0026-895X/07/7101-92–100$20.00
MOLECULAR PHARMACOLOGY
Copyright © 2007 The American Society for Pharmacology and Experimental Therapeutics
Mol Pharmacol 71:92–100, 2007

Vol. 71, No. 1
30163/3162707
Printed in U.S.A.

Amino Acid Residues in the $P2X_7$ Receptor that Mediate Differential Sensitivity to ATP and BzATP

Mark T. Young,[1] Pablo Pelegrin, and Annmarie Surprenant

Department of Biomedical Science, University of Sheffield, Sheffield, United Kingdom

Received August 26, 2006; accepted October 10, 2006

ABSTRACT

Agonist properties of the $P2X_7$ receptor ($P2X_7R$) 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 analog 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 $P2X_7R$ to delineate regions or specific residues that may be responsible for the unique actions of these agonists at the $P2X_7R$. We measured membrane currents in response to ATP and BzATP at wild-type rat and mouse $P2X_7R$, at chimeric $P2X_7Rs$, and at mouse $P2X_7Rs$ bearing point mutations. Wild-type rat $P2X_7R$ was 10 times more sensitive to ATP and 100 times more sensitive to BzATP than wild-type mouse $P2X_7R$. We found that agonist EC_{50} values were determined solely by the ectodomain of the $P2X_7R$. 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^{284}, in the rat $P2X_7R$ 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^{127} and Asn^{284} from rat to mouse. Thus, single amino acid differences between species can account for large changes in agonist effectiveness and differentiate between the two widely used agonists at $P2X_7$ receptors.

The $P2X_7$ receptors belong to a family of cation-permeable membrane proteins gated by extracellular ATP. They can be distinguished from other family members ($P2X_1$–$P2X_6$) 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 Da (North, 2002). Second, activation by extracellular ATP rapidly engages a series of cytoskeletal and mitochondrial alterations, which include actin/α-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; Morelli et al., 2003; Verhoef et al., 2003; Pfeiffer et al., 2004; Elliott et al., 2005; Ferrari et al., 2006). Third, the $P2X_7$ receptor in immune cells of monocyte/macrophage lineage becomes upregulated 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-1β, release of tumor necrosis factor α, and activation of nuclear factor-κB (North, 2002; Ferrari et al., 2006). Finally, studies using mice in which the $P2X_7$ receptor has been deleted further support a role in inflammatory processes (Solle et al., 2001; Labasi et al., 2002; Chessell et al., 2005).

$P2X_7$ 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_{50} value (>300 μM) is approximately 100-fold greater than for other P2X receptors (North, 2002). Third, the ATP analog 2′,3′-*O*-(4-benzoyl-benzoyl)ATP (BzATP) is considerably more potent that ATP itself at $P2X_7$ receptors, whereas at other P2X receptors it is less potent (North, 2002; Baraldi et al., 2004). Fourth, certain antagonists are selective for the $P2X_7$ receptor, although few have 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 $P2X_7$ receptors. The effectiveness

This work was supported by The Wellcome Trust and the Biotechnology and Biological Sciences Research Council.

M.T.Y. and P.P. contributed equally to this study.

[1] Current affiliation: Faculty of Life Sciences, University of Manchester, Manchester, United Kingdom.

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.
doi:10.1124/mol.106.030163.

ABBREVIATIONS: BzATP, 2′-3′-O-(4-benzoylbenzoyl) adenosine 5′-triphosphate; PCR, polymerase chain reaction; KN-62, 1-[*N*,*O*-bis(5-isoquinolinesulfonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine; KN-04, *N*-(1-(*p*-(5-isoquinolinesulfonyl)benzyl)-2-(4-phenylpiperazinyl)ethyl)-5-isoquinolinesulfonamide.

of BzATP relative to ATP, which often has been used as the primary distinguishing feature of the P2X$_7$ receptor, is not equally reliable among species (Surprenant et al., 1996; Chessell et al., 1998a,b; Hibell et al., 2000; Young et al., 2006). For example, isoquinolone derivatives such as KN-62 and KN-04 block human P2X$_7$ receptors with low nanomolar affinity but are without effect at rodent P2X$_7$ 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$_7$ receptors (Jiang et al., 2000).

The differences in properties between the P2X$_7$ receptors of different species are important for several reasons. First, quite different polymorphisms have been described in the human and mouse P2X$_7$ 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$_7$ 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 the use of P2X$_7$ receptor knockout mice as animal models of disease (e.g., neuropathic and inflammatory pain, Chessell et al., 2005; osteopenia, Li et al., 2005; and joint inflammation, Labasi et al., 2002) drives the need for a more precise knowledge of the properties of this receptor. In general, information concerning residues involved in agonist action at P2X receptors is extensive for P2X$_1$, P2X$_2$, and P2X$_4$ receptors but minimal for P2X$_7$ receptors (North, 2002; Vial et al., 2004; Zemkova et al., 2004).

The purpose of the present study was to identify residues in P2X$_7$ receptors that may be involved in agonist action. We recently noticed that the sensitivity to BzATP was different between rat and mouse P2X$_7$ receptors (Young et al., 2006). Of the 595 amino acids of the P2X$_7$ receptors, 88 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$_7$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$_7$ constructs (Surprenant et al., 1996; Chessell et al., 1998b) were subcloned in the same expression vector background (pcDNA3; Invitrogen, Paisley, UK) and bore C-terminal glutamic acid- glutamic acid epitope tags (EYMPME) to allow detection of protein expression by Western blotting. The 3′- and 5′-noncoding regions of the mouse P2X$_7$ construct was engineered to be identical with that of the rat P2X$_7$ construct to eliminate any differences in expression as a result of noncoding 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-nucleotide synthetic oligonucleotides designed with an in-frame 9-nucleotide 5′-adapter tail to introduce overlapping sequences to fuse chimeras between rat and mouse P2X$_7$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 HindIII and XbaI and replaced back in the HindIII-XbaI 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 (CEQ 2000 Dye Terminator; Beckman Coulter, Fullerton, CA), and protein expression was verified by Western blotting.

