

MOL No. 30163

## **Amino acid residues in the P2X<sub>7</sub> receptor that mediate differential sensitivity to ATP and BzATP**

Mark T. Young<sup>1</sup>, Pablo Pelegrin<sup>1</sup>, and Annmarie Surprenant

Department of Biomedical Science, University of Sheffield, Addison Building Western Bank, Sheffield S10 2TN, UK

MOL No. 30163

Running title: ATP and BzATP selectivity at mouse and rat P2X<sub>7</sub> receptors

Corresponding author: A. Surprenant at Department of Biomedical Science, Addison Building Western Bank, University of Sheffield, Sheffield S10 2TN, UK. phone: 44-114-222-2366; fax: 44-114-222-2360  
email: a.surprenant@sheffield.ac.uk

**Number of text pages: 21**

**Number of tables: 1**

**Number of figures: 6**

**Number of references: 41**

**Words in Abstract: 244**

**Words in Introduction: 723**

**Words in Discussion: 950**

**Abbreviations used:** ATP, adenosine 5'-triphosphate; BzATP, 2'-3'-O-(4-benzoylbenzoyl) adenosine 5'-triphosphate; HEK293, human embryonic kidney cell line; PVDF, polyvinylidene difluoride

## Abstract

Agonist properties of the P2X<sub>7</sub> receptor (P2X<sub>7</sub>R) differ strikingly from other P2X receptors in two main ways: high concentrations of ATP (> 100 μM) are required to activate the receptor and the ATP analogue, 2',3'-O-(4-benzoyl-benzoyl)ATP (BzATP), is both more potent than ATP and evokes a higher maximum current. However, there are striking species differences in these properties. We sought to exploit the large differences in ATP and BzATP responses between rat and mouse P2X<sub>7</sub>R in order to delineate regions, or specific residues, that may be responsible for the unique actions of these agonists at the P2X<sub>7</sub>R. We measured membrane currents in response to ATP and BzATP at wildtype rat and mouse P2X<sub>7</sub>R, at chimeric P2X<sub>7</sub>Rs, and at mouse P2X<sub>7</sub>Rs bearing point mutations. Wildtype rP2X<sub>7</sub>R was 10 times more sensitive to ATP and 100 times more sensitive to BzATP than wildtype mP2X<sub>7</sub>R. We found that agonist EC<sub>50</sub> values were determined solely by the ectodomain of the P2X<sub>7</sub>R. Two segments (residues 115-136 and 282-288) when transposed together converted mouse sensitivities to those of rat. Point mutations through these regions revealed a single residue, asparagine<sup>284</sup> in the rat P2X<sub>7</sub>R, that fully accounted for the 10-fold difference in ATP sensitivity, whereas the 100-fold difference in BzATP sensitivity required the transfer of both Lys<sup>127</sup> and Asn<sup>284</sup> from rat to mouse. Thus, single amino acid differences between species can account for large changes in agonist effectiveness, and also differentiate between the two widely used agonists at P2X<sub>7</sub> receptors.

MOL No. 30163

The P2X<sub>7</sub> receptors belong to a family of cation-permeable membrane proteins gated by extracellular ATP. They can be distinguished from other family members (P2X<sub>1</sub> - P2X<sub>6</sub>) by several properties. First, receptor activation is followed in several seconds by the appearance of a permeation pathway that allows passage of molecules up to 900 daltons (North, 2002). Second, activation by extracellular ATP rapidly engages a series of cytoskeletal and mitochondrial alterations which include actin/ $\alpha$ -tubulin rearrangements, phosphatidylserine translocation, mitochondrial swelling and loss of mitochondrial membrane potential, and membrane blebbing (Mackenzie et al., 2001, 2005; Le Feuvre et al., 2002; Verhoef et al., 2003; Morelli et al., 2003; Pfeiffer et al., 2004; Elliott et al., 2005; Ferrari et al., 2006). Third, the P2X<sub>7</sub> receptor in immune cells of monocyte/macrophage lineage becomes up-regulated and functionally active in response to inflammatory stimuli (Guerra et al., 2003; Ferrari et al., 2006); its activation there engages cascades that culminate in processing and release of interleukin-1 $\beta$ , release of tumor necrosis factor  $\alpha$ , and activation of NF- $\kappa$ B (North, 2002; Ferrari et al., 2006). Finally, studies using mice in which the P2X<sub>7</sub> receptor has been deleted further support a role in inflammatory processes (Solle et al., 2001; Labasi et al., 2002; Chessell et al., 2005).

P2X<sub>7</sub> receptors can also be readily distinguished from other family members when membrane ionic current is measured directly. First, they are more potently inhibited by extracellular calcium and/or magnesium. Second, they are unusually insensitive to ATP; the EC<sub>50</sub> (>300  $\mu$ M) is about 100-fold higher than for other P2X receptors (North, 2002). Third, the ATP analog 2',3'-O-(4-benzoyl-benzoyl)ATP (BzATP) is considerably more potent than ATP itself at P2X<sub>7</sub> receptors, whereas at other P2X receptors it is less potent (North, 2002; Baraldi et al., 2004). Fourth, certain antagonists are selective for the P2X<sub>7</sub> receptor, although few have yet been studied at the primary effect of membrane current (Humphreys et al., 1998; Jiang et al., 2000).

There are marked species differences in agonist and antagonist pharmacology for P2X<sub>7</sub> receptors. The effectiveness of BzATP relative to ATP, which has often been used as the primary distinguishing feature of the P2X<sub>7</sub> receptor, is not equally reliable among species (Surprenant et al., 1996; Chessell et al., 1998a; 1998b; Hibell et al., 2000; Young et al., 2006). For example, isoquinolone derivatives such as KN-62 and KN-04 block

MOL No. 30163

human P2X<sub>7</sub> receptors with low nanomolar affinity, but are without effect at rodent P2X<sub>7</sub> receptors even at high micromolar concentrations (Humphreys et al., 1998; Baraldi et al., 2004). Conversely, Brilliant Blue G is 20 times more potent at rat than human P2X<sub>7</sub> receptors (Jiang et al., 2000).

The differences in properties between the P2X<sub>7</sub> receptors of different species are important for several reasons. First, quite different polymorphisms have been described in the human and mouse P2X<sub>7</sub> receptors (Adriouch et al., 2002; Gu et al., 2004; Cabrini et al., 2005; Shemon et al., 2005); these may affect their properties in different ways. Second, pharmacological characterizations and structure-function studies of P2X<sub>7</sub> receptors have mainly been obtained from heterologous expression of the rat ortholog; such studies will be of limited value if properties of agonists and antagonists are very different from human. Third, increasing use of P2X<sub>7</sub> receptor knock-out mice as animal models of disease (e.g. neuropathic and inflammatory pain: Chessell et al., 2005; osteopenia: Li et al., 2005; joint inflammation: Labasi et al., 2002) drives the need for a more precise knowledge of the properties of this receptor. More generally, information concerning residues involved in agonist action at P2X receptors is extensive for P2X<sub>1</sub>, P2X<sub>2</sub> and P2X<sub>4</sub> receptors, but minimal P2X<sub>7</sub> receptors (North, 2002; Vial et al., 2004; Zemkova et al., 2004).

The purpose of the present study was to identify residues in P2X<sub>7</sub> receptors that may be involved in agonist action. We recently noticed that the sensitivity to BzATP was different between rat and mouse P2X<sub>7</sub> receptors (Young et al., 2006). Eighty-eight of the 595 amino acids of the P2X<sub>7</sub> receptors are different between mouse and rat (Fig. 1A). In the present study, we have investigated which of these might be responsible for the differences in effectiveness of ATP and BzATP. We have identified two residues in the ectodomain of P2X<sub>7</sub>R that can account for the differential agonist sensitivities: residue 127 which primarily influences BzATP affinity and residue 284 which can fully account for ATP sensitivity.

