7$ receptor were inserted into the corresponding positions of the human P2X$_7$ receptor.

The rat ECD and rat–human chimeric receptors could only be studied in sucrose buffer, as there was no detectable agonist-stimulated ethidium accumulation in NaCl buffer (data not shown). At both of these chimeric receptors, GW791343 increased responses to BzATP (Figures 2a and b). In contrast, GW791343 inhibited responses at the human–rat chimeric receptor in both sucrose and NaCl buffer (data not shown). These data suggest that the positive allosteric effect of GW791343 is determined by residues in the ECD and within the N-terminal 255 amino acids of the receptor.

The regions of the rat P2X$_7$ receptor responsible for the ability of GW791343 to increase agonist responses were further localized by studying the effect of GW791343 at chimeric receptors in which smaller domains of the extracellular region of the rat receptor were inserted into the human P2X$_7$ receptor (Figure 1). These studies were complicated by the fact that we could only study the domain 1, 2, 4 and 5 receptors in sucrose buffer, as responses in NaCl buffer were too small for quantitative analysis (data not shown). In contrast, the domain 3 receptor could only be studied in NaCl buffer (see below).

Despite these complications, it was evident that GW791343 inhibited responses at the domain 2, 4 and 5 receptors but increased responses at the domain 1 receptor when studied using BzATP in sucrose buffer (Figures 2c–f). In cells expressing the domain 2, 4 and 5 receptors, the inhibitory effects of GW791343 were all slightly different (Figure 2). At the domain 2 and 4 receptors, the lowest concentration of GW791343 tested (100 nM) significantly reduced the maximal effect of BzATP (Figures 2d and e, $P<0.05$, one-way ANOVA followed by Tukey's *post hoc* test). This contrasts with the lack of effect of 100 nM GW791343 at the human P2X$_7$ receptor (Figure 3a) and the domain 5 receptor (Figure 2f). GW791343 inhibited responses at the domain 3 receptor when studied in NaCl buffer, and the effect was similar to that observed at the wild-type human receptor (data not shown).

Amino acid 95 is a key residue in determining the species difference in effects of GW791343
To further explore the role of residues within domain 1 in modulating the effects of GW791343, a series of P2X$_7$ receptors were generated in which single residues of the human P2X$_7$ receptor were exchanged with the corresponding residue from the rat P2X$_7$ receptor. GW791343 was a

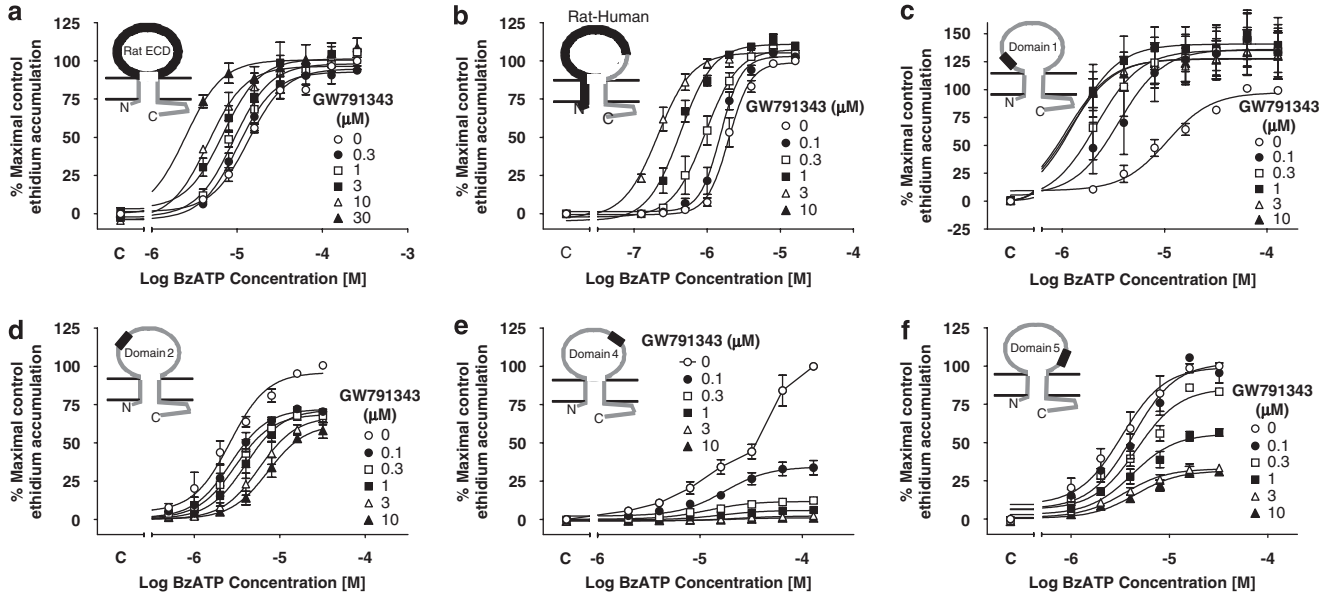

Figure 2 The effect of GW791343 on BzATP-induced ethidium accumulation in cells expressing human–rat recombinant chimeric P2X$_7$ receptors. All studies were performed at RT in sucrose buffer in cells pre-equilibrated for 40 min with the indicated concentrations of GW791343 before measuring BzATP responses. Studies were performed in HEK293 cells stably expressing the recombinant receptor (**b**) or in U-2 OS cells transiently transduced with recombinant P2X$_7$ receptors using BacMam virus (**a**, **c**–**f**). The effect of GW791343 is shown at (**a**) rat ECD, (**b**) rat-human, (**c**) domain 1, (**d**) domain 2, (**e**) domain 4 or (**f**) domain 5 receptors. Basal ethidium accumulation in the absence or presence of GW791343 is indicated on the X-ordinate as C. The data are the mean ± s.e.mean of 3–4 separate experiments.

