Dissecting the Facilitator and Inhibitor Allosteric Metal Sites of the P2X<sub>4</sub> Receptor Channel

CRITICAL ROLES OF CYS<sup>132</sup> FOR ZINC POTENTIATION AND ASP<sup>138</sup> FOR COPPER INHIBITION<sup>†</sup>

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Zinc and copper are atypical modulators of ligand-gated ionic channels in the central nervous system. We sought to identify the amino acids of the rat P2X<sub>4</sub> receptor involved in trace metal interaction, specifically in the immediate linear vicinity of His<sup>140</sup>, a residue previously identified as being critical for copper-induced inhibition of the ATP-evoked currents. Site-directed mutagenesis replaced conspicuous amino acids located within the extracellular domain region between Thr<sup>123</sup> and Thr<sup>146</sup> for alanines. cDNAs for the wild-type and the receptor mutants were expressed in <i>Xenopus laevis</i> oocytes and examined by the two-electrode technique. Cys<sup>132</sup>, but not Cys<sup>126</sup>, proved crucial for zinc-induced potentiation of the receptor activity, but not for copper-induced inhibition. Zinc inhibited in a concentration-dependent manner the ATP-gated currents of the C132A mutant. Likewise, Asp<sup>138</sup>, but not Asp<sup>131</sup> was critical for copper and zinc inhibition; moreover, mutant D138A was 20-fold more reactive to zinc potentiation than wild-type receptors. Asp<sup>129</sup>, Asp<sup>131</sup>, and Thr<sup>133</sup> had minor roles in metal modulation. We conclude that this region of the P2X<sub>4</sub> receptor has a unique role as a ligand for divalent metals including trace metals although the nature of the modulation and the magnitude of these effects vary among the different P2X subunits (12–17). The role of divalent trace metals as neuromodulators is of interest as zinc and copper are both novel and atypical brain transmitters (18, 19) and novel intracellular second messengers (20). The notion that zinc and copper are stored in neurons and are released upon electrical depolarization further highlights their importance in brain excitability with ample physiological and pharmacological implications (21, 22).

Extracellular ATP and structurally related nucleotides act as novel cell messengers through the activation of P2X receptors, which belong to the family of ligand-gated ionic channels. In addition, nucleotides, and particularly pyrimidine nucleotides, such as UTP and UDP, act on metabotropic P2Y receptors, members of the G protein-coupled receptor family. Seven subtypes of P2X channels have been identified and have been shown to be involved in a variety of neuronal pathways including pain transmission, the urination reflex, vas deferens contraction favoring sperm migration, etc. (1). These receptors are unique among ligand-gated ionic channels because each receptor subunit has only two transmembrane domains, with both the C and N termini facing the cytosol (2, 3). Moreover, recent studies using atomic force microscopy (4) provided topological evidence of the channel conformation and established that the functional P2X receptor channels are trimers, composed of either homo or heterotrimERIC subunits (4–6). Site-directed mutagenesis has provided pivotal information about specific P2X properties: the channel pore, agonist binding residues, receptor desensitization and allosteric modulation (7–16). As with other ligand-gated ionic channels, the P2X receptors are modulated by divalent metals including trace metals although the nature of the modulation and the magnitude of these effects vary among the different P2X subunits (12–17). The role of divalent trace metals as neuromodulators is of interest as zinc and copper are both novel and atypical brain transmitters (18, 19) and novel intracellular second messengers (20). The notion that zinc and copper are stored in neurons and are released upon electrical depolarization further highlights their importance in brain excitability with ample physiological and pharmacological implications (21, 22).

The P2X<sub>4</sub> receptor is an interesting model of an ionic channel differentially modulated by divalent trace metals. In a series of studies, Acuña-Castillo et al. (16) and Coddou et al. (23, 24) reported that zinc potentiated the ATP-evoked currents, whereas copper exerts an inhibitory effect on the activity of this receptor. Furthermore, single site-directed mutagenesis of each of the three extracellular histidine residues of the P2X<sub>4</sub> receptor revealed that only histidine 140 plays a key role in the inhibitory modulation by copper and high zinc concentrations (13). The replacement of His<sup>140</sup> by an alanine (H140A mutant) was not only resistant to the copper-induced inhibition of the ATP-gated receptor activity but evidenced a dramatic increase in the zinc-induced potentiation. Zinc potentiated more than 20-fold the ATP-evoked currents in the H140A mutant; the metal evidenced in this mutant a sigmoid concentration-response dependence instead of the bell-shaped zinc curve described in the wild-type receptor. This finding brought forth the hypoth-
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...that this residue does not form a disulfide bridge with other cysteines as has been suggested for other P2X subtypes. In our view, Cys$^{132}$ plays a relevant role in zinc modulation, and contributes to the molecular basis of trace metal modulation of the P2X$_4$ receptor channel.

