

**The penultimate arginine of the carboxy terminus
determines slow desensitization in a P2X receptor from the
cattle tick *Boophilus microplus***

Selvan Bavan, Louise Farmer, Shire K. Singh, Volko A. Straub, Felix D. Guerrero
and Steven J. Ennion

Department of Cell Physiology and Pharmacology, University of Leicester, Leicester LE1 9HN, UK.
(S.B., L.F., S.K.S., V.A.S., S.J.E); USDA-ARS, Knipling Bushland US Livestock Insect Research
Laboratory, 2700 Fredericksburg Road, Kerrville, TX 78028, USA. (F.D.G.)

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Corresponding author: Dr Steven J. Ennion
Department of Cell Physiology and Pharmacology,
University of Leicester,
Leicester,
United Kingdom
LE19HN.
Tel: 0116 229 7134.
FAX: 0116-252-5045.
Email: se15@le.ac.uk

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Abbreviations:

EC₅₀, Concentration of agonist evoking 50% of the maximum response.

pEC₅₀, -log₁₀ of the EC₅₀ value.

I₂₀, Percent peak current amplitude after 20 seconds of ATP application.

T.10%, Time taken for peak current to decay by 10%.

SNP, Single nucleotide polymorphism.

ABSTRACT

P2X ion channels have been functionally characterized from a range of eukaryotes. Whilst these receptors can be broadly classified into fast and slow desensitizing, the molecular mechanisms underlying current desensitization are not fully understood. Here we describe the characterization of a P2X channel from the cattle tick *Boophilus microplus* (*BmP2X*) displaying extremely slow current kinetics, little desensitization during ATP application and marked run-down in current amplitude between sequential responses. ATP (EC_{50} 67.1 μ M) evoked concentration dependent currents at *BmP2X* which were antagonized by suramin (IC_{50} 4.8 μ M) and potentiated by the antiparasitic drug amitraz. Ivermectin did not potentiate *BmP2X* currents but the mutation M362L conferred ivermectin sensitivity. To investigate the mechanisms underlying slow desensitization we generated intracellular domain chimeras between *BmP2X* and the rapidly desensitizing *HdP2X* receptor from *Hypsibius dujardini*. Exchange of N- or C-termini between these fast and slow desensitizing receptors altered the rate of current desensitization towards that of the donor channel. Truncation of the *BmP2X* C-terminus identified the penultimate residue (R413) as important for slow desensitization. Removal of positive charge at this position in the mutant R413A resulted in significantly faster desensitization which was further accentuated by the negatively charged substitution R413D. R413A and R413D however still displayed current run-down to sequential ATP application. Mutation to a positive charge (R413K) reconstituted the wild-type phenotype. This study identifies a new determinant of P2X desensitization where positive charge at the end of the C-terminal regulates current flow and further demonstrates that run-down and desensitization are governed by distinct mechanisms.

INTRODUCTION

P2X receptors are extracellular ATP gated ion channels that facilitate the ionotropic component of purinergic signaling (Surprenant and North, 2009). These channels form as homo or heteromeric trimers (Barrera et al., 2005; Nicke et al., 1998) with each monomer consisting of intracellular amino and carboxy termini, two transmembrane domains and a large extracellular region containing five disulfide bonds (Clyne et al., 2002; Ennion and Evans, 2002a) and the agonist binding site (Roberts et al., 2006). The recent determination of the crystal structure of zebrafish P2X4 at 3.1-Å resolution (Kawate et al., 2009) has allowed previous biochemical and mutagenic studies to be interpreted in a structural context (Browne et al., 2010; Young, 2009) and has provided the basis for the commencement of a rudimentary understanding of molecular mechanisms governing aspects of channel function such as ion permeation, channel gating, and agonist/antagonist binding. The zP2X4 structural model however does not include the intracellular N- and C-terminal domains as these were largely removed to aid crystallization (Kawate et al., 2009). It is clear from previous studies that these domains play important roles in regulating P2X channel function, particularly desensitization which manifests itself in two distinct forms. The first, often referred to as “run-down”, is a decline in current amplitude in sequential responses to repeated applications of ATP and has been observed in P2X1,3, 4 and 7 receptors. The second is a decline in current during the continued presence of agonist and is an integral part of the “time course” of an individual response. To avoid confusion, the former will subsequently be referred to as run-down and the latter simply as desensitization. Desensitization of ATP evoked currents in P2X receptors varies between different subtypes and receptors can be broadly classified into those that desensitize very rapidly during the continued presence of agonist, such as P2X1, P2X3 and *HdP2X* (Bavan et al., 2009), those that display moderate (several seconds) rates of desensitization, including P2X4, P2X5 and most other known invertebrate P2X channels, and those that desensitize slowly such as mammalian P2X2 and P2X7. The molecular mechanisms controlling desensitization are poorly understood and likely multifactorial

involving movement of intracellular, transmembrane and extracellular domains and possibly interactions with other proteins or intracellular messengers (Roberts et al., 2006; Stojilkovic et al., 2005).

Involvement of the transmembrane domains in P2X receptor desensitization was first demonstrated by chimeric P2X1-2 receptors (Werner et al., 1996). Mutation of extracellular domain residues can also profoundly alter current kinetics, for example, mutation of human P2X1 K68, a key residue in the ATP binding site, significantly slows the rate of desensitization (Ennion et al., 2000). Furthermore, chimeric P2X2-3 channels swapping parts of the extracellular domain indicate that the N-terminal half of this domain influences the stability of the desensitized conformation state (Zemkova et al., 2004). The intracellular N-terminal domain also plays a prominent role in regulating desensitization. This region contains a consensus protein kinase C site which is conserved in all known P2X receptors from *Dictyostelium* through to human. Disruption of this site by mutagenesis leads to an increased rate of desensitization in P2X2 (Boue-Grabot et al., 2000) and the already fast desensitizing P2X1 receptor (Ennion and Evans, 2002b). A desensitizing P2X2 splice variant (P2X2b) lacking 69 C-terminal amino acids first demonstrated that the intracellular C-terminal domain can also regulate desensitization (Brandle et al., 1997; Simon et al., 1997) and subsequent mutagenic studies narrowed the functional motif involved to a 6 amino acid region located near the second transmembrane domain (Koshimizu et al., 1998). The juxtamembrane C-terminus region of P2X4 has also been shown to play an important role in desensitization with an aromatic moiety at position 374 and an amino rather than a guanidino group at position 373 being essential for prolonged P2X4 currents (Fountain and North, 2006). Furthermore, exchange of the C-terminal domains in P2X2 and P2X3 receptors results in faster desensitization in P2X2 and slower desensitization in P2X3 (Paukert et al., 2001).

In this current study, we describe a novel invertebrate P2X receptor (*BmP2X*) cloned from the cattle tick *Boophilus microplus*. This receptor displays extremely slow desensitization properties which we utilize in chimeric and mutagenic studies to identify a new example by which the C-terminal domain can govern P2X receptor desensitization during ATP application by virtue of positive charge at the penultimate position. Our results also further indicate that desensitization during the continued presence of

agonist and run-down of currents between sequential applications are distinct processes regulated by different mechanisms.

MATERIALS AND METHODS

Identification and cloning of a *Boophilus microplus* P2X receptor. BLAST searches of the GenBank EST database identified partial 5' (accession: CK189412) and 3' (accession: CK189413) sequences from an EST clone (BEACR91) (Guerrero et al., 2005) which showed homology to the vertebrate P2X receptor family. The insert of this clone was sequenced and found to contain an open reading frame of 1242bp. This coding sequence was subsequently subcloned by PCR (Table 1 primer pair 1) into a pcDNA3 based *Xenopus* oocyte expression vector (Agboh et al., 2004) so as to introduce a mammalian Kozak sequence around the start codon. The inclusion of this sequence did not alter the coding sequence of the original clone and has previously been found to aid the expression of non-vertebrate P2X receptors in *Xenopus* oocytes (Agboh et al., 2004). The cloned insert was fully sequenced on both strands using vector and insert specific primers (Automated ABI sequencing service, University of Leicester, U.K.).