## Materials and Methods

**Cell culture, transfection and site-directed mutagenesis.** Both rat and mouse P2X<sub>7</sub> constructs (Surprenant et al., 1996; Chessell et al., 1998b) were sub-cloned in the same expression vector background (pcDNA3, Invitrogen, Paisley, UK) and bore C-terminal Glu-Glu epitope tags (EYMPME) to allow detection of protein expression by Western blotting. The 3' and 5'-non-coding regions of the mouse P2X<sub>7</sub> construct was engineered to be identical to that of the rat P2X<sub>7</sub> construct in order to eliminate any differences in expression due to non-coding sequences. Point mutations were generated from the above constructs using the PCR overlap extension method and Accuzyme proof-reading DNA polymerase (Bioline, London, UK). Single chimeras (Fig. 1B) were produced using 21 nucleotides synthetic oligonucleotides designed with an in-frame 9 nucleotides 5'-adapter tail to introduce overlapping sequences to fuse chimeras between rat and mouse P2X<sub>7</sub>R sequences. These oligonucleotides were used in combination with the T7 sense and BGH antisense oligonucleotides annealing in the pcDNA3 expression vector sequence. Overlapping amplification products were purified from a 1% agarose gel electrophoresis and used in combination for a second PCR amplification using the T7 sense and BGH antisense oligonucleotides. Three consecutive overlapping PCR amplifications were necessary to produce double chimeras (Fig. 1B). Final T7/BGH amplified products were double-digested with *Hind*III and *Xba*I and replaced back in the *Hind*III-*Xba*I positions of the original vector. A high concentration of template vector in combination with a proof-reading DNA polymerase and a low number of cycling steps were used in all amplification reactions to minimize random mutations. All subcloned products were confirmed by sequencing (Beckman-Coulter CEQ 2000 Dye Terminator) and protein expression was verified by Western blotting.

HEK293 cells were transiently transfected using Lipofectamine 2000 (Invitrogen, Paisley, UK). Cells were plated onto 13mm glass coverslips and maintained in Dulbecco's modified Eagle medium, supplemented with 10% heat inactivated fetal calf serum and 2mM L-glutamine at 37°C in a humidified 5% CO<sub>2</sub> incubator. We were concerned that differences in expression levels between constructs (Young et al., 2006) might change the pharmacological properties of the currents. Reducing the rat P2X<sub>7</sub>R

MOL No. 30163

cDNA concentration by 10-fold (from the standard 1 µg/ml to 0.1 µg/ml) resulted in approximately 50% reduction in maximum currents to ATP or BzATP without a significant change in agonist EC<sub>50</sub> (Fig. 2A). Therefore, in all subsequent experiments we used the standard (1 µg/ml cDNA) concentration for all transfections.

**Protein solubilisation, deglycosylation and Western blotting.** Confluent cells were washed with PBS and pelleted. Cell pellets were lysed in PBS containing 1% Triton X-100 and antiproteases (Complete, Roche, Lewes, UK) for 1 hour at 4°C, followed by centrifugation at 16000 x g for 10 minutes to pellet debris. Total protein samples were removed and assayed for protein content using the Bio-Rad Protein Assay kit (Bio-Rad, Hemel Hempstead, UK). SDS-PAGE sample buffer was added and the samples were boiled for 2 minutes at 100°C to denature the protein. Where appropriate, deglycosylation was performed by incubating protein samples (100µg) for 1 hour at 37°C with 500 units of PNGase F (NEB, Herts, UK) according to the manufacturer's instructions. Samples were separated on 8% polyacrylamide gels according to standard methods and transferred to PVDF membranes. Western blotting was performed according to standard protocols and proteins were visualised using anti Glu-Glu primary antibody (Bethyl Laboratories, Cambridge, UK) and HRP-conjugated secondary antibody (DA-KOCytomation, Ely, UK), both at 1:2000 dilution, followed by detection using the ECL-plus kit (Amersham, Bucks, UK) and Kodak Bio-Max MS film (Sigma, Poole, UK).

**Electrophysiological recordings.** Whole-cell recordings were made 24-48 hours after transfection using an EPC9 patch clamp amplifier (HEKA Elektronik, Lambrecht, Germany). Membrane potential was held at -60mV. Recording pipettes (5-7 MΩ) were pulled from borosilicate glass (World Precision Instruments, Sarasota, Florida) and filled with an intracellular solution that consisted of (in mM): 145 NaCl, 10 ethylene glycol-bis-(2-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), 10 *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES). The external solution contained (in mM): 147 NaCl, 10 HEPES, 13 glucose, 2 KCl, 2 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>. Agonists were applied in divalent-free solution; cells were otherwise superfused with normal external solution. Osmolarity and pH values of all solutions were 295-310 mOsm/l and 7.3 respectively. All experiments were performed at room temperature. Agonists were applied using a RSC 200 fast-flow delivery system (BioLogic Science

MOL No. 30163

Instruments, Grenoble, France). Agonists were applied for 5- or 10-second duration to obtain steady-state responses. Concentration-response curves to ATP and BzATP were obtained by first obtaining a maximum response to agonist, as marked run-up of response was observed at both rat and mouse P2X<sub>7</sub> receptors, (Surprenant et al., 1996; Chessell et al., 1998b; Young et al., 2006), and then either applying decreasing or increasing concentrations. In either case, similar curves were obtained, provided that a maximum response had been obtained beforehand. Concentration-response curves were plotted using KaleidaGraph (Synergy Software, Reading, Pennsylvania) and Prism v3.0a Software (Graphpad Prism; www.graphpad.com) using the Hill equation provided in Prism.

## Results

**Comparison of mouse and rat P2X<sub>7</sub> receptors.** Although all P2X<sub>7</sub>Rs are potently inhibited by extracellular divalent cations, there is a significant species difference with Mg<sup>++</sup> and Ca<sup>++</sup> being approximately 10-fold more potent to inhibit human than rat P2X<sub>7</sub>Rs (Surprenant et al., 1996; Rassendren et al., 1997). In preliminary experiments, we found that mouse P2X<sub>7</sub>R was also more sensitive (by approximately 5-fold) to inhibition by Mg<sup>++</sup> and Ca<sup>++</sup> than was rat P2X<sub>7</sub>R (data not shown). Therefore, all agonist responses were recorded in the divalent-free cation solution in order to rule out possible contributions of differential divalent cation sensitivity to agonist concentration-responses. We first compared ATP and BzATP concentration-response curves from wildtype rat and mouse P2X<sub>7</sub>Rs, using equal amounts of cDNA for transfection, and using a 10-fold lower concentration of rP2X<sub>7</sub>R cDNA which we have previously found results in similar protein expression of rat and mouse P2X<sub>7</sub>Rs (Young et al., 2006). Typical currents recorded from cells transfected with equal cDNA concentrations are shown in Fig. 2A and results from all experiments are shown in Fig. 2B, C. Reducing the rP2X<sub>7</sub>R cDNA concentration by 10-fold resulted in approximately 50% reduction in maximum currents to ATP or BzATP (Fig. 2B) without a significant change in agonist EC<sub>50</sub> values or BzATP:ATP maximum current ratio or EC<sub>50</sub> ratio (Fig. 2D, E). The maximum agonist-evoked currents recorded from cells transfected with mP2X<sub>7</sub>R were



MOL No. 30163

approximately the same as those recorded from cells transfected with the 10-fold lower rP2X<sub>7</sub>R cDNA concentration (Fig. 2B, C). In agreement with earlier studies on human and rat P2X<sub>7</sub>Rs (Surprenant et al., 1996; Wiley et al., 1998; Hibell et al., 2000), we found ATP to be a partial agonist relative to BzATP, with maximum BzATP-evoked currents that were 30 - 45% greater than maximum ATP-evoked currents at both rat and mouse P2X<sub>7</sub>R (Fig. 2D). EC<sub>50</sub> values for BzATP and ATP at the rat P2X<sub>7</sub>R (3.6 μM and 123 μM, respectively) were several-fold lower than at the mouse P2X<sub>7</sub>R (285 μM and 936 μM) (Table 1). These values yield a striking difference in the BzATP:ATP EC<sub>50</sub> ratio, which was 34 at the rP2X<sub>7</sub>R but only 3.3 at the mouse P2X<sub>7</sub>R (Fig. 2E).

