0026-895X/08/7301-252–259$20.00
MOLECULAR PHARMACOLOGY

Mol Pharmacol 73:252–259, 2008

Vol. 73, No. 1
39651/3291971
Printed in U.S.A.

Identification of Key Residues Coordinating Functional Inhibition of $P2X_7$ Receptors by Zinc and Copper[S]

Xing Liu, Annmarie Surprenant, Hong-Ju Mao, Sebastien Roger, Rong Xia, Helen Bradley, and Lin-Hua Jiang

Institute of Membrane and Systems Biology, Faculty of Biological Sciences, University of Leeds, Leeds, United Kingdom (X.L., H.-J.M., R.X., H.B., L.-H.J.); and Department of Biomedical Science, University of Sheffield, United Kingdom (A.S., S.R.)

Received July 4, 2007; accepted October 23, 2007

ABSTRACT

$P2X_7$ receptors are distinct from other ATP-gated P2X receptors in that they are potently inhibited by submicromolar concentrations of zinc and copper. The molecular basis for the strong functional inhibition by zinc and copper at this purinergic ionotropic receptor is controversial. We hypothesized that it involves a direct interaction of zinc and copper with residues in the ectodomain of the $P2X_7$ receptor. Fourteen potential metal interacting residues are conserved in the ectodomain of all mammalian $P2X_7$ receptors, none of which is homologous to previously identified sites in other P2X receptors shown to be important for functional potentiation by zinc. We introduced alanine substitutions into each of these residues, expressed wild-type and mutated receptors in human embryonic kidney 293 cells, and recorded resulting ATP and BzATP-evoked membrane currents. Agonist concentration-response curves were similar for all 12 functional mutant receptors. Alanine substitution at His^{62} or Asp^{197} strongly attenuated both zinc and copper inhibition, and the double mutant [H62A/D197A] mutant receptor was virtually insensitive to inhibition by zinc or copper. Thus, we conclude that zinc and copper inhibition is due to a direct interaction of these divalent cations with ectodomain residues of the $P2X_7$ receptor, primarily involving combined interaction with His^{62} and Asp^{197} residues.

The $P2X_7$ receptor (previously termed P2Z) is the last member (Surprenant et al., 1996) of the ATP-gated P2X receptor family and is found primarily on hematopoietic lineage cells, such as lymphocytes, monocytes, and macrophages, as well as brain microglial cells and astrocytes (North, 2002; Duan and Neary, 2006). Studies using different approaches, including gene knockout, have underscored $P2X_7$ receptors as a crucial component in a diversity of biological processes, such as inflammation, elimination of intracellular pathogens, release of pro-inflammatory cytokines by macrophages, and release of neurotransmitters by astrocytes, and chronic inflammatory and neuropathic pain (e.g., Lammas et al., 1997; Solle et al., 2001; Chessell et al., 2005; Duan and Neary, 2006; Ferrari et al., 2006).

$P2X_7$ receptors show several unique functional features that differ strikingly from those of other members of the P2X receptor family (North, 2002). $P2X_7$ receptors display remarkable functional plasticity; brief activation opens small cation permeable channels, whereas prolonged stimulation induces formation of "large molecule" permissive pores, rapid membrane and mitochondrial morphological changes, cytoskeletal rearrangement, and eventual cell death (Surprenant et al., 1996; North, 2002; Morelli et al., 2003). The most upstream signaling event consequent to the $P2X_7$ receptor activation, the ionic current, also shows properties uniquely different from those of other P2X receptors. Currents are activated by submillimolar concentrations of ATP, which are 10 to 100 times higher than those required for activation of other P2X receptors. Furthermore, $P2X_7$ receptors are potently inhibited by submicromolar concentrations of zinc and copper, whereas other P2X receptors are strongly potentiated or unaffected (Wildman et al., 1998, 1999a,b; Xiong et al., 1999; Acuña-Castillo et al., 2000; Clyne et al., 2002; Lorca et al., 2005). $P2X_7$ receptors are also substantially inhibited by magnesium and calcium, albeit with much less potency than zinc and copper. Earlier studies examining the effects of calcium and magnesium have led to the view that ATP^{4-} is the effective agonist form of ATP and these divalent cations inhibit the $P2X_7$ receptors by reducing the effective agonist concentrations (Cockcroft and Gomperts,

This work was supported by the Biotechnology and Biological Sciences Research Council (BBSRC) (L.-H.J., A.S.), the Royal Society (H.-J. M., L.-H.J.), a UK Overseas Research Scholarship (R.X.), and a BBSRC doctoral training grant (H.B.).

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

doi:10.1124/mol.107.039651.

[S] The online version of this article (available at http://molpharm.aspetjournals.org) contains supplemental material.

ABBREVIATIONS: BzATP, 2′,3′-O-(4-benzoylbenzoyl) adenosine 5′-triphosphate; HEK, human embryonic kidney.

1979). This view is still prevailing and implied in the inhibition by other divalent cations such as zinc and copper (North, 2002). Our previous indirect evidence demonstrates that the divalent cation that induces inhibition of $P2X_7$ currents is due primarily to direct binding to and subsequent allosteric modulation of the $P2X_7$ receptor (Virginio et al., 1997).

Histidine, cysteine, lysine, aspartic acid, and glutamic acid residues are all known to be capable of coordinating zinc inhibition/potentiation of different ion channels, such as $GABA_C$ receptors (Wang et al., 1995), glycine receptors (Laube et al., 2000), *N*-methyl-D-aspartate receptors (Paoletti et al., 2000), and acid-sensing ion channels (Chu et al., 2004). Sequence analysis of the ectodomain of the seven P2X receptor subunits led us to the identification of 14 potential zinc interacting residues that are conserved in mouse, rat, and human $P2X_7$ receptors but not the other P2X receptor family members. We introduced alanine substitution by site-directed mutagenesis into these 14 positions in the rat $P2X_7$ receptor and found that mutation of His^{62} or Asp^{197} residues strongly attenuated the inhibition by zinc and copper. Thus, the present study provides direct evidence supporting the view that the potent functional inhibition of the $P2X_7$ receptor by zinc and copper results primarily from an interaction with the receptor, in which His^{62} and Asp^{197} residues are critical.

Materials and Methods

Cell and Molecular Biology. Human embryonic kidney (HEK293) cells were cultured and transfected with plasmids as described previously (Jiang et al., 2001). Wild-type and mutant rat $P2X_7$ receptors were engineered to contain a C-terminal EYMPME epitope (EE tag). Tagging had no detectable effect on the functional properties of wild-type $P2X_7$ receptors (L.-H. Jiang and A. Surprenant, unpublished data). Alanine substitution was introduced by site-directed mutagenesis and confirmed by sequencing.