Human embryonic kidney 293 cells were transiently transfected using Lipofectamine 2000 (Invitrogen, Paisley, UK). Cells were plated onto 13-mm glass coverslips and maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum and 2 mM L-glutamine at 37°C in a humidified 5% CO_2 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$_7$R cDNA concentration by 10-fold (from the standard 1 to 0.1 μg/ml) resulted in approximately 50% reduction in maximum currents to ATP or BzATP without a significant change in agonist EC$_{50}$ values (Fig. 2A). Therefore, in all subsequent experiments we used the standard (1 μg/ml cDNA) concentration for all transfections.

Protein Solubilization, Deglycosylation, and Western Blotting. Confluent cells were washed with phosphate-buffered saline and pelleted. Cell pellets were lysed in phosphate-buffered saline

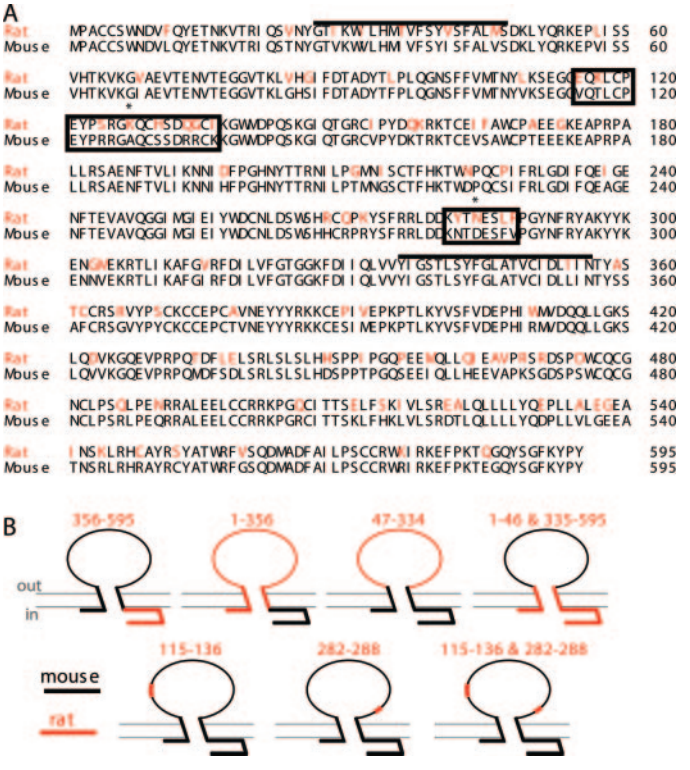

Fig. 1. Alignment of rat and mouse P2X$_7$R sequences and design of experimental approach. A, amino acid differences between species are shown in red boldface type; transmembrane domains indicated by black lines; boxed residues (115–136 and 282–288) show regions of lowest conservation in the ectodomain. B, schematic representation of chimeras where red and black represent rat and mouse P2X$_7$R sequence, respectively, and are shown in this, and all subsequent figures, as rat P2X$_7$R residues inserted into mouse P2X$_7$R background.

containing 1% Triton X-100 and antiproteases (Complete; Roche, Lewes, UK) for 1 h at 4°C followed by centrifugation at 16,000*g* for 10 min to pellet debris. Total protein samples were removed and assayed for protein content using the Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Hemel Hempstead, UK). SDS-polyacrylamide gel electrophoresis sample buffer was added, and the samples were boiled for 2 min at 100°C to denature the protein. Where appropriate, deglycosylation was performed by incubating protein samples (100 μg) for 1 h at 37°C with 500 units of PNGase F (New England Biolabs, Herts, UK) according to the manufacturer's instructions. Samples were separated on 8% polyacrylamide gels according to standard methods and transferred to polyvinylidene difluoride membranes. Western blotting was performed according to standard protocols, and proteins were visualized using anti glutamic acid- glutamic acid primary antibody (Bethyl Laboratories, Cambridge, UK) and horseradish peroxidase-conjugated secondary antibody (Dako UK Ltd., 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, Dorset, UK).

Electrophysiological Recordings. Whole-cell recordings were made 24 to 48 h after transfection using an EPC9 patch-clamp amplifier (HEKA Electronik, Lambrecht, Germany). Membrane potential was held at −60 mV. Recording pipettes (5–7 MΩ) were pulled from borosilicate glass (World Precision Instruments, Sarasota, FL) and filled with an intracellular solution that consisted of 145 mM NaCl, 10 mM EGTA, and 10 mM HEPES. The external solution contained 147 mM NaCl, 10 mM HEPES, 13 mM glucose, 2 mM KCl, 2 mM $CaCl_2$, and 1 mM $MgCl_2$. Agonists were applied in divalent-free solution; cells were otherwise superfused with normal external solution. Osmolarity and pH values of all solutions were 295 to 310 mOsM and 7.3, respectively. All experiments were performed at room temperature. Agonists were applied using a RSC 200 fast-flow delivery system (BioLogic Science Instruments, Grenoble, France). Agonists were applied for 5- or 10-s duration to obtain steady-state responses. Concentration-response curves to ATP and BzATP were obtained by first obtaining a maximum response to agonist, because marked run-up of response was observed at both rat and mouse $P2X_7$ 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 (Abelbeck/Synergy Software, Reading, PA) and Prism version 3.0a software (GraphPad Software Inc., San Diego, CA) using the Hill equation provided in Prism.