**Introducing segments of rat P2X<sub>7</sub>R into mouse P2X<sub>7</sub>R.** Rat and mouse P2X<sub>7</sub>R sequences are 84% identical with 88 specific amino acid differences; most of the non-conservative differences are found in two distinct regions of the ectodomain or in the intracellular C-terminal domain (Fig. 1A). Of the 22 non-conserved amino acids in the ectodomain, 8 are found in the region encompassing residues 115-136, 4 are found between residues 282 and 288 and the remaining half are scattered throughout the extracellular loop (Fig. 1A). We therefore made a series of chimeric constructs depicted in Fig. 2B in order to examine the effects of transposing rat ectodomain, C-terminus, residues 115-136 and 282-288 (alone and in combination) into the mouse P2X<sub>7</sub>R on agonist-evoked responses.

Neither agonist concentration-response curves nor agonist-evoked kinetics were altered by transposing rat intracellular C-terminus onto mouse P2X<sub>7</sub>R, or vice versa (Fig. 3A, C). Transposition of rat ectodomain onto mouse P2X<sub>7</sub>R resulted in ATP and BzATP EC<sub>50</sub> values that were the same as for wildtype rat P2X<sub>7</sub>R (Fig. 3C) but deactivation kinetics were several-fold slower (Fig. 3B). No responses were recorded from cells in which the mouse ectodomain was transposed into the rat P2X<sub>7</sub>R. The chimeric mouse P2X<sub>7</sub>R containing residues 115-136 of rat P2X<sub>7</sub>R resulted in a pronounced leftward shift of the BzATP concentration response curve without any alteration in the ATP-evoked responses (Fig. 4A; Table 1). When residues 282-288 of rat P2X<sub>7</sub>R were inserted into mouse P2X<sub>7</sub>R, there was a small leftward shift in the BzATP concentration-response curve but a large leftward shift in the ATP concentration response (Fig. 4B; Table 1). Substitution of both regions of rat P2X<sub>7</sub>R into mouse P2X<sub>7</sub>R resulted in agonist-evoked

MOL No. 30163

concentration-response curves and EC<sub>50</sub> values that were not significantly different from wildtype rat P2X<sub>7</sub>R (Fig. 4C; Table 1).

**Introducing single amino acids of rat P2X<sub>7</sub>R into mouse P2X<sub>7</sub>R.** We next substituted individually each rat P2X<sub>7</sub>R residue in these regions into the mouse receptor, and examined agonist responses in these mutant receptors. ATP-evoked responses were not different from wildtype mouse P2X<sub>7</sub>R for any mutation except at residue 284, where substitution of asparagine (rat P2X<sub>7</sub>R) for aspartate (mouse P2X<sub>7</sub>R) resulted in ATP concentration response and EC<sub>50</sub> that was not significantly different from wildtype rat P2X<sub>7</sub>R (Fig. 5A; Table 1). In contrast, the sensitivity of the mouse receptor to BzATP was clearly increased over wildtype by substitutions of the equivalent rat residue at several positions. These were D284N ( $p < 0.01$ ); A127K ( $p < 0.01$ ), S130H ( $p < 0.1$ ), R134G ( $p < 0.05$ ) and K136I ( $p < 0.05$ ). The largest difference was seen for mouse P2X<sub>7</sub>[A127K], in which case the EC<sub>50</sub> for BzATP (80  $\mu$ M) was intermediate between that for the rat (3  $\mu$ M) and mouse (285  $\mu$ M) receptor (Fig. 5B, Table 1).

The double substitution fully converted the mouse receptor (P2X<sub>7</sub>[A128K,D284N]) to the sensitivity of the rat receptor with respect to both ATP and BzATP EC<sub>50</sub> (Fig. 5A, Table 1) and was the only construct BzATP:ATP potency ratio was the same as that for wildtype rat P2X<sub>7</sub>R (Fig. 5C). However, we noticed that at this receptor BzATP was not able to produce as great a maximal current as ATP, which is the opposite for either of the wildtype receptors. Maximum BzATP-evoked currents were approximately 30% greater than maximum ATP-evoked currents at both mouse and rat P2X<sub>7</sub>R (Fig. 2D), but in the mouse P2X<sub>7</sub>[A128K,D284N] receptor the ATP-evoked currents were  $36 \pm 5\%$  ( $n = 5$ ) greater than the maximal currents evoked by BzATP.

**Involvement of N-glycosylation.** The substitution of Asp<sup>284</sup> with asparagine at the mouse P2X<sub>7</sub>R generates a potential N-glycosylation acceptor sequence (NESF). The wildtype rat P2X<sub>7</sub>R also contains a similar potential acceptor sequence at this position (NESL). Because it has been well demonstrated at other P2XRs that adding N-glycosylation sites significantly increases, while removing N-glycosylation sites decreases, protein expression (Newbolt et al., 1998; Torres et al., 1998; Rettinger et al., 2001; Chaumont et al., 2004), we assayed protein expression in wildtype, chimeric and mP2X<sub>7</sub>D284N receptor and asked whether this site was, indeed, glycosylated. As found

MOL No. 30163

previously (Young et al., 2006), transfection with equal concentrations of rat and mouse P2X<sub>7</sub>R cDNA yielded protein level ratios of approximately 3:1 (Fig. 6A). The presence of rat P2X<sub>7</sub>R ectodomain, but not N-terminus, C-terminus nor transmembrane domains, yielded protein levels not significantly different from wildtype rat P2X<sub>7</sub>R, while presence of mouse ectodomain yielded low protein expression equivalent to wildtype mouse P2X<sub>7</sub>R (Fig. 6A). We then removed N-glycan chains from wildtype mouse and rat P2X<sub>7</sub>R and mouse P2X<sub>7</sub>-D284N receptor using PNGase F and examined molecular mass by SDS-PAGE. Wildtype rat P2X<sub>7</sub>R, mouse P2X<sub>7</sub>-D284N receptor, and mouse P2X<sub>7</sub>R bands were detected at 78 kDa, 78 kDa and 75 kDa respectively (Fig. 6B). After PNGase F treatment all receptors were detected at approximately 65 kDa (Fig. 6B). This result is consistent with Asn<sup>284</sup> being glycosylated in the wildtype rat receptor and the mouse P2X<sub>7</sub>[D284N] receptor.

## Discussion

The sequence relatedness between the mouse and rat P2X<sub>7</sub> receptors (84.9%) is substantially less than for the other P2X receptors (for 1 through six 97.5, 97.2, 99.2, 94.7, 94.5 and 92.6% identity). This may underlie the large species difference in agonist sensitivity, which have generally not been described for other (homomeric) members of the P2X receptor family. The differences are somewhat clustered, and in two parts of the ectodomain the identity is only 68% (115-136) and 43% (282-288)(boxes, Fig. 1A). Unlike other P2X receptors, where mutations in transmembrane domains have been demonstrated to significantly alter ATP concentration-response curves (Haines et al., 2001), we found agonist potency to be determined solely by the ectodomain of the P2X<sub>7</sub> receptor. We then identified two amino acids which are not conserved in any of the other P2X receptors, one in each of these ectodomain segments, that could account for the differential ATP:BzATP agonist sensitivity at the P2X<sub>7</sub> receptor.