Electrophysiological Recording. Whole-cell recordings were carried out using an Axopatch 200B (Molecular Devices, Sunnyvale, CA) or EPC10 (HEKA, Lambrecht/Pfalz, Germany) amplifier at room temperature as described previously (Jiang et al., 2001). Cells were held at −60 mV. Patch pipettes were fabricated from borosilicate glass capillaries with a resistance of 4 to 6 MΩ. Standard external solution contained 147 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES, and 13 mM glucose. Intracellular solution contained 145 mM NaCl, 10 mM EGTA, and 10 mM HEPES. Copper chloride and zinc sulfate salts were dissolved in standard external solution. Application of the agonists, zinc and copper was carried out via a rapid solution changer RSC-160 system (Bio-Logic Science Instruments, Claix, France), which has limitations in studying very fast kinetics (Spelta et al., 2002), possibly contributing to the variable rebound currents. All the chemicals were purchased commercially (Sigma, St. Louis, MO).

Immunocytochemistry. Transfected cells were stained as described previously (Jiang et al., 2001) using mouse primary anti-EE antibody at a dilution of 1:1000 (BAbCO, Richmond, CA) and secondary fluorescein isothiocyanate-conjugated anti-mouse IgG antibody at a dilution of 1:200 (Sigma), or using rabbit anti-EE antibody at a dilution of 1:1000 (Bethyl Laboratories, Montgomery, TX) and secondary Alexa Fluor 488 anti-rabbit IgG antibody at a dilution of 1:2000 (Invitrogen, Carlsbad, CA). The images were captured using a Zeiss AxioVert 200M confocal microscope and LSM510 META software (Carl Zeiss GmbH, Jena, Germany).

Data Analysis. All data, where appropriate, are presented as mean ± S.E.M. The EC_{50} values for the agonists BzATP and ATP were determined by least-squares curve fitting to the Hill equation: $I/I_{max} = 1/[1+ (EC_{50}/[A])^{n_H}]$, where I is the current as a fraction of the maximum (I_{max}), [A] is the agonist concentration, and n_H is the Hill coefficient. Likewise, the IC_{50} values for the inhibitors zinc and copper were derived by fitting to $I/I_0 = 1/[1 + ([B]/IC_{50})^{n_H}]$, where I is the current as a fraction of the control current (I_0) in the absence of the inhibitors under examination and [B] is the inhibitor concentration. It is noteworthy that in the present study (except Fig. 1C), zinc or copper was coapplied with agonists for 4 s, and in most experiments, the inhibition occurred quickly and reached steady state by the end of the 4-s application. Figures show the curves fitted to the mean of all experiments. Curve fit was done using Origin software (OriginLab Corp, Northampton, MA). Statistical analysis was carried out using Student's *t* test.

Results

Zinc Inhibition of BzATP-Evoked Currents at the Wild-Type $P2X_7$ Receptor. We used BzATP at 30 μM, which evoked 30 to 50% of maximum current, and three

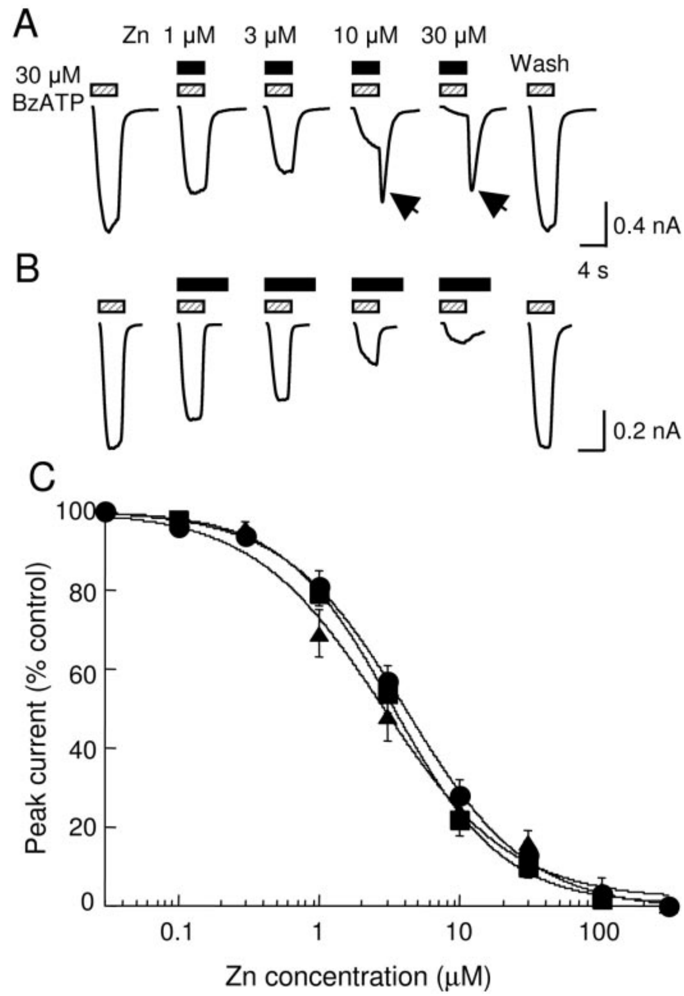

Fig. 1. Inhibition of $P2X_7$ receptor currents by zinc and rebound currents. A and B, representative BzATP-evoked currents in the presence of increasing concentrations of zinc. Note that there were robust rebound currents accompanying simultaneous washout of zinc and BzATP, as indicated by the arrows (the first protocol, A), which were absent in washout of BzATP in the persistent presence of zinc (the second protocol, B). C, zinc concentration-current response curves from all protocols as shown in A and B and described in text (n = 3–8 for each point). ●, data from the first protocol (IC_{50} = 4.6 ± 0.9 μM, n = 8); ■, data from the second protocol (IC_{50} = 5.9 ± 1.2 μM, n = 4); ▲, data from preapplication of zinc (IC_{50} = 5.6 ± 1.4 μM, n = 4). These values were not significantly different from each other.

distinct protocols to examine zinc inhibition of the BzATP-evoked currents: simultaneous application and washout of agonist and zinc (Fig. 1A), simultaneous application of agonist/zinc with sustained presence of zinc upon washout of BzATP (Fig. 1B), or preapplication of zinc for 30 to 90 s followed by either of the former protocols (Fig. 1C). The zinc inhibition curves were not significantly different using any of these protocols (IC_{50} = 4.6 ± 0.9 μM, n = 8; 5.9 ± 1.2 μM, n = 4, and 5.6 ± 1.4 μM, n = 4, respectively). It is noteworthy that we observed rebound currents upon simultaneous washout of agonist and zinc irrespective of the duration of preapplication of zinc (Fig. 1A). These rebound currents were particularly prominent at high concentrations of zinc but were never observed upon washing agonist in the persistent presence of zinc (Fig. 1B). No quantitative characterization of the rebound currents was attempted because there was significant variation in their amplitude that was probably due to the limited solution exchange rate of the system (see *Materials and Methods*). However, the presence of rebound currents upon simultaneous agonist/antagonist washout suggests that they result from the faster dissociation of zinc from its binding site than that of BzATP from its binding site. This further supports our hypothesis that zinc interacts directly with sites on the receptor protein rather than acting by chelating free ATP^{4-}. We thus turned to site-directed mutagenesis studies to seek direct evidence to corroborate this view.

