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Identification of key residues coordinating functional inhibition of $P2X_7$ receptors by zinc and copper*

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Running title: key residues in Zn and Cu inhibition of $P2X_7$ receptor

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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. There are 14 potential metal interacting residues conserved in the ectodomain of all mammalian $P2X_7$ receptors, none of which are 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 HEK293 cells and recorded resulting ATP and BzATP-evoked membrane currents. Agonist concentration-response curves were similar for all the twelve 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.

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The $P2X_7$ receptor (previously termed P2Z) is the last member (Surprenant et al., 1996) of the ATP-gated P2X receptor family and is primarily found 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 el., 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, while 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; Morelli et al., 2003; North, 2002). The most upstream signaling event consequent to the $P2X_7$ receptor activation, the ionic current, also shows uniquely different properties from other P2X receptors. Currents are activated by submillimolar concentrations of ATP, which are 10-100 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, 1999b, Xiong et al., 1999; Acuno-Catillo 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

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agonist form of ATP and these divalent cations inhibit the $P2X_7$ receptors by reducing the effective agonist concentrations (Cockcroft and Gomperts, 1979). This view is still prevailing and implied in the inhibition by other divalent cations such as zinc and copper (North, 2002). We have previously presented indirect evidence demonstrating that the divalent cation induces inhibition of $P2X_7$ currents is primarily due 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), NMDA 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 which supports 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 comprise a C-terminal EYMPME epitope (EE tag). Tagging had no detectable effect on the functional properties of wild

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type $P2X_7$ receptors (L-HJ and AS's 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 or HEKA EPC10 amplifier at room temperature as previously described (Jiang et al., 2001). Cells were held at -60 mV. Patch pipettes were fabricated from borosilicate glass capillaries with a resistance of 4-6 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 sulphate 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 (Biologic Science Instruments, France), which has limitations in studying very fast kinetics (Spelta et al., 2002), possibly contributing to the variable rebound currents. All the chemicals were commercially purchased (Sigma).

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) 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) and secondary Alexa Fluor 488 anti-rabbit IgG antibody at a dilution of 1:2000 (Molecular Probes). The images were captured using a Zeiss AxioVert 200M confocal microscope and LSM510 META software.

Data analysis. All data, where appropriate, are presented as mean ± SEM. The EC_{50} values for the agonists BzATP and ATP were determined by least squares curve fitting to the Hill's equation: $I/I_{max} = 1/[1+ (EC_{50}/[A])^n]$, where I is the current as a fraction of the maximum (I_{max}), [A] is the agonist concentration, and n is the Hill coefficient.

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Similarly, the IC_{50} values for the inhibitors zinc and copper were derived by fitting to $I/I_0 = 1/[1+([B]/IC_{50})^n]$, where I is the currents as a fraction of the control current (I_0) in the absence of the inhibitors under examination and [B] is the inhibitor concentration. Of note, in the present study (except Fig.1C), zinc or copper was co-applied 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 (OriginLab, 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 30-50% of maximum current (see below), and three 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 pre-application of zinc for 30-90 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). Interestingly, we observed rebound currents upon simultaneous washout of agonist and zinc irrespective of the duration of pre-application 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 which was probably due to the limited solution exchange rate of the system (see Materials and Methods). However, the presence of

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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 (5 histidines, 3 glutamic acids, 2 aspartic acids and 4 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.

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Effects of zinc. Firstly 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 (discussed later). 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 to 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, [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

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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 (Fig.2A and Fig.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 and 12 functional mutant receptors, using similar protocols 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 rebound currents upon simultaneous washout of BzATP/copper from the wild type or mutant receptors (e.g., Fig.4A and Fig.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 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 of 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 approximate 70%. The offset kinetics of

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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 did not observe any rebound current upon simultaneous washout of ATP with zinc or copper at the wild type or any of the mutant receptors (Fig.6). Similarly to the results obtained using BzATP as a agonist, we found that both zinc and copper inhibition of ATP-evoked currents were potently attenuated or abolished for the [H62A], [D197A] and [H62A/D197A] $P2X_7$ mutant receptors (Fig.6). Additionally, there was a small, nonetheless significant, attenuation of both zinc and copper inhibition of the [H201A] and [H267A] receptors currents (Fig.6), which was not observed when BzATP was used as the agonist (*cf.* Fig.2 and Fig.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 the direct evidence against the long-standing view that divalent cation inhibition of $P2X_7$ receptors results from chelation of ATP^{4-} itself 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 fairly 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. Furthermore, the rebound currents recorded upon simultaneous washout of BzATP and zinc lend further support. Copper inhibition (IC_{50} ~ 2-5 μM) was more potent than zinc inhibition (IC_{50} ~ 5-10 μM), and rebound currents were observed only upon washout of zinc with BzATP but not ATP. The simplest