Site directed mutagenesis. Point mutations in the *Bmp2X* plasmid were introduced using the QuikChange™ Mutagenesis Kit (Stratagene, U.S.A.) according to the manufacturer's instructions. Methionine at position 362 was mutated to leucine, tyrosine 411 was mutated to alanine and arginine 413 was mutated to lysine, aspartic acid and alanine. A series of truncation mutants were also generated by introducing a premature stop codon (denoted as Δ) at positions 388, 394, 400, 408, 410, 412, 413 and 414. Introduction of the correct mutation and the absence of spontaneous mutations was confirmed by DNA sequencing on both strands.

Generation of *Bmp2X-Hdp2X* chimeras. Six chimeric constructs were generated in which one or both of the amino and carboxy intracellular domains of the slow desensitizing *Bmp2X* receptor and the fast desensitizing *Hdp2X* receptor were interchanged (Fig. 3). The positions of transmembrane domains TM1 and TM2 in *Hdp2X* and *Bmp2X* were predicted using TMPred (Hofmann and Stoffel, 1993) and TopPredII (Claros and von Heijne, 1994) programs. Since the two prediction programs varied slightly in assignment of start and end positions for TM domains, the splice residue for cloning was chosen slightly away from the outermost prediction so as to avoid the possibility of disrupting transmembrane sequence.

The N-terminal regions interchanged consisted of residues 1-28 and 1-40 for *BmP2X* and *HdP2X* respectively whereas the C-terminal regions consisted of residues 378-414 for *BmP2X* and 371-480 for *HdP2X*. Intracellular domains were spliced to residue 29 (N-terminus) and/or residue 377 (C-terminus) in *BmP2X* and residues 41 and 370 in *HdP2X*. Chimeras were generated using the technique of seamless cloning. PCR primer pairs (Table 1), one of which incorporated an *EarI* restriction site, an enzyme which cuts outside of its recognition sequence, were designed to allow amplification of the two component parts of the chimera which were subsequently ligated together after *EarI* digestion to produce a seamless join at the desired position. Both wild-type *BmP2X* and *HdP2X* sequences contain a single internal *EarI* site and this was disrupted by site directed mutagenesis so as to leave the coding sequence unchanged prior to commencing chimera generation. For chimeras A and C the acceptor wild-type P2X plasmid was first used in a PCR reaction to amplify the sequence from the N-terminus to the end of TM2. The reverse primer incorporates an *EarI* restriction site so that the overhang remaining on the bottom strand after *EarI* digestion of the PCR product corresponds to the inverse complement of the codon at the desired splice position. The C-terminal region from the donor P2X plasmid was amplified in a separate PCR reaction where the forward primer incorporated an *EarI* site that would leave a top strand overhang corresponding to the codon of the splice position. The two *EarI* digested PCR products were subsequently ligated together and blunt end cloned into expression vector. A similar strategy in reverse was used to generate N-terminal exchange chimeras B and D. Chimeras containing a double intracellular domain exchange (E and F) were generated using chimeras B and D respectively as the acceptor sequence. PCR reactions consisted of 10ng plasmid template, 12.5 pmoles each primer, 0.25mM nucleotides, 1x HF buffer (Stratagene) and 1.25 units *PfuUltra*TM DNA polymerase (Stratagene). Thermal cycling consisted of 95 °C for 2 minutes followed by 20 cycles of 95°C for 30 s, 55 °C for 30 sec and 72°C for 1 min 10 sec. All chimeric constructs were verified by sequencing on both strands.

Electrophysiological recordings. Sense strand cRNA was generated from linearized P2X plasmids using a T7 mMessage mMachineTM kit (Ambion, U.S.A.). Manually defolliculated stage V *Xenopus* oocytes were injected with 50ng cRNA and stored at 18°C in ND96 buffer (96 mM NaCl, 2 mM

KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 5 mM sodium pyruvate, 5 mM HEPES, pH 7.6) prior to recording 3-7 days later. Two-electrode voltage clamp recordings were made from P2X expressing *Xenopus* oocytes at room temperature using an Axoclamp 900A amplifier with a Digidata 1440A data acquisition system and pClamp 10 acquisition software (Molecular Devices U.S.A.). Oocytes were clamped at -60 mV and recording solution consisted of ND96 with BaCl₂ (1.8mM) substituting CaCl₂ in order to prevent the activation of endogenous oocyte calcium activated chloride channels. Microelectrodes were filled with 3 M KCl and had a resistance of 0.2 MΩ. ATP was applied from a nearby U-tube perfusion system whereas suramin (Bayer, U.K.), ivermectin and amitraz were bath perfused and also present at the appropriate concentration in the U-tube application of ATP. In order to control for the run-down in responses displayed by *BmP2X* between sequential applications of ATP, concentration response data were normalized to a bracketing concentration of 100 μM ATP applied prior to and subsequent to the test concentration with a five minute recovery period between applications. Current properties were analyzed using Clampfit 10.2 software (Molecular Devices USA.). Desensitization in wild-type, chimeric and mutant channels was quantified using the parameters of I₂₀ (% peak current amplitude after 20 seconds of ATP application) and T.10% (time taken for peak current to decay by 10%). T.10% was chosen rather than the more conventional T.50% since wild-type and some mutant channel currents did not decay by 50% even after prolonged (up to 6 minutes tested) application of agonist. Run-down was quantified by recording peak amplitudes after sequential ATP applications with a five minute recovery period between the end of one application and the start of the next. Data are presented as mean ± S.E.M. Differences between means were tested using one way analysis of variance (Kruskal-Wallis) with Dunn's multiple comparison post test (Graphpad Prism software). Concentration response data were fitted with the equation $Y = ((X)^H \cdot M) / ((X)^H + (EC_{50})^H)$ where Y = response, X = agonist concentration, H is the Hill coefficient, M is maximum response and EC₅₀ is the concentration of agonist evoking 50% of the maximum response. pEC₅₀ is the -log₁₀ of the EC₅₀ value.

Unless otherwise stated, all chemicals, nucleotides and drugs were obtained from Sigma, U.K.

RESULTS

Sequence analysis of BmP2X. The EST clone BEACR91 was found to contain a full length open reading frame (Submitted to GenBank as Accession Number: HQ333533) encoding 414 amino acids with homology to the human P2X1-7 receptor family ranging from 30.6% (P2X7) to 43.6% identity (P2X4). Prediction of membrane topology using TMPred (Hofmann and Stoffel, 1993) and TopPredII (Claros and von Heijne, 1994) programs suggests a typical P2X topology with intracellular carboxy and amino termini, two transmembrane domains and a large extracellular loop. Typical features of vertebrate P2X receptors such as 10 extracellular cysteine residues, a consensus protein kinase C phosphorylation site in the amino terminal domain, and positive and aromatic residues thought to be involved in ATP binding are also conserved in *BmP2X*.