Effects of Alanine Substitution on Functional Expression. Sequence analysis of the ectodomains of the seven P2X receptor subunits identified 14 potential interacting residues (five histidines, three glutamic acids, two aspartic acids, and four lysines) uniquely located in the ectodomain of the $P2X_7$ receptor but not $P2X_{1-6}$ receptors (Supplemental Fig. 1). This analysis was based on the discriminating actions at the $P2X_7$ receptor versus other P2X receptors and the known roles of histidine, cysteine, lysine, aspartic acid, and glutamic acid in zinc-mediated inhibition/potentiation of several ion channels (see Introduction). We substituted the individual residues with alanine, expressed the resultant mutant receptors in HEK293 cells, and measured the BzATP-evoked currents by patch clamp recording. The current amplitudes to maximum concentration of BzATP were not significantly different from wild-type currents at 11 of the mutant receptors (Table 1). Either no currents or minimal (< 50 pA) currents were recorded at the $P2X_7$ mutant receptors [K54A] and [D156A], and immunostaining using an anti-EE epitope Ab showed significantly reduced membrane localization (Supplemental Fig. 2). Therefore, these mutant receptors were not further investigated. The [H201A] mutant receptor showed significantly lower maximum current amplitudes (4-fold lower) without a significant shift in the agonist EC_{50} (Table 1). Of the 12 functional mutant receptors examined in this study, only one, [K145A], showed a significantly different BzATP EC_{50} value, which was shifted to the right by approximately 4-fold (Table 1). These results suggest that alanine substitution at all sites introduced minimal global conformational changes.

Effects of Zinc. First, we examined the wild-type and 12 functional mutant channel currents elicited by 30 μM BzATP (producing ~20–50% of the maximum current as shown in Supplemental Fig. 3, except [K145A] at which 200 μM BzATP was used). We used this concentration of BzATP to minimize the potential functional regulation by endogenous P2Y receptors (see *Discussion*). Furthermore, we used 30 μM zinc (resulting in nearly maximum inhibition) in the presence of BzATP to ask which residues, upon substitution with alanine, would give rise to pronounced reduction or loss of zinc inhibition. No significant differences in zinc inhibition of the BzATP-evoked currents were observed at 10 of the 12 mutant receptors examined (Fig. 2); however, we noted that zinc inhibition at the [H201A] mutant receptor showed much greater variability compared with the other receptors (Fig. 2B). Upon simultaneous washout of BzATP and zinc, rebound currents continued to be present at all of these mutant receptors and were not qualitatively different from those observed for the wild-type receptor. In contrast, zinc inhibition of BzATP-evoked currents was strongly attenuated (by 65–85%) at both [H62A] and [D197A] mutant receptors (Fig. 2). We constructed zinc inhibition curves for the wild-type receptor and [H62A], [H201A], and [D197A] mutant $P2X_7$ receptors (Fig. 3). IC_{50} values in these experiments were 6.6 ± 0.7 μM (n = 15), 5.9 ± 1.4 μM (n = 4), 52 ± 2.6 μM (n = 10), and 171 ± 27 μM (n = 7) for the wild-type receptor and [H201A], [D197A], and [H62A] mutant receptors, respectively. We also generated a $P2X_7$ receptor double mutant, [H62A/D197A], and found that the maximum current amplitude was reduced approximately 3-fold; however, the BzATP concentration response curve was shifted significantly to the left at this mutant receptor, resulting in an approximately 4-fold decrease in BzATP EC_{50} value (Table 1). In contrast to these relatively modest alterations in receptor function, the [H62A/D197A] double mutant receptor was virtually resistant to inhibition by zinc at concentrations up to 100 μM (Fig. 3). In addition, no rebound currents were observed upon simultaneous washout of BzATP and zinc for the [H62A] and [H62A/D197A] mutant receptors; however, rebound currents were reduced, but not abolished, for the [D197A] mutant receptor (Figs. 2A and 3A).

Effects of Copper. Copper also inhibits $P2X_7$ receptors with even greater potency than zinc (Virginio et al., 1997). Here, we also examined copper inhibition of BzATP-evoked currents for the wild-type receptor and 12 functional mutant receptors, using protocols similar to those for zinc inhibition (i.e., 30 μM BzATP and 10 μM copper, a concentration having nearly maximum inhibition). We observed little or no re-

TABLE 1
Summary of BzATP EC_{50} values and maximal currents of wild-type and mutant $P2X_7$ receptors

Receptors	BzATP EC_{50}	n_H	Maximum Currents	Cell No
	μM		*nA*	
WT	53 ± 4	1.9 ± 0.2	1.8 ± 0.4	9
H62A	68 ± 9	2.1 ± 0.5	1.4 ± 0.2	10
H85A	79 ± 6	1.6 ± 0.1	1.8 ± 0.6	6
H201A	38 ± 3	1.9 ± 0.4	0.5 ± 0.1*	8
H219A	42 ± 7	1.9 ± 0.2	2.1 ± 0.5	6
H267A	32 ± 2	2.4 ± 0.3	1.5 ± 0.3	4
E70A	57 ± 8	2.7 ± 0.3	1.8 ± 0.3	4
K110A	98 ± 7	4.4 ± 0.7	2.1 ± 0.6	5
E115A	65 ± 15	2.2 ± 0.5	1.1 ± 0.1	5
K137A	35 ± 4	2.4 ± 0.3	1.5 ± 0.3	4
K145A	196 ± 11*	3.5 ± 0.1	0.6 ± 0.1	6
D197A	88 ± 7	2.7 ± 0.2	3.6 ± 0.7	7
E255A	33 ± 10	1.8 ± 0.3	1.3 ± 0.2	4
H62A/D197A	14 ± 1*	2.1 ± 0.2	0.4 ± 0.1*	6

* $P < 0.01$, compared with the wild-type receptor (WT).

bound currents upon simultaneous washout of BzATP/copper from the wild-type or mutant receptors (e.g., Figs. 4A and 5A). Otherwise, results with copper were very similar to those observed for zinc. For the [H62A] and [D197A] but not the other mutant receptors, we observed significantly attenuated inhibition by copper and an even greater decrease in copper inhibition at the [H62A/D197A] double mutant receptor (Fig. 4). We then constructed the copper concentration inhibition curves for the wild-type receptor and the [H62A], [D197A] and [H62A/D197A] mutant receptors, which produced IC_{50} values of 2.3 ± 0.2 μM (n = 13), 12 ± 1.6 μM (n = 8), 17 ± 1.6 μM (n = 8), and 114 ± 25 μM (n = 9) respectively (Fig. 5).