The first main finding of the present study is that, of all the differences between rat and mouse P2X<sub>7</sub> receptor ectodomain residues, a single amino acid is largely responsible for the difference in sensitivity to ATP. Thus, introducing asparagine in place of aspartate at position 284 in the mouse P2X<sub>7</sub>R changed the EC<sub>50</sub> from 936 μM to 146 μM, which is close to the value for the wild type rat receptor (123 μM, Table 1). The

MOL No. 30163

change in sensitivity associated with the aspartate to asparagine substitution (from -O<sup>-</sup> to -NH<sub>2</sub>) is striking. The asparagine is situated within a sequence commonly found at sites of N-linked glycosylation (N-X-S; NESL in rat P2X<sub>7</sub>; NESF in mouse P2X<sub>7</sub>[D284N]). We found evidence that the mutated mouse P2X<sub>7</sub>[D284N] receptor was glycosylated at this position, because the molecular mass was about 3 kDa higher than the wild type mouse receptor. The mass corresponded to that of the wild type rat receptor (78 kDa). We note that the wild type rat receptor and mouse receptor both carry the same five potential N-linked glycosylation sites (Asn<sup>74</sup>, Asn<sup>100</sup>, Asn<sup>106</sup>, Asn<sup>187</sup>, Asn<sup>241</sup>) while only rat P2X<sub>7</sub>R carries a sixth (Asn<sup>284</sup>) glycosylation site. Thus, the wildtype rat P2X<sub>7</sub>R and the mouse P2X<sub>7</sub>[D284N] mutated receptor each carry the same N-linked glycosylation sites. In both cases, treatment with PNGase F reduced the molecular mass to about 65 kDa which is similar to the calculated molecular weight (68.5 kDa) of the receptor. The large effect that this aspartate to asparagine substitution has on the potency of ATP could plausibly indicate that the attached sugar moiety participates directly in ATP binding. An alternative explanation is that the glycan impedes the conformational change leading from binding to gating; this seems less likely because it is the form of the P2X<sub>7</sub> receptor *with* the attached sugar at this position (mouse P2X<sub>7</sub>[D284N], or rat P2X<sub>7</sub>) that is more sensitive to ATP than the form without it (mouse P2X<sub>7</sub>). Rettinger et al. (2000) and Roberts and Evans (2006) have previously found that, in the case of the P2X<sub>1</sub> receptor, glycosylation affects the potency of ATP. Depending on the glycosylated residue the difference in potency was small (P2X<sub>1</sub>[N210Q], about 3-fold) or large (P2X<sub>1</sub>[N290A], 60-fold), but in either case it was also the form of the receptor without attached sugar that was less sensitive. In the P2X<sub>1</sub> receptor, the asparagine was situated close to the centre of the ectodomain (Asn<sup>210</sup> of rat P2X<sub>1</sub>; Roberts & Evans, 2004). The subject asparagine of the present study, at position 284, is situated in region that is poorly conserved among P2X receptors. Thus, the present results indicate that it plays a key role in determining the unique response to ATP observed at the P2X<sub>7</sub> receptor.

The second main finding of the present work was that the sensitivity to BzATP was almost 100-fold different between rat and mouse receptors, 10 times greater than the difference for ATP, and this BzATP differential was largely influenced by residue 127 (lysine in rat and alanine in mouse P2X<sub>7</sub>R). The residue Asp<sup>284</sup> also had an effect on the

MOL No. 30163

BzATP sensitivity because substitution of Asp for Asn at this position did increase the potency of BzATP, but by only about three-fold (Table 1). A much greater further increase of about 30-fold was observed when the additional A127K mutation was introduced into the mouse receptor. The observation that the effects of ATP and BzATP are differentially affected by two point mutations might imply that the residues are involved directly in agonist binding rather than in the subsequent gating conformational changes (which might be expected to be more in common for distinct agonists). If this is the case, then one might ask why an alanine to lysine substitution might affect the action of BzATP but not ATP. The obvious difference between the two agonists is the presence of the two (O-linked) aromatic moieties at the 2'(3') position. One might speculate that the cationic lysine residue in the binding site might interact with the pi electron cloud on one or other of the aromatic rings, providing a contribution to binding energy that would be unique to BzATP.

Irrespective of the detailed mechanism of the differences between ATP and BzATP, the present results provide a stark qualitative reminder of the criticality of species differences in ATP receptor pharmacology. Important differences in antagonist effectiveness have been reported for P2X<sub>1</sub> receptors (chick *vs* human: Soto et al; 2003), P2X<sub>4</sub> receptors (human *vs* rat: Buell et al., 1996; Soto et al., 1996) and for P2X<sub>7</sub> receptors (human *vs* rat: Humphreys et al., 1998; Jiang et al., 2000; Baraldi et al., 2004 and mammalian *vs* non-mammalian: Lopez-Castejon et al., 2006). However, differences among species in agonist sensitivity have not been widely described. The findings therefore suggest that caution should be exercised in cross species extrapolation on studies on P2X<sub>7</sub> receptors, where BzATP is in very widespread use as an experimental agonist.

## References

- Adriouch S, Dox C, Welge V, Seman M, Koch-Notle F, and Haag F (2002) Cutting edge: a natural P451L mutation in the cytoplasmic domain impairs the function of the mouse P2X<sub>7</sub> receptor. *J Immunol* **169**:4108-4112.
- Baraldi PG, Di Virgilio F, and Romagnoli R (2004) Agonists and antagonists acting at P2X<sub>7</sub> receptor. *Curr Top Med Chem* **4**:1707-1717.
- Buell G, Lewis C, Collo G, North RA, and Surprenant A (1996) An antagonist-insensitive P2X receptor expressed in epithelia and brain. *EMBO J* **15**:55-62.
- Chaumont S, Jiang LH, Penna A, North RA and Rassendren F (2004) Identification of a trafficking motif involved in the stabilization and polarization of P2X receptors. *J Biol Chem* **279**(28):29628-29638.
- Cabrini G, Falzoni S, Forchap SL, Pellegatti P, Balboni A, Agostini P, Cuneo A, Castoldi G, Baricordi OR, and Di Virgilio F (2005) A His-155 to Tyr polymorphism confers gain-of-function to the human P2X<sub>7</sub> receptor of human leukemic lymphocytes. *J Immunol* **175**(1):82-89.
- Chessell IP, Hatcher JP, Bountra C, Michel AD, Hughes JP, Green P, Egerton J, Murfin M, Richardson J, Peck WL, Grahames CB, Casula MA, Yiangou Y, Birch R, Anand P, and Buell GN (2005) Disruption of the P2X<sub>7</sub> purinoceptor gene abolishes chronic inflammatory and neuropathic pain. *Pain* **114**(3):386-396.
- Chessell IP, Michel AD, and Humphrey PP (1998a) Effects of antagonists at the human recombinant P2X<sub>7</sub> receptor. *Br J Pharmacol* **124**(6):1314-1320.
- Chessell IP, Simon J, Hibell AD, Michel AD, Barnard EA, and Humphrey PP (1998b) Cloning and functional characterisation of the mouse P2X<sub>7</sub> receptor. *FEBS Lett* **439**(1-2):26-30.
- Elliott JI, Surprenant A, Marelli-Berg FM, Cooper JC, Cassidy-Cain RL, Wooding C, Linton K, Alexander DR, and Higgins CF (2005) Membrane phosphatidylserine distribution as a non-apoptotic signalling mechanism in lymphocytes. *Nat Cell Biol* **7**(8):808-816.
- Ferrari D, Pizzirani C, Adinolfi E, Lemoli RM, Curti A, Idzko M, Panther E, and Di Virgilio F (2006) The P2X<sub>7</sub> receptor: a key player in IL-1 processing and release. *J Immunol* **176**:3877-3883.
- Gu BJ, Sluyter R, Skarratt KK, Shemon AN, Dao-Ung LP, Fuller SJ, Barden JA, Clarke AL, Petrou S, and Wiley JS (2004) An Arg307 to Gln polymorphism within the ATP-binding site causes loss of function of the human P2X<sub>7</sub> receptor. *J Biol Chem* **279**(30):31287-31295.
- Guerra AN, Fisette PL, Pfeiffer ZA, Quinchia-Rios BH, Prabhu U, Aga M, Denlinger LC, Guadarrama AG, Abozeid S, Sommer JA, Proctor RA, and Bertics PJ (2003) Purinergic receptor regulation of LPS-induced signaling and pathophysiology. *J Endotoxin Res* **9**(4):256-263.
- Haines WR, Migita K, Cox JA, Egan TM, and Voigt MM (2001) The first transmembrane domain of the P2X receptor subunit participates in the agonist-induced gating of the channel. *J Biol Chem* **276**(35):32793-32798.
- Hibell AD, Kidd EJ, Chessell IP, Humphrey PP, and Michel AD (2000) Apparent species differences in the kinetic properties of P2X<sub>7</sub> receptors. *Br J Pharmacol* **130**(1):167-173.

