Identification of Key Residues Coordinating Functional Inhibition of P2X$_7$ Receptors by Zinc and Copper

Xing Liu, Annnmarie 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.
CaCl2, 10 mM HEPES, and 13 mM glucose. Intracellular solution View that the potent functional inhibition of the P2X7 receptor in the present study provides direct evidence supporting the critical.

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\(_\text{A}\) receptors (Wang et al., 1995), glycine receptors (Laube et al., 2000), N-methyl-D-aspartate receptors (Paolletti et al., 2000), and acid-sensing ion channels (Chu et al., 2004). Sequence analysis of the ectodomain of the seven P2X receptors were engineered to contain a C-terminal EYMPME epitope (EE tag). Tagging had no detectable effect on the functional properties of wild-type P2X7 receptors (L.-H. Jiang and A. Surpreant, 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 15 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 rapid events. 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:

\[
III_{\text{max}} = \frac{I}{I_0} = \frac{1 + \left(\frac{[A]}{EC_{50}}\right)^n}{1 + \left(\frac{[B]}{IC_{50}}\right)^n}
\]

where \(I\) is the current as a fraction of the maximum (\(I_{\text{max}}\)), \([A]\) is the agonist concentration, and \(n_{\text{H}}\) is the Hill coefficient. Likewise, the IC\(_{50}\) values for the inhibitors zinc and copper were derived by fitting to

\[
III_{\text{max}} = \frac{I}{I_0} = \frac{1 + \left(\frac{[B]}{IC_{50}}\right)^n}{1 + \left(\frac{[B]}{IC_{50}}\right)^n}
\]

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 ($IC_{50} = 4.6 \pm 0.9 \mu M, n = 8; 5.9 \pm 1.2 \mu M, n = 4$, and $5.6 \pm 1.4 \mu M, n = 4$, 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$^-$. 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 receptor [K54A] and [D197A], 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 $\mu M$ BzATP (producing ~20–50% of the maximum current as shown in Supplemental Fig. 3, except [K145A] at which 200 $\mu M$ 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 $\mu M$ 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 $\pm$ 0.7 $\mu M$ ($n = 15$), 5.9 $\pm$ 1.4 $\mu M$ ($n = 4$), 52 $\pm$ 2.6 $\mu M$ ($n = 10$), and 171 $\pm$ 27 $\mu M$ ($n = 7$) 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 [H201A/D197A] double mutant receptor was virtually resistant to inhibition by zinc at concentrations up to 100 $\mu M$ (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 $\mu M$ BzATP and 10 $\mu M$ copper, a concentration having nearly maximum inhibition). We observed little or no re-

<table>
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<th>Receptors</th>
<th>BzATP EC$_{50}$</th>
<th>$n_M$</th>
<th>Maximum Currents</th>
<th>Cell No</th>
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<tr>
<td>WT</td>
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<tr>
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* $P < 0.01$, 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 = 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 P2X7 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 P2X7 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] P2X7 mutant receptors (Fig. 6). In addition, there was a small but nonetheless significant attenuation of

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**Fig. 2.** Mutational effects on inhibition of BzATP-evoked P2X7 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 P2X7 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 P2X7 receptors. A, representative BzATP-evoked currents in the presence of increasing concentrations of zinc for wild type (WT), [D197A], [H62A], and [H62A/D197A] mutant P2X7 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 His62 and Asp197, located in the ectodomain of the P2X7 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 P2X7 receptors results from chelation of ATP4−, which is considered to be the effective agonist (North, 2002). Indeed, single or combined mutation of His62 and Asp197 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 P2X7 receptor by divalent cations. The rebound currents recorded upon simultaneous washout of BzATP and zinc lend further support. Copper inhibition (IC50 ~ 2–5 μM) was more potent than zinc inhibition (IC50 ~ 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 ≥ 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 P2X2 and P2X4 receptor currents (see Introduction). Similar mutational analysis by
Nagaya et al. (2005) in the ectodomain of P2X<sub>2</sub> receptors identified His<sup>120</sup> and His<sup>213</sup> as the key residues mediating zinc facilitation of P2X<sub>2</sub> receptor. Subsequently, a series of elegant experiments on concatenated P2X<sub>2</sub> trimers convincingly demonstrated that these two residues are located closely at the interface between subunits and form an inter-subunit zinc binding site. There is no information regarding how close His<sup>62</sup> and Asp<sup>197</sup> residues are in the P2X<sub>7</sub> 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<sub>A</sub> (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<sup>62</sup> and Asp<sup>197</sup> may be closely positioned. His<sup>62</sup> is located between Lys<sup>64</sup>, the critical residue for P2X<sub>7</sub> 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<sup>62</sup> and Asp<sup>197</sup> 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<sup>197</sup>) in coordinating zinc and copper inhibition of the P2X<sub>7</sub> 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<sup>62</sup> and Asp<sup>197</sup> 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.](image-url)
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 P2X7 receptor. We demonstrated that Lys127 and Asn284 together are required to maintain BzATP potency, whereas Asn284 alone is involved in ATP potency. We therefore suggest that these findings may indicate overlapping but distinct regions of the P2X7 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 (His62 and Asp197), 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 P2X7 receptor by the endogenous P2Y receptors (Schachter et al., 1997) as shown for the P2X2 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 P2X7 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 P2X7 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, although 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 P2X7 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 P2X7 receptors 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 P2X7 receptors. P2X7 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 P2X7 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 P2X7 receptor, primarily involving combined interaction with His62 and Asp197. 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


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: lh.jiang@leeds.ac.uk