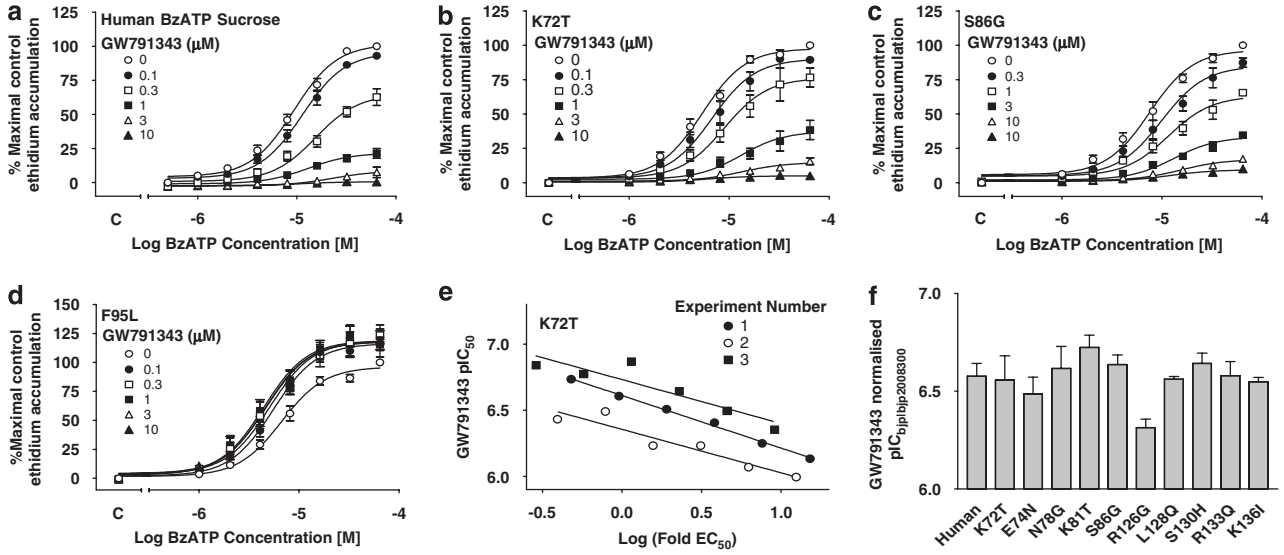

Figure 3 The effect of GW791343 on BzATP-induced ethidium accumulation in cells expressing human–rat recombinant chimeric P2X$_7$ receptors. All studies were performed at RT in sucrose buffer in U-2 OS cells transiently transduced with recombinant P2X$_7$ receptors using BacMam virus and pre-equilibrated for 40 min with the indicated concentrations of GW791343 before measuring BzATP responses. The effect of GW791343 is shown at (**a**) human, (**b**) K72T, (**c**) S86G and (**d**) F95L receptors. Basal ethidium accumulation in the absence or presence of GW791343 is indicated on the *x* ordinate as C. (**e**) The data from (**b**) have been analysed to calculate the pIC$_{50}$ of GW791343 at each concentration of agonist. Agonist concentration is expressed relative to agonist EC$_{50}$ such that log (fold EC$_{50}$) represents logarithm [agonist concentration/agonist EC$_{50}$]. The data were fitted to a straight line by linear regression. From this analysis, the pIC$_{50}$ at the agonist EC$_{50}$ (the normalized pIC$_{50}$) was determined as described in Materials and methods. (**f**) Normalized pIC$_{50}$ values for GW791343 at human and mutant receptors. There were no significant differences in normalized pIC$_{50}$ between any of the single-point mutants ($P>0.05$, one-way ANOVA followed by Tukey's *post hoc* test). The data are the mean ± s.e.mean of three separate experiments.

potent antagonist at the K72T, E74N, N78G, K81T and S86G single-point mutant receptors (Figures 3b, c and f). Interestingly, in contrast to the studies using the larger domain swaps (Figure 2), the antagonist effect of GW791343, when observed, was similar to the wild-type human receptor for all of the single-point mutant receptors (Figures 3a–c). The relationship between pIC$_{50}$ and (logarithm [agonist concentration/agonist EC$_{50}$]) was linear for all of the single-point mutant receptors (Figure 3e, data for K72T and data not shown) and the slopes of the lines of best fit were not significantly different between the various receptors. To compare potency of GW791343 between the various mutant receptors, the data from each experiment were fitted using linear regression, and the pIC$_{50}$ value at the agonist EC$_{50}$ (normalized pIC$_{50}$) was determined from the fit as described in Materials and methods. Graphically, the normalized pIC$_{50}$ corresponds to the pIC$_{50}$ value at the intercept of the line of best fit with 0 on the *x* axis (Figure 3e). For the K72T, E74N, N78G, K81T and S86G mutant receptors, the normalized pIC$_{50}$ values were similar to that at the wild-type human receptor (Figure 3f, $P>0.05$, one-way ANOVA followed by Tukey's *post hoc* test).

In contrast, GW791343 increased responses to BzATP at the F95L mutant receptor (Figure 3d), although the magnitude of this effect was not as marked as at the rat ECD, rat–human or domain 1 receptor (Figures 2a–c).

The effects of GW791343 were also evaluated at five single-point mutant receptors in which residues from domain 2 of the human receptor were exchanged with the corresponding residues from the rat receptor (R126G, L128Q, S130H, R133Q, K136I). At all of these mutant receptors, GW791343 produced similar antagonist effects to those observed at the wild-type human receptor (data not shown). The normalized pIC$_{50}$ values of GW791343 for the five single-point mutant receptors in domain 2 were similar to that observed at the wild-type receptor (Figure 3f, $P>0.05$, one-way ANOVA followed by Tukey's *post-hoc* test). The mean pIC$_{50}$ at the R126G mutant receptor was slightly lower than at the human receptor, but this difference was not statistically significant (Figure 3f).

Amino acid 95 contributes to the species differences in the effects of SB203580

We have previously shown that SB203580 is a weak antagonist of the human P2X$_7$ receptor (pIC$_{50}$ 5.5–6.5) but has no activity at the rat P2X$_7$ receptor at concentrations up to 10 μM (Michel *et al*., 2006a). Furthermore, at a chimeric human–rat P2X$_7$ receptor containing the first 255 amino acids of the human receptor, SB203580 was an antagonist, but it had no effect at a chimeric receptor containing the first 255 amino acids of the rat receptor (Michel *et al*., 2006a). Consistent with this, SB203580 blocked BzATP responses at the domain 5 receptor in which amino acids 264–304 were exchanged from the rat into the human receptor (data not shown) and the effect was similar to that observed at the wild-type human receptor (Figure 4a). SB203580 also blocked BzATP responses at the domain 2 and 4 receptors (Figures 4c and d), although its effects at the domain 2 receptor (Figure 4c) were less than that observed at the wild-type human receptor (Figure 4a). SB203580 did not produce any

