Histidine 140 Plays a Key Role in the Inhibitory Modulation of the P2X$_4$ Nucleotide Receptor by Copper but Not Zinc*

Claudio Coddou‡, Bernardo Morales‡‡, Jorge González‡, Marta Grauso‡, Felipe Gordillo‡, Paulina Bull‡, François Rassendren‡, and J. Pablo Huidobro-Toro‡‡

From the ‡Centro de Regulación Celular y Patología J. V. Luco, Instituto Milenio Biología Fundamental y Aplicada, MIFAB, Departamentos de Fisiología y Genética Molecular y Microbiología, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Santiago 1, Chile, §Departamento de Ciencias Biológicas, Facultad de Química y Biología, Universidad de Santiago de Chile, Santiago 1, Chile, and ¶Institut de Genetique Humaine, UPR 1142 CNRS, 141 rue de la Cardonille, 34396 Montpellier, France

To elucidate the role of extracellular histidines in the modulation of the rat P2X$_4$ receptor by trace metals, we generated single, double, and triple histidine mutants for residues 140, 241, and 286, replacing them with ala- nines. cDNAs for the wild-type and receptor mutants were expressed in Xenopus laevis oocytes and in human embryonic kidney 293 cells and examined by the two electrode and patch clamp techniques, respectively. Whereas copper inhibited concentration-dependently the ATP-gated currents in the wild-type and in the single or double H241A and H286A receptor mutants, all receptors containing H140A were insensitive to copper in both cell systems. The characteristic bell-shaped concentration-response curve of zinc observed in the wild-type receptor became sigmoid in both oocytes and human embryonic kidney cells expressing the H140A mutant; in these mutants, the zinc potentiation was 2.5–4-fold greater than in the wild-type. Results with the H140T and H140R mutants further support the importance of a histidine residue at this position. We conclude that His-140 is critical for the action of copper, indicating that this histidine residue, but not His-241 or His-286, forms part of the inhibitory allosteric metal-binding site of the P2X$_4$ receptor, which is distinct from the putative zinc facilitator binding site.

The notion that trace metals such as zinc or copper are atypical brain messengers has attracted much attention in view of their emerging role in the modulation of brain excitability (1). The importance of trace metals in synaptic activity is highlighted by the description that both copper and zinc are stored in synaptic vesicles from where they are released by electrical depolarization, reaching a high micromolar concentration at the synaptic cleft (2–4). Trace metals are known to modulate a wide variety of brain ionotropic receptors such as glycine, N-methyl-D-aspartate, γ-aminobutyric acid, nicotinic, and the novel nucleotide receptors (P2X) family (5–9). The P2X purinoceptors are homomeric or heteromeric membrane channels gated by extracellular ATP and related synthetic nucleotides; North (10) recently reviewed the principles of the molecular physiology of this family of receptors. Within the P2X receptor family, the P2X$_4$ is the most widely distributed in the central nervous system, including the cerebellum and the CA1 region of the hippocampus, where it has been proposed to play a role in glutamatergic synapses (11).

The P2X$_4$ receptor is an interesting model of an ionic channel differentially modulated by trace metals. Acuña-Castillo et al. (12) and Coddou et al. (13) reported that zinc potentiates the ATP-evoked currents whereas copper has an inhibitory effect on the activity of this receptor. Based on these findings, Acuña-Castillo et al. (12) proposed that trace metals modulate the activity of the P2X$_4$ receptor via two separate metal-binding sites. One of these sites has a preferential selectivity for copper and is characterized by a non-competitive inhibition of the ATP-gated channel activity. The second site shows preference for zinc and apparently leads to an increase in affinity for the binding of ATP. Copper and zinc also modulate other members of the P2X family of receptors, but their effects depend on the receptor subtype. For example, both zinc and copper facilitate the ATP-evoked responses on the P2X$_2$ receptor (14, 15) whereas in the P2X$_7$ receptor, copper inhibits the ATP-gated currents, whereas zinc is virtually without effect (13). These findings further support the notion that the P2X purinoceptors may have separate metal-binding sites to modulate the channel activity.

This research was aimed at identifying structural determinants for the binding of copper and to assess the hypothesis that copper and zinc bind to separate, independent metal-binding sites in the receptor. Based on the notion that histidine residues are commonly found in consensus copper-binding motifs (16), as extensively studied in superoxide dismutase, where the imidazole ring is essential to coordinate copper (17), and both histidines and cysteines have been defined in zinc-binding motifs (18), we were challenged to clarify the role of the three histidines of the rat P2X$_4$ receptor in the modulatory action of trace metals. Interestingly, the three rat P2X$_4$ histidine residues are located extracellularly. To tentatively identify putative residues involved in the copper inhibitory modulation, we preliminarily used chemicals to selectively alkylate histidine and cysteine residues. Next, we performed site-directed mutagenesis to create single, double, and triple mutants where the histidine residues (His-140, His-241, and His-286) of the receptor were replaced by alanines. The cDNAs coding for the wild-type and mutated receptors were expressed in two cell systems; the modulation by copper or zinc was examined in either Xe-
Expression of P2X<sub>4</sub> Receptors and Electrophysiological Recordings from *X. laevis* Oocytes