EXPERIMENTAL PROCEDURES

ATP tri-sodium salt, hydrogen, penicillin-streptomycin and ivermectin were purchased from Sigma-Aldrich. Copper, zinc, and mercury chlorides were obtained from Merck (Darmstadt, Germany). (2-(trimethylammonium)-ethyl)Methanethiosulfonate bromide (MTSET)$^4$ was obtained from Toronto Research Chemical Inc. (Ontario, Canada). The salts used to prepare the incubation media were purchased from Sigma-Aldrich or Merck. Samples of the triple-distilled water used in buffer preparation, were analyzed for electrode conductivity; metal contamination was less than 0.01 ppm. Trace metal determinations were performed by applying for 10 s, increasing concentrations of the nucleotide ranging between 1 and 1000 μM. Curves were normalized against the concentration of ATP that evoked the maximal response. For metal modulation experiments, at least

4 The abbreviations used are: MTSET, (2-(trimethylammonium)-ethyl)methanethiosulfonate bromide; wt, wild-type; EC$_{50}$, median effective concentration; $n_H$, Hill coefficient; EC$_{SO}$, median inhibitory concentration; $I_{max}$, maximal current.
5 control ATP applications were performed; the average of all the control currents was used as the standard response (100%). This procedure allowed us to determine the variation between the control responses, which never exceeded 10%. The ATP median effective concentration (EC$_{50}$) was interpolated from each concentration-response curve. Likewise, the maximal ATP current ($I_{\text{max}}$) was obtained from each ATP concentration-response curve. Each protocol was performed in at least two separate batches of oocytes from different frogs; each experiment was repeated at least in four separate oocytes. Special care was taken to complete each protocol in a single oocyte; with incomplete protocols being discarded, to favor correct statistical analysis. ATP and metals solutions were prepared daily before usage.

**Metal Characterization Protocols**—These protocols describe the general outline of the experiments performed in wild-type and mutant receptors. Care was exercised to run the whole protocol in a single oocyte allowing each oocyte as its own internal control, particularly when concentration-response curves were performed.

**Metal Selectivity**—We examined the modulator role of copper and zinc chloride salts on the ATP-gated currents of the P2X$_4$ wild-type and mutant receptors. Reversal of the metal effect was mandatory prior to testing other metal concentrations, or examining the effect of another metal in the same oocyte. The recovery of the ATP-evoked currents following additions of the metals was controlled in all cases by the subsequent application of ATP challenges until the full original current was attained.

**Metal and Ligand Concentration Studies**—The metal concentration dependence was assessed by quantifying the ATP-gated currents in the absence and later in the presence of 0.1–300 $\mu$M copper and zinc. In these assays, each oocyte served as its own control; reversal of the metal action was carefully controlled as mentioned above. To examine the action of zinc, experiments used a concentration of the nucleotide that elicits only a 5% of the maximal ATP response (EC$_{5}$), because this concentration was attained. To examine the action of copper, the EC$_{5}$ for the wild-type P2X$_4$ receptor is 1 $\mu$M while for the C132A and D138A mutants this value is 3 $\mu$M. To assess the copper-induced inhibition, these protocols were systematically performed using the EC$_{50}$ for each receptor (see Table 1). These ATP concentrations were established previously as optimal to test the effect of each metal (16).

To investigate how copper or zinc modified the ATP concentration-response curve, ATP concentration-response curves were performed in the absence, and later, in the presence of either 10 $\mu$M copper or 10 $\mu$M zinc. For these protocols, the metals were pre-applied for 1-min and next co-applied with ATP. Sets of at least 4–6 oocytes from separate batches were studied; complete ATP concentration-response protocols were performed per oocyte.