Effects of Zinc and Copper on ATP-Evoked Currents. Finally, we repeated these experiments using 1 mM ATP (producing ~20–50% of the maximum current of the wild-type and mutant receptors) as the agonist. It was noted that both zinc and copper were slightly less potent in inhibiting the wild-type $P2X_7$ receptors activated by ATP than by BzATP; the IC_{50} values for zinc (19 ± 1.1 μM, n = 4) and copper (6.4 ± 1.9 μM, n = 4) were 2–3 fold greater than those observed with BzATP as the agonist. The concentrations of zinc (100 μM) and copper (10 μM) used in these experiments resulted in inhibition of the wild-type receptor currents by approximately 70%. The offset kinetics of ATP at the $P2X_7$ receptor were significantly faster than those observed with BzATP, most likely because BzATP has a higher affinity for this receptor. We also observed no rebound current upon simultaneous washout of ATP with zinc or copper at the wild-type or any of the mutant receptors (Fig. 6). Similar to the results obtained using BzATP as a agonist, we found that zinc and copper inhibition of ATP-evoked currents was potently attenuated or abolished for the [H62A], [D197A], and [H62A/D197A] $P2X_7$ mutant receptors (Fig. 6). In addition, there was a small but nonetheless significant attenuation of

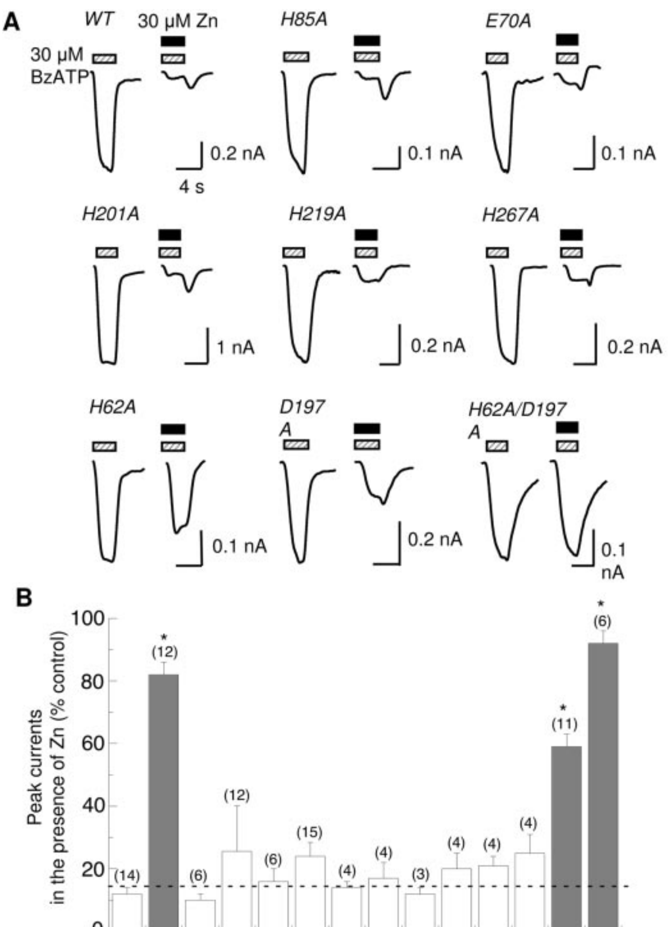

Fig. 2. Mutational effects on inhibition of BzATP-evoked $P2X_7$ receptor currents by zinc. A, representative BzATP-evoked currents in the absence or presence of 30 μM zinc at wild-type (WT) and indicated mutant $P2X_7$ receptors. BzATP concentration was 30 μM except at [K145A] where 200 μM was used. B, summary of the results for wild-type and all the mutant receptors examined. The number of cells examined in each receptor is indicated in brackets. The inhibition was significantly reduced for the [D197A], [H62A], and [H62A/D197A] mutant receptors. *, p < 0.01, compared with WT.

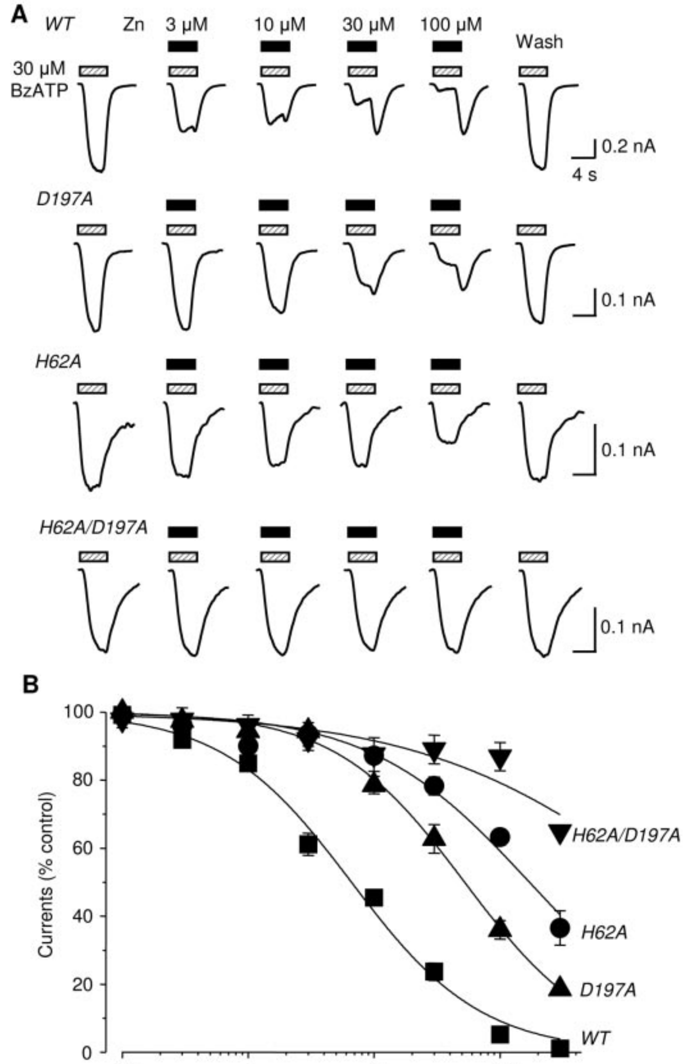

Fig. 3. Zinc concentration-current response curves for wild-type and mutant $P2X_7$ receptors. A, representative BzATP-evoked currents in the presence of increasing concentrations of zinc for wild type (WT), [D197A], [H62A], and [H62A/D197A] mutant $P2X_7$ receptors. B, concentration-current response curves from experiments as shown in A (n = 3–15 for each point). The IC_{50} values are 6.6 ± 0.7 μM (n = 15), 52 ± 2.6 μM (n = 10), and 171 ± 27 μM (n = 7) for the WT receptor, [D197A], and [H62A] mutant receptors. The Hill slopes are not significantly different between the WT and mutant receptors.

both zinc and copper inhibition of the [H201A] and [H267A] receptors currents (Fig. 6) that was not observed when BzATP was used as the agonist (compare Figs. 2 and 4).