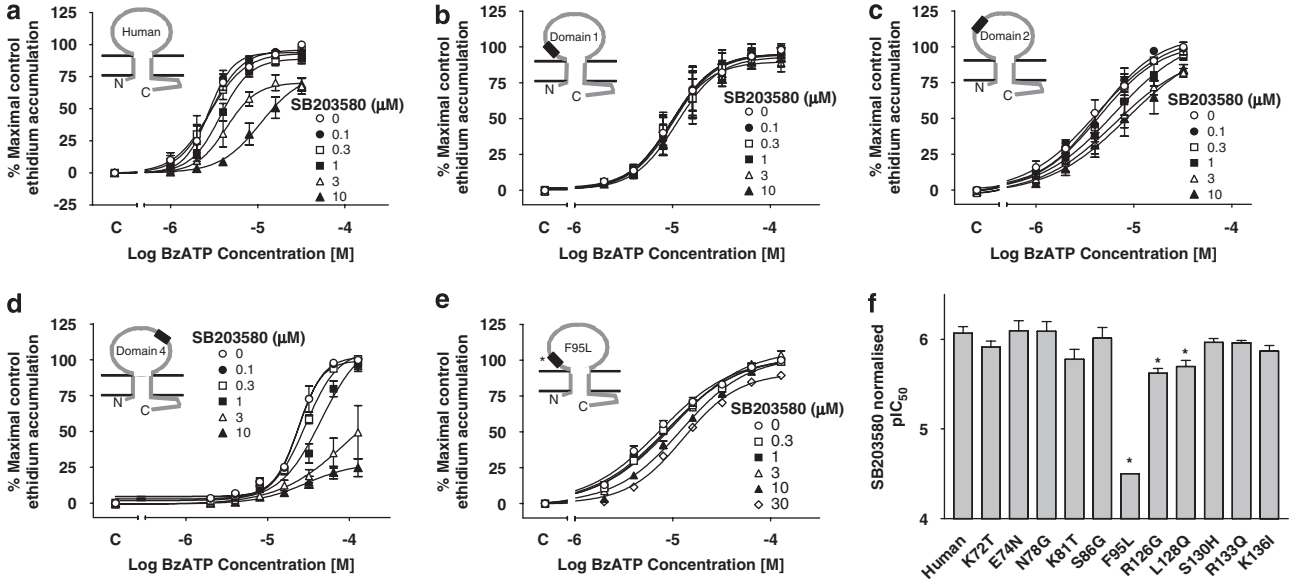

Figure 4 The effect of SB203580 on BzATP-induced ethidium accumulation in cells expressing human–rat recombinant chimeric $P2X_7$ receptors. All studies were performed at RT in sucrose buffer in U-2 OS cells transiently transduced with recombinant $P2X_7$ receptors using BacMam virus and pre-equilibrated for 40 min with the indicated concentrations of SB203580 before measuring BzATP responses. The effects of SB203580 are shown at (**a**) human wild type, (**b**) domain 1, (**c**) domain 2, (**d**) domain 4 or (**e**) F95L receptors. Basal ethidium accumulation in the absence or presence of SB203580 is indicated on the *x* ordinate as C. (**f**) Normalized pIC_{50} values for SB203580 at the indicated single-point mutant receptors were determined as described in Materials and methods. *Significantly different from normalized pIC_{50} determined at human receptor, $P<0.05$, one-way ANOVA followed by Tukey's *post hoc* test. The data are the mean ± s.e.mean of 3–7 separate experiments.

detectable antagonist effect at the domain 1 receptor at concentrations up to 10 μM (Figure 4b).

When the single-point mutant receptors from within domain 1 and 2 were examined, the effect of SB203580 was greatly reduced at the F95L mutant receptor, although there was some antagonist effect at both 10 and 30 μM (Figure 4e). In contrast, at the other single-point mutant receptors from within domain 1 and 2, the antagonist effects of SB203580 were similar to those observed at the wild-type human receptor (data not shown). Furthermore, the normalized pIC_{50} values were similar to that determined at the wild-type human receptor ($P>0.05$, one-way ANOVA followed by Tukey's *post hoc* test) with the exceptions of the R126G and L128Q mutant receptors (Figure 4f). However, the reduction in pIC_{50} at the latter receptors was minimal (pIC_{50} values at human, R126G and L128Q receptors were 6.07 ± 0.08, 5.62 ± 0.06 and 5.70 ± 0.07, respectively).

The antagonist effects of SB203580 were attenuated at the domain 3 receptor such that the compound only produced antagonist effects at 10 and 30 μM (data not shown), and the normalized pIC_{50} of 5.06 ± 0.12 was significantly lower than the normalized pIC_{50} value of 6.26 ± 0.04 at the human wild-type receptor ($P<0.05$, one-way ANOVA followed by Tukey's *post hoc* test). We only constructed one single-point mutant receptor from within domain 3. This was the H155Y mutation, which also exists as a natural SNP in the human receptor (Cabrini *et al.*, 2005). This mutant was constructed in a human receptor also containing an H270R single SNP, which complicates comparisons. However, SB203580 effects at the H155Y–H270R receptor were similar to those observed at the wild-type human receptor (data not shown), and the normalized pIC_{50} value of 5.99 ± 0.04 was not significantly different to the normalized pIC_{50} value of 6.26 ± 0.04 at the human wild-type receptor ($P>0.05$, one-way ANOVA followed by Tukey's *post hoc* test).

Effect of KN62 at chimeric receptors

KN62 is an effective antagonist of the human $P2X_7$ receptor (Figure 5a) but had no effect at the rat $P2X_7$ receptor at concentrations up to 10 μM (data not shown). In studies on the rat–human $P2X_7$ receptor and the rat ECD $P2X_7$ receptor, KN62 had no effect, but at the human-rat $P2X_7$ receptor, KN62 was an antagonist (data not shown). This suggested that the residues responsible for the antagonist effects of KN62 were in the ECD and within the first 255 amino acids of the receptor.

KN62 was an antagonist at the domain 1, 2, 4 and 5 receptors (Figures 5b–d and data not shown), although its effects were reduced at the domain 1 and 2 receptors. In these receptors, KN62 produced more limited shifts in the BzATP concentration–effect curve and did not suppress maximal responses to the same extent as observed in studies on the wild-type human $P2X_7$ receptor (Figures 5a–c). The effects of KN62 were similarly modified at the domain 3 receptor when studied in a NaCl buffer (data not shown), although the normalized pIC_{50} value at the domain 3 receptor (8.32 ± 0.16) was not significantly different ($P>0.05$, Student's *t*-test) from that determined at the wild-type human receptor (7.92 ± 0.02).

In studies on the single-point mutant receptors from within domains 1 and 2, KN62 antagonist effects were almost identical to those at the human receptor for the K72T, E74N, N78G, K81T, R126G, L128Q, S130H, R133Q and K136I