**MTSET Studies**—Consistent with the role of free thiol groups in the extracellular receptor domain, we used a non-permeable SH reactive agent to chemically modify SH groups in the external surface of the receptor domain. Trimethylammoniummethanethiosulfonate (MTSET) was used as a prototype alkylthiosulfonate, an agent that covalently alkylates free thiol groups of cysteine residues. Oocytes were treated with 1 mM MTSET, applied for 3-min period. The effect of zinc, copper, and ivermectin were tested before and after the application of MTSET, in the same oocyte. These protocols were repeated in 4–5 different oocytes from separate oocyte batches.

**Statistical Data Analysis**—Curve fitting was performed with GraphPad software (San Diego, CA). ATP and zinc EC$_{50}$ values, ATP Hill coefficient ($n_H$), copper median inhibitory concentration (IC$_{50}$), and the $I_{\text{max}}$ were obtained from each concentration-response curve; values were derived after adjusting experimental values to a sigmoid curve generated using Graph Pad software (San Diego, CA). Statistical studies included the Mann-Whitney test; we had previously determined the convenience of non-parametric analysis procedures in our statistical evaluations (16).

## RESULTS

**Site-directed Mutagenesis of Critical Amino Acid Residues in the Thr$^{123}$-Thr$^{146}$ Region of the P2X$_4$ Receptor**—To examine the role of key amino acid residues in the vicinity of His$^{140}$, an amino acid previously identified to play a critical role in copper-induced modulation (13), we systematically mutated for alanines selected amino acids localized in the Thr$^{123}$-Thr$^{146}$ region (Fig. 1A). As candidates for mutagenesis we choose the following amino acids: aspartic acids, Asp$^{129}$, Asp$^{131}$, and Asp$^{138}$, commonly described in protein metal-binding motifs and two cysteines, Cys$^{126}$ and Cys$^{137}$, which through their sulfhydryl groups could interact as metals ligands (25), particularly zinc. In addition, we also mutated 3 threonines and a serine; based on a theoretical computational model developed by P. Bull. All these P2X$_4$ receptor mutants were functional; their ATP concentration-response protocols were systematically performed using the EC$_{50}$ for each receptor (see Table 1). These ATP concentrations were established previously as optimal to test the effect of each metal (16).

![Figure 1](image-url)
dramatically modified from a biphasic curve in the wild-type receptor. The zinc concentration-response curve in C132A was further detailed, showing the interaction of this receptor mutant with trace metals. The zinc concentration-response curve in C132A was dramatically modified from a biphasic curve in the wild-type receptor phenotype (Fig. 2A), to a flat curve with negative slope and an estimated IC_{50} of 18.2 ± 10.1 μM, demonstrating the inhibitory activity of zinc. Representative recordings shown in Fig. 2A evidence the zinc-induced reversible inhibition of the ATP-gated currents in oocytes expressing C132A mutant instead of the potentiation observed in the wild-type receptor.

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Table 1 summarizes the main parameters examined in the single mutants tested, along with several double mutations and a triple mutant.

Modulator Effect of Zinc and Copper in Wild-type and the Receptor Mutants—Each mutant was examined independently to evaluate the modulatory activity of 10 μM zinc and 10 μM copper; a summary of these results is shown in Fig. 1, B and C.

Zinc-induced potentiation was completely abolished in the mutant C132A, demonstrating the critical role of this residue for the modulator action of zinc but not copper. The zinc potentiation was also significantly reduced, although not abolished, in the mutant T133A (Fig. 1B, p < 0.01). Additionally, the modulator activity of 10 μM zinc was almost 2-fold larger in mutants D131A and D138A, mimicking the observation previously reported for the H140A mutant (Fig. 1B). In the rest of the mutants examined, the modulator action of zinc was within the experimental variation, not manifesting statistical differences to the wild-type receptor. With regard to the modulator activity of copper, mutant D138A was resistant to the 10 μM copper-induced inhibition of the ATP-gated currents; as indicated above, the modulator action of zinc was significantly augmented 2-fold (Fig. 1B). Mutant D129A demonstrated a significant 50% reduction in copper-induced inhibition (p < 0.05, Fig. 1C), while the modulator activity of zinc was conserved (Fig. 1B). The other mutants examined did not evidence significant deviations from the wild-type phenotype.