BmP2X is an ATP gated ion channel. ATP evoked large (~15 μ A) inward currents at recombinant *BmP2X* receptors expressed in *Xenopus* oocytes (Fig. 1A). These currents displayed unusually slow kinetics taking 4.5 ± 0.7 seconds to reach peak and 17.0 ± 2.4 seconds to decay by 10% during the continued presence of agonist (n = 24). Even after prolonged application of agonist (up to 6 minutes) currents did not decay by 50% making measurement of T.50% (time for current to decay by 50%) impractical due to changes in holding current required to clamp oocytes at -60 mV over prolonged periods of large membrane currents. Despite this low level of current desensitization during the continued presence of agonist, a marked run-down in current amplitude was observed between sequential applications of agonist with current amplitudes ~12% of their original amplitude after 8 sequential 40 second applications of 100 μ M ATP 5 minutes apart (Fig. 1C). The decline in amplitude between the 2nd and 8th applications was best fit with a single exponential ($\tau = 8.2 \pm 1.3$ min, n = 24 oocytes). The first ATP application was omitted from this fit as oocytes did not have a prior five minute recovery period at this time point. ATP had an EC₅₀ of 67.1 μ M with a Hill slope of 1.5 ± 0.03 (Fig. 1D). Adenosine, ADP and UTP (tested at 1mM) did not evoke currents at *BmP2X*. The general P2 receptor antagonist suramin inhibited ATP evoked *BmP2X* currents with an IC₅₀ of 4.8 μ M (Fig. 1E). However, there was a suramin

resistant component to the *BmP2X* current that persisted at the highest concentration of suramin tested (300 μ M).

Wild-type *BmP2X* is not potentiated by ivermectin but the mutation M362L confers ivermectin sensitivity. The macrocyclic lactone ivermectin is a broad spectrum antiparasitic agent used in human and veterinary medicine and has previously been shown to potentiate ATP activated currents in human and rat P2X4 (Khakh et al., 1999; Priel and Silberberg, 2004), the *SchP2X* receptor from the blood fluke *Schistosoma mansoni* (Agboh et al., 2004) and the *HdP2X* receptor from the tardigrade *Hypsibius dujardini* (Bavan et al., 2009). Since ivermectin is an effective treatment against *Boophilus microplus* infestation in cattle (Cramer et al., 1988a; Cramer et al., 1988b), we were interested to determine whether *BmP2X* currents were similarly affected by ivermectin. Wild-type *BmP2X* receptors show a marked run-down in current amplitude between sequential ATP applications which may mask any subtle effects of ivermectin. We therefore normalized 100 μ M ATP responses in the presence of either 3 or 10 μ M ivermectin to ATP responses five minutes before and five minutes after the test application. Ivermectin however had no significant effect on ATP evoked currents at both concentrations tested (Fig. 2B and C). Ivermectin acts as an allosteric modulator of P2X4 by interacting with the transmembrane regions (Silberberg et al., 2007) and several predominantly nonpolar residues lying on the same side of their respective helices have been identified that when mutated to cysteine disrupt ivermectin sensitivity (Jelinkova et al., 2008). From alignment of P2X transmembrane sequences, the sequence motif (G(L/I)(G/A)LL) in TM2 is seen to be present only in those P2X channels known to be ivermectin sensitive (*SmpP2X*, *HdP2X*, *hP2X4* and *rP2X4*) (Fig. 2A). The first and last residues in this motif have previously been shown to be essential for ivermectin sensitivity (Jelinkova et al., 2008). Since the wild-type *BmP2X* receptor differed from this motif by only one amino acid (M362), we mutated this residue to leucine and tested for ivermectin sensitivity. Ivermectin potentiated ATP evoked currents at the M362L mutant by ~40% at both 3 and 10 μ M ($P < 0.01$) (Fig. 2 B and C). The M362L mutation had no effect on current run-down between sequential agonist application (data not shown) but was slightly less sensitive to ATP with an EC_{50} of 117.5 μ M (Fig. 2D). The triazapentadiene compound amitraz is also widely used

to treat tick infestation in cattle and we therefore also tested the effects of this compound on *Bmp2X*. Amitraz alone (100 μ M) did not evoke membrane currents in *Xenopus* oocytes expressing *Bmp2X* receptors (data not shown). However, when amitraz was co-applied with 100 μ M ATP, membrane currents were potentiated by $22.5 \pm 8\%$ and $93.9 \pm 31\%$ at 1 and 100 μ M amitraz respectively (Fig. 2F).

The contribution of the intracellular domains in determining desensitization properties of *Bmp2X* and *hdp2X* receptors. The slow desensitization properties of ATP evoked currents in *Bmp2X* receptors are in marked contrast to the exceptionally fast desensitization displayed by the *Hdp2X* receptor previously cloned from the tardigrade species *Hypsibius dujardini* (Bavan et al., 2009). In order to assess the contribution of the intracellular domains in determining these markedly contrasting desensitization kinetics, a series of six chimeric channels were generated by exchanging intracellular N- and C-termini between *Bmp2X* and *Hdp2X* (Fig. 3). All six chimeras produced functional ATP-gated channels (Fig. 3). However, maximal current amplitudes were significantly lower than the respective wild-type channels with some chimeras (A,C and D) producing currents less than 100 nA (Table 3). Nevertheless, clear changes in desensitization properties were apparent and these were quantified using the parameters of 10-90% rise time, I_{20} (% peak current after 20 seconds ATP application) and $T_{10\%}$ (time taken for the current to decay by 10%). With the exception of chimera A, ATP was slightly more potent at chimeric channels when compared to their respective wild-type (Fig. 4C, Table 2). Marginal (< 5 fold) shifts in ATP potency were observed for all chimeras and in each case 1mM ATP produced a maximal response. Chimera A, consisting of *Bmp2X* with the C-terminus replaced with that of *Hdp2X* displayed a rise time (269 ± 14 ms (n = 23)) ~3.5 times faster ($P < 0.01$) than wild-type *Bmp2X* (Table 3). Chimera A currents initially desensitized rapidly with a mean $T_{10\%}$ of 364 ± 37 ms followed by a much slower rate of desensitization reflected in an I_{20} value of $34.8 \pm 1.7\%$. Similarly, replacement of the C-terminus of *Hdp2X* with that of *Bmp2X* in chimera C also resulted in a change in current kinetics towards that of the C-terminal donor channel with rise time, I_{20} (Fig. 4A) and T_{10} (Fig. 4 B) values all significantly slower ($P < 0.01$) than *Hdp2X* and similar to those of *Bmp2X* (Table 3). Exchange of N-terminal domain regions in chimeras B and D also resulted in phenotypic changes in current kinetics towards those of the donor

channel with chimera B becoming faster than *BmP2X* and chimera D becoming slower than *HdP2X* (Table 3). Despite the very small (~22nA) current amplitude displayed by chimera D it was clear that, similar to chimera A, this channel also displayed an initial rapid phase of desensitization ($T_{10\%} = 61 \pm 7$ ms ($n = 16$)) followed by a slower rate of desensitization which resulted in an I_{20} value of $52.7 \pm 3.5\%$. Given the large shifts in desensitization properties resulting from exchange of a single intracellular N- or C-terminal domains, it was perhaps unexpected that exchange of both intracellular termini together in chimeras E and F resulted in a less pronounced change when compared to the single domain swap chimeras. Nevertheless, chimera E displayed $T_{10\%}$ and I_{20} values that were significantly ($P < 0.01$) faster than wild-type *BmP2X* and chimera F was significantly slower than wild-type *HdP2X* (Table 3, Fig. 4A and B).

Replacement of the intracellular domains disrupts the marked current run-down displayed by *BmP2X*. In contrast to *BmP2X*, which displays a marked run-down in current amplitude between repeated applications of agonist (Fig. 1 B and C), *HdP2X* shows little run-down in current amplitude with a five minute recovery period between sequential applications of agonist (Fig. 5 B). We therefore investigated current run-down after 6 sequential applications of ATP, 5 minutes apart, in chimeras A-F in order to assess the potential roles of intracellular domains in determining these differing phenotypes. Similar to wild-type *HdP2X*, a small decrease in current amplitude between first and second responses was observed in chimeras A,B,D,E and F (Fig. 5). Subsequent applications however produced stable responses and the amplitude of the 2nd response was not significantly different to the 6th response ($P > 0.05$) (Fig. 5 B). The amplitude of responses in chimera C similarly did not show run-down between sequential applications and current amplitudes increased slightly during the course of the 6 applications.

