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RESEARCH PAPER

Identification of the amino acid residues in the extracellular domain of rat $P2X_7$ receptor involved in functional inhibition by acidic pH

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Background and purpose: $P2X_7$ receptors are potently inhibited by extracellular acidification. The underlying molecular basis remains unknown. This study aimed to examine the role of extracellular histidine, lysine, aspartic acid and glutamic acid residues in the functional inhibition of rat $P2X_7$ receptors by acidic pH.
Experimental approach: We introduced point mutations into rat $P2X_7$ receptor by site-directed mutagenesis, expressed wild type (WT) and mutant receptors in human embryonic kidney (HEK293) cells and, using patch clamp recording, characterized the effects of acidic pH on BzATP [2′-3′-*O*-(4-benzoylbenzoyl) adenosine 5′-triphosphate]-evoked ionic currents.
Key results: Reducing extracellular pH, that is, increasing extracellular proton concentrations, inhibited BzATP-evoked currents in cells expressing WT $P2X_7$ receptors, with IC_{50} value (half-maximal antagonist or inhibitor concentration) for protons of 0.2 $\mu mol \cdot L^{-1}$. The major effect of acidification was suppression of the maximal current response without altering the agonist sensitivity. Five residues in the receptor extracellular domain (His^{85}, Lys^{110}, Lys^{137}, Asp^{197} and His^{219}) were mutated to alanine and current inhibition by protons assessed. Compared with WT, the H85A, H219A, K137A mutants were two- to threefold more sensitive, whereas the K110A and D197A mutants were 2.5- and 9-fold less sensitive. Double-alanine substitution of Lys^{110} and Asp^{197} resulted in 23-fold decreased sensitivity to inhibition by protons. Furthermore, charge neutralization (K110M, K110F, D197N and D197F), but not charge conserving mutation (K110R and D197E), attenuated the inhibition of currents by protons.
Conclusions and implications: Functional inhibition of rat $P2X_7$ receptors by acidic pH was variably affected by the extracellular His^{85}, Lys^{110}, Lys^{137}, Asp^{197} and His^{219} residues, with the Asp^{197} residue being most critical for this inhibition.
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Keywords: acidification; at $P2X_7$ receptor; heterologous expression; mutagenesis

Abbreviations: BzATP, 2′-3′-*O*-(4-benzoylbenzoyl) adenosine 5′-triphosphate; HEK293, human embryonic kidney cell line; EC_{50}, half-maximal agonist concentration; IC_{50}, half-maximal antagonist or inhibitor concentration

Introduction

P2X receptors are a family of ATP-gated ion channels formed by assembly of homo/hetero-trimers from seven subunits ($P2X_{1-7}$) (North, 2002; nomenclature conforms to Alexander *et al.*, 2008). Each subunit has a large extracellular domain flanked by two transmembrane segments (TM1 and TM2), and intracellular N- and C-termini. The $P2X_7$ receptor is the last and also the unique member of the P2X receptor family, as it not only forms a Ca^{2+}-permeable cationic channel that is opened by brief application of agonist but also induces formation of pores passing large inorganic molecules up to 900 Da in response to sustained stimulation (Surprenant *et al.*, 1996; Rassendren *et al.*, 1997). $P2X_7$ receptors are predominantly localized on immune cells such as macrophages, mast cells, monocytes and lymphocytes, and also on non-neuronal cells (such as microglia and astrocytes) in the brain and spinal cord (Surprenant *et al.*, 1996; Collo *et al.*, 1997; Duan and Neary, 2006). There is compelling evidence that $P2X_7$ receptors play crucial roles in immune responses and inflammation (MacKenzie *et al.*, 2001; Solle *et al.*, 2001; Labasi *et al.*, 2002; Ferrari *et al.*, 2006), neuron-glia communication (Zhang *et al.*, 2007), inflammatory and neuropathic pain (Chessell *et al.*, 2005; Honore *et al.*, 2006), neuronal damage (Choi *et al.*, 2007) and autoimmune encephalomyelitis (Matute *et al.*, 2007).

Extracellular acidosis occurs at sites of inflammation and infection, and also in injured or malignant tissues (Edlow and Sheldon, 1971; Nielson *et al.*, 1981; Simmen and Blaser, 1993) or in the surroundings of neurons undergoing intense activation (Chesler and Kaila, 1992). P2X receptors are functionally

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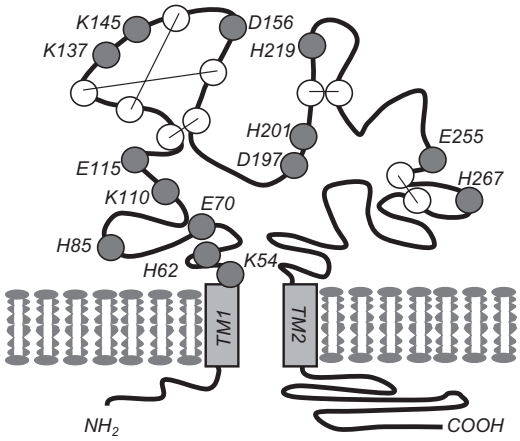