The C132A Mutant—In view of the novelty of the results derived from the C132A mutant, and to further confirm and study the nature of its resistance to zinc, we investigated in further detail the interaction of this receptor mutant with trace metals. The zinc concentration-response curve in C132A was dramatically modified from a biphasic curve in the wild-type receptor phenotype (Fig. 2B), to a flat curve with negative slope and an estimated IC_{50} of 18.2 ± 10.1 μM, demonstrating the inhibitory activity of zinc. Representative recordings shown in Fig. 2A evidence the zinc-induced reversible inhibition of the ATP-gated currents in oocytes expressing C132A mutant instead of the potentiation observed in the wild-type receptor.

**Table 1**

| Receptor          | EC_{50} (μM) | n_{H} | I_{max} (μA) |
|------------------|--------------|-------|-------------|
| P2X_{4} wild-type | 11.4 ± 2.8   | 1.4   | 4.9 ± 0.8   |
| T123A            | 22.3 ± 6.4   | 1.2   | 3.3 ± 0.6   |
| S124A            | 24.6 ± 4.8   | 1.0   | 3.3 ± 0.7   |
| CI26A            | 15.4 ± 4.6   | 1.9   | 9.0 ± 2.8   |
| D129A            | 33.3 ± 8.6   | 1.4   | 3.4 ± 0.9   |
| D131A            | 17.5 ± 6.4   | 1.2   | 6.4 ± 1.7   |
| CI32A            | 36.1 ± 13.2  | 1.3   | 3.4 ± 1.6   |
| T133A            | 13.3 ± 3.7   | 0.9   | 4.1 ± 1.3   |
| D138A            | 36.8 ± 6.5   | 0.9   | 6.2 ± 1.8   |
| D138N            | 19.1 ± 8.7   | 1.1   | 5.6 ± 2.2   |
| H140A            | 61.2 ± 9.5   | 1.4   | 4.3 ± 0.6   |
| T146A            | 29.0 ± 7.0   | 1.7   | 2.8 ± 1.1   |
| T123/T146        | 13.7 ± 2.3   | 1.4   | 4.7 ± 0.8   |
| S124/T146        | 19.2 ± 13.2  | 0.8   | 2.7 ± 1.0   |
| T123A/S124A/T146A| 24.6 ± 4.1   | 0.9   | 4.5 ± 0.9   |

*p < 0.05, compared with the values obtained with the wild-type P2X_{4} receptor.

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A 1-min pre-application of 10 μM zinc in the C132A mutant inhibited the 3 μM ATP-evoked currents; the inhibitor action of zinc was concentration-dependent (Fig. 2B). In further proof of the two independent sites of metal action in the P2X_{4} receptor, the C132A mutant showed a copper inhibition curve identical to the wild-type phenotype (Fig. 2C). Consonant with these results, 10 μM zinc did not modify the ATP concentration-response curve, while 10 μM copper inhibited non-competitively the ATP curve, much as in the wild-type receptors (Fig. 2D). Interestingly, the C126A mutant showed a wild-type phenotype to the modulation by zinc, evidencing the classical biphasic zinc interaction curve (Fig. 2C) or the non-competitive copper-induced inhibition (data not shown). Taken together, these results allow the conclusion that Cys^{132} is critical for zinc-induced potentiation, but not for copper inhibition.

Based on the notion that Cys^{132} is part of the zinc-facilitator site, we reasoned that the facilitator action of zinc would be eliminated, at least in part, by reagents such as MTSET, which alkylate free sulfhydryl groups. Oocyte treatment with MTSET halved significantly the maximal zinc-induced potentiation from 5.9 ± 0.4- to 3.1 ± 0.2-fold (p < 0.01) in wild-type P2X_{4} receptors without altering the magnitude of the ATP-evoked currents (Fig. 3A). The ATP IC_{50} and maximal response after MTSET-treatment were 19.8 ± 4.0 μM and 6.4 ± 1.9 μA respectively (n = 5, data not shown). MTSET treatment was irreversible; 45 min after the treatment we consistently observed the reduction of the zinc-induced potentiation. As a
further control for this set of experiments, MTSET treatment did not modify the ivermectin-induced potentiation (Fig. 3B) or copper inhibition (Fig. 3C). 3 μM IVM potentiated 3.7 ± 0.6, indicating that this agent has a smaller potentiation than that of zinc. After MTSET treatment, the IVM potentiation was 4.4 ± 1.4-fold, a value that did not differ from the non-treated oocytes. These results indicate that the chemical modification of the sulphydryl of extracellular cysteines affected exclusively the zinc-induced modulation, but not the copper-induced modulation.