MOL No. 30163

- Humphreys BD, Virginio C, Surprenant A, Rice J, and Dubyak G (1998) Isoquinolines as antagonists of the P2X<sub>7</sub> nucleotide receptor: high selectivity for the human versus rat receptor homologues. *Mol Pharmacol* **54**:22-32.
- Jiang LH, Mackenzie AB, North RA, and Surprenant A. (2000) Brilliant blue G selectively blocks ATP-gated rat P2X<sub>7</sub> receptors. *Mol Pharmacol* **58**:82-68.
- Labasi JM, Petrushova N, Donovan C, McCurdy S, Lira P, Payette MM, Brissette W, Wicks JR, Audoly L, and Gabel CA (2002) Absence of the P2X<sub>7</sub> receptor alters leukocyte function and attenuates an inflammatory response. *J Immunol* **168**(12):6436-6445.
- Le Feuvre RA, Brough D, Iwakura Y, Takeda K, and Rothwell NJ (2002) Priming of macrophages with lipopolysaccharide potentiates P2X<sub>7</sub>-mediated cell death via a caspase-1-dependent mechanism, independently of cytokine production. *J Biol Chem* **277**(5):3210-3218.
- Li J, Liu D, Ke HZ, Duncan RL, and Turner CH (2005) The P2X<sub>7</sub> nucleotide receptor mediates skeletal mechanotransduction. *J Biol Chem* **280**:42952-42959.
- Lopez-Castejon G, Young MT, Meseguer J, Surprenant A, and Mulero V (2006) Characterization of ATP-gated P2X<sub>7</sub> receptors in fish provides new insights into the mechanism of release of the leaderless cytokine interleukin 1 $\beta$ . *Mol Immunol* (in press).
- MacKenzie A, Wilson HL, Kiss-Toth E, Dower SK, North RA, and Surprenant A (2001) Rapid secretion of interleukin-1beta by microvesicle shedding. *Immunity* **15**(5):825-835.
- Mackenzie AB, Young MT, Adinolfi E, and Surprenant A (2005) Pseudoapoptosis induced by brief activation of ATP-gated P2X<sub>7</sub> receptors. *J Biol Chem* **280**(40):33968-33976.
- Morelli A, Chiozzi P, Chiesa A, Ferrari D, Sanz JM, Falzoni S, Pinton P, Rizzuto R, Olson MF, and Di Virgilio F (2003) Extracellular ATP causes ROCK I-dependent bleb formation in P2X<sub>7</sub> transfected HEK293 cells. *Mol Biol Cell* **14**:2655-2664.
- Newbolt A, Stoop R, Virginio C, Surprenant A, North RA, Buell G, and Rassendren F (1998) Membrane topology of an ATP-gated ion channel (P2X receptor). *J Biol Chem* **273**:15177-15182.
- North RA (2002) Molecular physiology of P2X receptors. *Physiol Rev* **82**(4):1013-1067.
- Pfeiffer ZA, Aga M, Prabhu U, Watters JJ, Hall DJ, and Bertics PJ (2004) The nucleotide receptor P2X<sub>7</sub> mediates actin reorganization and membrane blebbing in RAW 264.7 macrophages via p38 MAP kinase and Rho. *J Leukoc Biol* **75**(6):1173-1182.
- Rassendren F, Buell GB, Virginio C, Collo G, North RA, and Surprenant A (1997) The permeabilizing ATP receptor, P2X<sub>7</sub>. Cloning and expression of a human cDNA. *J Biol Chem* **272**:5482-5486.
- Rettinger J, Aschrafi A, and Schmalzing G (2000) Roles of individual N-glycans for ATP potency and expression of the rat P2X<sub>1</sub> receptor. *J Biol Chem* **275**(43):33542-33547.
- Roberts JA and Evans RJ (2004) ATP binding at human P2X<sub>1</sub> receptors. Contribution of aromatic and basic amino acids revealed using mutagenesis and partial agonists. *J Biol Chem* **279**(10):9043-9055.

MOL No. 30163

- Roberts JA and Evans RJ (2006) Contribution of conserved polar glutamine, asparagine and threonine residues and glycosylation to agonist action at human P2X<sub>1</sub> receptors for ATP. *J Neurochem* **96**:843-852.
- Shemon AN, Sluyter R, Fernando SL, Clarke AL, Dao-Ung LP, Skarratt KK, Saunders BM, Tan KS, Gu BJ, Fuller SJ, Britton WJ, Petrou S, and Wiley JS (2005) A Thr<sup>357</sup> to Ser polymorphism in homozygous and compound heterozygous subjects causes absent or reduced P2X<sub>7</sub> function and impairs ATP-induced mycobacterial killing by macrophages. *J Biol Chem*. **281**:2079-2086.
- Solle M, Labasi J, Perregaux DG, Stam E, Petrushova N, Koller BH, Griffiths RJ, and Gabel CA (2001) Altered cytokine production in mice lacking P2X(7) receptors. *J Biol Chem* **276**(1):125-132.
- Soto F, Garcia-Guzman M, Gomez-Hernandez JM, Hollmann M, Karschin C, and Stuhmer W (1996) P2X<sub>4</sub>: an ATP-activated ionotropic receptor cloned from rat brain. *Proc Natl Acad Sci U S A* **93**:3684-3688.
- Soto F, Krause U, Borchardt K, and Ruppelt A (2003) Cloning, tissue distribution and functional characterization of the chicken P2X<sub>1</sub> receptor. *FEBS Lett* **533**:54-58.
- Surprenant A, Rassendren F, Kawashima E, North RA, and Buell G (1996) The cytolytic P2Z receptor for extracellular ATP identified as a P2X receptor (P2X<sub>7</sub>). *Science* **272**(5262):735-738.
- Torres GE, Egan TM, and Voigt MM (1998) N-Linked glycosylation is essential for the functional expression of the recombinant P2X<sub>2</sub> receptor. *Biochemistry* **37**(42):14845-14851.
- Verhoef PA, Estacion M, Schilling W, and Dubyak GR (2003) P2X<sub>7</sub> receptor-dependent blebbing and the activation of Rho-effector kinases, caspases, and IL-1 beta release. *J Immunol* **170**(11):5728-5738.
- Vial C, Roberts JA, and Evans RJ (2004) Molecular properties of ATP-gated P2X receptor ion channels. *Trends Pharmacol Sci* **25**:487-493.
- Wiley JS, Gargett CE, Zhang W, Snook MB, and Jamieson GP (1998) Partial agonists and antagonists reveal a second permeability state of human lymphocyte P2Z/P2X<sub>7</sub> channel. *Am J Physiol* **275**:C1224-1231.
- Young MT, Pelegrin P, and Surprenant A (2006) Identification of Thr<sup>283</sup> as a key determinant of P2X<sub>7</sub> receptor function. *Br J Pharmacol* (in press).
- Zemkova H, He ML, Koshimizu TA, and Stojilkovic SS (2004) Identification of ectodomain regions contributing to gating, deactivation, and resensitization of purinergic P2X receptors. *J Neurosci* **24**(31):6968-6978.