Truncation of the C-terminus increases the rate of *BmP2X* desensitization but does not prevent current run-down. Replacement of the C-terminal domain in the fast desensitizing *HdP2X* channel with that of the slowly desensitizing *BmP2X* (chimera C) was sufficient to convert *HdP2X* into a slowly desensitizing channel with $T_{10\%}$ decay time, 10-90% rise time and I_{20} values not significantly different from *BmP2X* (Table 3). This suggests that the C-terminal domain plays a prominent role in

determining the slow current desensitization properties of *BmP2X* and in order to investigate this further we created a series of truncation mutations by introducing premature stop codons in the C-terminal domain of *BmP2X*. As an initial screen, stop codons were introduced at positions 408, 400, 394 and 388. Truncation at the first 3 of these positions resulted in functional channels with peak amplitudes $> 7 \mu\text{A}$ and 10-90% rise time, $T_{10\%}$ and I_{20} values significantly ($P < 0.01$) faster than the wild-type *BmP2X* channel (Table 3, Fig. 6). The truncation Y388 Δ however produced a non functional channel. Removal of just the six terminal amino acids from the C-terminal domain by the mutation E408 Δ was sufficient to produce marked speeding in the desensitization properties of the channel (Fig. 6). We therefore generated a further four truncation mutants within this 6 amino acid region to assess which residues were required for the slow desensitization properties of the wild-type *BmP2X* channel. Removal of the terminal serine residue in the mutation S414 Δ resulted in no significant change in current kinetics with 10-90% rise times, $T_{10\%}$ and I_{20} values all similar to wild-type (Table 3, Fig. 6C and D). Truncation at position 413, removing the terminal arginine and serine residues, however did result in a speeding of current desensitization with, $T_{10\%}$ and I_{20} values significantly ($P < 0.01$) faster than wild-type. This speeding in desensitization was more pronounced when truncations were made further upstream at positions 412 and 410 resulting in channels with very similar properties to the truncation mutants K400 Δ and E408 Δ . Similar to wild-type, all functional truncation mutants displayed a run-down in peak current amplitude after sequential application of 1mM ATP with a 5 minute recovery period resulting in current amplitudes $\sim 12\%$ of their original values after 6 sequential applications (Fig. 6E).

Positive charge at position 413 is required for the slow desensitization of *BmP2X*.

Truncations of the intracellular C-terminal domain identified the end of this region as being important in determining the slow desensitization properties of *BmP2X* since truncations upstream of position 413 disrupted the slow desensitizing phenotype. We therefore mutated R413 to alanine, aspartic acid or lysine in order to determine whether charge at this position was an important factor and also mutated Y411 to alanine since this nearby residue partially met the criteria for a consensus tyrosine phosphorylation site. ATP evoked currents in the Y411A mutant were not significantly different to those of the wild-type

channel (Fig. 7, Table 3) indicating that potential phosphorylation at this position does not play a role in determining the slow desensitizing phenotype. However, mutation of arginine 413 to alanine, thereby neutralizing the positive charge at this position, resulted in a speeding of desensitization such that T.10% and I₂₀ values were significantly ($P < 0.01$) faster than wild-type. Converting the positive charge at this position to a negative charge further increased the initial rate of desensitization with mutant R413D displaying a T.10% ~4 times faster than R413A and ~20 times faster than wild-type. The current remaining after 20 seconds ATP application (I₂₀) however was very similar between the R413A and R413D with both mutants displaying approximately half the current of the wild-type channel at this time point (Table 3). Maintaining the positive charge at position 413 by mutation to lysine (mutant R413K) resulted in a wild-type phenotype with 10-100% rise time, T.10% and I₂₀ values not significantly different from *BmP2X* (Fig. 7, Table 3) demonstrating that it is positive charge rather than the arginine *per se* at this position that determines the slow desensitization properties of *BmP2X*. Run-down after sequential ATP applications was still apparent in R413 and Y411A mutants with each, similar to wild-type, displaying peak current amplitudes ~15% of their original values after 6 sequential responses (Fig. 7D). The mutants R413A and R413D however initially showed an increase in current amplitude between the first and second responses before run-down occurred at a rate similar to wild-type between the 3rd and 6th responses (Fig. 7D). Interestingly a similar phenomenon was also observed with the R413Δ truncation mutant (Fig. 6E).

DISCUSSION

Functional evidence that *BmP2X* corresponds to an ATP gated channel establishes the existence of P2X receptors in the phylum Arthropoda. This is of significance regarding our understanding of the evolution of purinergic signaling since P2X receptors are absent in the fully sequenced genomes of several other arthropods such as *Anopheles gambiae*, *Drosophila* and *Apis mellifera* suggesting a selective loss of P2X receptors within this phylum has occurred in insects.

The ectoparasite *Boophilus microplus* represents a significant problem to livestock production in tropical and sub-tropical regions through direct detrimental effects of blood feeding and also by the transmission of disease (Young et al., 1988). Our initial interest in *BmP2X* stemmed from the possibility that this channel could represent a target for the antiparasitic drug ivermectin. Whilst *BmP2X* proved to be ivermectin insensitive (Fig. 2C), key residues within TM2 showed homology with ivermectin sensitive P2X receptors (Fig. 2A). One obvious difference was *BmP2X* M362 which is a leucine in ivermectin sensitive P2X receptors. As a *BmP2X* gene SNP could have resulted in this difference we generated the mutant M362L. Leucine at position 362 conferred ivermectin sensitivity in *BmP2X* confirming previous studies (Jelinkova et al., 2008) highlighting the importance of this residue in ivermectin binding. However, the effect of ivermectin on *BmP2X* M362L was relatively modest (~40% potentiation) casting doubt on whether a potential SNP at this position could be of relevance to the development of ivermectin resistance in *Boophilus microplus* (Perez-Cogollo et al., 2010). The ectoparasiticide amitraz is also widely used to treat tick infestation and is thought to act via parasite muscle octopamine receptors (Chen et al., 2007). Amitraz is typically applied to cattle at a concentration of 0.025% (~850 μ M) (Eamens et al., 2001; Mekonnen, 2001). This concentration would be sufficient to affect *BmP2X* function as 100 μ M amitraz approximately doubled ATP evoked currents (Fig. 2F). It is therefore possible that *BmP2X* potentiation is an additional target for the parasiticide action of amitraz, particularly in view of the fact that P2X receptors are known to play important roles in muscle contractility (Surprenant and North, 2009).

The extremely slow kinetics of *BmP2X* led us to utilize this receptor as a model to probe mechanisms of desensitization. Chimeras produced by swapping N- and C-terminal domains between the fast desensitizing *HdP2X* and slow desensitizing *BmP2X* were used to investigate the importance of intracellular domains in determining current desensitization and run-down. Whilst interpretation of these chimeric studies is complicated by difficulties in distinguishing between effects caused by introduction of a new domain and those resulting from removal of the replaced domain, the overall pattern that emerged was that both N- and C-termini both play important roles in determining desensitization. However, the extent to which a particular domain exerts its effect depends upon the context in which it is placed. For example, considering the 2 chimeras that resulted in the most complete fast-to-slow or slow-to-fast phenotype reversal (chimeras B and C), the N-terminal domain of the fast desensitizing *HdP2X* appears to exert a dominant fast desensitizing effect on the slow *BmP2X* C-terminal domain (chimera B). However, when the slow *BmP2X* C-terminal is placed in the context of the fast desensitizing *HdP2X* (chimera C), it is the slow *BmP2X* C-terminal that dominates over the fast *HdP2X* N-terminal. A composite phenotype of fast and slow desensitizing components resulted when either the *HdP2X* C-terminal was placed in the context of *BmP2X* or the *BmP2X* N-terminal was placed in the context of *HdP2X* (chimeras A and D respectively). Taken together these findings suggest that whilst both terminal domains of both receptors can modulate desensitization, in *HdP2X* it is the N-terminal domain that is most important for fast desensitization whereas in *BmP2X* it is the C-terminal domain that is most important for slow desensitization. When both N- and C-termini were exchanged together, a similar slow-to-fast (chimera E) and fast-to-slow (chimera F) reversal was observed. However, the effects of double N- and C-terminal exchange were not as pronounced as those in single exchanges suggesting that in addition to potentially interacting with each other, the intracellular domains may also couple with other regions of the receptor, as has been proposed for P2X2 (He et al., 2002) and that these interactions are disrupted more by exchange of a single intracellular domain than by exchange of both domains together. Chimeras generated by exchange of transmembrane regions linked to N- or C-terminal domains between the fast desensitizing P2X1 (or P2X3) and the slow desensitizing P2X2 receptor previously demonstrated that transmembrane