Figure 1 Amino acid residues in the extracellular domain of the rat $P2X_7$ receptor. Schematic presentation of the rat $P2X_7$ receptor subunit, in which the 10 conserved cysteine residues, indicated in open circles, are thought to form disulfide bonds. The residues in the extracellular domain investigated in this study are marked as filled circles.

regulated by acidic pH and the consequences depend on the receptor subtypes. Thus, reduction in extracellular pH facilitates $P2X_2$ and $P2X_{2/3}$ receptors but inhibits $P2X_1$, $P2X_3$, $P2X_4$ and $P2X_7$ receptors (King *et al.*, 1996; 1997; Li *et al.*, 1996; 1997; Stoop *et al.*, 1997; Wildman *et al.*, 1997; 1998; 1999a,b; Virginio *et al.*, 1997). Site-directed mutagenesis studies have identified receptor-specific histidine (His) residues in the extracellular domain of the receptors that mediates functional modulation of $P2X_2$ (His^{319}), $P2X_3$ (His^{206}) and $P2X_4$ (His^{286}) receptors (Clarke *et al.*, 2000; Clyne *et al.*, 2002; Gerevich *et al.*, 2007). However, the extracellular histidine residues seem to play no or only a minor role in inhibiting $P2X_7$ receptors (Acuna-Castillo *et al.*, 2007). It is known that lysine (Lys), aspartic acid (Asp) and glutamic acid (Glu) residues, as well as histidine, can coordinate proton binding and mediate functional modulation of pH-sensitive ion channels and receptors (Rho and Park, 1998; Wilkins *et al.*, 2005; Mott *et al.*, 2008). There are several such residues that are specifically present in the extracellular domain of $P2X_7$ receptors. This study, combining site-directed mutagenesis and patch clamp current recording, examined the role of these residues in the rat $P2X_7$ receptor (Fig. 1). We showed that Lys^{110} and particularly Asp^{197} were important in the functional inhibition of rat $P2X_7$ receptor by acidic pH.

Methods

Constructs, cell culture and transfection

Alanine mutations in rat and mouse $P2X_7$ receptors tagged with a C-terminal EYMPME epitope were introduced by site-directed mutagenesis and confirmed by sequencing. Human embryonic kidney (HEK293) cells were used to express $P2X_7$ receptors. Cell maintenance and transfection were carried out as described previously (Liu *et al.*, 2008).

Patch clamp current recording and dye uptake assay

Whole-cell patch clamp recording was performed using an Axopatch 200B amplifier (Axon) at room temperature as described previously (Liu *et al.*, 2008). Internal solution contained (in $mmol \cdot L^{-1}$) 145 NaCl, 10 HEPES and 10 EGTA, pH 7.3, and standard external solution contained (in $mmol \cdot L^{-1}$) 147 NaCl, 2 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 HEPES and 13 glucose. External solutions were adjusted to the desired pH with concentrated NaOH or HCl, with pH 7.3 for standard external solution. Agonist application or change in external pH was via a RSC160 system (Biologic, France). BzATP [2′-3′-*O*-(4-benzoylbenzoyl) adenosine 5′-triphosphate] concentrations used, except specified, are close to EC_{50} for each receptor (Liu *et al.*, 2008), and listed in Table 1.

Ethidium dye uptake assay was performed using a Nikon confocal microscope with excitation/emission at 543/590 nm. Prior to recording, cells were perfused with 25 $\mu mol \cdot L^{-1}$ ethidium bromide for 5 min in external solutions at the indicated pH. Fluorescence was recorded at 5 s intervals for 1 min, before superfusion application of 100 $\mu mol \cdot L^{-1}$ BzATP. Fluorescence was measured over another 9 min, during which it reached the maximum. For each set of experiments, 30–50 isolated cells were analysed to obtain the average maximum values and the rates of dye uptake (Pelegrin and Surprenant, 2007).

Data analysis

Data are presented as mean ± SEM. The mean EC_{50} values for BzATP were obtained by least square fitting the data from each cell to the Hill equation: $I/I_{max} = 100/(1 + (EC_{50}/[BzATP])^n)$, where I represents currents evoked by BzATP expressed as % of the maximal current, I_{max}, for each cell, EC_{50} is the concentration evoking half of maximal current, and n is the Hill coefficient. Similarly, the mean IC_{50} values were determined by fitting the data from each cell to $I/I_0 = 100/(1 + ([proton]/IC_{50})^n)$, where I is the agonist-evoked currents in solutions with indicated pH or proton concentrations, expressed as % of the maximal current, I_0, for each cell, IC_{50} is the concentration inhibiting half of maximal current, and n is the Hill coefficient. The smooth curves shown in the figures were derived by fitting the mean data. Curve fit was carried out using Origin program (OriginLab, Northampton, MA). Comparison was made using Student's *t*-test.