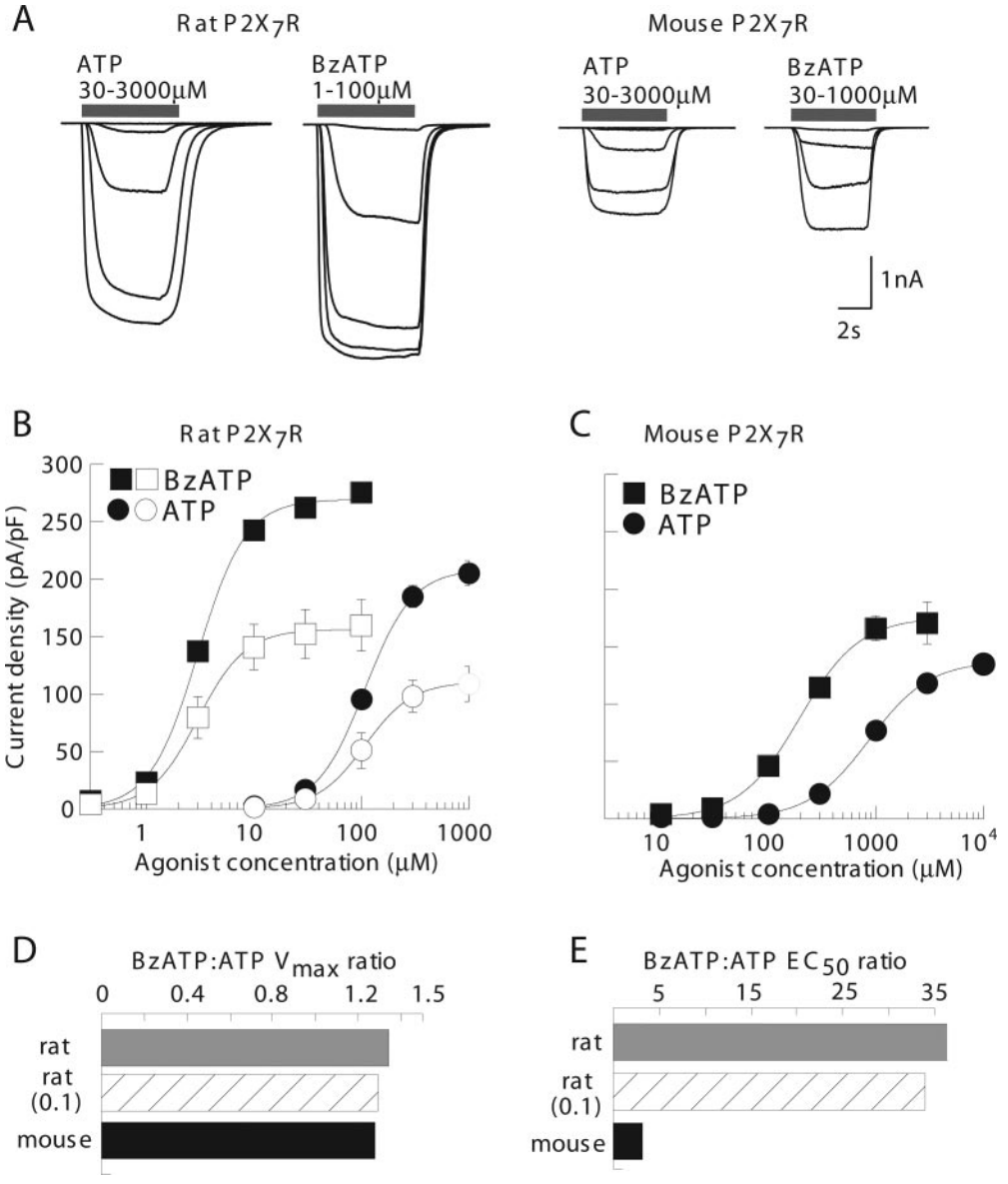

Fig. 2. Comparison of agonist actions at wild-type rat and mouse $P2X_7R$. A, representative currents recorded from cells expressing rat or mouse $P2X_7R$ in response to increasing concentrations of ATP or BzATP as indicated. B, summary of all experiments as illustrated in A for wild-type rat $P2X_7R$ 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 wild-type mouse $P2X_7R$ (1 μg/ml cDNA transfected). D, mean ratio of maximum current amplitudes to BzATP relative to ATP shown for rat and mouse $P2X_7R$; these were similar for both species and for low and high expression levels. E, mean ratio of EC_{50} values of BzATP relative to ATP; low or high expression of rat $P2X_7R$ showed the same approximate 35-fold difference, whereas mouse $P2X_7R$ showed only 4-fold difference.

Results

Comparison of Mouse and Rat $P2X_7$ Receptors. Although all $P2X_7$Rs are potently inhibited by extracellular divalent cations, there is a significant species difference with Mg^{2+} and Ca^{2+} being approximately 10-fold more potent to inhibit human than rat $P2X_7$Rs (Surprenant et al., 1996; Rassendren et al., 1997). In preliminary experiments, we found that mouse $P2X_7$R was also more sensitive (by approximately 5-fold) to inhibition by Mg^{2+} and Ca^{2+} than was rat $P2X_7$R (data not shown). Therefore, all agonist responses were recorded in the divalent-free cation solution to rule out possible contributions of differential divalent cation sensitivity to agonist concentration responses. We first compared ATP and BzATP concentration-response curves from wild-type rat and mouse $P2X_7$Rs using equal amounts of cDNA for transfection and using a 10-fold lower concentration of $rP2X_7$R cDNA, which we have found previously results in similar protein expression of rat and mouse $P2X_7$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. 2, B and C. Reducing the $rP2X_7$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_{50} values or BzATP/ATP maximum current ratio or EC_{50} ratio (Fig. 2, D and E). The maximum agonist-evoked currents recorded from cells transfected with $mP2X_7$R were approximately the same as those recorded from cells transfected with the 10-fold lower $rP2X_7$R cDNA concentration (Fig. 2, B and C). In agreement with earlier studies on human and rat $P2X_7$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 to 45% greater than maximum ATP-evoked currents at both rat and mouse $P2X_7$R (Fig. 2D). EC_{50} values for BzATP and ATP at the rat $P2X_7$R (3.6 and 123 μM, respectively) were several-fold lower than at the mouse $P2X_7$R (285 and 936 μM) (Table 1). These values yield a striking difference in the BzATP/ATP EC_{50} ratio, which was 34 at the $rP2X_7$R but only 3.3 at the mouse $P2X_7$R (Fig. 2E).