domains play an important role in desensitization (Werner et al., 1996). The *Bm-HdP2X* chimeras utilized in this current study however involved exchange of only intracellular domains and therefore demonstrate that both these regions can modulate desensitization independently from their respective transmembrane sequence. This is also supported by the naturally occurring P2X2b (Brandle et al., 1997; Koshimizu et al., 1998; Simon et al., 1997) and mouse P2X2e (Koshimizu and Tsujimoto, 2006) splice variants, mutation of the protein kinase C site in the N-terminal domain (Boue-Grabot et al., 2000), mutation of a P2X4 C-terminal domain lysine residue (Fountain and North, 2006), and exchange of the C-terminal domains in P2X2 and P2X3 receptors (Paukert et al., 2001).

The intracellular C-terminal domains are highly variable in length between different P2X receptors and share little sequence similarity. The functional significance of this divergence is unclear but likely arises from the evolution of different regulatory mechanisms to control channel function. To further investigate the significance of the *BmP2X* C-terminal domain in regulating slow desensitization, we first generated a series of truncation mutants (Fig. 6). Similar to zebrafish P2X4 (Kawate et al., 2009), *BmP2X* channel function was quite tolerant to C-terminal truncation with all truncations up to and including E394 producing functional channels (Fig. 6). This is in contrast to human P2X4 where deletion of the terminal 15 amino acids prevented function (Fountain and North, 2006). No ATP evoked currents were observed in the Y388 Δ truncation suggesting that the minimal C-terminal domain length required for *BmP2X* function lies between residues 388 and 394. All functional *BmP2X* truncations before the terminal serine at position 414 resulted in a speeding in the rate of desensitization (Fig. 6B), suggesting that the very end of the C-terminal domain is important in determining the slow desensitization properties of *BmP2X*. Indeed, disruption of the positive charge at the penultimate residue (R413) in the mutants R413A and R413D significantly increased the speed of channel desensitization (Fig. 7A). The fact that substitution for lysine at this same position (R413K) resulted in a wild-type phenotype demonstrates that it is the positive charge at this position that is crucial for slow desensitization rather than arginine *per se*. Amongst human, rat and mouse P2X1-7 and the known invertebrate P2X receptors, *BmP2X* is the only channel with an arginine at the penultimate position. A positively charged C-terminal lysine residue has also been shown to be

important for slow desensitization in P2X₄, however, in this case it is the amino group rather than the positive charge that is critical (Fountain and North, 2006).

In addition to desensitization during the continued presence of agonist, *BmP2X* responses also displayed a marked run-down in current amplitude between sequential applications of agonist. The lack of run-down in the *BmP2X* based chimeras A,B and E suggests that, in addition to regulating desensitization during the continued presence of agonist, intracellular domains may also play a role in the mechanism of run-down between sequential responses, possibly by influencing the rate of receptor internalization (Dutton et al., 2000) and trafficking (Bobanovic et al., 2002). However, the *BmP2X* N- or C-termini when added to the *HdP2X* core did not confer run-down suggesting that the intracellular domains are not the sole determinants. Furthermore, chimeric channels generally had smaller current amplitudes than wild-type channels making direct comparison of run-down difficult. C-terminal truncations (Fig. 6E) and R413 point mutants (Fig 7. D) did display peak current amplitudes that were comparable to wild-type *BmP2X* (Table 3). In these mutant channels run-down and desensitization appeared to be independent processes since a marked increase in desensitization was not accompanied by a significant alteration in the rate of run-down after 6 sequential ATP applications. It was however interesting that truncation R413 Δ and the point mutants R413A and R413D (but not R413K) initially showed an increase in current amplitude between first and second responses before run-down occurred at a rate similar to wild-type (Figs. 6E and 7D). The reason for this is unclear but given that R413 plays a key role in desensitization, it could suggest that whilst desensitization and run-down are largely governed by distinct mechanisms, positive charge at position 413 could also have a minor influence on run-down.

In summary, this study presents a new member of the P2X receptor family, *BmP2X* and identifies a novel example by which P2X desensitization is regulated by positive charge at the end of the C-terminus. *BmP2X* may also be of importance to the cattle industry either as a potential target for new antiparasitic agents or as an epitope for vaccines against tick infestation.

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AUTHOR CONTRIBUTIONS

Participated in research design: Ennion

Conducted experiments: Bavan, Farmer, Singh, Guerrero, Ennion

Performed data analysis: Bavan, Farmer, Singh, Ennion

Wrote or contributed to the writing of the manuscript: Straub, Guerrero, Ennion

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FOOTNOTES

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Reprint requests to: Dr Steven J. Ennion, Department of Cell Physiology and Pharmacology, University of Leicester, Leicester, United Kingdom, LE19HN. Email: se15@le.ac.uk

FIGURE LEGENDS

Figure 1. *BmP2X* is a slowly desensitizing ATP gated ion channel. Membrane currents were recorded by two-electrode voltage clamp in *Xenopus* oocytes expressing *BmP2X* receptors. A) Example current traces in response to a 40 second application (solid black bar) of 1 mM ATP (left) or 1mM ADP (right). B) Consecutive responses from the same oocyte demonstrating a marked run down in current amplitude with sequential 40 second applications of 100 μ M ATP and a five minute recovery period between the end of one application and the start of the next. C) Mean data expressed as % peak current amplitude of the first response for consecutive ATP applications as described in B (n = 24 oocytes). Dotted line shows a single exponential fit (excluding time point zero), τ = 8.2 minutes. D) Concentration response curve for ATP in *BmP2X* expressing oocytes. Mean currents (\pm S.E.M) were normalized to responses given by 100 μ M ATP, EC_{50} = 67.1 μ M (n = 7 oocytes). E) Inhibition curve for mean responses to 100 μ M ATP in the presence of different concentrations of suramin (IC_{50} = 4.8 μ M) (n = 7).

Figure 2. The mutation M362L confers ivermectin sensitivity in *BMP2X*. A) Amino acid alignment of the region surrounding transmembrane domain 2 (TM2) in *BmP2X*, *Schistosoma mansoni* P2X (*SmP2X*) (Agboh et al., 2004), *Hypsibius dujardini* P2X (*HdP2X*) (Bavan et al., 2009), rat P2X4 and human P2X1-7 receptors. Ivermectin sensitive receptors are highlighted by gray background. A TM2 motif (G(L/I)(G/A)LL) present only in those receptors sensitive to ivermectin is highlighted in bold. B) Example two-electrode voltage clamp recordings from wild-type and M362L *BmP2X* expressing *Xenopus* oocytes. ATP (100 μ M) evoked currents were recorded from the same cell in the presence and absence of 3 μ M ivermectin (+IVM) (applications indicated by bars). A five minute recovery period was allowed between applications and ivermectin was bath perfused in the five minutes preceding the second recording as well as being present in the ATP application. C) Mean data for ATP (100 μ M) responses in the presence and absence of ivermectin (3 or 10 μ M) as described in B. Ivermectin responses were normalized to the mean of the bracketing ATP responses 5 minutes prior and 5 minutes after the

ivermectin response. ** denotes $P < 0.01$ and ns $P > 0.05$ in comparison to the wild-type control response ($n = 10-15$). D) ATP concentration response curve for M362L ($n = 8$). The wild-type *BmP2X* curve (gray) is also shown for comparison. E) Example wild-type *BmP2X* current traces to sequential applications (5 minutes apart) of ATP (100 μM) in the presence and absence of 100 μM amitraz (+AMZ). F) Mean data ($n = 12-15$) for ATP (100 μM) responses in the presence and absence of amitraz (1 or 10 μM) (protocol as described in B).