Materials

All chemicals, including BzATP and ethidium bromide, were purchased from Sigma, culture media and transfection reagent (lipofectamine2000) from Invitrogen and HEK293 cells from American Type Culture Collection.

Results

Characterization of effects of extracellular pH on WT rat $P2X_7$ receptor

Figure 2A shows a set of representative inward current recordings evoked by BzATP in a cell expressing wild-type (WT) rat $P2X_7$ receptor in extracellular solutions with pH values ranging from pH 8.5 to pH 5.5. Currents were slightly increased by mild alkalinization (pH 8.0–8.5), compared with control pH 7.5. Currents were progressively attenuated by reducing pH from 7.5 to 5.5 and almost completely abolished

Table 1 Effects of extracellular acidic pH on wild type (WT) and mutant P2X$_7$ receptors

Receptors	*BzATP used (μmol·L^{-1})*	*Proton IC$_{50}$ (μmol·L^{-1})*	n$_H$	*Cell No.*
WT	50	0.17 ± 0.02	1.37 ± 0.07	10
H62A	50	0.15 ± 0.03	1.14 ± 0.14	5
H85A	50	0.06 ± 0.01*	1.55 ± 0.03*	4
H201A	50	0.11 ± 0.01	1.19 ± 0.01*	3
H219A	30	0.05 ± 0.004*	1.69 ± 0.17	3
H267A	30	0.11 ± 0.002	1.91 ± 0.26	3
E70A	50	0.10 ± 0.01	2.12 ± 0.25	3
K110A	100	0.52 ± 0.11**	1.15 ± 0.18	7
K110R	30	0.20 ± 0.01	1.16 ± 0.07	3
K110M	50	0.42 ± 0.07*	1.35 ± 0.45	4
K110F	100	1.13 ± 0.12*	0.71 ± 0.04**	4
E115A	50	0.14 ± 0.05	1.22 ± 0.09	3
K137A	30	0.09 ± 0.003*	1.76 ± 0.03**	3
K145A	200	0.12 ± 0.01	1.29 ± 0.08	3
D197A	100	1.76 ± 0.45**	0.90 ± 0.06**	8
D197E	30	0.14 ± 0.01	1.29 ± 0.14	3
D197N	50	5.51 ± 0.86**	1.01 ± 0.29	3
D197F	10	0.67 ± 0.07*	1.03 ± 0.07*	4
E255A	30	0.12 ± 0.02	1.20 ± 0.06	3
D197A/K110A	200	4.71 ± 1.02**	1.12 ± 0.15	7

Data shown in the table are the mean ± SEM results from the numbers of cells studied (cell No.). *P < 0.01, **P < 0.001 compared with WT.
A, alanine; BzATP, 2′-3′-*O*-(4-benzoylbenzoyl) adenosine 5′-triphosphate; E, glutamic acid; D, aspartic acid; F, phenylalanine; H, histidine; IC$_{50}$, half-maximal antagonist or inhibitor concentration; K, lysine; M, methionine; N, asparagine; R, arginine.

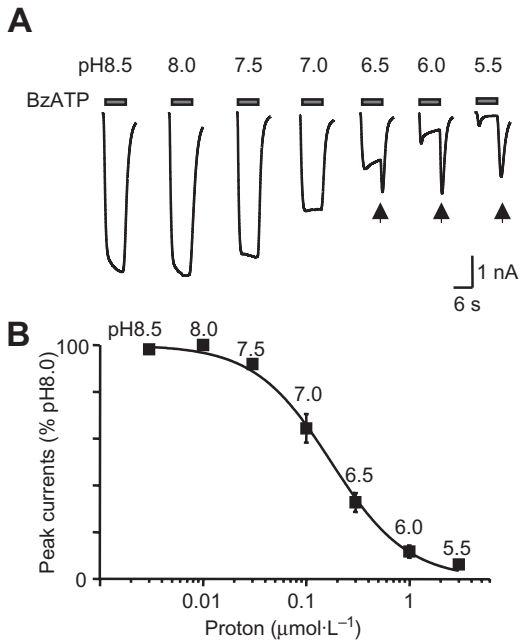

Figure 2 Concentration-dependent effect of protons on rat P2X$_7$ receptor-mediated currents. A. Representative BzATP-evoked currents in extracellular solutions with pH ranging from 8.5 to 5.5 in a cell expressing wild-type rat P2X$_7$ receptors. The arrows denote the rebound currents upon simultaneous washout of BzATP and change back to control pH 7.5. B. Proton concentration–current relationship curve, derived from data obtained from experiments shown in (A). n = 3–5 for each data point. BzATP, 2′-3′-*O*-(4-benzoylbenzoyl) adenosine 5′-triphosphate.

by strong acidification to pH 5.5. The onset of inhibition was fast, as a steady state was readily reached within 1–2 s (Fig. 2A). Simultaneous washing of BzATP and changing back to the control pH (7.5) resulted in strong rebound currents (indicated by arrows in Fig. 2A), indicating that the reversal of inhibition was rapid, or at least faster than dissociation of BzATP from the receptor. The concentration–response curve yielded an IC$_{50}$ value for protons of 0.2 ± 0.02 μmol·L^{-1} (n = 10) (Fig. 2B and Table 1).