Introducing Segments of Rat $P2X_7$R into Mouse $P2X_7$R. Rat and mouse $P2X_7$R sequences are 84% identical with 88 specific amino acid differences; most of the nonconservative differences are found in two distinct regions of the ectodomain or in the intracellular C-terminal domain (Fig. 1A). Of the 22 nonconserved amino acids in the ectodomain, 8 are found in the region encompassing residues 115 to 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 to examine the effects of transposing rat ectodomain, C terminus, and residues 115 to 136 and 282 to 288 (alone and in combination) into the mouse $P2X_7$R on agonist-evoked responses.

Neither agonist concentration-response curves nor agonist-evoked kinetics was altered by transposing rat intracellular C terminus onto mouse $P2X_7$R or vice versa (Fig. 3, A and C). Transposition of rat ectodomain onto mouse $P2X_7$R resulted in ATP and BzATP EC_{50} values that were the same as for wild-type rat $P2X_7$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 $P2X_7$R. The chimeric mouse $P2X_7$R containing residues 115 to 136 of rat $P2X_7$R resulted in a pronounced leftward shift of the BzATP concentration-response curve without any alteration in the ATP-evoked responses (Fig. 4A and Table 1). When residues 282 to 288 of rat $P2X_7$R were inserted into mouse $P2X_7$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 and Table 1). Substitution of both regions of rat $P2X_7$R into mouse $P2X_7$R resulted in agonist-evoked concentration-response curves and EC_{50} values that were not significantly different from wild-type rat $P2X_7$R (Fig. 4C and Table 1).

Introducing Single Amino Acids of Rat $P2X_7$R into Mouse $P2X_7$R. We next substituted individually each rat $P2X_7$R residue in these regions into the mouse receptor and examined agonist responses in these mutant receptors. ATP-evoked responses were not different from wild-type mouse $P2X_7$R for any mutation except at residue 284, at which substitution of asparagine (rat $P2X_7$R) for aspartate (mouse $P2X_7$R) resulted in ATP concentration response and EC_{50} value that was not significantly different from wild-type rat $P2X_7$R (Fig. 5A and Table 1). In contrast, the sensitivity of the mouse receptor to BzATP was clearly increased over wild-type 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 $P2X_7$[A127K], in which case the EC_{50} value for BzATP (80 μM) was intermediate between that for the rat (3 μM) and mouse (285 μM) receptor (Fig. 5B and Table 1).

TABLE 1
EC_{50} values for key $P2X_7$ constructs used in this study

Construct	ATP EC_{50}	BzATP EC_{50}
	μM	
Rat $P2X_7$	123 ± 4 (21)	3.6 ± 0.2 (19)
Mouse $P2X_7$	936 ± 21 (18)	285 ± 16 (18)
Mouse with rat $P2X_7$[1–356]	125 ± 10 (5)	2.2 ± 0.2 (5)
Mouse with rat $P2X_7$[356–595]	1251 ± 202 (5)	306 ± 24 (3)
Mouse with rat $P2X_7$[47–334]	58 ± 0.8 (5)	1.4 ± 0.1 (5)
Mouse with rat $P2X_7$[115–136]	634 ± 27 (5)	33 ± 2.4 (5)
Mouse $P2X_7$[A127K]	815 ± 56 (10)	80 ± 7 (9)
Mouse with rat $P2X_7$[282–288]	162 ± 11 (9)	89 ± 4 (9)
Mouse $P2X_7$[D284N]	146 ± 11 (8)	94 ± 7 (10)
Mouse with rat $P2X_7$[115–136/282–288]	74 ± 5 (8)	2.9 ± 0.1 (9)
Mouse $P2X_7$[A127K/D284N]	112 ± 11 (5)	5 ± 1 (6)

Numbers in parentheses indicate total number of experiments.

The double substitution fully converted the mouse receptor ($P2X_7$[A128K,D284N]) to the sensitivity of the rat receptor with respect to both ATP and BzATP EC_{50} (Fig. 5A and Table 1) and was the only construct BzATP/ATP potency ratio was the same as that for wild-type rat $P2X_7R$ (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 wild-type receptors. Maximum BzATP-evoked currents were approximately 30% greater than maximum ATP-evoked currents at both mouse and rat $P2X_7R$ (Fig. 2D), but in the mouse $P2X_7$[A128K,D284N] receptor, the ATP-evoked currents were 36 ± 5% (n = 5) greater than the maximal currents evoked by BzATP.

Involvement of *N*-Glycosylation. The substitution of Asp^{284} with asparagine at the mouse $P2X_7R$ generates a potential *N*-glycosylation acceptor sequence. The wild-type rat $P2X_7R$ 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, whereas removing *N*-glycosylation sites decreases, protein expression (Newbolt et al., 1998; Torres et al., 1998; Rettinger et al., 2000; Chaumont et al., 2004), we assayed protein expression in wild-type, chimeric, and $mP2X_7D284N$ receptor and asked whether this site was, indeed, glycosylated. As found previously (Young et al., 2006), transfection with equal concentrations of rat and mouse $P2X_7R$ cDNA yielded protein level ratios of approximately 3:1 (Fig. 6A). The presence of rat $P2X_7R$ ectodomain, but not N terminus, C terminus, or transmembrane domains, yielded protein levels not significantly different from wild-type rat $P2X_7R$, whereas the presence of mouse ectodomain yielded low protein expression equivalent to wild-type mouse $P2X_7R$ (Fig. 6A). We then removed *N*-glycan chains from wild-type mouse and rat $P2X_7R$ and mouse $P2X_7$-D284N receptor using PNGase F and examined molecular mass by SDS-polyacrylamide gel electrophoresis. Wild-type rat $P2X_7R$, mouse $P2X_7$-D284N receptor, and mouse $P2X_7R$ bands were detected at 78, 78, 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^{284} being glycosylated in the wild-type rat receptor and the mouse $P2X_7$[D284N] receptor.