Figure 3. Changes in desensitization properties after exchange of intracellular domains in chimeric channels. Example membrane currents in response to a 30 second application of 1mM ATP (black bars) are shown for wild-type and intracellular domain chimeras between *BmP2X* (gray) and *HdP2X* (white) channels.

Figure 4. Quantification of current desensitization in *Xenopus* oocytes expressing wild-type and chimeric *Bm-HdP2X* channels. A) I_{20} (% peak current amplitude remaining after 20 seconds ATP application) values for membrane currents in response to a 30 second application of 1mM ATP ($n = 15-25$). B) T.10% (time taken for the current to decay by 10% from peak amplitude) values for same traces as in A) (note log scale of y axis). ** and †† denote a significant difference ($P < 0.01$) to wild-type *BmP2X* and *HdP2X* respectively (* = $P < 0.05$). C) Concentration response curves for wild-type and chimeric channels (see table 2).

Figure 5. Replacement of intracellular domains in *BmP2X* chimeras prevents the marked current run down observed in WT *BmP2X*. A) Example consecutive responses to a 30 second application of 1mM ATP (solid bars) with a five minute recovery period between applications in *Xenopus* oocytes expressing chimeras consisting of a *BmP2X* core (chimeras A,B and E). The marked run down in current amplitude to sequential ATP applications displayed by wild-type *BmP2X* is abolished in these chimeras. B) Mean

peak current amplitudes to sequential application of ATP (1mM, 5 minutes apart) in wild-type *BmP2X* and *HdP2X* (dashed lines) and chimeric channels (n = 5-7).

Figure 6. Truncation of the *BmP2X* C-terminal tail increases the rate of current desensitization. A) *BmP2X* C-terminal domain sequence indicating positions of truncation mutants (arrows). Predicted TM2 domain sequence is highlighted in gray. B) Example current traces to a 40 second application of 1mM ATP (black bar) recorded from *Xenopus* oocytes expressing *BmP2X* C-terminal truncation mutants. C) and D) Mean I_{20} and T.10% values for truncation mutants (n = 12-18). ** denotes a significant difference to wild-type ($P < 0.01$). NF in the case of Y388 Δ denotes a non functional channel. E) Mean peak current amplitudes to sequential application of ATP (1mM, 5 minutes apart) in wild-type (WT) *BmP2X* (dashed line) and truncation mutants (n = 7-9).

Figure 7. Mutation of arginine 413 demonstrates that positive charge in this position is essential for the slow desensitizing phenotype of wild-type *BmP2X*. A) Example current traces recorded from *Xenopus* oocytes expressing Y411 and R413 mutant *BmP2X* channels. Removal of positive charge in mutants R413A and R413D speeds the rate of current desensitization whereas slow desensitization is restored in mutant R413K. B) and C) Mean I_{20} and T.10% values for truncation mutants (n = 8-15). ** denotes a significant difference to wild-type ($P < 0.01$). D) Mean peak current amplitudes to sequential application of ATP (1mM, 5 minutes apart) in wild-type (WT) *BmP2X* (dashed line) and point mutants (n = 7-8).

TABLES

Table 1

Oligonucleotide primers

	Forward primer (5'-3')	Reverse primer (5'-3')	Region
1 <i>BmP2X</i>	GCCGCCACCATGGGCTTGAAGTGGC	GCATACACTTGAGTGCTCG	1-414
2 <i>BmP2X</i> N-TM2	GCCGCCACCATGGGCTTGAAC	AACTCTTC CACAGCAGGCAGTTAAGAACAACAAG	1-378
3 <i>HdP2X</i> N-TM2	GCCGCCACCATGACGAATTTCACTAATACG	AACTCTTC ACTTCAGGAAGTCCGTTAAAAAGTC	1-371
4 <i>BmP2X</i> TM1-C	AACTCTTC ACGTATCGGCGTGCTCAACAGGCTC	AGTGCTCGTTAGCATGCTCC	28-414
5 <i>HdP2X</i> TM1-C	AACTCTTC AAAGTTGGGCGCCATCAATCGGAC	GGAGTTTTGGTAGCGCACAG	40-480
6 <i>BmP2X</i> N	Forward primer 2 above	AACTCTTC ACTTTTTGTTGCCGATGTGGAC	1-28
7 <i>HdP2X</i> N	Forward primer 3 above	AACTCTTC AACGAGTGCTGTAGACCTTGAC	1-40
8 <i>BmP2X</i> C	AACTCTTC AAAGCGTCGTGATCTGTACAAG	Reverse primer 4 above	378-414
9 <i>HdP2X</i> C	AACTCTTC AAAGCGTCGTGATCTGTACAAG	Reverse primer 5 above	371-480

Primer pair 1: Amplification of the *BmP2X* wild-type sequence to introduce a mammalian Kozak sequence. Primer pairs 2-9: *BmP2X-HdP2X* chimera generation, N and C signify amino and carboxy termini respectively, TM transmembrane domain. Region refers to the amino acid positions encoded in the resulting PCR product. *Earl* endonuclease recognition sites incorporated into primers are in bold.

Table 2

ATP Concentration response data

Channel	EC ₅₀ (μ M)	pEC ₅₀	Hill Slope
WT <i>BmP2X</i>	67.1	4.17 \pm 0.03	1.5 \pm 0.03
<i>BmP2X</i> M362L	117.5	3.93 \pm 0.03	2.7 \pm 0.03
<i>HdP2X</i>	25.5	4.59 \pm 0.04	1.2 \pm 0.01
Chimera A	100.2	4.00 \pm 0.06	1.0 \pm 0.12
Chimera B	34.8	4.46 \pm 0.07	0.9 \pm 0.11
Chimera C	5.2	5.29 \pm 0.05	0.7 \pm 0.06
Chimera D	12.1	4.92 \pm 0.06	0.5 \pm 0.04
Chimera E	7.2	5.14 \pm 0.06	1.1 \pm 0.13
Chimera F	6.9	5.16 \pm 0.04	0.8 \pm 0.05