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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 $P2X_2$ and $P2X_4$ receptor currents (see Introduction). Similar mutational analysis by Hume and his colleagues in the ectodomain of $P2X_2$ receptors has 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 have convincingly demonstrated that these two residues are located closely at the interface between subunits and form an inter-subunit zinc binding site (Nagaya et al., 2005). 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 do (or do not) form inter- or intra-subunit zinc/copper binding sites. Interestingly, 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 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 small

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side-chain containing residue) may to some extent facilitate the channel gating. Consistent with this idea, occupation by divalent cations would disfavour 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 non-histidine residue (Asp^{197}) in coordinating zinc and copper inhibition of the $P2X_7$ receptor, similar to zinc inhibition of the NMDA receptor (Paoletti et al., 2000).

We observed modest, but significant differences depending on the agonists used: Firstly, zinc and copper were 2-3 fold more potent at inhibiting BzATP-evoked currents than ATP-evoked currents. Secondly, 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 the agonist, but in addition we also observed significant attenuation at the [H201A] and [H267A] mutant receptors (*cf.* Fig.2B, Fig.4B and Fig.7). Recently, we identified two residues in the ectodomain that could account for the differential agonist (BzATP vs ATP) sensitivity at the $P2X_7$ receptor. We demonstrated that Lys^{127} and Asn^{284} together are required to maintain BzATP potency, while Asn^{284} alone is involved in ATP potency. We therefore suggest 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 additional residues 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

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$P2X_1$ 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, a paper by Acuna-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* 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. Acuna-Castillo et al showed that the [H201] and [H267A] mutant receptors had either a significant reduction in or loss of inhibition by copper while 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 when compared to [H62A] and [D197A] mutants (the latter mutant was not studied by Acuna-Castillo et al) (Fig.6). The inhibition became variable and insignificantly different from the wild type receptor when BzATP was the agonist (Fig.2B), probably due to the relatively higher potency of zinc and copper (discussed above). 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 Acuna-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

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mammalian cells (e.g., Surprenant et al., 1996), but not in oocytes (Petrou et al., 1997; Klapperstuck et al., 2000). In addition, loss of function associated with a naturally occurring mutation E496A in monocytes and lymphocytes (Gu et al., 2000) 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 tens of micromolar concentrations (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 multi-functional ionotropic purinergic receptor can be activated by agonist, and in particular modulated by trace metals.

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FIGURE LEGENDS

Fig.1 Inhibition of $P2X_7$ receptor currents by zinc and rebound currents

A-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 = 3-9 for each point). Circles are data from the first protocol (IC_{50} = 4.6 ± 0.9 μM, n = 8), squares are data from the second protocol (IC_{50} = 5.9 ± 1.2 μM, n = 4), and triangles are data from pre-application of zinc (IC_{50} = 5.6 ± 1.4 μM, n = 4). These values were not significantly different from each other.

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 to 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.

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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.

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 to 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.

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 to WT. Note the differential inhibition of [H62A] and [D197A] mutant receptors by zinc and copper.

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TABLE 1

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

Receptors	*BzATP EC_{50} (μM)*	*n_H*	*Max currents (nA)*	*Cell no*
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 to the wild type receptor (WT).