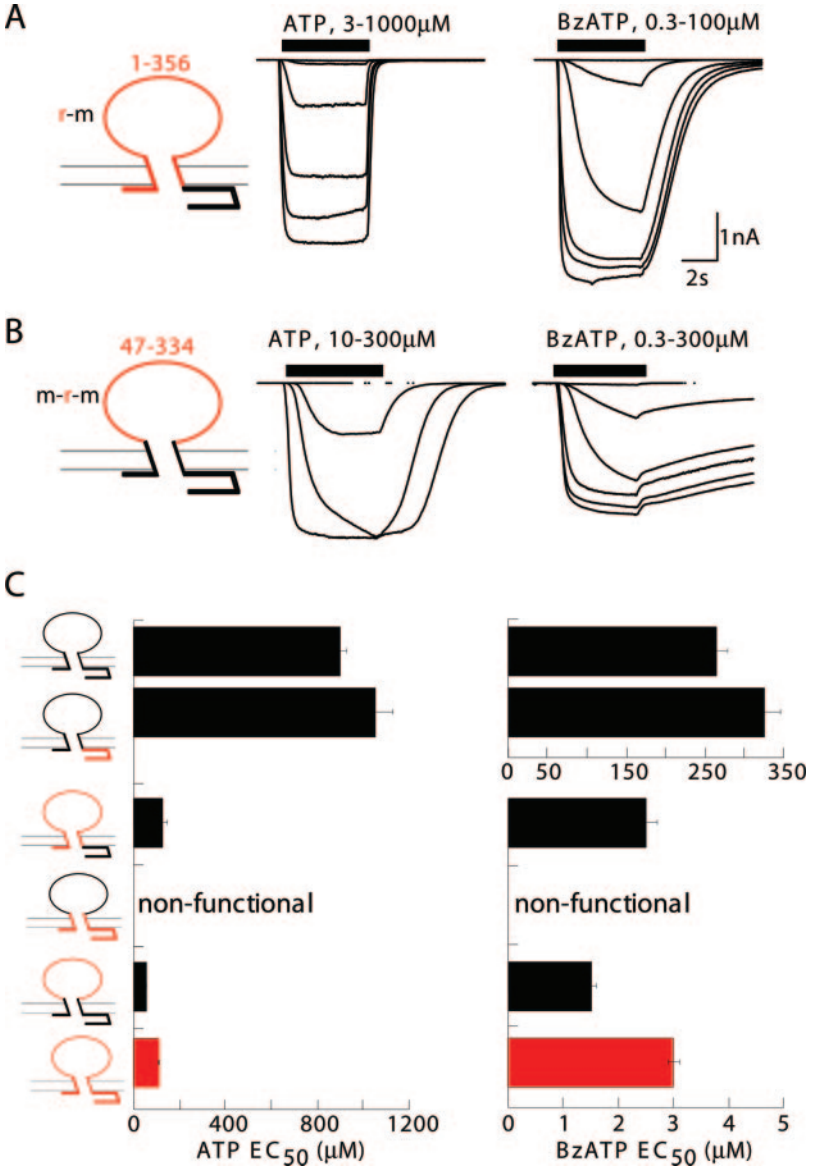

Fig. 3. The ectodomain is solely responsible for agonist potency at $P2X_7R$. A and B, examples of currents recorded from rat $P2X_7R$ containing mouse C terminus (A) and from mouse $P2X_7R$ containing rat ectodomain (B). Note the prolonged deactivation kinetics upon removal of BzATP at the mouse $P2X_7R$ containing rat ectodomain (B); no other chimeric or mutant receptor showed altered kinetics of onset or offset. C, summary of EC_{50} values for ATP and BzATP obtained from chimeric receptors; note difference in x-axis scale for BzATP to better depict the 100-fold difference between rat and mouse receptors.

Discussion

The sequence relatedness between the mouse and rat P2X$_7$ receptors (84.9%) is substantially less than for the other P2X receptors (for 1 through 6, 97.5, 97.2, 99.2, 94.7, 94.5, and 92.6% identity, respectively). 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) (Fig. 1A, boxes). Unlike other P2X receptors, in which 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$_7$ receptor. We then identified two amino acids that 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$_7$ receptor.

The first main finding of the present study is that, of all the differences between rat and mouse P2X$_7$ 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$_7$R changed the EC_{50} value from 936 to 146 μM, which is close to the value for the wild-type rat receptor (123 μM, Table 1). The change in sensitivity associated with the aspartate-to-asparagine substitution (from -O^- to -NH_2) is striking. The asparagine is situated within a sequence commonly found at sites of N-linked glycosylation (N-X-S; NESL in rat P2X$_7$; *N*-glycosylation acceptor sequence in mouse P2X$_7$[D284N]). We found evidence that the mutated mouse P2X$_7$[D284N] receptor was glycosylated at this position, because the molecular mass was approximately 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^{74}, Asn^{100}, Asn^{106}, Asn^{187}, Asn^{241}), whereas only rat P2X$_7$R carries a sixth (Asn^{284}) glycosylation site. Thus, the wild-type rat P2X$_7$R