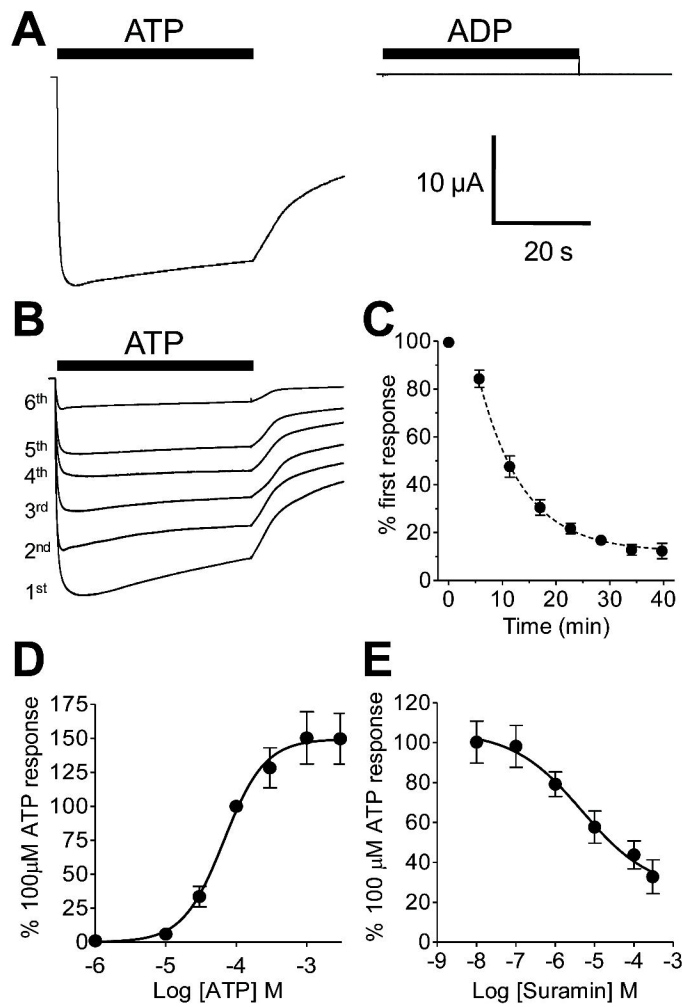
Table 3

Properties of ATP evoked currents in wild-type, mutant and chimeric P2X channels

Channel	Peak current (nA)	10-90% Rise time (ms)	100-90% Decay time (ms) (T.10%)	% Peak current after 20 s (I_{20})
WT <i>Bm</i> P2X	-14677 ± 882 ††	951 ± 129 ††	17016 ± 2420 ††	86.6 ± 1.7 ††
WT <i>Hd</i> P2X	-1808 ± 141 **	65 ± 2 **	46 ± 3 **	0.1 ± 0.08 **
Chimera A	-77 ± 6 **††	269 ± 14 **††	364 ± 37 **††	34.8 ± 1.7 **††
Chimera B	-1282 ± 165 **	281 ± 8 **††	237 ± 10 **††	2.9 ± 0.2 **††
Chimera C	-63 ± 4 **††	1730 ± 192 ††	10020 ± 1391 ††	82.4 ± 1.5 ††
Chimera D	-22 ± 1 **††	195 ± 12 **	61 ± 7 **	52.7 ± 3.5 **††
Chimera E	-5441 ± 538	1072 ± 70 ††	1388 ± 92 **††	10.6 ± 1.1 **††
Chimera F	-196 ± 12 **††	482 ± 39 ††	1882 ± 249 **††	54.7 ± 1.7 ††
S414Δ	-14056 ± 628 ††	1515 ± 230 ††	16244 ± 1374 ††	88.6 ± 1.6 ††
R413Δ	-11720 ± 883 ††	588 ± 75 ††	6469 ± 766 **††	68.3 ± 3.3 **††
L412Δ	-8600 ± 1043 **††	454 ± 39 ††	700 ± 41 **††	5.1 ± 1.1 **††
F410Δ	-8670 ± 857 **††	491 ± 31 ††	764 ± 41 **††	8.4 ± 1.1 **††
E408Δ	-15812 ± 1152 ††	358 ± 23 **††	1118 ± 113 **††	13.2 ± 1.7 **††
K400Δ	-7078 ± 864 **††	276 ± 35 **††	620 ± 77 **††	9.8 ± 1.7 **††
E394Δ	-14263 ± 1194 ††	328 ± 14 **††	1337 ± 140 **††	26.1 ± 2.6 **††
Y388Δ	NF	NF	NF	NF
Y411A	-14379 ± 580 ††	731 ± 113 ††	16207 ± 4615 ††	79.0 ± 3.8 ††
R413K	-11640 ± 1168 ††	1500 ± 313 ††	15455 ± 3934 ††	84.5 ± 3.0 ††
R413A	-7805 ± 1476 **††	624 ± 91 ††	2964 ± 396 **††	43.4 ± 5.7 **††
R413D	-7435 ± 871 **††	275 ± 24 **††	758 ± 50 **††	40.0 ± 3.2 **††

Values are means ± S.E.M. . * ($P < 0.05$) and ** ($P < 0.01$) denote a significant difference to wild-type *Bm*P2X. † ($P < 0.05$) and †† ($P < 0.01$) denote a significant difference to wild-type *Hd*P2X. Y388Δ was non-functional (NF). $n \geq 15$ for each channel.