MOL No. 30163

### Footnotes

This work was supported by The Wellcome Trust and the BBSRC. We thank E. Martin and L. Collinson for cell and molecular biology technical support.

Reprint and materials requests to: A. Surprenant, Department of Biomedical Science, Addison Building Western Bank, University of Sheffield, Sheffield S10 2TN, U.K.  
email: [a.surprenant@sheffield.ac.uk](mailto:a.surprenant@sheffield.ac.uk)

MTY present address: Faculty of Life Sciences, University of Manchester, Michael Smith Building, Manchester M13 9PT, U.K.

<sup>1</sup> These authors contributed equally to this study.

### Figure legends.

Figure 1. Alignment of rat and mouse P2X<sub>7</sub>R sequences and design of experimental approach. A, Amino acid differences between species are shown in red bold type; transmembrane domains indicated by black lines; boxed residues (115-136 and 282-288) show regions of lowest conservation in the ectodomain. B, Schematic representation of chimeras where red and black represent rat and mouse P2X<sub>7</sub>R sequence respectively, and are shown in this, and all subsequent figures, as rat P2X<sub>7</sub>R residues inserted into mouse P2X<sub>7</sub>R background.

Figure 2. Comparison of agonist actions at wildtype rat and mouse P2X<sub>7</sub>R. A, Representative currents recorded from cells expressing rat or mouse P2X<sub>7</sub>R in response to increasing concentrations of ATP or BzATP as indicated. B, Summary of all experiments as illustrated in A for wildtype rat P2X<sub>7</sub>R for cells in which standard cDNA concentration (1 µg/ml, filled symbols) or 10-fold lower concentration (0.1 µg/ml, open symbols) was used for transfection. C, Agonist concentration-response curves obtained for wildtype mouse P2X<sub>7</sub>R (1 µg/ml cDNA transfected). D, Mean ratio of maximum current amplitudes to BzATP relative to ATP shown for rat and mouse P2X<sub>7</sub>R; these were similar for both species and for low and high expression levels. E, Mean ratio of EC<sub>50</sub> values of BzATP relative to ATP; low or high expression of rat P2X<sub>7</sub>R showed the same approximate 35-fold difference while mouse P2X<sub>7</sub>R showed only 4-fold difference.

Figure 3. The ectodomain is solely responsible for agonist potency at P2X<sub>7</sub>R. A, B, Examples of currents recorded from rat P2X<sub>7</sub>R containing mouse C-terminus (A) and from mouse P2X<sub>7</sub>R containing rat ectodomain (B). Note the prolonged deactivation kinetics upon removal of BzATP at the mouse P2X<sub>7</sub>R containing rat ectodomain (B); no other chimeric or mutant receptor showed altered kinetics of onset or offset. C, Summary of EC<sub>50</sub> values for ATP and BzATP obtained from chimeric receptors; note difference in x-axis scale for BzATP in order to better depict the 100-fold difference between rat and mouse receptors.

MOL No. 30163

Figure 4. Small subregions in the ectodomain account for differences between mouse and rat sensitivity to ATP and BzATP. A – C, concentration-response curves for ATP (left hand graphs) and BzATP (right hand graphs) from wildtype rat P2X<sub>7</sub>R (red filled circles), mouse P2X<sub>7</sub>R (black squares) and chimeric receptors (open circles). A, When residues 115-136 from rat P2X<sub>7</sub>R were inserted into mouse P2X<sub>7</sub>R, no change in ATP-evoked responses occurred while BzATP response showed a 10-fold leftward shift. B, Insertion of rat P2X<sub>7</sub>R residues 282-288 resulted in a significant leftward shift in both agonist concentration-response curves with the most pronounced effect occurring for the ATP dose-response curve. C, Insertion of both these regions of rat P2X<sub>7</sub>R into mouse P2X<sub>7</sub>R resulted in ATP concentration-response that was slightly shifted to the left of wildtype rat P2X<sub>7</sub>R and a BzATP response that was not different from wildtype rat P2X<sub>7</sub>R.

Figure 5. Asparagine<sup>284</sup> solely governs ATP potency differential while lysine<sup>127</sup> with asp<sup>284</sup> largely govern BzATP potency differential at P2X<sub>7</sub>R. Histograms of ATP (A) and BzATP (B) EC<sub>50</sub> values obtained from mouse P2X<sub>7</sub>R carrying point mutations in the regions between 115-136 and 282-288. In each case the residue substituted was that found in wildtype rat P2X<sub>7</sub>R. Stippled bars indicate values significantly different from wildtype mP2X<sub>7</sub>R. A, ATP EC<sub>50</sub> at mP2X<sub>7</sub>[D284N] was shifted 10-fold to the left of wildtype mP2X<sub>7</sub>R and was not significantly different from wildtype rat P2X<sub>7</sub>R while the BzATP EC<sub>50</sub> was shifted only 1.6-fold to the left at this mutation. B, Mutations at residues 127, 130, 134 and 136 also shifted the BzATP EC<sub>50</sub> significantly to the left with A127K mutation showing the largest effect. The mouse P2X<sub>7</sub>[A127K/D284N] double mutation yielded agonist EC<sub>50</sub> values that were not significantly different from wildtype rat P2X<sub>7</sub>R. C, Histograms of BzATP:ATP EC<sub>50</sub> ratios for chimeric and mutant receptors as indicated. Ratios were similar to wildtype rat P2X<sub>7</sub>R only when both A127K and D284N mutations were present.

Figure 6. Protein expression and N-linked glycosylation at mouse and rat P2X<sub>7</sub>Rs. A, Total protein expression of constructs as illustrated. Equal protein (10 µg) was loaded per lane and confirmed by comparing β-actin levels. P2X<sub>7</sub>R protein was detected using

MOL No. 30163

the anti-glu-glu epitope tag antibody. The ectodomain, but not transmembrane or intracellular domains, determined levels of P2X<sub>7</sub>R protein expression. B, Western blot of native and deglycosylated receptors. Both rat P2X<sub>7</sub>R and mouse P2X<sub>7</sub>R-D284N exhibited higher molecular weight bands (78 kDa) than mouse P2X<sub>7</sub>R (75 kDa). Treatment with PNGase F gave rise to products of the same lower mass of approximately 65 kDa, with an additional lower mass band (at 60 kDa) that may be due to protein degradation. C, Schematic indicating the two important residues conferring ATP (284N) and BzATP (127K+284N) sensitivity at rat and mouse P2X<sub>7</sub>Rs.

MOL No. 30163

**Table 1: EC<sub>50</sub> values for key P2X<sub>7</sub> constructs used in this study.**

Numbers in parentheses indicate total number of experiments.

<b>Construct</b>	<b>ATP EC<sub>50</sub> (μM)</b>	<b>BzATP EC<sub>50</sub> (μM)</b>
rat P2X <sub>7</sub>	123 ± 4 (21)	3.6 ± 0.2 (19)
mouse P2X <sub>7</sub>	936 ± 21 (18)	285 ± 16 (18)
mouse with rat P2X <sub>7</sub> [1-356]	125 ± 10 (5)	2.2 ± 0.2 (5)
mouse with rat P2X <sub>7</sub> [356-595]	1251 ± 202 (5)	306 ± 24 (3)
mouse with rat P2X <sub>7</sub> [47-334]	58 ± 0.8 (5)	1.4 ± 0.1 (5)
mouse with rat P2X <sub>7</sub> [115-136]	634 ± 27 (5)	33 ± 2.4 (5)
mouse P2X <sub>7</sub> [A127K]	815 ± 56 (10)	80 ± 7 (9)
mouse with rat P2X <sub>7</sub> [282-288]	162 ± 11 (9)	89 ± 4 (9)
mouse P2X <sub>7</sub> [D284N]	146 ± 11 (8)	94 ± 7 (10)
mouse with rat P2X <sub>7</sub> [115-136/282-288]	74 ± 5 (8)	2.9 ± 0.1 (9)
mouse P2X <sub>7</sub> [A127K/D284N]	112 ± 11 (5)	5 ± 1 (6)