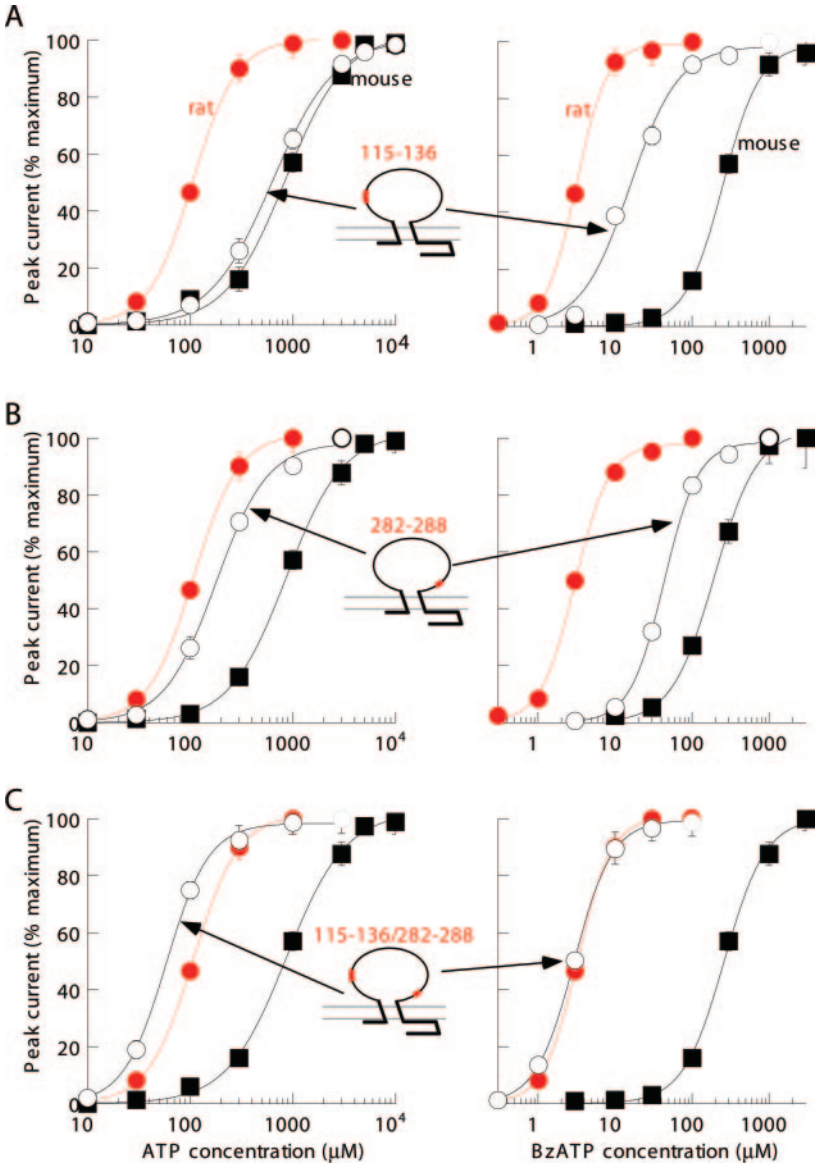

Fig. 4. Small subregions in the ectodomain account for differences between mouse and rat sensitivity to ATP and BzATP. A to C, concentration-response curves for ATP (left graphs) and BzATP (right graphs) from wild-type rat P2X$_7$R (red-filled circles), mouse P2X$_7$R (■), and chimeric receptors (○). A, when residues 115 to 136 from rat P2X$_7$R were inserted into mouse P2X$_7$R, no change in ATP-evoked responses occurred, whereas BzATP response showed a 10-fold leftward shift. B, insertion of rat P2X$_7$R residues 282 to 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$_7$R into mouse P2X$_7$R resulted in ATP concentration response that was slightly shifted to the left of wild-type rat P2X$_7$R and a BzATP response that was not different from wild-type rat P2X$_7$R.

and the mouse $P2X_7$[D284N] mutated receptor each carry the same *N*-linked glycosylation sites. In both cases, treatment with PNGase F reduced the molecular mass to approximately 65 kDa, which is similar to the calculated molecular mass (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 $P2X_7$ receptor *with* the attached sugar at this position (mouse $P2X_7$[D284N] or rat $P2X_7$) that is more sensitive to ATP than the form without it (mouse $P2X_7$). Rettinger et al. (2000) have found previously that, in the case of the $P2X_1$ receptor, glycosylation affects the potency of ATP. Although the difference in potency was small at the $P2X_1$ receptor (approximately 3-fold), it was also the form of the receptor without attached sugar ($P2X_1$[N210Q]) that was less sensitive. In the $P2X_1$ receptor, the asparagine was situated close to the center of the ectodomain (Asn^{210} of rat $P2X_1$;