Discussion

The key finding from this study is the identification of residues His^{62} and Asp^{197}, located in the ectodomain of the $P2X_7$ receptor, that are critical for inhibition by the divalent cations zinc and copper. This is of much conceptual significance, because it provides direct evidence against the longstanding view that divalent cation inhibition of $P2X_7$ receptors results from chelation of ATP^{4-}, which is considered to be the effective agonist (North, 2002). Indeed, single or combined mutation of His^{62} and Asp^{197} results in minimal effects on agonist sensitivity (Table 1) but strongly reduces zinc and copper inhibition, which quite conclusively excludes such an interpretation and substantiates the idea that direct binding is the primary mechanism underlying the inhibition of the $P2X_7$ receptor by divalent cations. The rebound currents recorded upon simultaneous washout of BzATP and zinc lend further support. Copper inhibition ($IC_{50} \sim$ 2–5 μM) was more potent than zinc inhibition ($IC_{50} \sim$ 5–10 μM), and rebound currents were observed only upon washout of zinc with BzATP but not ATP. The simplest explanation could be that the rebound currents depend upon the relative dissociation rate of the divalent cation and the agonist used. Thus, if the dissociation rate of ATP is > BzATP and that of zinc > BzATP $\geq$ copper, rebound currents could occur only with the BzATP/zinc combination.

Zinc and copper in submicromolar concentrations have either opposite effects or no actions at other P2X receptors. For example, zinc potently facilitates the $P2X_2$ and $P2X_4$ receptor currents (see Introduction). Similar mutational analysis by

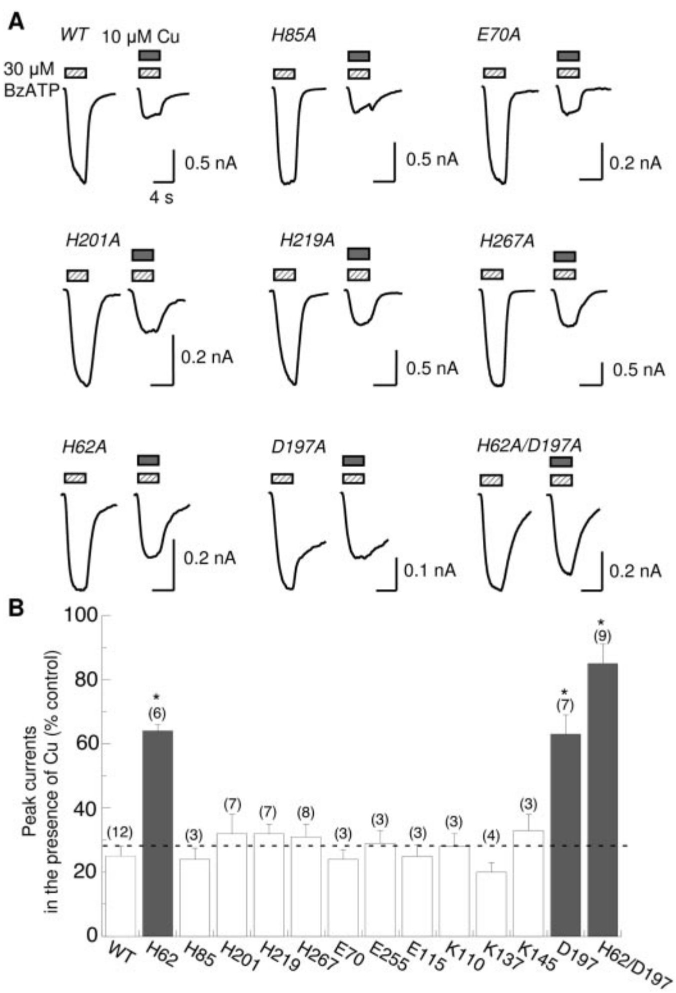

Fig. 4. Mutational effects on inhibition of BzATP-evoked $P2X_7$ receptor currents by copper. A, representative BzATP-evoked currents in the absence or presence of 10 μM copper at wild-type (WT) and indicated mutant $P2X_7$ receptors. B, summary of the results for all experiments as illustrated in A. The number of cells examined in each receptor is indicated in brackets. Significant attenuation in copper inhibition was seen at [D197A], [H62A] and [H62A/D197A] mutant receptors. *, $p < 0.01$, compared with WT.

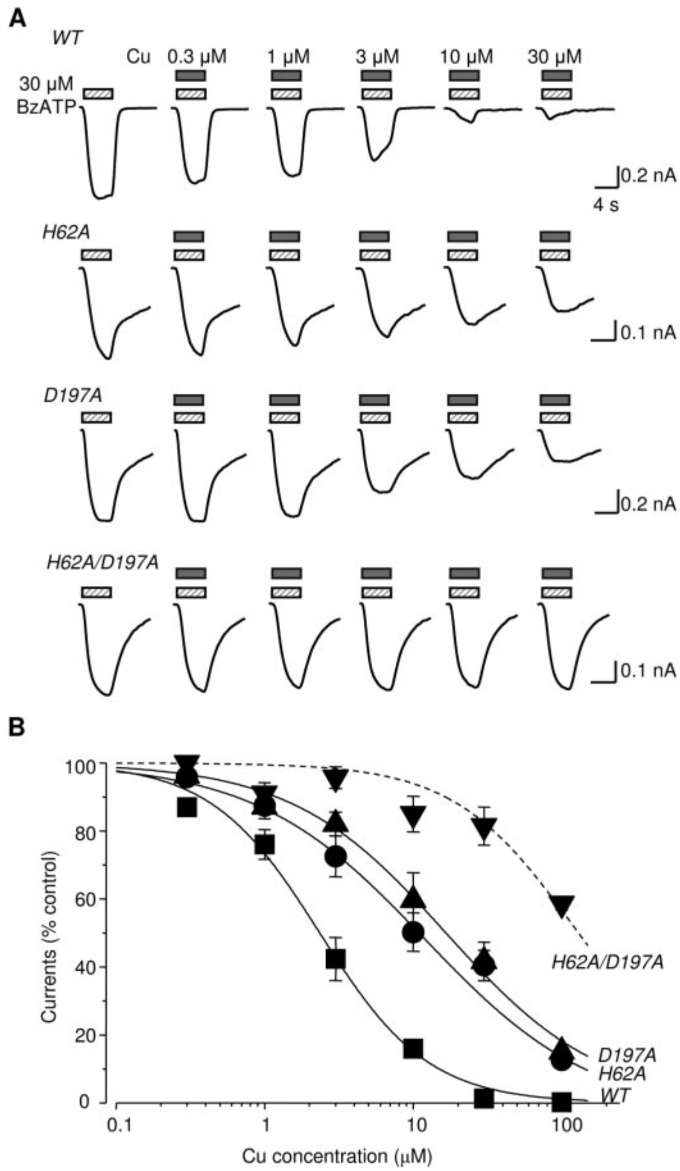

Fig. 5. Copper concentration-current response curves for wild-type and mutant $P2X_7$ receptors. A, representative BzATP-evoked current responses to increasing concentrations of copper at wild-type (WT), [H62A], [D197A], and [H62A/D197A] mutant $P2X_7$ receptors. B, concentration-current response curves from experiments shown in A (n = 5–13 for each point). The IC_{50} values are 2.3 ± 0.2 μM (n = 13), 12 ± 1.6 μM (n = 8), 17 ± 1.6 μM (n = 8), and 114 ± 25 μM (n = 9) for the WT, [H62A], [D197A], and [H62/D197A] mutant receptors. The Hill slopes are not significantly different between the WT and mutant receptors.