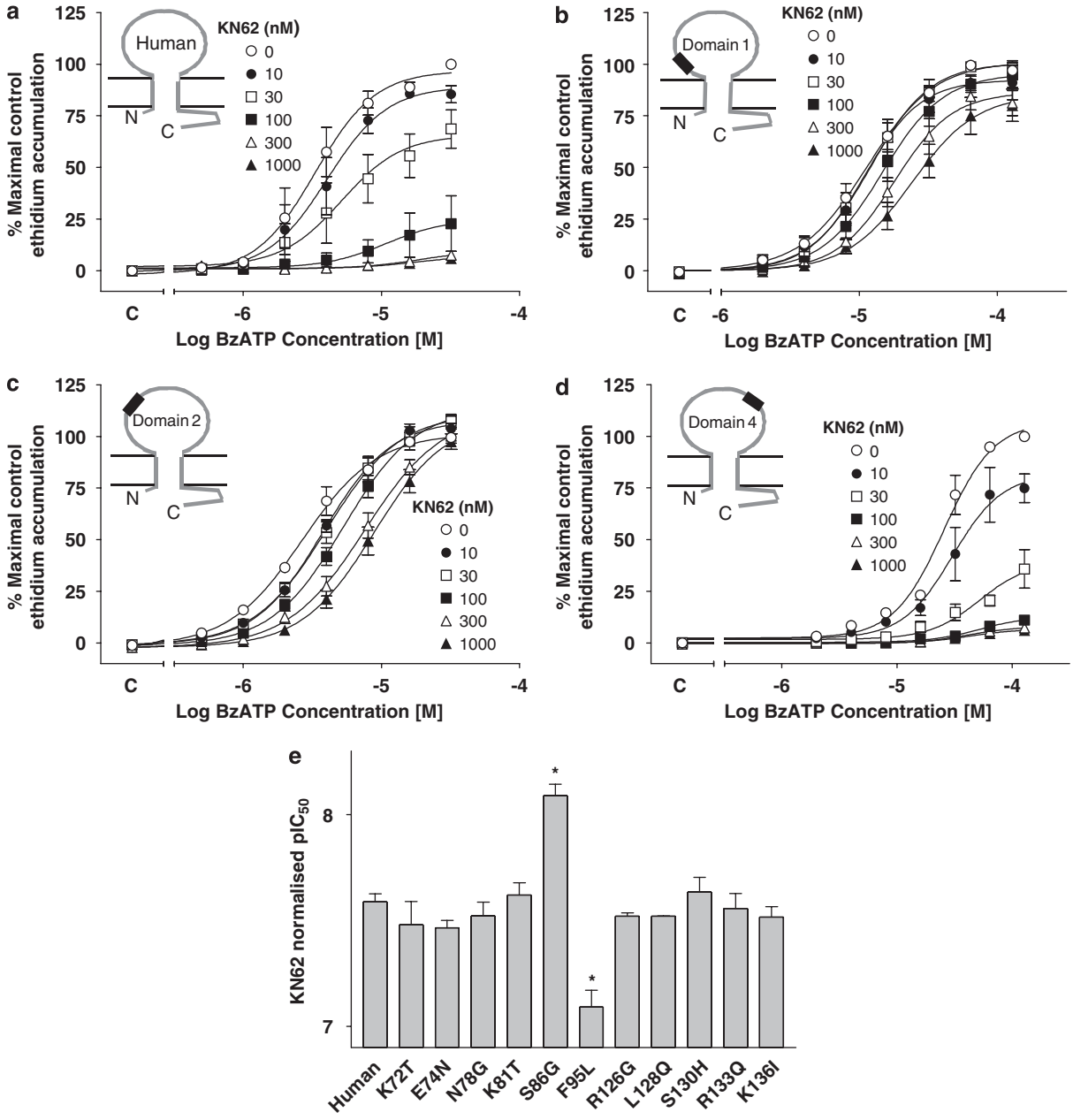

Figure 5 The effect of KN62 on BzATP-induced ethidium accumulation in cells expressing human–rat recombinant chimeric P2X$_7$ receptors. Studies were performed at RT in sucrose buffer in U-2 OS cells transiently transduced with recombinant P2X$_7$ receptors using BacMam virus and then pre-equilibrated for 40 min with the indicated concentrations of KN62 before measuring BzATP responses. The effect of KN62 is shown at (**a**) wild-type human, (**b**) domain 1, (**c**) domain 2 or (**d**) domain 4 receptors. Basal ethidium accumulation in the absence or presence of KN62 is indicated on the *x* ordinate as C. (**e**) Normalized pIC$_{50}$ values for KN62 at the indicated single-point mutant receptors were determined as described in Materials and methods. *Significantly different from normalized pIC$_{50}$ determined at the human receptor, $P<0.05$, one-way ANOVA followed by Tukey's *post hoc* test. The data are the mean ± s.e.mean of 3–6 separate experiments.

mutants and there was no significant difference in normalized pIC$_{50}$ when compared to the human receptor for any of these mutant receptors (Figure 5e, $P>0.05$, one-way ANOVA followed by Tukey's *post hoc* test). However, at the F95L receptor, the effect of GW791343 on BzATP responses (data not shown) was very similar to that observed at the domain 1 receptor (Figure 5b), and this was associated with a significant decrease in normalized pIC$_{50}$ compared with the human receptor (Figure 5e). In contrast, the normalized pIC$_{50}$ of KN62 at the S86G mutant was higher than at the human P2X$_7$ receptor (Figure 5e).

We did not evaluate the effects of GW791343 or SB203580 in binding studies as the potency of GW791343 is similar at rat and human P2X$_7$ receptors, while SB203580 only produces a small inhibition of binding (Michel *et al*., 2007, 2008). However, KN62 discriminated between the human and rat P2X$_7$ receptor in binding studies, as it had no effect at the rat P2X$_7$ receptor but inhibited binding at the human