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Fig.1

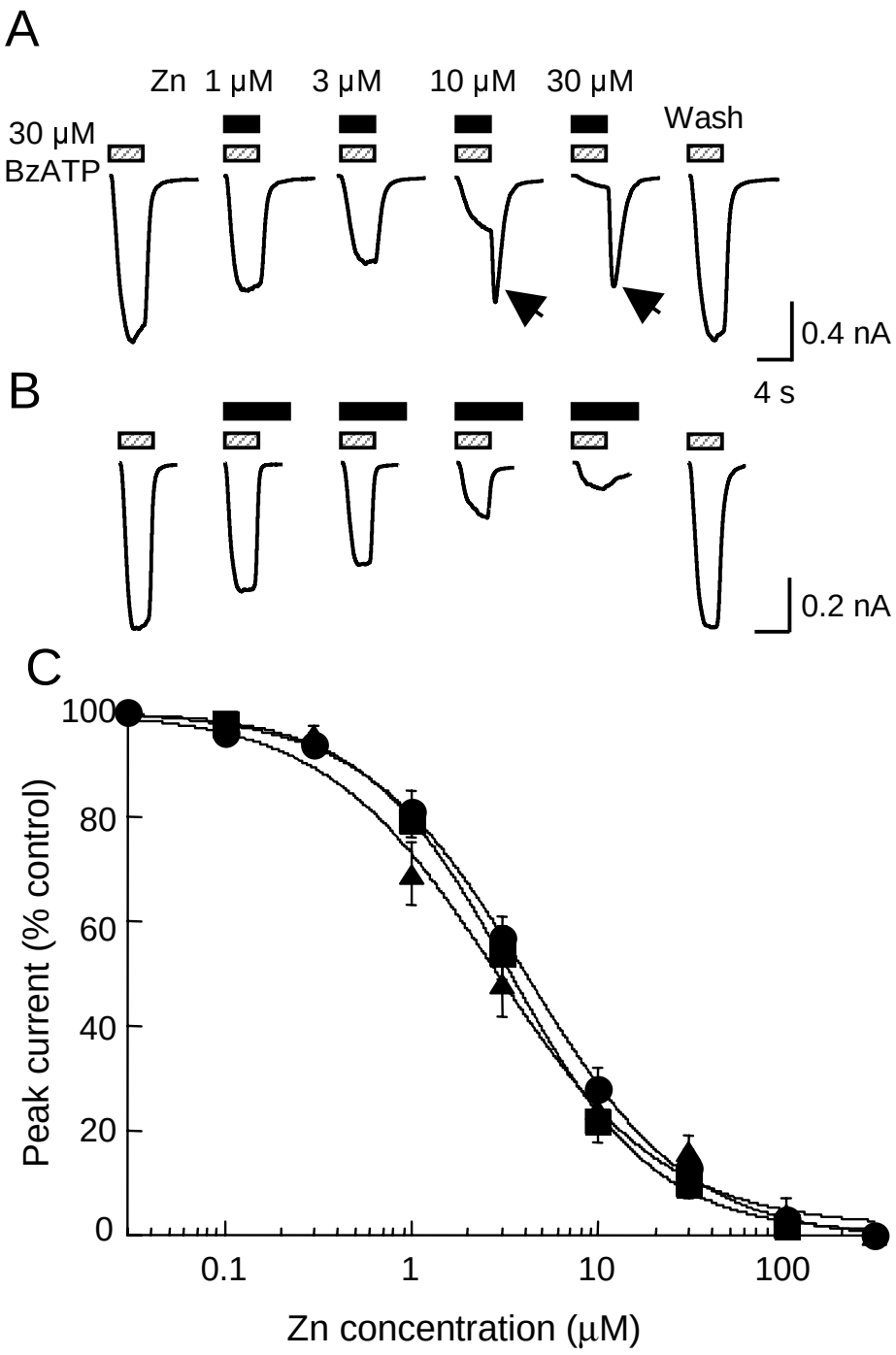