Nagaya et al. (2005) in the ectodomain of $P2X_2$ receptors identified His[120] and His[213] as the key residues mediating zinc facilitation of $P2X_2$ receptor. Subsequently, a series of elegant experiments on concatenated $P2X_2$ trimers convincingly demonstrated that these two residues are located closely at the interface between subunits and form an intersubunit zinc binding site. There is no information regarding how close His[62] and Asp[197] residues are in the $P2X_7$ receptor, but similar approaches may be expected to determine whether these two key residues form interior intrasubunit zinc/copper binding sites. It is noteworthy that our functional characterization (Table 1) showed that alanine substitution of both residues together ([H62A/D197A] double mutant), but not individually, resulted in a significant increase in the agonist sensitivity. This is reminiscent of the mutational effects on the close apposition and direct interaction between the N-terminal ligand binding domain and the extracellular loops between transmembrane segments in the pore forming domain, which underlie the gating of the $GABA_A$ (Kash et al., 2003) and nACh receptors (Lee and Sine, 2005). Although the mutational effects on agonist sensitivity are not fully understood, one possibility is that His[62] and Asp[197] may be closely positioned. His[62] is located between Lys[64], the critical residue for $P2X_7$ receptor activation (Wilkinson et al., 2006), and the first transmembrane domain, which is also implicated in channel gating (Jiang et al., 2001). An alternative explanation is that replacement of both His[62] and Asp[197] with alanine (a residue that contains a small side chain) may facilitate, to some extent, the channel gating. Consistent with this idea, occupation by divalent cations would disfavor channel gating and thereby results in a rightward shift and suppression of maximal receptor responses in the agonist concentration response curves as previously observed (Virginio et al., 1997). Finally, the present study has also shown a critical role of the nonhistidine residue (Asp[197]) in coordinating zinc and copper inhibition of the $P2X_7$ receptor, similar to zinc inhibition of the *N*-methyl-D-aspartate receptor (Paoletti et al., 2000).

We observed modest, but significant differences depending on the agonists used: first, zinc and copper were 2- to 3-fold more potent at inhibiting BzATP-evoked currents than ATP-evoked currents. Second, when BzATP was used to evoke currents, only single and double mutations of His[62] and Asp[197] resulted in significant attenuation of zinc and copper inhibition. These mutant receptors also exhibited the strongest attenuation of zinc and copper inhibition when ATP was

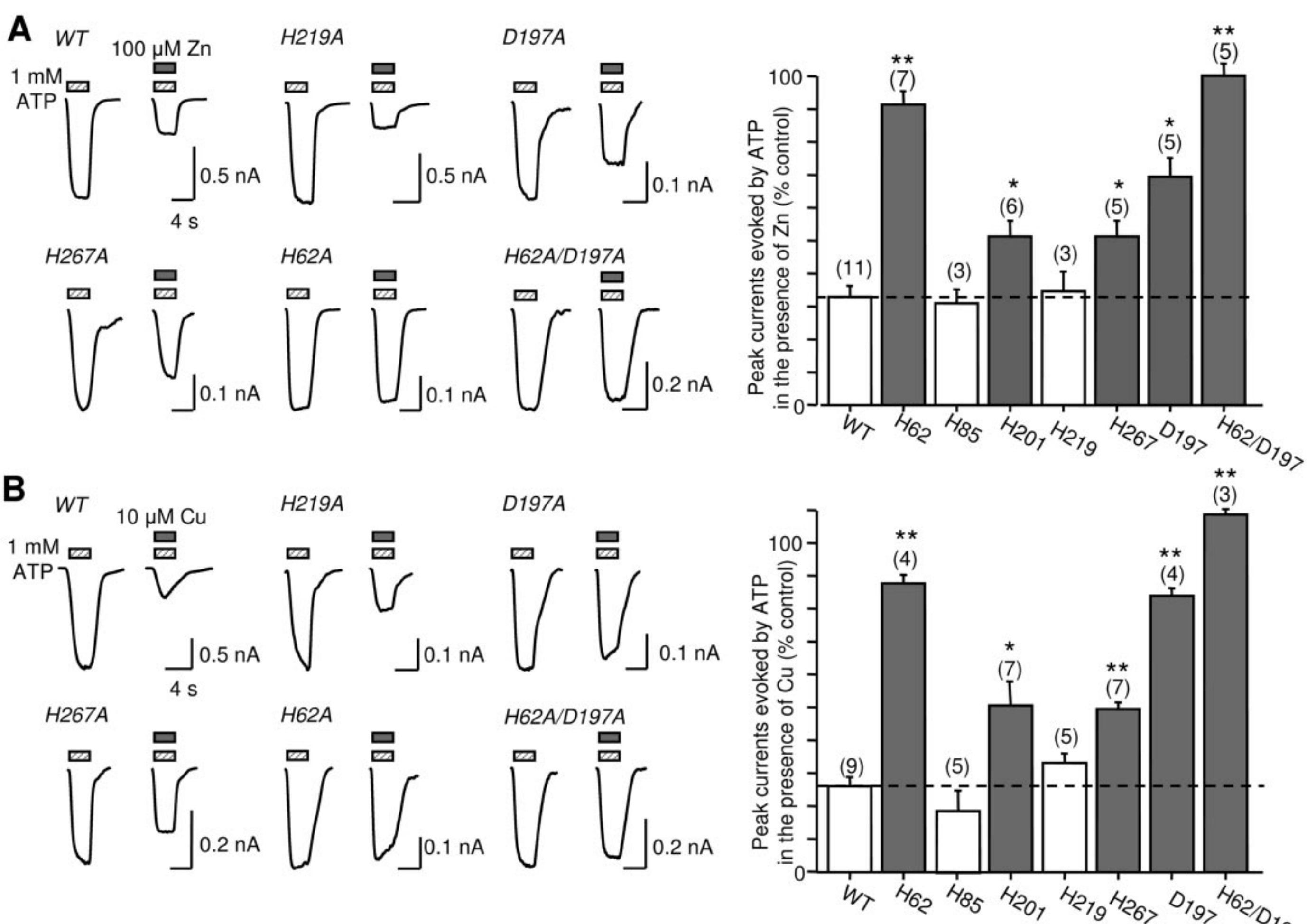

Fig. 6. Inhibition of ATP-evoked $P2X_7$ receptor currents by zinc and copper. A, left, representative currents of wild-type (WT) and mutant $P2X_7$ receptors activated by ATP (1 mM) in the absence and presence of zinc (100 μM). Right, summary of the results as shown on the left. B, left, representative currents of wild-type (WT) and mutant $P2X_7$ receptors activated by ATP (1 mM) in the absence and presence of copper (10 μM). Right, summary of the results as shown on the left. The number of cells examined in each case is indicated in brackets. *, $p < 0.05$; **, $p < 0.01$, compared with WT. Note the differential inhibition of [H62A] and [D197A] mutant receptors by zinc and copper.