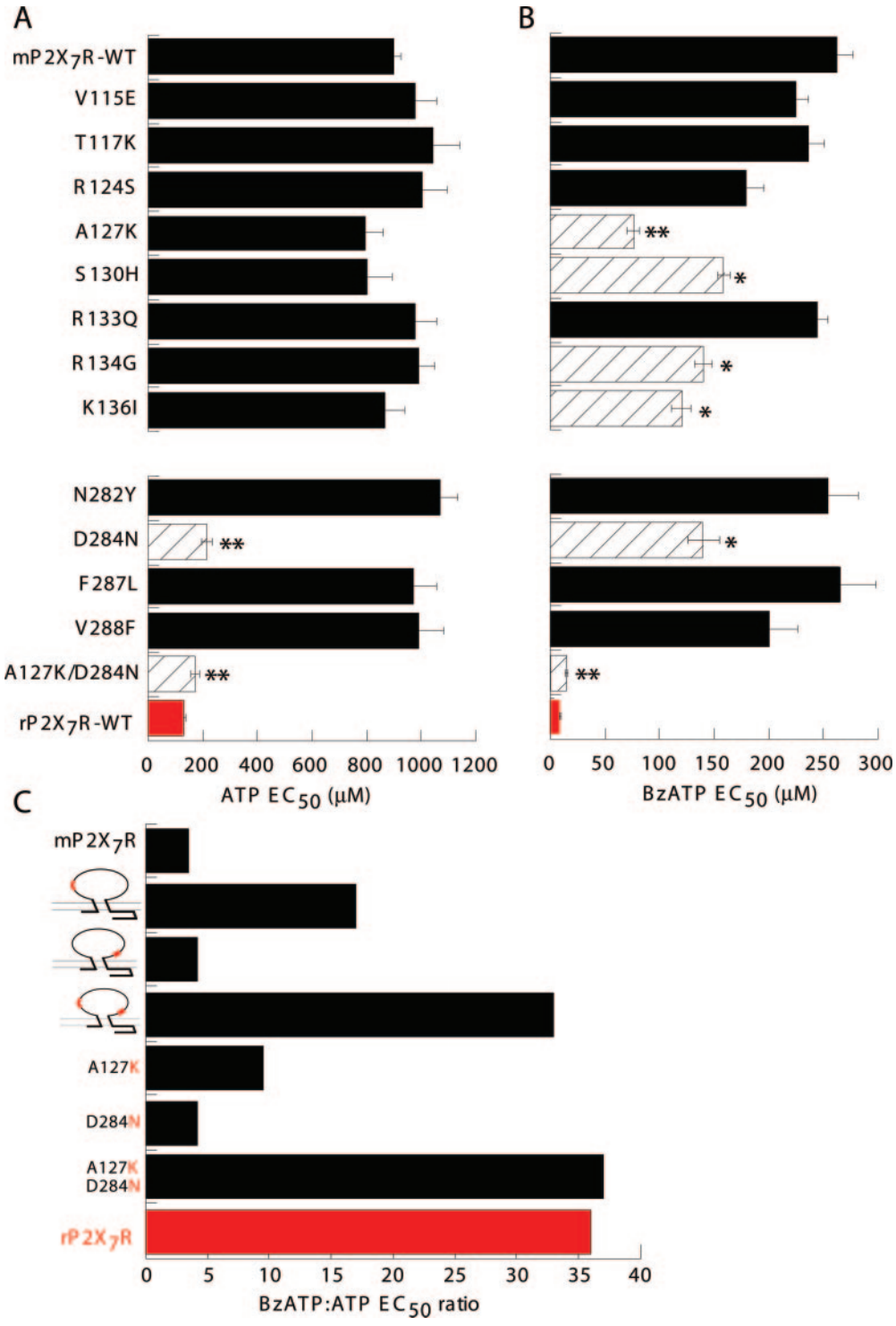

Fig. 5. Asparagine^{284} solely governs ATP potency differential whereas lysine^{127} with Asp^{284} largely governs BzATP potency differential at $P2X_7R$. Histograms of ATP (A) and BzATP (B) EC_{50} values obtained from mouse $P2X_7R$ carrying point mutations in the regions between 115 to 136 and 282 to 288. In each case, the residue substituted was that found in wild-type rat $P2X_7R$. Stippled bars indicate values significantly different from wild-type $mP2X_7R$. A, ATP EC_{50} value at $mP2X_7$[D284N] was shifted 10-fold to the left of wild-type $mP2X_7R$ and was not significantly different from wild-type rat $P2X_7R$, whereas the BzATP EC_{50} value 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_{50} value significantly to the left, with A127K mutation showing the largest effect. The mouse $P2X_7$[A127K/D284N] double mutation yielded agonist EC_{50} values that were not significantly different from wild-type rat $P2X_7R$. C, histograms of BzATP/ATP EC_{50} ratios for chimeric and mutant receptors as indicated. Ratios were similar to wild-type rat $P2X_7R$ only when both A127K and D284N mutations were present.

Roberts and 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$_7$ 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$_7$R). The residue Asp^{284} also had an effect on the BzATP sensitivity because substitution of aspartic acid for asparagine at this position did increase the potency of BzATP, but by only approximately 3-fold (Table 1). A much greater further increase of approximately 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 π electron cloud on one or another 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$_1$ receptors (chick versus human: Soto et al., 2003), P2X$_4$ receptors (human versus rat: Buell et al., 1996; Soto et al., 1996), and for P2X$_7$ receptors (human versus rat: Humphreys et al., 1998; Jiang et al., 2000; Baraldi et al., 2004; and mammalian versus nonmammalian: Lopez-Castejon et al., 2007). 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$_7$ receptors, in which BzATP is in very widespread use as an experimental agonist.

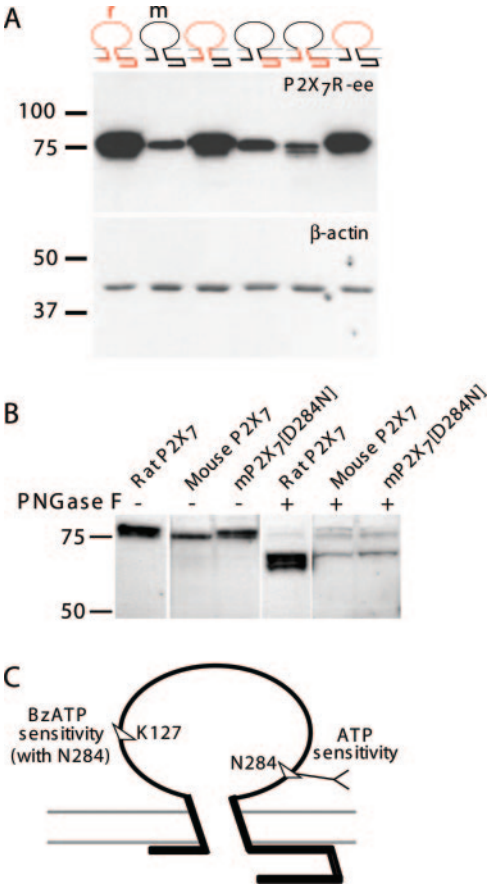

Fig. 6. Protein expression and *N*-linked glycosylation at mouse and rat P2X$_7$Rs. A, total protein expression of constructs as illustrated. Equal protein (10 μg) was loaded per lane and confirmed by comparing β-actin levels. P2X$_7$R protein was detected using the anti-glutamic acid-glutamic acid epitope tag antibody. The ectodomain, but not transmembrane or intracellular domains, determined levels of P2X$_7$R protein expression. B, Western blot of native and deglycosylated receptors. Both rat P2X$_7$R and mouse P2X$_7$R-D284N exhibited higher molecular mass bands (78 kDa) than mouse P2X$_7$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$_7$Rs.

Acknowledgments

We thank E. Martin and L. Collinson for cell and molecular biology technical support.