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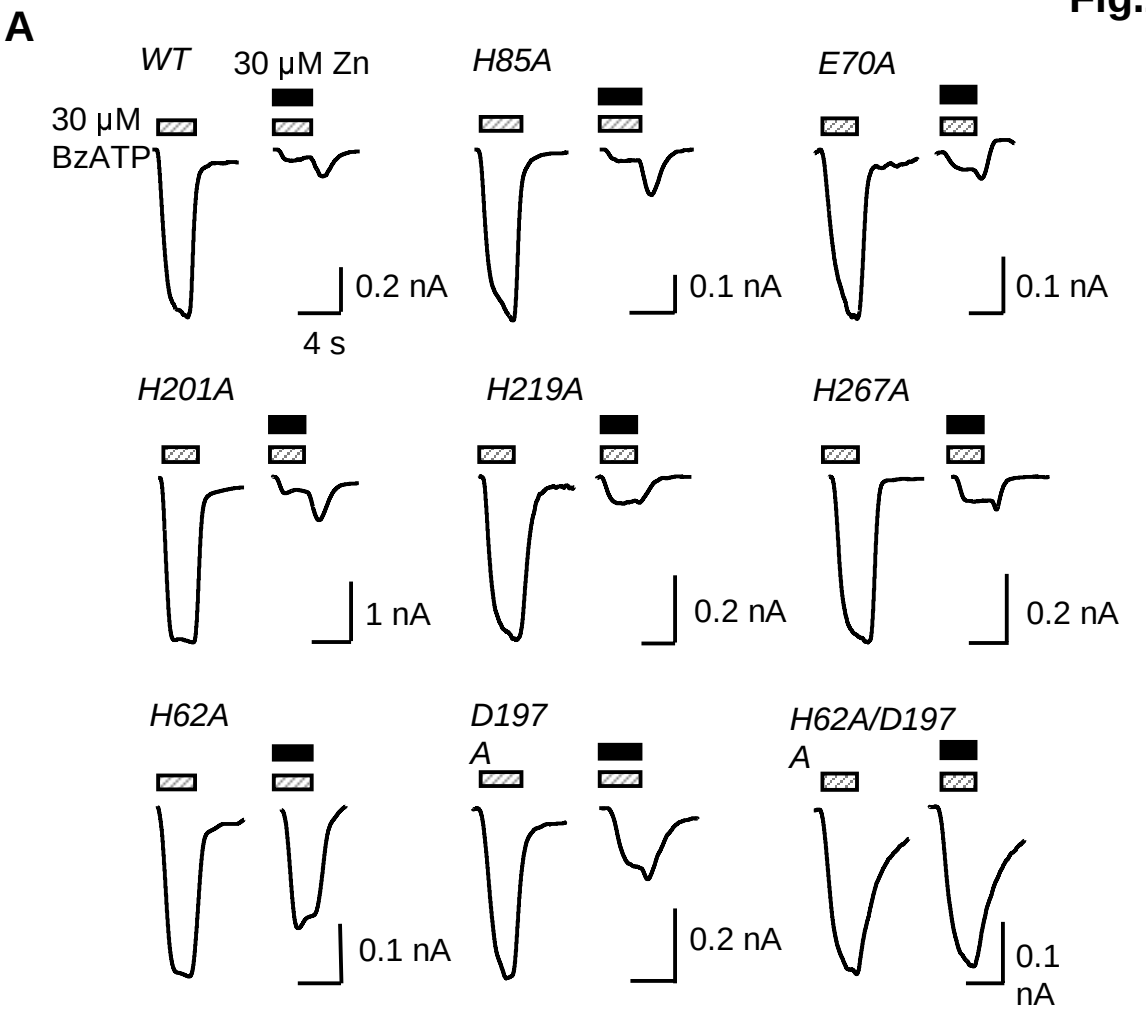
Fig.2