the agonist, but we also observed significant attenuation at the [H201A] and [H267A] mutant receptors (compare Figs. 2B, 4B, and 7). We have identified two residues in the ectodomain that could account for the differential agonist (BzATP versus ATP) sensitivity at the $P2X_7$ receptor. We demonstrated that Lys^{127} and Asn^{284} together are required to maintain BzATP potency, whereas Asn^{284} alone is involved in ATP potency. We therefore suggest that these findings may indicate overlapping but distinct regions of the $P2X_7$ receptor that bind BzATP and ATP (Young et al., 2007). If binding of BzATP involves residues in addition to those required for ATP binding, it is reasonable to imagine a similar overlapping but distinct binding of divalent cations in the presence of each of these agonists. In any event, the actions of zinc and copper share the same two key residues (His^{62} and Asp^{197}), both of which are required to inhibit currents evoked by either agonist. Another possible contribution to the modest agonist-dependent difference seen here is the potential regulation of the $P2X_7$ receptor by the endogenous P2Y receptors (Schachter et al., 1997) as shown for the $P2X_1$ receptor (Vial et al., 2004). However, such an effect should be minimal when BzATP is used (Wilson et al., 2002).

During the preparation of the present study, an article by Acuña-Castillo et al. (2007) appeared in which they examined the role of histidine residues in zinc and copper inhibition of ATP-evoked rat $P2X_7$ receptor currents in *Xenopus laevis* oocytes expressing single alanine mutant receptors. In agreement with our findings, this group also observed no significant effects on agonist sensitivity by mutating the histidine residues and reached the same conclusion that zinc and copper inhibition of the $P2X_7$ receptor has little to do with changes in the active form of the agonist. However, their results differ considerably from ours in terms of the contribution these residues make. Acuña-Castillo et al. (2007) showed that the [H201] and [H267A] mutant receptors had either a significant reduction in or loss of inhibition by copper, whereas the [H219A] and [H267A] mutant receptors were both insensitive to inhibition by zinc. We also demonstrated a reduced zinc or copper inhibition of the [H201A] and [H267A] mutant receptors when ATP was used as the agonist, but clearly the reduction was far less pronounced compared with [H62A] and [D197A] mutants (the latter mutant was not studied by Acuña-Castillo et al., 2007) (Fig. 6). The inhibition became variable and insignificantly different from the wild-type receptor when BzATP was the agonist (Fig. 2B), probably because of the relatively higher potency of zinc and copper (see *Results*). The most surprising difference between the two studies is that we found the most prominent decrease in zinc and copper inhibition of $P2X_7$ receptor currents at the [H62A] mutant receptor, regardless of whether we used BzATP (Figs. 2–5) or ATP (Fig. 6), whereas Acuña-Castillo et al. (2007) observed no effects evoked by ATP. Inconsistent results have been noted for $P2X_7$ receptors expressed in mammalian cells and oocytes. The most noticeable difference is that just as in native $P2X_7$ receptor expressing cells, formation of the large dye uptake pore was persistently observed when the $P2X_7$ receptors were expressed in mammalian cells (e.g., Surprenant et al., 1996) but not in oocytes (Petrou et al., 1997; Klapperstück et al., 2000). In addition, loss of function associated with a naturally occurring mutation E496A in monocytes and lymphocytes (Gu et al., 2001) was confirmed in HEK293 cells (Cabrini et al., 2005). However, the [E496A] mutant receptor was functional and insignificantly differed from the wild-type receptor in oocytes as well as in HEK293 cells in another study (Boldt et al., 2003). Thus, utilization of the expression cells and in particular different cells (mammalian cell versus oocyte) may contribute to the discrepancy observed in the different studies.

Inhibition of $P2X_7$ receptors by zinc and copper has significant physiological relevance. For example, $P2X_7$ receptors are expressed in brain astrocytes and glial cells where they are increasingly known to engage in neural signaling processes such as neurotransmitter release (Duan and Neary, 2006). Local concentrations of zinc and copper released from nerve terminals are in the range of 10 to 100 μM (Kardos et al., 1989; Li et al., 2001) and can easily reach astrocytes and glial cells, which are in close vicinity and thus inhibit $P2X_7$ receptors. $P2X_7$ receptors are highly expressed in immune cells and important to the physiology and pathology of these cells (see Introduction). Under conditions of infection and/or inflammation, copper is found at micromolar concentrations and is essential for both T-cell proliferation and the ability of neutrophils to generate superoxide to kill ingested microorganisms (Percival, 1998). The present study has revealed, at the molecular level, how zinc and copper modulate the $P2X_7$ receptor.

In summary, we have provided direct evidence showing that zinc and copper inhibition is due to a direct interaction with ectodomain residues of the $P2X_7$ receptor, primarily involving combined interaction with His^{62} and Asp^{197}. The present study enhances our understanding of how this multifunctional ionotropic purinergic receptor can be activated by agonist, and in particular modulated by trace metals.

Acknowledgments

We are grateful to Dr. C. Milligan for critical comments on the manuscript.

References

Acuña-Castillo C, Coddou C, Bull P, Brito J, and Huidobro-Toro JP (2007) Differential role of extracellular histidines in copper, zinc, magnesium and proton modulation of the $P2X_7$ purinergic receptor. *J Neurochem* **101:**17–26.

Acuña-Castillo C, Morales B, and Huidobro-Toro JP (2000) Zinc and copper modulate differentially the $P2X_4$ receptor. *J Neurochem* **74:**1529–1537.

Boldt W, Klapperstück M, Buttner C, Sadtler S, Schmalzing G, and Markwardt F (2003) Glu496Ala polymorphism of human $P2X_7$ receptor does not affect its electrophysiological phenotype. *Am J Physiol Cell Physiol* **284:**C749–C756.

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.

Chu XP, Wemmie JA, Wang WZ, Zhu XM, Saugstad JA, Price MP, Simon RP, and Xiong ZG (2004) Subunit-dependent high-affinity zinc inhibition of acid-sensing ion channels. *J Neurosci* **24:**8678–8689.