Figure 1

A

Rat	MPACCSWNDV <b>FQ</b> YETNKVTRIQSVNYGT <b>IKWILHMTVFSYVSPALMS</b> DKLYQRKEP <b>L</b> ISS 60
Mouse	MPACCSWNDV <b>LQ</b> YETNKVTRIQS <b>T</b> NYGT <b>VKWLHMI</b> VFSY <b>ISFALV</b> SDKLYQRKEP <b>V</b> ISS 60
Rat	VHTKVKG <b>V</b> AEVTENVTEGGVTK <b>L</b> VHGIFD <b>TADY</b> T <b>LP</b> LQGNSSFFVMTNY <b>LK</b> SEGV <b>EQK</b> LCP 120
Mouse	VHTKVKG <b>I</b> AEVTENVTEGGVTK <b>L</b> GHSIFD <b>TADY</b> T <b>FP</b> LQGNSSFFVMTNY <b>VK</b> SEGV <b>VOT</b> LCP 120
Rat	EYPS <b>SRGKQCHSDQ</b> GIK <b>G</b> WMDPQSKGIQTGR <b>CIP</b> YD <b>Q</b> KRKTCE <b>IF</b> AWCP <b>A</b> E <b>E</b> GKEAP <b>R</b> PA 180
Mouse	EYPR <b>RGAQCS</b> SD <b>RR</b> CK <b>K</b> GWMDPQSKGIQTGR <b>CV</b> YD <b>K</b> TRKTCE <b>VS</b> AWCP <b>T</b> E <b>E</b> EKEAP <b>R</b> PA 180
Rat	LLRSAENFTVL <b>I</b> KNN <b>I</b> DFPGHNYT <b>T</b> RN <b>I</b> LP <b>G</b> M <b>N</b> I <b>S</b> CTFHKT <b>W</b> N <b>P</b> Q <b>C</b> IFR <b>L</b> GDIF <b>Q</b> E <b>I</b> G <b>E</b> 240
Mouse	LLRSAENFTVL <b>I</b> KNN <b>I</b> H <b>F</b> PGHNYT <b>T</b> RN <b>I</b> LP <b>T</b> M <b>G</b> SCTFHKT <b>W</b> D <b>P</b> Q <b>C</b> SIFR <b>L</b> GDIF <b>Q</b> E <b>A</b> G <b>E</b> 240
Rat	NFTEVAVQGGIMGIEI <b>Y</b> WDCN <b>L</b> DSWS <b>HRC</b> Q <b>P</b> KYSFRRLD <b>D</b> K <b>Y</b> T <b>N</b> ES <b>L</b> EPGYNFRYAKY <b>Y</b> K 300
Mouse	NFTEVAVQGGIMGIEI <b>Y</b> WDCN <b>L</b> DSWS <b>HCR</b> P <b>R</b> YSFRRLD <b>D</b> K <b>N</b> I <b>D</b> ES <b>F</b> EPGYNFRYAKY <b>Y</b> K 300
Rat	EN <b>G</b> MEK <b>R</b> TL <b>I</b> KA <b>F</b> GV <b>R</b> FD <b>I</b> L <b>V</b> FG <b>T</b> GG <b>K</b> FD <b>I</b> I <b>Q</b> L <b>V</b> V <b>I</b> GS <b>T</b> LS <b>Y</b> F <b>G</b> LAT <b>V</b> CI <b>D</b> L <b>I</b> NT <b>Y</b> A <b>S</b> 360
Mouse	EN <b>N</b> VEK <b>R</b> TL <b>I</b> KA <b>F</b> GI <b>R</b> FD <b>I</b> L <b>V</b> FG <b>T</b> GG <b>K</b> FD <b>I</b> I <b>Q</b> L <b>V</b> V <b>I</b> GS <b>T</b> LS <b>Y</b> F <b>G</b> LAT <b>V</b> CI <b>D</b> L <b>L</b> INT <b>Y</b> S <b>S</b> 360
Rat	<b>T</b> CCRS <b>G</b> V <b>P</b> YS <b>K</b> CC <b>C</b> EP <b>C</b> AVNE <b>Y</b> Y <b>R</b> KK <b>C</b> E <b>P</b> I <b>V</b> EPK <b>P</b> TL <b>K</b> Y <b>S</b> VF <b>D</b> EPH <b>I</b> W <b>M</b> VD <b>Q</b> LL <b>G</b> KS 420
Mouse	<b>A</b> FCRS <b>G</b> V <b>V</b> Y <b>P</b> Y <b>K</b> CC <b>C</b> EP <b>C</b> T <b>V</b> NE <b>Y</b> Y <b>R</b> KK <b>C</b> E <b>S</b> I <b>M</b> EPK <b>P</b> TL <b>K</b> Y <b>S</b> VF <b>D</b> EPH <b>I</b> W <b>M</b> VD <b>Q</b> LL <b>G</b> KS 420
Rat	L <b>Q</b> D <b>V</b> K <b>G</b> Q <b>E</b> VP <b>R</b> P <b>Q</b> T <b>D</b> F <b>L</b> E <b>L</b> S <b>R</b> L <b>S</b> L <b>S</b> L <b>H</b> S <b>P</b> P <b>I</b> P <b>G</b> Q <b>P</b> E <b>M</b> Q <b>L</b> L <b>Q</b> I <b>E</b> A <b>V</b> P <b>R</b> S <b>R</b> D <b>S</b> P <b>D</b> W <b>C</b> Q <b>G</b> 480
Mouse	L <b>Q</b> V <b>V</b> K <b>G</b> Q <b>E</b> VP <b>R</b> P <b>Q</b> M <b>D</b> F <b>S</b> D <b>L</b> S <b>R</b> L <b>S</b> L <b>S</b> L <b>H</b> D <b>S</b> P <b>P</b> T <b>P</b> G <b>Q</b> S <b>E</b> E <b>I</b> Q <b>L</b> L <b>H</b> E <b>V</b> A <b>P</b> <b>K</b> S <b>G</b> D <b>S</b> P <b>S</b> W <b>C</b> Q <b>G</b> 480
Rat	N <b>C</b> L <b>P</b> S <b>Q</b> L <b>P</b> EN <b>R</b> R <b>A</b> LE <b>E</b> L <b>C</b> CR <b>R</b> K <b>P</b> G <b>Q</b> C <b>I</b> T <b>T</b> S <b>E</b> L <b>F</b> S <b>K</b> I <b>V</b> L <b>S</b> R <b>E</b> A <b>L</b> Q <b>L</b> L <b>L</b> L <b>L</b> Y <b>Q</b> E <b>P</b> L <b>L</b> A <b>L</b> E <b>G</b> E <b>A</b> 540
Mouse	N <b>C</b> L <b>P</b> S <b>R</b> L <b>P</b> E <b>Q</b> R <b>R</b> A <b>L</b> E <b>E</b> L <b>C</b> CR <b>R</b> K <b>P</b> <b>G</b> R <b>C</b> I <b>T</b> T <b>S</b> <b>K</b> L <b>F</b> H <b>K</b> L <b>V</b> L <b>S</b> R <b>D</b> T <b>L</b> Q <b>L</b> L <b>L</b> L <b>L</b> Y <b>Q</b> D <b>P</b> L <b>L</b> V <b>L</b> G <b>E</b> A 540
Rat	<b>I</b> NS <b>K</b> LR <b>H</b> C <b>A</b> Y <b>R</b> S <b>Y</b> AT <b>W</b> R <b>F</b> V <b>S</b> Q <b>D</b> M <b>A</b> D <b>F</b> A <b>I</b> L <b>P</b> S <b>C</b> CR <b>W</b> <b>K</b> I <b>R</b> KE <b>F</b> P <b>K</b> T <b>Q</b> G <b>Q</b> Y <b>S</b> G <b>F</b> K <b>Y</b> P <b>Y</b> 595
Mouse	<b>T</b> NS <b>R</b> LR <b>H</b> R <b>A</b> Y <b>R</b> C <b>Y</b> AT <b>W</b> R <b>F</b> G <b>S</b> Q <b>D</b> M <b>A</b> D <b>F</b> A <b>I</b> L <b>P</b> S <b>C</b> CR <b>W</b> <b>I</b> R <b>KE</b> F <b>P</b> K <b>T</b> E <b>G</b> Q <b>Y</b> S <b>G</b> F <b>K</b> Y <b>P</b> Y 595