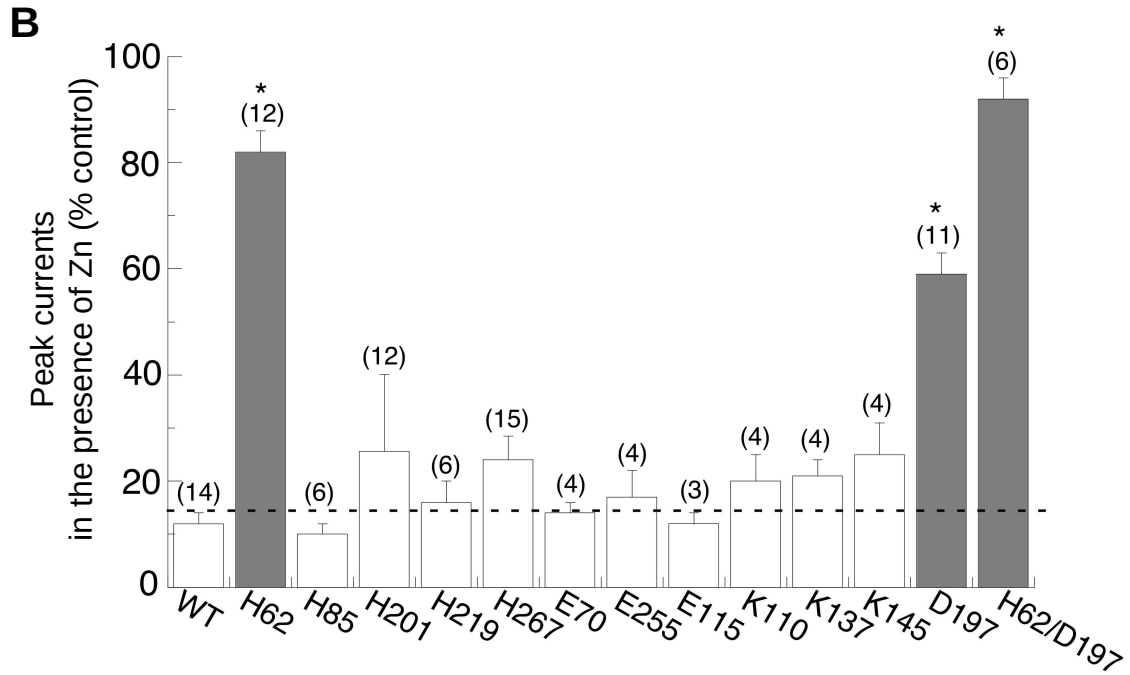


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Fig.3