Clyne JD, LaPointe LD, and Hume RI (2002) The role of histidine residues in modulation of the rat P2X(2) purinoceptor by zinc and pH. *J Physiol* **539:**347–359.

Cockcroft S and Gomperts BD (1979) Activation and inhibition of calcium-dependent histamine secretion by ATP ions applied to rat mast cells. *J Physiol* **296:**229–243.

Duan S and Neary JT (2006) P2X(7) receptors: properties and relevance to CNS function. *Glia* **15:**738–746.

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, Zhang W, Worthington RA, Sluyter R, Dao-Ung P, Petrou S, Barden JA, and Wiley JS (2001) A Glu-496 to Ala polymorphism leads to loss of function of the human $P2X_7$ receptor. *J Biol Chem* **276:**11135–11142.

Jiang LH, Rassendren F, Spelta V, Surprenant A, and North RA (2001) Amino acid residues involved in gating identified in the first membrane-spanning domain of the rat $P2X_2$ receptor. *J Biol Chem* **276:**14902–14908.

Kash TL, Jenkins A, Kelley JC, Trudell JR, and Harrison NL (2003) Coupling of agonist binding to channel gating in the GABA(A) receptor. *Nature* **421:**272–275.

Kardos J, Kovacs I, Hajos F, Kalman M, and Simonyi M (1989) Nerve endings from rat brain tissue release copper upon depolarization: A possible role in regulating neuronal excitability. *Neurosci Lett* **103:**139–144.
Klapperstück M, Buttner C, Bohm T, Schmalzing G, and Markwardt F (2000) Characteristics of $P2X_7$ receptors from human B lymphocytes expressed in Xenopus oocytes. *Biochim Biophys Acta* **1467:**444–456.
Laube B, Kuhse J, and Betz H (2000) Kinetic and mutational analysis of Zn^{2+} modulation of recombinant human inhibitory glycine receptors. *J Physiol* **522:**215–230.
Lammas DA, Stober C, Harvey CJ, Kendrick N, Panchalingam S, and Kumararatne DS (1997) ATP-induced killing of mycobacteria by human macrophages is mediated by purinergic P2Z(P2X7) receptors. *Immunity* **7:**433–444.
Lee WY and Sine SM (2005) Principal pathway coupling agonist binding to channel gating in nicotinic receptors. *Nature* 438:243–247.
Li Y, Hough CJ, Suh SW, Sarvey JM, and Frederickson CJ (2001) Rapid translocation of Zn^{2+} from presynaptic terminals into postsynaptic hippocampal neurons after physiological stimulation. *J Neurophysiol* **86:**2597–2604.
Lorca RA, Coddou C, Gazitua MC, Bull P, Arredondo C, and Huidobro-Toro JP (2005) Extracellular histidine residues identify common structural determinants in the copper/zinc $P2X_2$ receptor modulation. *J Neurochem* **95:**499–512.
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.
Nagaya N, Tittle RK, Saar N, Dellal SS, and Hume RI (2005) An intersubunit zinc binding site in rat $P2X_2$ receptors. *J Biol Chem* **280:**25982–25993.
North RA (2002) Molecular physiology of P2X receptors. *Physiol Rev* **82:**1013–1067.
Paoletti P, Perin-Dureau F, Fayyazuddin A, Le Goff A, Callebaut I, and Neyton J (2000) Molecular organization of a zinc binding N-terminal modulatory domain in a NMDA receptor subunit. *Neuron* **28:**911–925.
Percival SS (1998) Copper and immunity. *Am J Clin Nutr* **67**:1064S–1068S.
Petrou S, Ugur M, Drummond RM, Singer JJ, and Walsh JV Jr (1997) $P2X_7$ purinoceptor expression in Xenopus oocytes is not sufficient to produce a pore-forming P2Z-like phenotype. *FEBS Lett* **411:**339–345.
Schachter JB, Sromek SM, Nicholas RA, and Harden TK (1997) HEK293 human embryonic kidney cells endogenously express the $P2Y_1$ and $P2Y_2$ receptors. *Neuropharmacology* **36:**1181–1187.
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.
Spelta V, Jiang LH, Surprenant A, and North RA (2003) Kinetics of agonist actions at rat P2X2/3 heteromeric receptors. *Br J Pharmacol* **135:**1524–1530.
Surprenant A, Rassendren F, Kawashima E, North RA, and Buell G (1996) The cytolytic P2Z receptor for extracellular ATP identified as a P2X receptor ($P2X_7$). *Science* **272:**735–738.
Vial C, Tobin AB, and Evans RJ (2004) G-protein-coupled receptor regulation of $P2X_1$ receptors does not involve direct channel phosphorylation. *Biochem J* **382:**101–110.
Virginio C, Church D, North RA, and Surprenant A (1997) Effects of divalent cations, protons and calmidazolium at the rat $P2X_7$ receptor. *Neuropharmacology* **36:**1285–1294.
Wang TL, Hackam A, Guggino WB, and Cutting GR (1995) A single histidine residue is essential for zinc inhibition of GABA ρ1 receptors. *J Neurosci* **15:**7684–7691.
Wildman SS, King BF, and Burnstock G (1998) Zn^{2+} modulation of ATP-responses at recombinant $P2X_2$ receptors and its dependence on extracellular pH. *Br J Pharmacol* **123:**1214–1220.
Wildman SS, King BF, and Burnstock G (1999a) Modulation of ATP-responses at recombinant $rP2X_4$ receptors by extracellular pH and zinc. *Br J Pharmacol* **126:**762–768.
Wildman SS, King BF, and Burnstock G (1999b) Modulatory activity of extracellular H^+ and Zn^{2+} on ATP-responses at $rP2X_1$ and $rP2X_3$ receptors. *Br J Pharmacol* **128:**486–492.
Wilkinson WJ, Jiang LH, Surprenant A, and North RA (2006) Role of ectodomain lysines in the subunits of the heteromeric $P2X_{2/3}$ receptor. *Mol Pharmacol* **70:**1159–1163.
Wilson HL, Wilson SA, Surprenant A, and North RA (2002) Epithelial membrane proteins induce membrane blebbing and interact with the $P2X_7$ receptor C terminus. *J Biol Chem* **277:**34017–34023.
Xiong K, Peoples RW, Montgomery JP, Chiang Y, Stewart RR, Weight FF, and Li C (1999) Differential modulation by copper and zinc of $P2X_2$ and $P2X_4$ receptor function. *J Neurophysiol* **81:**2088–2094.
Young MT, Pelegrin P, and Surprenant A (2007) Amino acid residues in the $P2X_7$ receptor that mediate differential sensitivity to ATP and BzATP. *Mol Pharmacol* **71:**92–100.

Address correspondence to: Dr. Lin-Hua Jiang, Institute of Membrane and Systems Biology, Faculty of Biological Sciences, University of Leeds, Leeds LS2 9JT, UK. E-mail: l.h.jiang@leeds.ac.uk