Effects of acidic pH on rat and mouse WT and mutant P2X$_7$ receptors

We examined five histidines, three glutamic acids, two aspartic acids and four lysines in the extracellular domain of rat P2X$_7$ receptor (Fig. 1). Although Asp[156] is replaced with glutamic acid in human P2X$_7$ receptors, and Glu[115] and Asp[197] are replaced with valine and histidine respectively in mouse P2X$_7$ receptors, these residues are specific and conserved in human, rat and mouse P2X$_7$ receptors. While alanine substitution of two residues (Lys[54] and Asp[156]) led to complete loss of function, all 12 other alanine mutants were functional and showed similar agonist sensitivity to that of the WT receptor, with the exception of the H201A mutant that was approximately fourfold less sensitive (Liu *et al.*, 2008).

To examine the role of these residues in functional inhibition by acidic pH, we used BzATP, at concentrations approximating to the EC$_{50}$ for each receptor, and compared BzATP-evoked currents in extracellular solutions with pH 8.0 and pH 6.5 (Fig. 3A). There was no significant difference in the inhibition of BzATP-evoked currents for seven out of the 12 mutants (Fig. 3B). However, the degree of inhibition was significantly increased by mutating His[85], His[219] or Lys[137] to alanine, and markedly decreased by substituting Lys[110] and particularly Asp[197], with alanine (Fig. 3B). These results suggest that the sensitivity of rat P2X$_7$ receptors to protons involves these five residues, among which Asp[197] is most critical. We also carried out similar experiments on WT mouse P2X$_7$ receptors, where the Asp[197] in human P$_2$X$_7$ receptors is replaced by His[197], and the corresponding mutant H197A