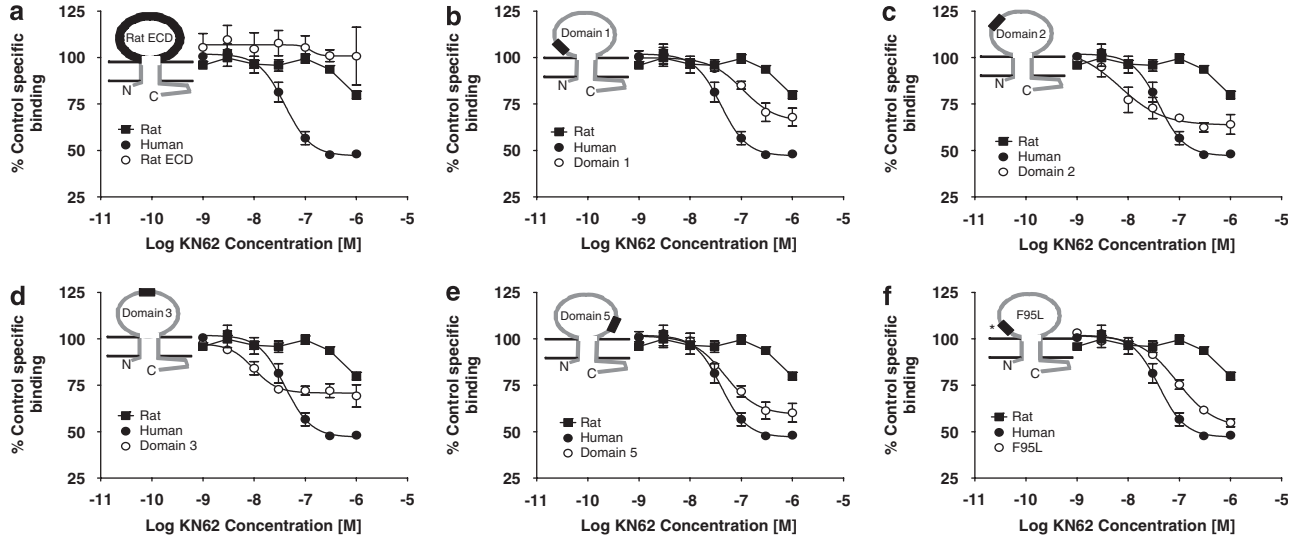

Figure 6 The potency of KN62 to inhibit [^{3}H]-compound-17 binding to membranes prepared from cells expressing human–rat recombinant chimeric P2X$_7$ receptors. Membranes were prepared from U-2 OS cells transiently transduced with recombinant P2X$_7$ receptors using BacMam virus. The radioligand concentration was 2 nM and specific binding was defined with 10 μM compound-17. The effect of KN62 at wild-type rat and human receptors is compared with effects at (**a**) rat-ECD, (**b**) domain 1, (**c**) domain 2, (**d**) domain 3, (**e**) domain 5 or (**f**) F95L receptors. The data are the mean ± s.e.mean of four separate experiments.

P2X$_7$ receptor (Figure 6). Consequently, we determined whether radioligand-binding studies could provide more quantitative information on the effect(s) of KN62 at the various chimeric receptors. However, the data were not easy to interpret. Thus, although KN62 had similar effects at the rat and rat ECD P2X$_7$ receptors (Figure 6a), and its effects were reduced at the domain 1 and F95L receptors (Figures 6b and f), the effects of KN62 were also modified at the domain 2, domain 3 and domain 5 receptors (Figures 6c–e). Indeed, the only domain at which KN62 effects were not modified was the domain 4 receptor where the KN62 inhibition curve was identical to that at the human receptor (data not shown). Interestingly, KN62 potency was increased, but maximal inhibition decreased, at the domain 2 and 3 receptors (Figures 6c and d), although the maximal inhibition was slightly reduced at the domain 5 receptor (Figure 6e).

Effect of PPADS at chimeric receptors

Pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid is a slowly reversible antagonist of the P2X$_7$ receptor (Chessell *et al*., 1998). However, it appears to interact at the ATP-binding site and there appears to be little interaction with the other antagonists such as SB203580 (AD Michel, unpublished data) or GW791343 (Michel *et al*., 2008). PPADS is also selective for human over rat receptors (Hibell *et al*., 2001) and so was an interesting compound to evaluate at the various chimeric P2X$_7$ receptors.

The potency of PPADS at the domain 4 receptor was similar to that measured at the human P2X$_7$ receptor (Figure 7a, $P>0.05$, one-way ANOVA followed by Tukey's *post hoc* test). In contrast, the potency of PPADS was reduced at the domain 1, 2 and 5 receptors (Figure 5a, $P<0.05$, one-way ANOVA followed by Tukey's *post hoc* test), although the reduction in potency at the domain 1 receptor was modest. The decrease in potency of PPADS at the domain 2 and 5 receptors was more marked, although the normalized pIC$_{50}$ values were significantly greater than at the rat receptor ($P<0.05$, one-way ANOVA followed by Tukey's *post hoc* test).

Although PPADS potency was slightly reduced at the domain 1 receptor, there was no significant effect of any of the single-point mutations within this domain on PPADS potency (Figure 7b). In the case of the domain 2 single-point mutant receptors, the potency of PPADS was most markedly reduced at the R126G mutant, although the normalized pIC$_{50}$ was significantly greater than at the rat receptor ($P<0.05$, one-way ANOVA followed by Tukey's *post hoc* test). PPADS potency at the S130H and K136I receptors was also significantly lower than at the human receptor, although the differences in potency were small (normalized pIC$_{50}$ values at human, S130H and K136I receptors were 8.34 ± 0.18, 8.13 ± 0.02 and 7.92 ± 0.06, respectively). We did not construct any single-point mutant receptors from within domain 5.

Studies on the domain 3 receptor were complicated by the need to perform the studies in NaCl buffer, by the use of different expression systems and by the use of a different form of the human receptor for some of the studies. Nevertheless, the potency of PPADS was slightly, but significantly, higher ($P<0.05$, Student's *t*-test) at the domain 3 receptor (normalized pIC$_{50}$ = 7.75 ± 0.02) than at the wild-type human receptor (normalized pIC$_{50}$ = 7.42 ± 0.03) when studied in HEK293 cells following stable expression. When studied in U-2 OS cells after transient expression, the normalized pIC$_{50}$ values of PPADS at the wild-type, H270R and H155Y-H270R receptors were 7.18 ± 0.03, 7.21 ± 0.02 and 7.17 ± 0.03, respectively, and were not significantly different from each other ($P>0.05$, one-way ANOVA followed by Tukey's *post hoc* test), suggesting that the residue

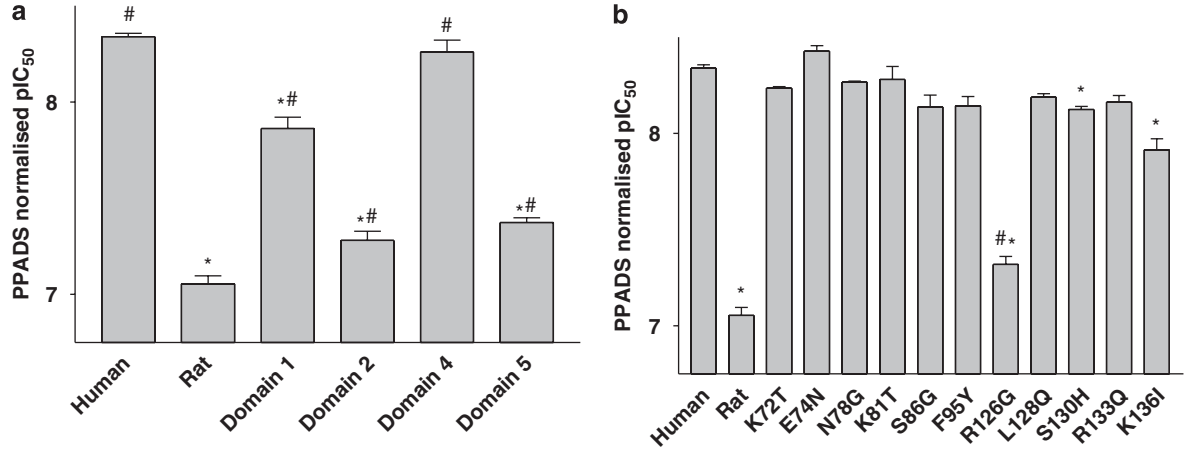

Figure 7 The potency of PPADS to block BzATP-induced ethidium accumulation in cells expressing human–rat recombinant chimeric P2X$_7$ receptors. (**a**) Data from studies using domain 1, 2, 4 and 5 receptors are shown, whereas panel **b** shows data obtained using single-point mutations from within domain 1 and 2. Studies were performed at RT in sucrose buffer in U-2 OS cells transiently transduced with recombinant P2X$_7$ receptors using BacMam virus and then pre-equilibrated for 40 min with PPADS before measuring BzATP responses. The normalized pIC$_{50}$ was calculated as described in Materials and methods for each receptor. *Significantly different from normalized pIC$_{50}$ determined at human receptor, $P<0.05$, one-way ANOVA followed by Tukey's *post hoc* test. #Significantly different from normalized pIC$_{50}$ determined at rat receptor, $P<0.05$, one-way ANOVA followed by Tukey's *post hoc* test.