Figure 1



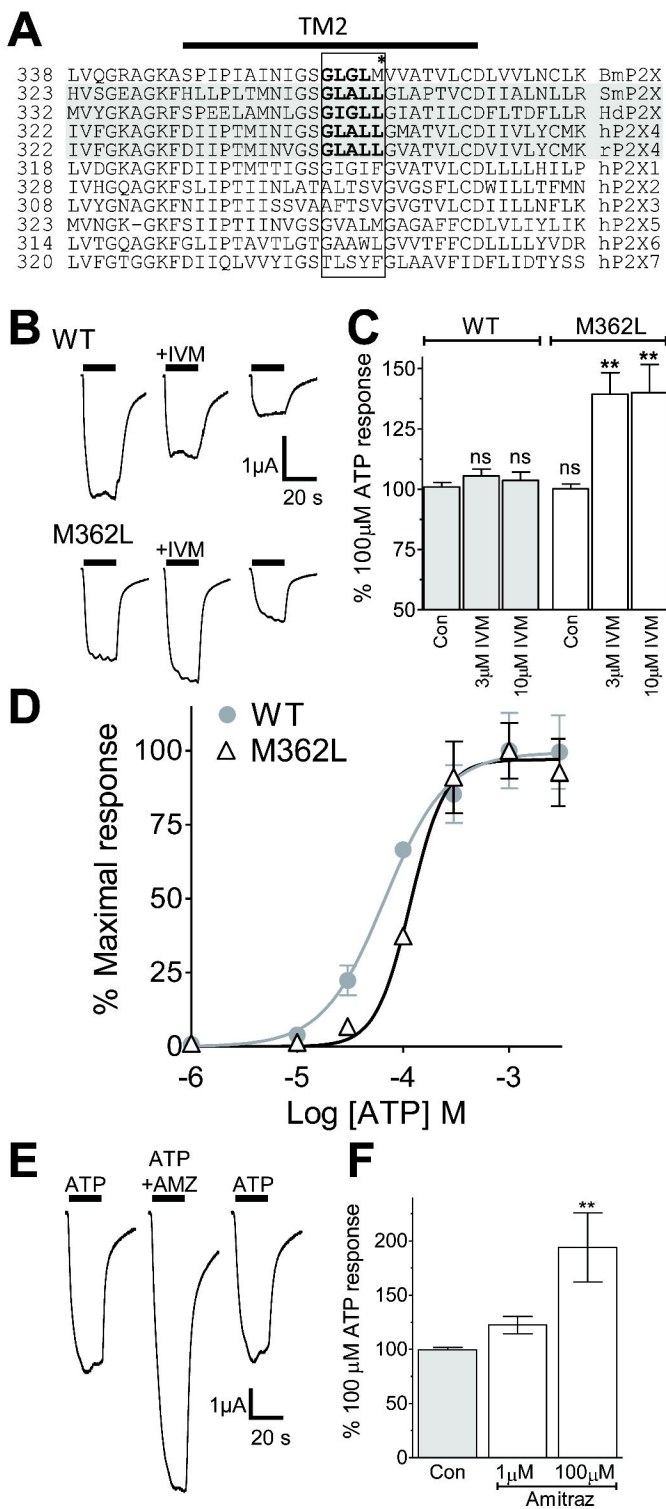


Figure 3

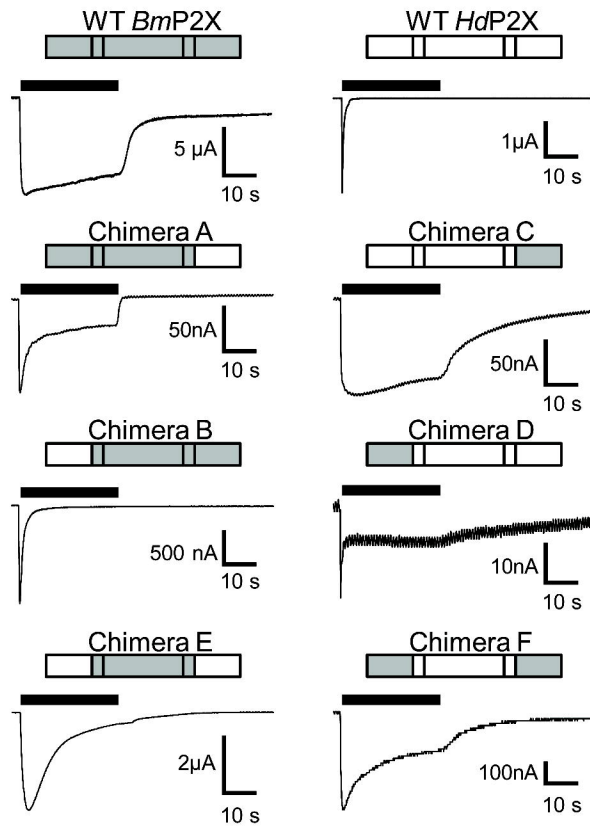


Figure 5

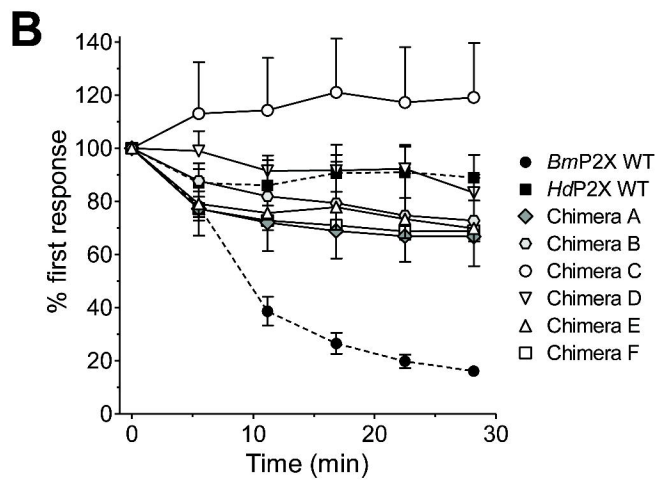
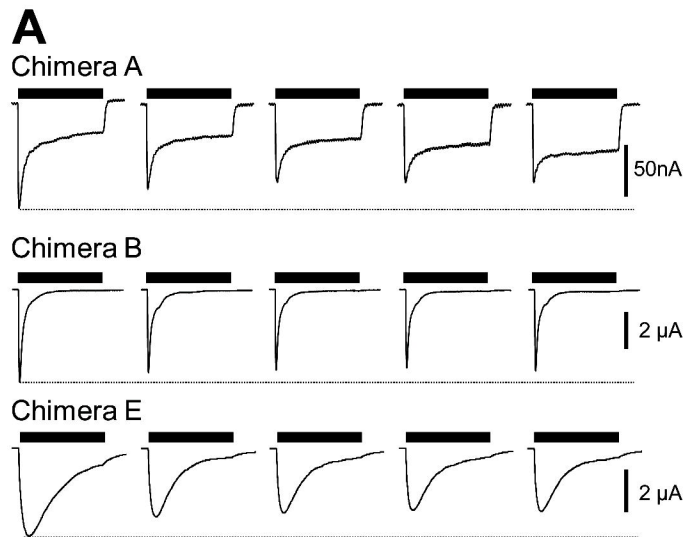


Figure 6

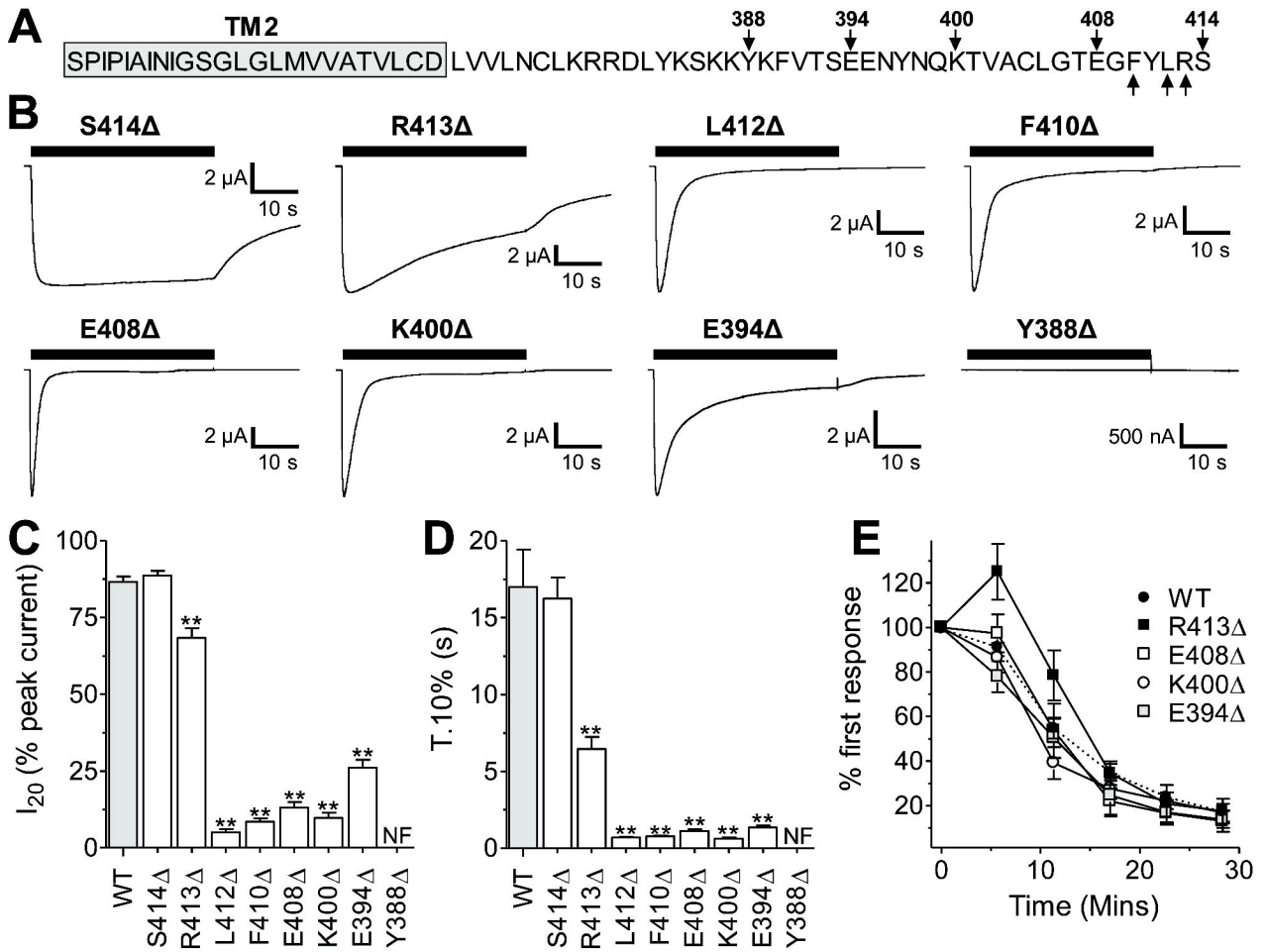


Figure 7