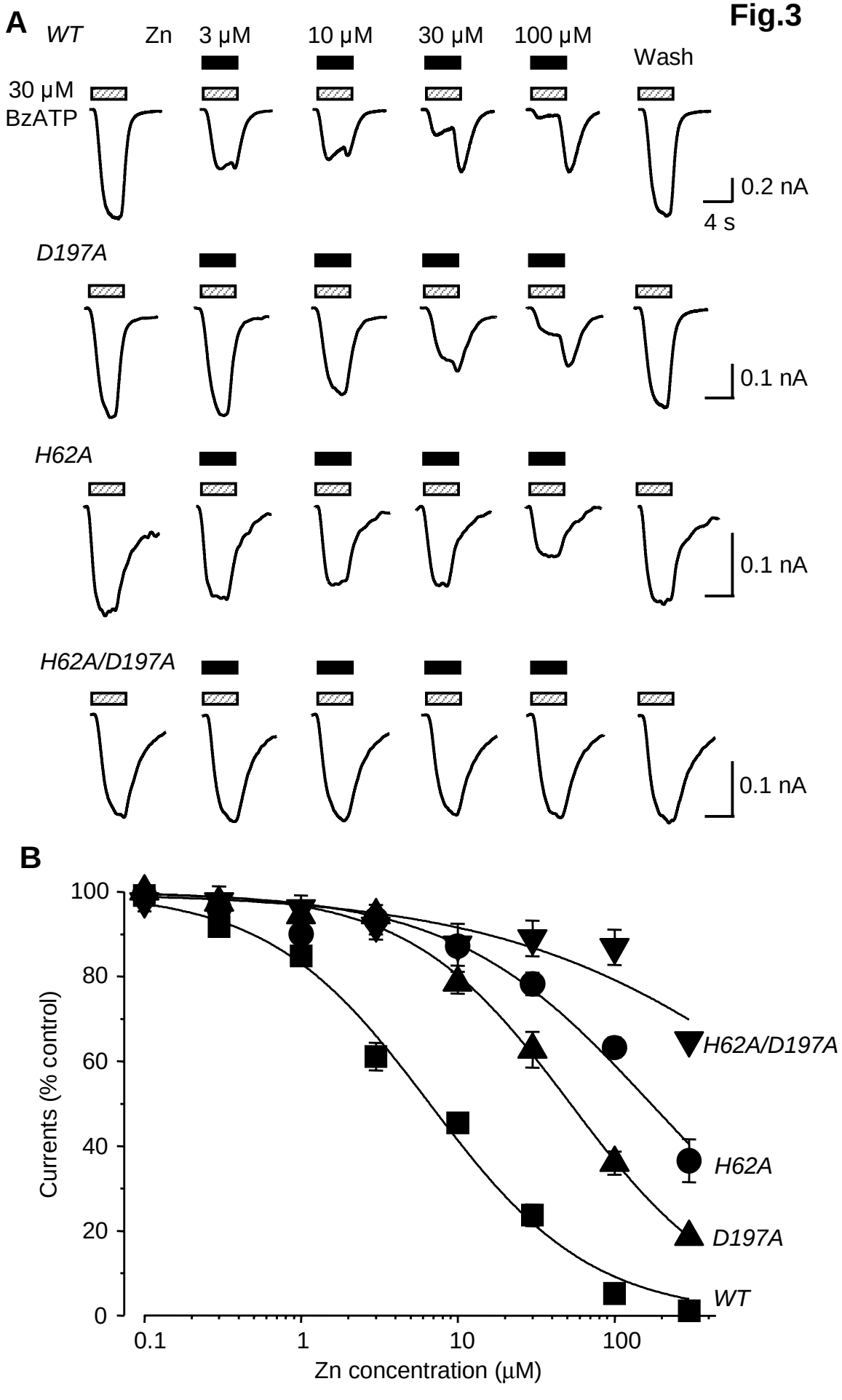

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Fig.4

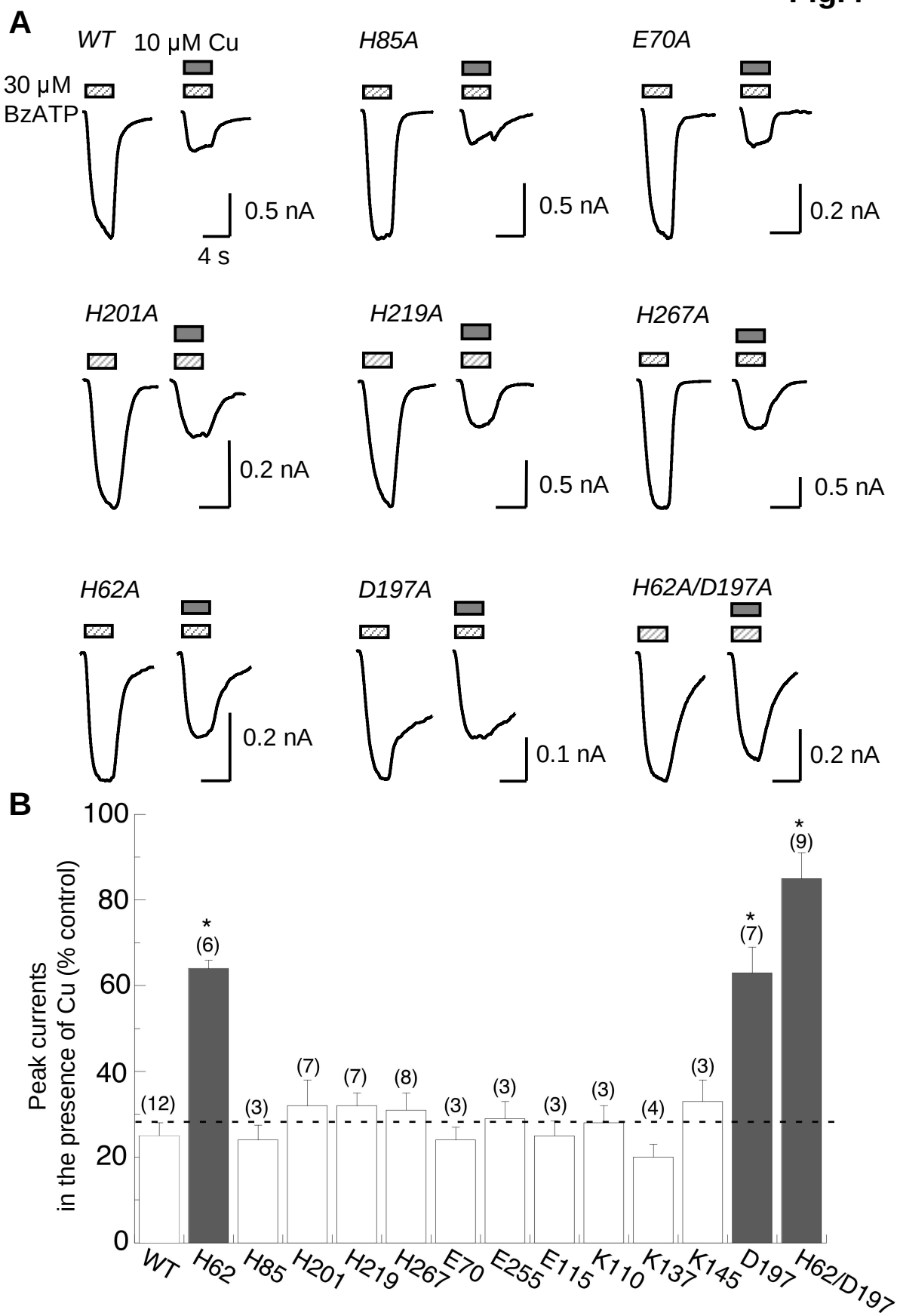