at position 155 does not contribute to the slight differences in potency between the domain 3 and the wild-type human receptor. Note that in these studies, using NaCl buffer, PPADS potency is lower than in sucrose buffer. However, the human versus rat species difference in potency was maintained (data not shown).

Effect of antagonists at the rat L95F receptor

The rat L95F receptor exhibited a high degree of tonic activity in sucrose buffer and so studies were performed in NaCl buffer. GW791343 increased responses at the wild-type rat receptor (Michel *et al*., 2008) but was a weak antagonist of the rat L95F receptor (Figures 8a and d). SB203580 has no effect at the rat receptor (Michel *et al*., 2006a) but was an antagonist of the rat L95F receptor (Figures 8b and e). For SB203580, the antagonist effects at the L95F receptor were not as marked as observed at the human receptor (cf. Figures 4a and 8b). This was also true for GW791343 (cf. Figures 3a and 8a). KN62 has no effect at the rat receptor (Hibell *et al*., 2001 and data not shown) and had little effect on the L95F receptor, although it did produce a modest inhibition of responses to the lower concentrations of ATP (Figures 8c and f).

Increase in agonist potency at the domain 3 P2X$_7$ receptor

There were differences in agonist potency or effect at several of the mutant receptors constructed. This was most pronounced at the domain 3 receptor where agonist potency was higher than at the other receptors studied. Indeed, this receptor could not be examined in sucrose buffer as it appeared to be constitutively activated, presumably by endogenously released ATP. Thus, there was a high level of basal ethidium uptake that could be blocked by apyrase (1 unit mL^{-1}) or P2X$_7$ receptor antagonists such as decavanadate (10 μM) or compound-17 (1 μM) (data not shown).

In NaCl buffer, BzATP also possessed high potency at the domain 3 receptor with a pIC$_{50}$ of 5.94 ± 0.09. The pEC$_{50}$ value for BzATP at the domain 3 receptor was significantly higher than at the rat or human receptors ($P<0.05$, one-way ANOVA followed by Tukey's *post hoc* test) where pEC$_{50}$ values were 5.30 ± 0.08 and 4.43 ± 0.05, respectively (Fonfria *et al*., 2008). The maximal response produced by BzATP at the domain 3 receptor was similar to that produced by ATP (97.3 ± 1.07% of the ATP maximum; $P>0.05$, one-way ANOVA followed by Tukey's *post hoc* test). This contrasts with effects at the wild-type human receptor where BzATP produces a smaller maximal effect than ATP (Fonfria *et al*., 2008). In this study, the ATP pEC$_{50}$ at the domain 3 receptor was 4.16 ± 0.08, and this was significantly higher than at the rat or human receptors ($P<0.05$, one-way ANOVA followed by Tukey's *post hoc* test) where pEC$_{50}$ values were 3.66 ± 0.02 and 3.06 ± 0.04, respectively (Fonfria *et al*., 2008).

The differences in agonist potency were also observed in binding studies where ADP and αβ-methylene-ATP (data not shown) as well as ATP, BzATP, adenosine-5′-O-(3-thiotriphosphate) and 2-methylthio-ATP were 3- to 10-fold more potent at the domain 3 receptor than at the rat P2X$_7$ receptor and between 16- and 100-fold more potent than at the human P2X$_7$ receptor (Figures 9a–d). Lysolipids can increase agonist potency at the P2X$_7$ receptor by at least 100-fold (Michel and Fonfria, 2007), raising the possibility that the domain 3 residue was mimicking the effects of lipids in some way. However, palmitoyl L-α-lysophosphatidylcholine (lysoPC) increased ATP potency at the domain 3 receptor and the effect was comparable with its effects at the human receptor (Figures 9e and f). These studies were complicated by the reduction in binding produced by lysoPC. This probably reflects its ability to augment the inhibitory effects on radioligand binding of endogenous ATP present in the membranes, as the inhibitory effect of lysoPC was not observed in the presence of 1 unit mL^{-1} of the ATP degrading enzyme, apyrase (data not shown).