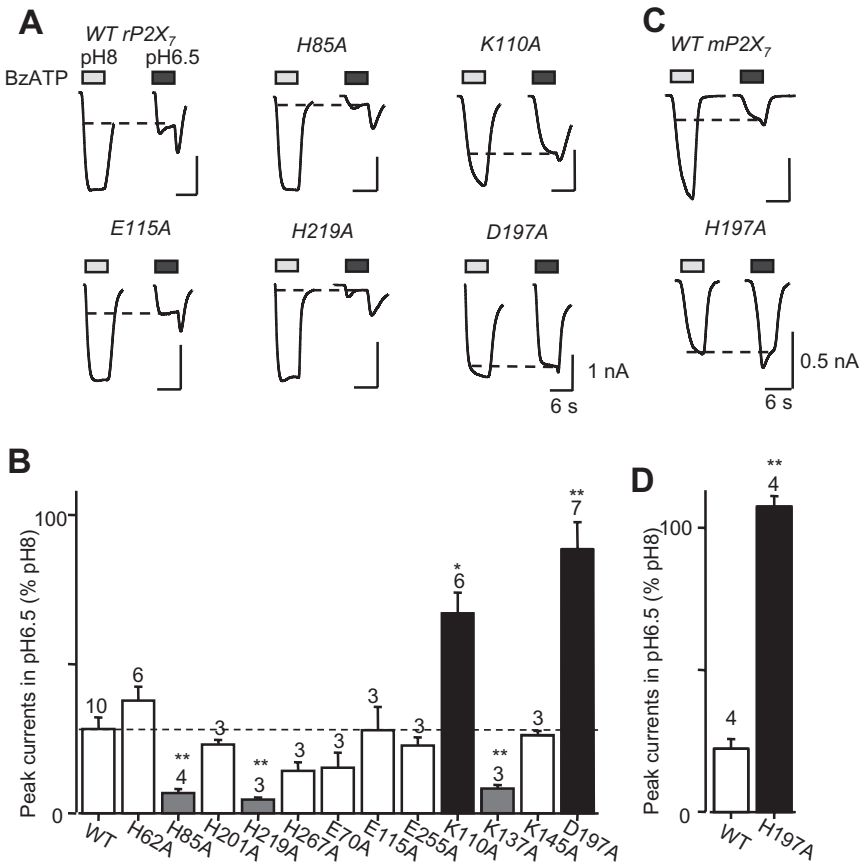

Figure 3 Effects of acidic pH on rat and mouse P2X$_7$ receptor-mediated currents. A. Representative currents evoked by BzATP at concentrations approximating to the EC$_{50}$ for WT and each indicated mutant rat P2X$_7$ receptors in extracellular solutions with pH 8.0 and pH 6.5. B. Summary of data from wild type (WT) and mutant receptors. C. Representative currents in extracellular solutions with pH 8.0 and pH 6.5, evoked by BzATP (300 μmol·L^{-1}) that was approximately the EC$_{50}$ value for WT and H197A mutant mouse P2X$_7$ receptors. D. Summary of data from WT and H197A mutant receptors. The currents at pH 6.5 were expressed as % of those at pH 8.0. The number of cells examined in each case is indicated above each bar. *P < 0.01, **P < 0.001, compared with WT. BzATP, 2′-3′-*O*-(4-benzoylbenzoyl) adenosine 5′-triphosphate; EC$_{50}$, half-maximal agonist concentration.

receptor. The BzATP-evoked currents for WT mouse receptors were strongly inhibited by pH 6.5, and substitution of His[197] with alanine abolished this inhibition (Fig. 3C,D).

We next constructed full proton concentration–current response relationship curves. Figure 4 illustrates BzATP-evoked currents in the extracellular solutions with pH ranging from 7.5 to 5.5 for WT, K110A and D197A mutant rat P2X$_7$ receptors. The IC$_{50}$ values for protons were 0.5 ± 0.1 μmol·L^{-1} and 1.8 ± 0.5 μmol·L^{-1} for the K110A and D197A mutants, representing a 2.5-fold decrease and a ninefold decrease in the sensitivity to protons. In contrast, mutation of His[85], His[219] or Lys[137] rendered the resultant mutant receptors threefold, 3.4-fold and twofold more sensitive (Table 1). This Table also shows IC$_{50}$ and Hill coefficient values obtained from these types of experiments at all rat P2X$_7$ mutants examined in this study.

We further generated a double mutant, in which both Lys[110] and Asp[197] residues were mutated to alanine. In standard extracellular solution, the K110A/D197A mutant receptor was approximately threefold less sensitive to BzATP (EC$_{50}$: 187 ± 5 μmol·L^{-1}, n = 4 for K110A/D197A; 57 ± 3 μmol·L^{-1}, n = 12, for WT) and the maximal currents were also significantly augmented (3.0 ± 0.2 nA, n = 4 for K110A/D197A; 1.9 ± 0.3 nA, n = 12 for WT; P < 0.05). Nonetheless, the most prominent change for the K110A/D197A mutant was a 23-fold reduction in the sensitivity to protons, with IC$_{50}$ values of 4.7 ± 1.0 μmol·L^{-1} (Fig. 4B).

We also constructed BzATP concentration–current relationship curves in solutions with pH 7.5 and pH 6.0 to investigate underlying mechanisms of modulation of rat P2X$_7$ receptors by acidic pH. At the WT receptor, change from pH 7.5 to pH 6.0 dramatically suppressed the maximal currents (Fig. 5A) with no significant effect on the BzATP sensitivity (Fig. 5B). The K110A mutant was similar to WT (data not shown). In contrast, for both the D197A and D197A/K110A mutants, the maximal currents in pH 7.5 and pH 6.0 were not significantly different (Fig. 5C,D), and there was very modest increase in the sensitivity to BzATP at pH 6.0 (Fig. 5D).

Role of charge in Lys[110] and Asp[197] in inhibition by acidic pH of rat P2X$_7$ receptors

We substituted Lys[110] with arginine (R), methionine (M) or phenylalanine (F), and Asp[197] with glutamic acid (E), asparagine (N) or phenylalanine, to examine the role of the charge. The EC$_{50}$ values for BzATP were 57 ± 3 μmol·L^{-1} for WT (n = 12), 32 ± 3.4 μmol·L^{-1} for K110R (n = 5),