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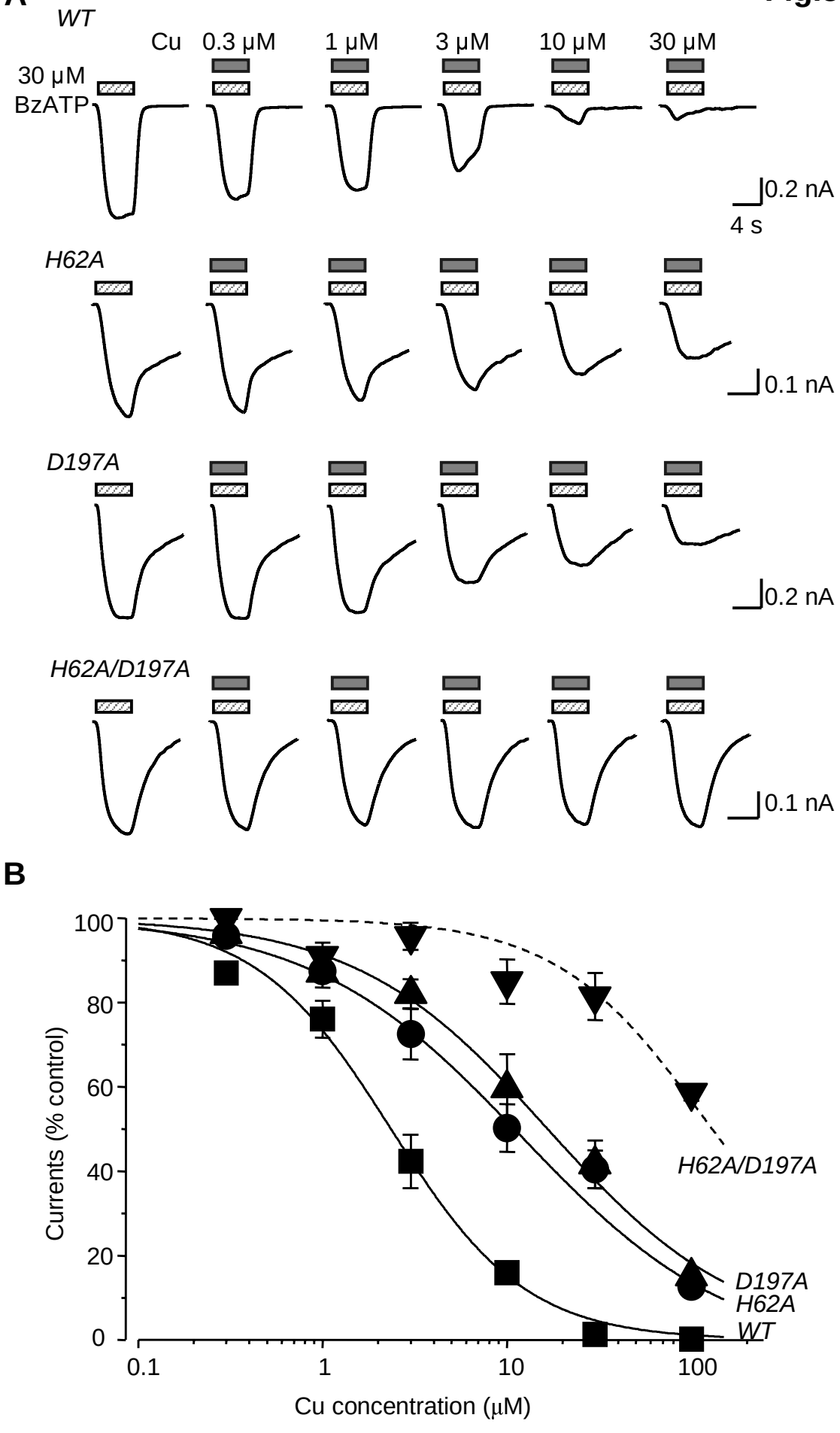
Fig.5