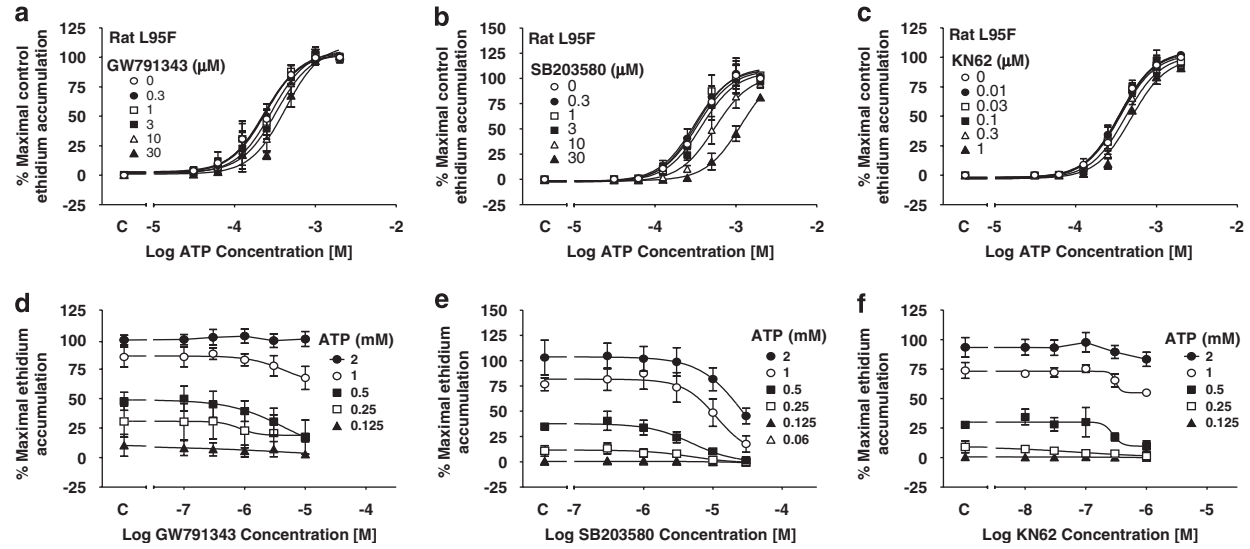

Figure 8 Antagonist effects on ATP-induced ethidium accumulation in cells expressing the rat L95F receptor. All studies were performed at RT in NaCl buffer in HEK293 cells stably expressing the recombinant receptor and pre-equilibrated for 40 min with the indicated concentrations of antagonist before measuring ATP responses. The effect on ATP responses is shown for (**a**) GW791343, (**b**) SB203580 or (**c**) KN62. (**d**) Transposition of the data shown in (**a**) to show effect of GW791343 on ATP responses. (**e**) Transposition of the data shown in (**b**) to show effect of SB203580 on ATP responses. (**f**) Transposition of the data shown in (**c**) to show effect of KN62 on ATP responses. Basal ethidium accumulation in the absence or presence of GW791343 is indicated on the *x* ordinate as C in (**a**–**c**). The data are the mean ± s.e.mean of 3–4 separate experiments.

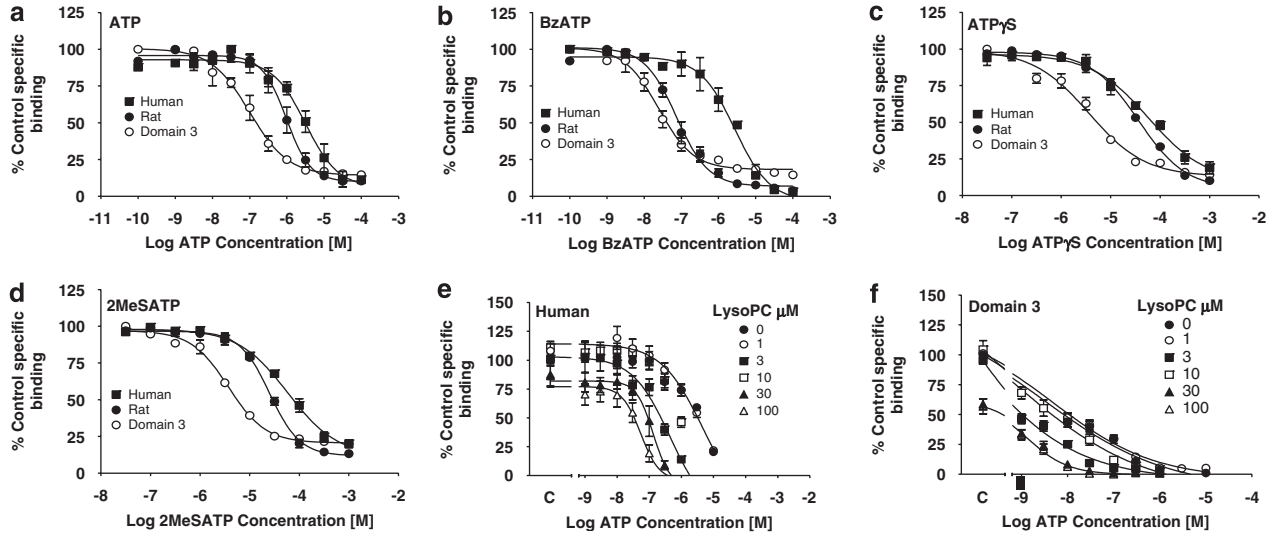

Figure 9 Inhibition by agonists of [^{3}H]-compound-17 binding to membranes prepared from cells expressing wild-type human, rat and domain 3 chimeric recombinant P2X$_7$ receptors. Membranes were prepared from HEK293 cells stably expressing the recombinant P2X$_7$ receptors. The radioligand concentration was 2 nM and specific binding was defined with 10 μM compound-17. The effect of agonists at the domain 3 receptor is compared with effects at the rat and human receptors for (**a**) ATP, (**b**) BzATP, (**c**) adenosine-5′-O-(3-thiotriphosphate) or (**d**) 2-methylthio-ATP. The effect of lysoPC on ATP inhibition of binding is shown in (**e**) the human wild-type receptor and (**f**) the domain 3 receptor. The effect of lysoPC on radioligand binding in the absence of ATP is indicated on the *x* ordinate as C. The data are the mean ± s.e.mean of three separate experiments.

Discussion

The main finding of this study is that the nature of the amino acid in position 95 of the P2X$_7$ receptor has a marked effect on the potency, or effects, of several P2X$_7$ receptor antagonists and so may be a key determinant of the species differences in effects of these antagonists.

GW791343 is an allosteric modulator of the P2X$_7$ receptor, being a negative allosteric modulator at human and a positive allosteric modulator at rat P2X$_7$ receptors (Michel *et al.*, 2008). Studies on the human chimeric and mutant receptors sequentially localized the amino acids responsible for this species difference in effect to the ECD, the first 255 amino acids, amino acids 72–95 and finally to amino acid 95

of the receptor. This residue was phenylalanine in the human $P2X_7$ receptor, at which GW791343 inhibited responses, but was leucine in the rat $P2X_7$ receptor where GW791343 increased responses to ATP. This amino acid was also important in the species selectivity of SB203580 and partially contributed to the species difference in the potency of KN62. We also generated complimentary data using the rat receptor where the corresponding L95F mutation appreciably modified the effects of both GW791343 and SB203580 and also produced a subtle change in the effects of KN62. Although there was compelling evidence that the residue at position 95 could greatly affect the species selectivity of the antagonists, it should be noted that exchanging this residue did not result in a complete species interconversion of antagonist effects even for GW791343. Thus, the increase in ATP responses produced by GW791343 at the human F95L receptor was not as marked as observed at the rat receptor, whereas the antagonist effect of GW791343 at the rat L95F receptor was less than observed at the human receptor.

It is not clear from this study exactly how the residue at amino acid 95 influenced antagonist effects or potency. Phenylalanine has been implicated in the binding of ATP to some P2X receptors but, on the basis of previous site-directed mutagenesis studies, it seems unlikely that amino acid 95 is part of the ATP-binding site (Vial *et al*., 2004). Furthermore, PPADS interacts at the ATP-binding site (Michel *et al*., 2006b), and its potency was not influenced by the amino acid at position 95. Similarly, the potency of ATP and BzATP differ between rat and human $P2X_7$ receptors, although only 3- to 10-fold, yet their potency estimates at the F95L human $P2X_7$ chimeric receptor were not significantly different from those at the human receptor (AD Michel, unpublished data).