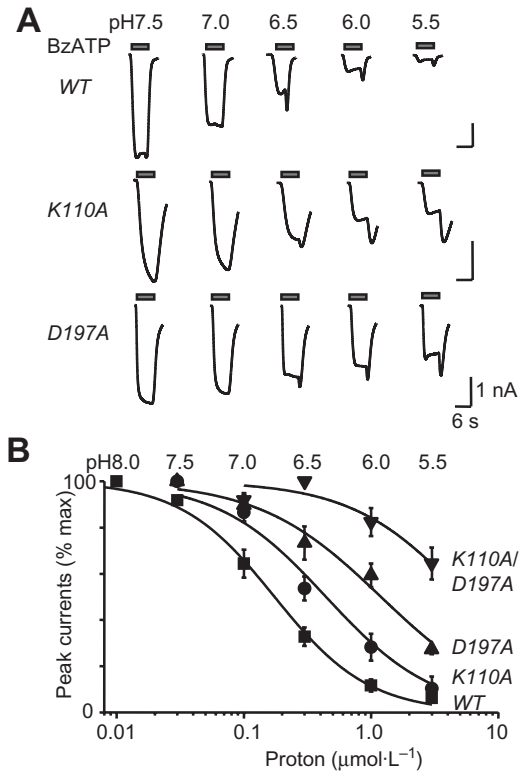

Figure 4 Proton concentration–current relationship curves for rat P2X$_7$ receptors. A. Representative BzATP-evoked currents in extracellular solutions with indicated pH for wild type (WT) and indicated mutant rat P2X$_7$ receptors. B. Proton concentration–current relationship curves, derived from data obtained from experiments shown in (A). n = 3–8 for each data point. BzATP, 2′-3′-*O*-(4-benzoylbenzoyl) adenosine 5′-triphosphate.

77 ± 8.6 μmol·L^{-1} for K110M (n = 5), 95 ± 3.2 μmol·L^{-1} for K110F (n = 4), 18 ± 2.5 μmol·L^{-1} for D197E (n = 4), 8.4 ± 0.4 μmol·L^{-1} for D197F (n = 4) and 57 ± 5.7 μmol·L^{-1} for D197N (n = 3) respectively. Thus, both charge-conserving and -neutralizing mutations caused less than threefold change in the BzATP sensitivity, with an exception of the D197F mutation, which resulted in a sevenfold increase in the BzATP sensitivity. The pH sensitivity of these mutants (Table 1) was not altered by charge-conserving mutations (K110R and D197E), but significantly attenuated by neutralizing mutations (K110M, K110F, D197F and D197N). These results, together with those from the alanine mutations, suggest that the charge of Lys[110] and Asp[197] is crucial for functional inhibition of rat P2X$_7$ receptors by protons.

Effects of acidic pH on WT and mutant rat P2X$_7$ receptor-mediated pore formation

Finally, we examined the effect of acidic pH on pore formation in cells expressing WT, K110A and D197A mutant rat P2X$_7$ receptors by comparing ethidium uptake in extracellular solutions with pH values of 7.3 and 5.5. In extracellular solution with pH 7.3, application of BzATP resulted in substantial dye accumulation in cells expressing the WT or D197A mutant receptors (Fig. 6A). The maximal dye uptake for the D197A mutant was significantly reduced compared with those for the WT. Surprisingly, there was very little dye uptake in cells expressing the K110A mutant (Fig. 6A). Change from pH 7.3 to pH 5.5 almost completely abolished the dye uptake in cells expressing the WT, but had no significant effect in cells expressing the D197A mutant (Fig. 6B,C).

Discussion

The P2X$_7$ receptors are profoundly inhibited by extracellular acidic pH, that is, an increase in extracellular proton concentrations. This study shows that acidic pH suppresses the maximal agonist-evoked response without altering the agonist sensitivity and several residues in the extracellular domain are involved in the inhibition by protons. Therefore, it provides a mechanistic and molecular understanding of the modulation of P2X$_7$ receptors by protons.

We have confirmed the potent inhibition of rat P2X$_7$ receptor-mediated currents by extracellular acidification (Fig. 2), previously reported by Virginio *et al* (1997). We have also demonstrated that acidic pH has a similar inhibitory action on mouse P2X$_7$ receptors (Fig. 3C,D). Here we observed that the onset of inhibition was rapid within seconds (Fig. 2A). The reversal was also rapid; the strong rebound currents resulting from simultaneously washing BzATP and restoring control pH (Fig. 2A) indicate that the dissociation of protons is at least faster than that of BzATP. The fast kinetics suggest an extracellular action of protons on the P2X$_7$ receptors, as was proposed for the other P2X receptors (Stoop *et al.*, 1997). Furthermore, comparison of BzATP concentration–current curves at pH 7.5 and pH 6.0 shows that acidic pH mainly suppresses the maximal current response, without significant alteration in the agonist sensitivity (Fig. 5), as observed with the P2X$_4$ receptor at which acidic pH is also inhibitory (Clarke *et al.*, 2000).

The major finding of this study is to show that Lys[110] and particularly Asp[197] are important in determining the inhibition of rat P2X$_7$ receptor by acidic pH. Extracellular histidine residues have been identified as mediating the functional facilitation or inhibition of P2X$_2$, P2X$_3$ and P2X$_4$ receptors by protons (Clarke *et al.*, 2000; Clyne *et al.*, 2002; Gerevich *et al.*, 2007). Consistent with a recent report (Acuna-Castillo *et al.*, 2007), we found that mutation of the five conserved extracellular histidine residues to alanine did not attenuate the inhibition by acidic pH. Interestingly, mutation of His[85] and His[219] instead caused a modest increase in the sensitivity to protons (Table 1). Lysine, aspartic acid and glutamic acid residues are also known to coordinate proton binding in pH-sensitive channels and receptors. Indeed, alanine substitution of Lys[110] and particularly Asp[197] substantially reduced the inhibition of BzATP-evoked currents (Figs 3 and 4) and dye uptake by acidic pH (Fig. 6). Surprisingly, the K110A mutation, despite its minimal effect on ion channel function (Liu *et al.*, 2008; Figs 3 and 4), led to almost complete loss of the pore formation, as indicated by the lack of dye uptake (Fig. 6A). The reasons for this are currently unclear.