The D138A Mutant—This mutant was copper-resistant, no significant inhibition was observed with metal concentrations up to 100 μM (Fig. 4B); in contrast, 10 μM copper elicited 75% current inhibition in the wild-type receptors. Further increasing the copper concentration to 300 μM, inhibited the currents ∼40%. Representative tracings illustrate the inhibitory modulation of 10 μM copper in oocytes transfected with either wild-type or the Asp<sup>138</sup> mutant (Fig. 4A). Consonant with the finding reported for the H140A mutant (13), which was also shown to be resistant to the action of copper, the zinc concentration-response curve was dramatically modified from a biphasic curve in wild-type receptors, to a sigmoid, in the D138A mutant (Fig. 4C). The maximal zinc-evoked potentiation in the mutant was at least 3-fold larger than in wild-type receptors (19.0 ± 2.1- versus 6.3 ± 0.7-fold increase, p < 0.01, n = 6). Moreover, when the ATP concentration-response curve was examined in the presence of 10 μM copper, the curve in the D138A mutant, as anticipated, was not shifted as compared with the wild-type receptors (36.8 ± 6.5 versus 29.5 ± 2.9 μM, Fig. 4A and B). As expected, 10 μM zinc displaced leftward the ATP concentration-response curve, reducing 3-fold the ATP EC<sub>50</sub> from 36.8 ± 6.5 to 12.0 ± 5.1, p < 0.05, Fig. 4D).

Moreover, in a further set of experiments we examined the consequence of replacing the negatively charged aspartic acid residue with asparagine (D138N) rather than the previously examined alanine. This approach partially recovered the ability of copper to inhibit the ATP-evoked currents; the copper IC<sub>50</sub> in this mutant was 22.4 ± 5.5 as compared with 5.4 ± 0.8 μM in the wild-type receptor (p < 0.05, Fig. 4B).

Relative Influence of Other Amino Acids in the Trace Metal Modulation—Besides C132A, other mutants that showed significant differences in the zinc-evoked potentiation were D131A and T133A (see Fig. 1B). Zinc concentration-response protocols revealed that both mutants conserved the biphasic metal concentration curve phenotype observed in wild-type receptors, although the maximal potentiation evoked by 10 μM zinc was 2-fold in the case of mutant D131A (p < 0.05) and decreased by half in the mutant T133A (p < 0.05; Fig. 5A). The D129A, but not the D131A mutant, showed a significant parallel rightward displacement of the copper concentration-response curve; its copper IC<sub>50</sub> was 24.9 ± 0.8 μM (p < 0.05) as compared with the wild-type receptor (IC<sub>50</sub> = 4.8 ± 0.8 μM, n = 5). The estimated copper IC<sub>50</sub> for the D138A mutant was 8.3 ± 2.9 μM (Fig. 5B).

DISCUSSION

A major aim of this investigation was to identify structural determinants of allosteric binding sites for trace metals in the
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**FIGURE 5. Metal modulation on the D129A, D131A, and T133A mutants.**
A, zinc concentration-response curves for the D129A (closed squares), D131A (open circles), and T133A (open triangles) mutants; the dashed line represents the curve for wild-type receptors. The maximal zinc-induced potentiations were 10.3 ± 0.5 (D131A), 4.0 ± 0.6 (D129A), and 3.5 ± 0.8 (T133A)-fold increase. B, copper concentration-response curves for the D129A (closed squares) and the D131A (open circles) mutant receptors. The dashed line represents the curve for wild-type receptors; the estimated IC$_{50}$ values were 24.9 ± 4.4 μM for D129A and 8.3 ± 2.9 μM for D131A, n = 4–6.