References

Adriouch S, Dox C, Welge V, Seman M, Koch-Nolte F, and Haag F (2002) Cutting edge: a natural P451L mutation in the cytoplasmic domain impairs the function of the mouse P2X$_7$ receptor. *J Immunol* **169:**4108–4112.
Baraldi PG, Di Virgilio F, and Romagnoli R (2004) Agonists and antagonists acting at P2X$_7$ 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 (Eur Mol Biol Organ) 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:**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$_7$ receptor of human leukemic lymphocytes. *J Immunol* **175:**82–89.
Chessell IP, Hatcher JP, Bountra C, Michel AD, Hughes JP, Green P, Egerton J, Murfin M, Richardson J, Peck WL, et al. (2005) Disruption of the P2X$_7$ purinoceptor gene abolishes chronic inflammatory and neuropathic pain. *Pain* **114:**386–396.
Chessell IP, Michel AD, and Humphrey PP (1998a) Effects of antagonists at the human recombinant P2X$_7$ receptor. *Br J Pharmacol* **124:**1314–1320.
Chessell IP, Simon J, Hibell AD, Michel AD, Barnard EA, and Humphrey PP (1998b) Cloning and functional characterisation of the mouse P2X$_7$ receptor. *FEBS Lett* **439:**26–30.
Elliott JI, Surprenant A, Marelli-Berg FM, Cooper JC, Cassady-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:**808–816.
Ferrari D, Pizzirani C, Adinolfi E, Lemoli RM, Curti A, Idzko M, Panther E, and Di Virgilio F (2006) The P2X$_7$ 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$_7$ receptor. *J Biol Chem* **279:**31287–31295.
Guerra AN, Fisette PL, Pfeiffer ZA, Quinchia-Rios BH, Prabhu U, Aga M, Denlinger LC, Guadarrama AG, Abozeid S, Sommer JA, et al. (2003) Purinergic receptor regulation of LPS-induced signaling and pathophysiology. *J Endotoxin Res* **9:**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:**32793–32798.
Hibell AD, Kidd EJ, Chessell IP, Humphrey PP, and Michel AD (2000) Apparent

species differences in the kinetic properties of $P2X_7$ receptors. *Br J Pharmacol* **130:**167–173.
Humphreys BD, Virginio C, Surprenant A, Rice J, and Dubyak G (1998) Isoquinolines as antagonists of the $P2X_7$ 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 $P2X_7$ receptors. *Mol Pharmacol* **58:**82–88.
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 $P2X_7$ receptor alters leukocyte function and attenuates an inflammatory response. *J Immunol* **168:** 6436–6445.
Le Feuvre RA, Brough D, Iwakura Y, Takeda K, and Rothwell NJ (2002) Priming of macrophages with lipopolysaccharide potentiates $P2X_7$-mediated cell death via a caspase-1-dependent mechanism, independently of cytokine production. *J Biol Chem* **277:**3210–3218.
Li J, Liu D, Ke HZ, Duncan RL, and Turner CH (2005) The $P2X_7$ nucleotide receptor mediates skeletal mechanotransduction. *J Biol Chem* **280:**42952–42959.
Lopez-Castejon G, Young MT, Meseguer J, Surprenant A, and Mulero V (2007) Characterization of ATP-gated $P2X_7$ receptors in fish provides new insights into the mechanism of release of the leaderless cytokine interleukin 1β. *Mol Immunol* **44:**1286–1299.
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:**825–835.
Mackenzie AB, Young MT, Adinolfi E, and Surprenant A (2005) Pseudoapoptosis induced by brief activation of ATP-gated $P2X_7$ receptors. *J Biol Chem* **280:**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 $P2X_7$ 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:**1013–1067.
Pfeiffer ZA, Aga M, Prabhu U, Watters JJ, Hall DJ, and Bertics PJ (2004) The nucleotide receptor $P2X_7$ mediates actin reorganization and membrane blebbing in RAW 264.7 macrophages via p38 MAP kinase and Rho. *J Leukoc Biol* **75:**1173–1182.
Rassendren F, Buell GB, Virginio C, Collo G, North RA, and Surprenant A (1997) The permeabilizing ATP receptor, $P2X_7$, 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 $P2X_1$ receptor. *J Biol Chem* **275:**33542–33547.
Roberts JA and Evans RJ (2004) ATP binding at human $P2X_1$ receptors. Contribution of aromatic and basic amino acids revealed using mutagenesis and partial agonists. *J Biol Chem* **279:**9043–9055.
Shemon AN, Sluyter R, Fernando SL, Clarke AL, Dao-Ung LP, Skarratt KK, Saunders BM, Tan KS, Gu BJ, Fuller SJ, et al. (2005) A Thr^{357} to Ser polymorphism in homozygous and compound heterozygous subjects causes absent or reduced $P2X_7$ 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:**125–132.
Soto F, Garcia-Guzman M, Gomez-Hernandez JM, Hollmann M, Karschin C, and Stuhmer W (1996) $P2X_4$: an ATP-activated ionotropic receptor cloned from rat brain. *Proc Natl Acad Sci USA* **93:**3684–3688.
Soto F, Krause U, Borchardt K, and Ruppelt A (2003) Cloning, tissue distribution and functional characterization of the chicken $P2X_1$ 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 (P2X7). *Science (Wash DC)* **272:**735–738.
Torres GE, Egan TM, and Voigt MM (1998) N-Linked glycosylation is essential for the functional expression of the recombinant $P2X_2$ receptor. *Biochemistry* **37:** 14845–14851.
Verhoef PA, Estacion M, Schilling W, and Dubyak GR (2003) $P2X_7$ receptor-dependent blebbing and the activation of Rho-effector kinases, caspases, and IL-1 beta release. *J Immunol* **170:**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/P2X7 channel. *Am J Physiol* **275:**C1224–C1231.
Young MT, Pelegrin P, and Surprenant A (2006) Identification of Thr^{283} as a key determinant of $P2X_7$ receptor function. *Br J Pharmacol* **149:**261–268.
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:**6968–6978.

Address correspondence to: Dr. Annmarie Surprenant, Department of Biomedical Science, Addison Building Western Bank, University of Sheffield, Sheffield S10 2TN, UK. E-mail: a.surprenant@sheffield.ac.uk