It seems unlikely that the residue at amino acid 95 exerts any direct effect on the binding of GW791343, as the affinity of GW791343, assessed using [^{3}H]-compound-17, did not differ greatly between human and rat receptors (Michel *et al*., 2008). Furthermore, GW791343 is an allosteric modulator of the $P2X_7$ receptor, and the main difference in GW791343 effect when exchanging phenylalanine with leucine was to change the compound from a negative to a positive allosteric modulator. Consequently, it seems more likely that amino acid 95 can modulate putative conformational changes that occur as a consequence of GW791343 binding. In the case of the human receptor, the residue is phenylalanine and this may result in conformational changes following GW791343 binding that lead to inhibition of channel opening, either by direct steric hindrance from the large phenylalanine residue or by an indirect effect on receptor flexibility. In the case of the rat receptor, where the residue is a smaller and potentially more flexible leucine, this may lead to the conformational changes induced by GW791343 facilitating, rather than inhibiting, channel opening. In the case of KN62 and SB203580, where decreases in potency were observed in the chimeric F95L human $P2X_7$ receptor, it may be that the large aromatic group of phenylalanine coordinates ligand binding better than leucine, although it seems more plausible that the efficacy, or extent of effect, of these allosteric antagonists is affected by the residue at position 95.

Interestingly, amino acid 95 is close to an amino-acid sequence (79–86) that is unique to the $P2X_7$ receptor (North, 2002). Although we did not observe any marked change in GW791343 or SB203580 effects at the two mutants from within this region, K81T or S86G, binding of compounds at such a region may be a plausible explanation for the considerable selectivity of most $P2X_7$ receptor antagonists over other P2X receptor types (see Introduction).

It seems likely that other residues in the $P2X_7$ receptor apart from those in domain 1 can affect the functional effects of GW791343 and SB203580. Thus, their inhibitory potency was also changed in several of the other domains studied. This may not be unexpected when exchanging large tracts of receptor between orthologues, as this may lead to quite large changes in tertiary structure of the receptor, and further studies would be required to determine if these effects can be localized to a single residue or result from a general change in receptor conformation. SB203580 effects were also reduced in the domain 2 and 3 receptors, but we could not identify a single residue responsible for this.

The situation with KN62 was more complicated. Its potency was reduced in domain 1 and also at the F95L chimera but its potency was also slightly increased in the S86G mutant. The significance of this increase in potency at the S86G receptor is not known, but it provides further evidence that this region of the receptor is important for the effects of allosteric antagonists. The effects of KN62 were also modified at the domain 2 receptor, although we could not identify a specific residue responsible for this change. It should also be noted that we have evaluated several other structurally diverse $P2X_7$ receptor antagonists and have found several compounds that differentiate between human and rat $P2X_7$ receptors, but their potency is not affected in either the F95L or R126G (see below) mutants, suggesting that there may be multiple regions of the receptor responsible for the species selectivity of antagonists (AD Michel, unpublished data).

The results with PPADS provided further information on the sites of the receptor responsible for antagonist effects. PPADS was an interesting compound to study, as mechanistic studies suggested that it interacts with the $P2X_7$ receptor in a distinct manner to KN62, GW791343 and SB203580 (Michel *et al*., 2006a, 2008), yet it also displays higher potency at human than at rat receptors. PPADS potency was modified in several of the chimeric receptors. There was a small reduction in potency at the domain 1 receptor, but this was not detected in any of the single-point mutant receptors within domain 1, including F95L. In the domain 2 receptor, PPADS potency was markedly reduced and this may be due to the amino acid at position 126. Interestingly, this residue is adjacent to a key amino acid at position 127, which is thought to contribute to the mouse and rat species differences in the potency of BzATP (Young *et al*., 2007). In the domain 5 receptor, PPADS potency was also reduced, although we did not construct single-point mutations within this region. Interestingly, residue 284 is localized within domain 5 and this is thought to be a further key residue responsible for the species differences in ATP and BzATP effects at mouse and rat receptors (Young *et al*., 2007). However, this residue is asparagine at both human and rat receptors, suggesting it is unlikely to be the amino acid responsible for the species differences in PPADS effects.

Given that PPADS appears to interact at the ATP-binding site (Michel *et al*., 2006b), it may be that residues close to amino acid 284 affect PPADS species selectivity. Certainly adjacent amino acids at positions 282, 285 and 288 differ between the human and the rat receptors.

There are limitations to the approach of exchanging large tracts of receptors between orthologues as illustrated by the data obtained with the domain 3 chimera. In this receptor, agonist potency was greatly increased. Indeed, the increase in ATP potency was so great that the receptor became tonically activated when studied in the sucrose assay buffer. This was, presumably, due to receptor activation by endogenously released ATP and was observed only in studies in sucrose buffer, as ATP has much higher potency in sucrose than in NaCl buffer (Michel *et al*., 1999). The increase in potency at the domain 3 receptor was not restricted to ATP and was also observed with other ATP analogues. The increased potency at the domain 3 receptor was also evident in ligand-binding studies and did not appear to reflect a simple switch in the pharmacological properties of the human receptor to those of the rat receptor, as agonist potency at the domain 3 receptor was even higher than at the rat receptor for all of the agonists studied. Interestingly, PPADS is thought to bind to the ATP-binding site, and PPADS potency was also increased, although only threefold, at the domain 3 receptor.

The reason(s) for the high agonist potency at the domain 3 receptor is not known and it would be interesting to determine if the change reflects the large exchange of protein between orthologues or can be localized to a single residue. In this respect, H155Y is a reported gain-of-function SNP in the human $P2X_7$ receptor (Cabrini *et al*., 2005), and the residue is tyrosine in the rat receptor. However, it did not appear to be responsible for any of the changes in agonist or antagonist potency, although this comparison was complicated by the use of a human receptor containing an additional single SNP (H270R) in these studies. The domain 3 receptor also includes amino acid 173, and a previous study demonstrated that a single-point mutation of the rat P2X7 receptor, in which the human glycine residue at position 173 was inserted into the rat receptor, resulted in a chimeric receptor at which BzATP was 10- to 30-fold higher than at the rat receptor (Thompson, 2001). Although this change in potency is in the opposite direction to that observed in this study, where exchange of rat sequence into the human receptor increased potency, it is consistent with this region of the receptor being important for defining agonist potency at the $P2X_7$ receptor.

In summary, we have identified a key residue at position 95, which seems to determine the nature of the allosteric effects of GW791343 and which is also partly responsible for the species difference in effects of SB203580 and KN62 but is not involved in the species selectivity of PPADS or in the species difference in agonist potency. A further residue at position 126 appeared to be important for the species difference in potency of PPADS, although residues in domain 5 also seemed to contribute to its species selectivity, perhaps implicating multiple regions of the receptor in the binding site for PPADS. Finally, we identified a further region of the $P2X_7$ receptor that affected agonist and PPADS potency at the $P2X_7$ receptor. This region is distinct from other areas of the receptor previously identified as being important for agonist binding and further study of residues within this domain may provide additional information on the interaction of agonists with the receptor.

Conflict of interest

The authors are employed by GlaxoSmithKiline.

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