P2X$_4$ receptor. We now report that Cys$^{132}$ is essential for the potentiation by zinc, while Asp$^{138}$ is necessary for copper and zinc inhibition. Conceptually, the interpretation of the results presented herein demonstrate that this receptor has two separate and independent allosteric sites for divalent trace metal modulation, which are localized in the extracellular domain. One is a facilitator site, involved in the action of zinc, which results in a potentiation of the ATP-gated currents and a distinct, inhibitor site, which interacts with copper, and large concentrations of zinc, accounting for a non-competitive inhibition of ATP-evoked currents. The emerging picture for the extracellular metal allosteric sites of the P2X$_4$ receptor is schematized in Fig. 6. Because trace metal coordination with proteins involves several amino acids (25, 26), we focused on identifying amino acid residues other than the previously identified His$^{140}$ (13).

Functional tests revealed that of the amino acids in the 123–146 sequence, only Cys$^{132}$ proved essential for zinc modulation, because its replacement for an alanine abrogated the metal modulation. Because the C132A mutant lacks an essential zinc ligand to interact at the facilitator site, the metal can now only act at the inhibitor site, an explanation that can account for the reduction of the ATP-gated currents. Critical to this interpretation and as a control of this experimental design, mutant C126A showed essentially a wild-type phenotype, demonstrating the topological relevance of the sulfhydryl group in Cys$^{132}$ at the zinc facilitator site. In contrast to the P2X$_4$ receptor, two extracellular histidines (His$^{120}$ and His$^{141}$) were identified as critical for zinc potentiation in the P2X$_4$ receptor (12, 14), accentuating the diversity the metal binding sites in these proteins. Moreover, in the P2X$_4$ receptor an intersubunit zinc binding site with moderate flexibility has been proposed (27, 28). Of the three extracellular histidines of the P2X$_4$ receptor, His$^{140}$ is part of the inhibitor site (13), His$^{141}$ is not involved in trace metal modulation (13) and His$^{246}$ is involved in proton sensing (9). Therefore, we anticipated that residues other than histidines, like Cys$^{132}$, conform the zinc facilitator site in the P2X$_4$ receptor. The two amino acids in the immediate vicinity of the primary sequence of the receptor, Asp$^{131}$ and Thr$^{133}$, modified zinc potentiation but did not suppress it, most likely indicating that they are indirectly involved in zinc potentiation.

The structural conservation of 10 cysteine residues in the extracellular loop of the P2X receptors (29) has led to the speculation that these amino acid residues are likely to be involved in disulfide bond formation. To address directly the role of disulfide bonds in the P2X$_4$ receptor, Ennion and Evans generated receptor mutants, which replaced each of the 10 extracellular cysteines with alanine and examined the resulting effects on channel function using electrophysiology and biochemical methods (30). In most of the single mutants, modest changes were observed in the ATP potency and maximal response. The C126A and C132A mutants were both functional and possessed similar properties as compared with the wild-type receptor. However, Ennion and Evans (30) labeled extracellular free cysteines with MTSEA-biotin, to confirm that the 10 cysteines of the P2X$_4$ receptor must be forming disulfide bonds (30). In the P2X$_2$ receptor, the corresponding Cys$^{124}$ (C126) and Cys$^{130}$ (C132) mutants resulted in a dramatically reduced EC$_{50}$ and I$_{max}$, indicating a structural role for these residues (31). As with the P2X$_2$ receptor, our results on the P2X$_4$ receptor also indicate that the replacement of either Cys$^{126}$ or Cys$^{132}$ for alanine

**FIGURE 6. Schematic model shows the potential extracellular metal sites within the P2X$_4$ receptor.** Left panel, in the facilitator site, the sulfhydryl group of Cys$^{132}$ is crucial for zinc-induced potentiation of the receptor activity. The neighboring residues, Asp$^{131}$ and Thr$^{133}$ are not critical, but could also contribute to the site stability. Right panel, at the inhibitor site, the carboxylic group of Asp$^{138}$ and His$^{140}$ are crucial for copper and zinc inhibition of the receptor activity, while Asp$^{129}$ may contribute to a lesser degree.
resulted in only minor variations in the ATP potency (Table 1), although mutant C132A was completely insensitive to zinc potentiation. Therefore, we deem that the sulfhydryl group of Cys$^{132}$ acts as a zinc ligand, critically necessary for the metal potentiation. This hypothesis is further supported by the observation that MTSET significantly reduced zinc-induced potentiation, without altering the ATP-gated currents. Altogether, these data indicate that the sulfhydryl group of Cys$^{132}$ is not involved in disulfide bonding. Moreover, since MTSET treatment did not modify copper inhibition or the ivermectin-induced potentiation, we also conclude that the facilitator site for zinc is distinct from the copper inhibitor site, as predicted in our working hypothesis. Ivermecitin is an antiparasitic drug derived from Streptomyces avermitilis, which facilitates the ATP-gated currents exclusively of the P2X$_4$ receptor (32), acting at residues located near the transmembrane domains (11).