B

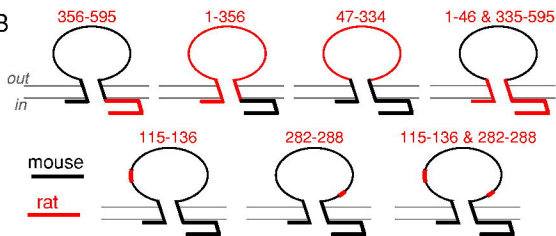


Figure 2

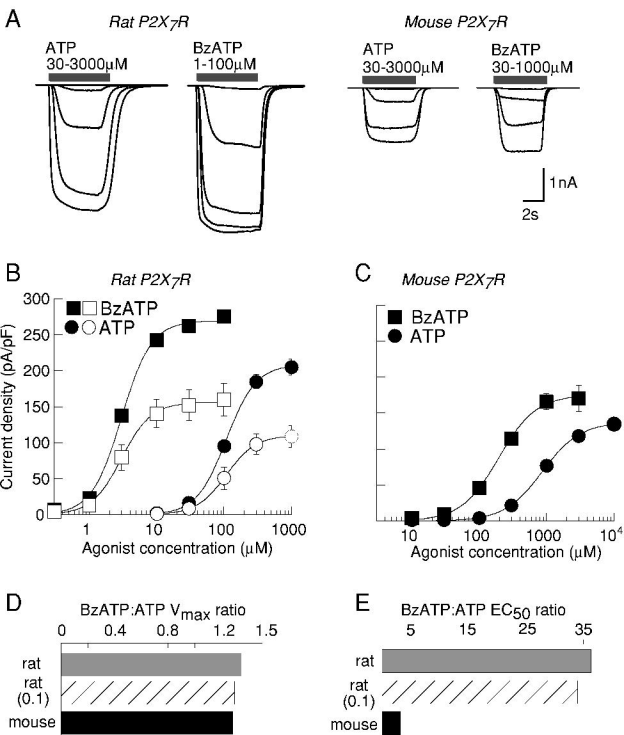


Figure 3

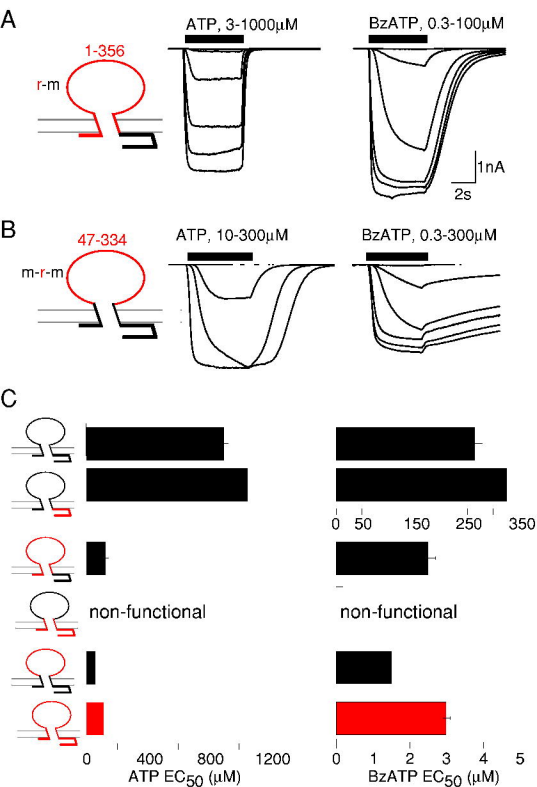




Figure 4

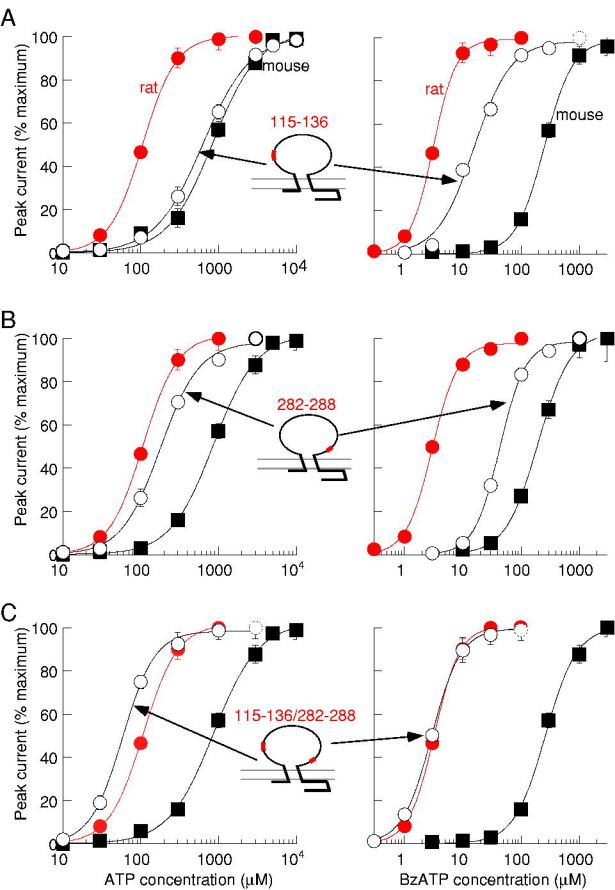


Figure 5

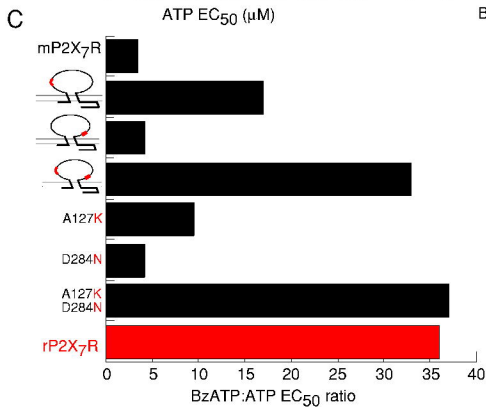
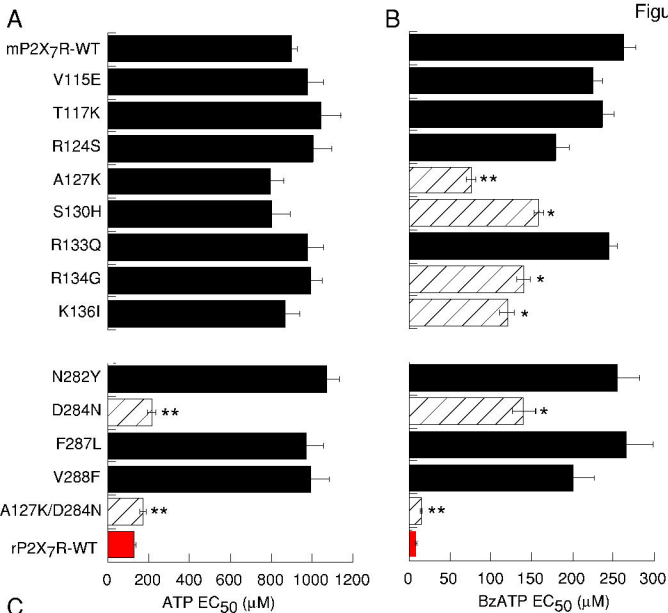


Figure 6