Alanine substitution of His[197] in the mouse P2X$_7$ receptor, which corresponds to Asp[197] in rat and human P2X$_7$ receptors, also prevented the inhibition of BzATP-evoked currents

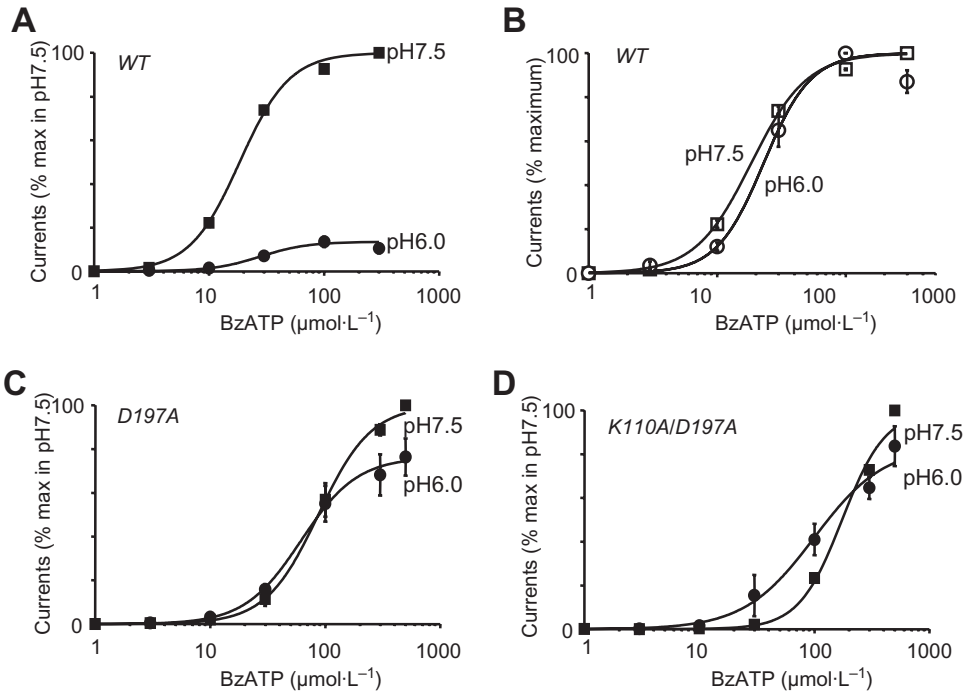

Figure 5 Effects of acidic pH on BzATP concentration–current relationship for rat P2X$_7$ receptors. BzATP concentration–current relationship curves were constructed for wild type (WT) (A, B), D197A (C) and K110A/D197A (D) in extracellular solutions with pH 7.5 and pH 6.0. All the currents were normalized to the maximal currents obtained at pH 7.5 (A, C and D) or the maximal currents at the respective pH (B). The curves were derived from fitting the mean data to the Hill equation. For WT, there was significant reduction in maximum currents (2.4 ± 0.5 nA, $n = 3$, pH 7.5; 0.3 ± 0.1 nA, $n = 4$, pH 6.0; $P < 0.05$) without altering the EC_{50} value for BzATP (18.4 ± 0.8 μmol·L^{-1}, $n = 3$, pH 7.5; 26.8 ± 3.1 μmol·L^{-1}, $n = 4$, pH 6.0; $P > 0.05$). For D197A and D197A/K110A mutants, the maximal currents at pH 7.5 and pH 6.0 were not significantly different ($P > 0.05$), but there was very modest increase in EC_{50} for BzATP (D197A: 91.4 ± 13.5 μmol·L^{-1}, $n = 5$, pH 7.5; 52.8 ± 6.0 μmol·L^{-1}, $n = 9$, pH 6.0; $P < 0.05$ and D197A/K110A: 174.3 ± 6.0 μmol·L^{-1}, $n = 5$, pH 7.5; 90.0 ± 17.2 μmol·L^{-1}, $n = 7$, pH 6.0; $P < 0.05$). BzATP, 2′-3′-*O*-(4-benzoylbenzoyl) adenosine 5′-triphosphate; EC_{50}, half-maximal agonist concentration.