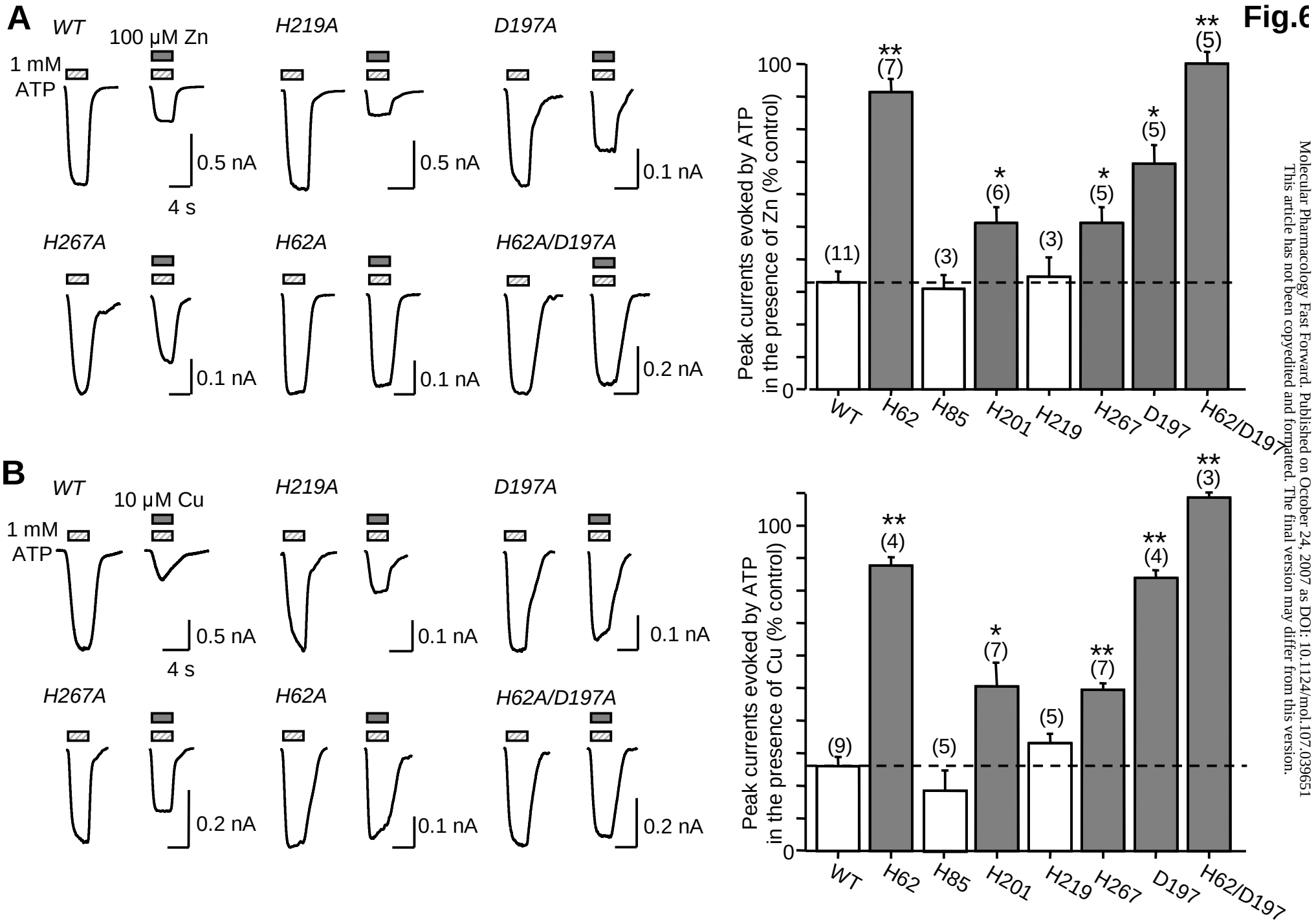


Fig.6