Treatment with dithiothreitol, a disulfide reducing agent neither significantly altered the ATP-evoked currents nor zinc modulation (data not shown). Although it was previously reported that reducing agents such as dithiothreitol or β-mercaptoethanol did not alter the pharmacology of the P2X$_4$ or the P2X$_2$ receptors (30, 31), this result may be accounted for the relative inaccessibility of these reagents to several sulfhydryl groups within these receptors.

The present study also identified Asp$^{138}$ as part of the inhibitor site. This residue and His$^{140}$ (13) are two essential ligands for copper and zinc coordination in the extracellular domain of the P2X$_4$ receptor. The finding that the zinc concentration-response curve in the D138A mutant was sigmoid, raised a second argument for a dual action of zinc in both modulator sites, although demonstrating preference for the facilitator site at lower metal concentrations. In the absence of the inhibitor site, as in the D138A or the H140A mutants, zinc interacts only with the facilitatory site, accounting for the large facilitation of the ATP-evoked response observed in these mutants. Interestingly, the replacement of Asp$^{138}$ by an asparagine resulted in only a partial recovery of copper inhibition, suggesting that a more conservative change could retain the function, as other residues also account for the metal effect, in this case, His$^{140}$.

Additionally we identified other amino acids that could play secondary roles in trace metal modulation. This may be the case with Asp$^{131}$, Thr$^{133}$, and Asp$^{129}$. Mutations of these residues either increase or decrease the maximal zinc potentiation (D131A, T133A) or decreased the copper potency (D129A) without changing the biphasic nature or the concentration-dependent inhibition.

Parallel studies from our laboratory have demonstrated the critical role of extracellular histidines in metal modulation and identified which of these residues are essential for copper and zinc modulation in the P2X$_2$ and the P2X$_4$ receptors (14, 15). Unfortunately the key residues identified as essential for copper and zinc modulation in these three P2X receptor channels are not linearly related. Therefore a better understanding of the three-dimensional structure of the receptor is essential to better define the structural determinants involved in the modulation by trace metals. The recent finding of an intracellular P2X receptor in the amoeba Dictyostelium discoideum and the elucidation of a new functional role for P2X receptors on intracellular organelles in osmoregulation, which was inhibited by nanomolar copper (33) highlights the importance of trace metals as modulators of the biology of P2X receptors.

Trace metals like zinc and copper are known to modulate voltage (34) and ligand-gated ionic channels including the glycinergic, N-methyl-D-aspartate, γ-aminobutyric acid, and nicotinic receptors (35–39). Because zinc and copper are stored in vesicles and are released upon nerve terminal depolarization (21, 22), reaching up to micromolar concentrations in the synapse, we hypothesize that trace metals may contribute to brain excitability. In this context the pioneer work of Hirzel et al. (40) highlights the role of trace metals in a recent in vivo study using transgenic mice. These authors found several abnormalities in mice carrying the glycine receptor mutation D80A; a substitution that selectively eliminated the potentiating effect of zinc on this receptor. This mutated glycine receptor was functional and demonstrated a wild-type phenotype, except for the response to zinc. Notwithstanding, the glycinerergic transmission was completely abnormal, demonstrating a series of behavioral abnormalities reinforcing that zinc is crucial for glycinerergic transmission (40). Altogether these reports underscore the significance of trace metals in brain excitability and highlight their role as novel and atypical brain messengers (18).

In summary these studies provides novel structural information for the role of amino acids involved in trace metal modulation. The identification of key amino acids selectively involved in modulation by either zinc or copper lay the structural foundations necessary to better understand the molecular basis of ionic channel modulation and its role in brain excitability. It has not escaped our attention that, in the near future, transgenic mice carrying selected mutations described in this study might be useful for testing the role of trace metals in neural transmission.

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