by acidic pH (Fig. 3C,D), suggesting that both aspartic acid and histamine at this position can mediate inhibitory modulation of P2X$_7$ receptors by acidic pH. Moreover, suppression by acidic pH of the maximal currents in BzATP concentration–current relationship curves was largely abolished in the D197A and K110A/D197A mutant rat P2X$_7$ receptors (Fig. 5). All the results consistently support the idea that these two residues and particularly Asp[197] are critical in determining the sensitivity of rat P2X$_7$ receptor to inhibition by protons. The apparent pK_a for rat P2X$_7$ receptors is 6.7 (Fig. 2B). The pK_a for free aspartic/glutamic acid, histidine and lysine are 4.4, 6.5 and 10 respectively (Stryer, 1995). Therefore, the sensitivity of rat P2X$_7$ receptor to protons is most likely to be coordinated by Asp[197] and Lys[110] and possibly involves His[85], His[219] and Lys[137]. In a recent study, we have shown that Asp[197] (as well as His[62] and, to less extent, His[201] and His[267]) is important for mediating the potent inhibition of rat P2X$_7$ receptors by zinc and copper (Liu *et al.*, 2008). Taken together, these studies have revealed that the inhibition of P2X$_7$ receptors by protons and trace metal ions, such as zinc and copper, engages a subset of distinct and yet overlapping residues.

P2X$_7$ receptors are widely and abundantly expressed in immune cells, and have crucial roles in several immune functions, including release of cytokines, cell proliferation and cell death (e.g. Baricordi *et al.*, 1999; MacKenzie *et al.*, 2001; Solle *et al.*, 2001; Labasi *et al.*, 2002; Adinolfi *et al.*, 2005; Tsukimoto *et al.*, 2006). Extracellular acidosis predominantly develops at sites of inflammation, infection, tissue injury, ischaemia and hypoxia (Edlow and Sheldon, 1971; Nielson *et al.*, 1981; Simmen and Blaser 1993) and, under these circumstances, the P2X$_7$ receptors are expected to be functionally suppressed. It is tempting to speculate that this tonic inhibition, which appears paradoxical, could represent a fine-tuning mechanism, which prevents or reduces unnecessary cell death and meanwhile allows the physiological events downstream of P2X$_7$ receptor activation, such as release of cytokines, to occur. There is increasing evidence in support of the proposition that extracellular acidosis does significantly affect the functions of immune cells, for example, lymphocyte cytotoxicity and proliferation, cytokine maturation and secretion (Lardner, 2001; Lang *et al.*, 2005; Martinez *et al.*, 2007). More research is, however, required to determine what are the potentially significant implications of such proton sensitivity for the immune functions mediated by P2X$_7$ receptors.

In summary, this study shows that acidic pH modulated rat P2X$_7$ receptor by inhibiting the maximal responses without altering agonist sensitivity and the inhibition involved His[85], Lys[110], Lys[137], Asp[197] and His[219] residues in the extracellular domain of the receptor. The Asp[197] residue appeared to be the most critical residue for such inhibition.

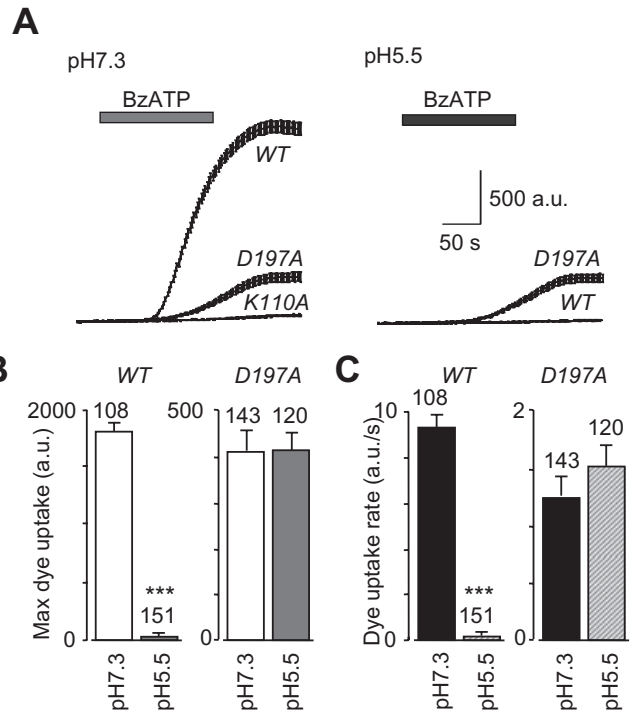

Figure 6 Effects of acidic pH on ethidium dye uptake mediated by rat P2X$_7$ receptors. A. Representative traces of ethidium bromide uptake in cells expressing rat wild type (WT), D197A and K110A mutant receptors in extracellular solutions with pH 7.3 (left panel) and pH 5.5 (right panel). B and C. Summary of the maximal values (B) and the rate (C) of dye uptake for WT and D197A mutant receptors. The number of cells examined in each case is indicated above each bar. ***$P < 0.0001$ compared with WT.

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Conflict of interest

The authors state no conflict of interest.

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