A segment of the ovary was surgically removed under anesthesia with *X. laevis* frogs; oocytes were manually defolliculated and next incubated with collagenase as detailed previously (12). Oocytes were injected intranuclearly with 3–5 ng of cDNA coding for either the wild-type or H140A, H241A, and H286A P2X<sub>4</sub> receptor proteins. After 36–48 h of incubation at 15 °C in Barth's solution (NaCl, 88 mM; KCl, 1 mM; NaHCO<sub>3</sub>, 2.4 mM; HEPES, 10 mM; MgSO<sub>4</sub>, 0.82 mM; Ca(NO<sub>3</sub>)<sub>2</sub>, 0.33 mM; Ca<sub>Cl</sub><sub>2</sub>, 0.91 mM; pH 7.5) containing 10 IU/liter penicillin/10 mg streptomycin and 2 mM pyruvate. Oocytes were incubated with collagenase as detailed previously (12). Oocytes were perfused with low ATP concentrations, which induce 5% of the maximal nucleotide concentration—ATP (0.1–3 μM) was used to evaluate the facilitator action of zinc in the H140A and H286A mutants. These concentrations of ATP were chosen based on previous findings (12) when we reported that the largest zinc potentiation was attained with low ATP concentrations, which induce 5% of the maximal nucleotide-evoked current (EC<sub>50</sub>). In all cases, ATP applications were spaced at regular 10-min intervals, minimizing desensitization and after attaining full reversibility of the zinc effect. The effect of zinc in the double or triple mutants was assessed likewise.

**ATP Concentration-response Protocols**

To study with more detail the mechanism of trace metal modulation, we next assessed whether the histidine residues with DEPC altered the inhibitory action of copper or the facilitator action of zinc. After routine applications of 10 μM ATP and testing of the inhibitory action of 10 μM copper or the facilitator action of 10 μM zinc, oocytes were treated for 5 min with 500 μM DEPC to modify the histidine residues. After this treatment, oocytes were perfused for 20–30 min to wash out the remaining DEPC prior to challenges with ATP or the metals. ATP concentration-response protocols (1–1000 μM) of ATP alone or ATP plus copper (1 min pre-incubated) were performed in separate oocytes. The ATP concentration-response curve was normalized against 500 μM ATP, a value close to the maximal current. The current gated by 500 μM ATP was obtained prior to the treatment with DEPC and the copper. Similar protocols were performed in separate oocytes using 300 μM NEM to selectively alkylate cysteine residues. As in the case of the DEPC-treated oocytes, oocytes were washed for 20–30 min from the residual chemical prior to testing with ATP and the trace metals. Results were normalized, as in the case of the DEPC treatment, against a standard of ATP obtained prior to applying the alkylating agent.

**Trace Metal Concentration-response Curves**

To examine the effect of copper, 0.1–600 μM of the metal was pre-applied for 1 min before a 15-s ATP pulse, at a concentration close to the receptor EC<sub>50</sub>, which in the wild-type receptor was 10 μM ATP. Care was taken to achieve full reversibility of the copper effect prior to testing with ATP. The median inhibitory concentration of copper (IC<sub>50</sub>) was interpolated from each metal concentration-response curve. A single oocyte was tested with at least four increasing concentrations of copper. If the relative potency of ATP was modified in the mutant receptors, the ATP concentration was adjusted to a value close to its EC<sub>50</sub> (60 μM ATP was used to evaluate the H140A mutant receptor, and 3 μM ATP was used for the H286 mutant). This protocol was also used to evaluate the potency of copper in the double or triple histidine mutants or the mutants that substituted His-140 by either threonine or arginine.

Parallel experiments were performed to assess whether the histidine mutants affected the facilitator action of zinc. In these protocols, the concentrations of ATP were applied 1 min prior to a challenge with 1 μM zinc to study whether zinc could affect the wild-type P2X<sub>4</sub> receptor or the H241A mutant. 3 μM ATP was used to evaluate the facilitator action of zinc in the H140A and H286A mutants. These concentrations of ATP were chosen based on previous findings (12) when we reported that the largest zinc potentiation was attained with low ATP concentrations, which induce 5% of the maximal nucleotide-evoked current (EC<sub>50</sub>). In all cases, ATP applications were spaced at regular 10-min intervals, minimizing desensitization and after attaining full reversibility of the zinc effect. The effect of zinc in the double or triple mutants was assessed likewise.

**ATP-gated Currents from the Whole Cell Configuration**

Transfection of HEK 293 Cells and the Recording of the ATP-gated Currents from the Whole Cell Configuration

HEK 293 cells were transiently transfected with 1 μg of cDNA coding for the wild-type, H140A, H241A, or H286A P2X<sub>4</sub> receptor protein. Cells grown to 50% confluence on 35-mm culture dishes were incubated with
cDNA mixed with 8 μl of LipofectAMINE in 1 ml of serum-free medium (Opti-MEM). After 3 h at 37 °C, the medium was replaced with Dulbecco’s modified Eagle’s medium, and 48 h later the cells were transferred to the experimental chamber containing recording buffer, which contained the following (in mM): 150 NaCl, 1 CaCl₂, 1 MgCl₂, 10 glucose, 10 HEPES, pH 7.3. Whole-cell ATP-dependent currents were obtained from single HEK 293 cells using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA). Patch pipettes (2–4 megohm) were filled with the following (in mM): 150 CsCl, 10 tetraethyl ammonium chloride, 10 EGTA, 10 HEPES, pH 7.3, and 275–285 mosM; pH was adjusted with CsOH. The junction potential (typically <5 mV) was compensated. Only cells with membrane potential more negative than −55 mV, access resistance <10 megohm (compensated at 80%), and input resistance >100 megohm were tested. The cells were discarded if the input or access resistance changed >0%. The ATP-dependent currents were recorded at a holding potential of −80 mV to augment the driving force of the ions that mediate the ATP-evoked currents and to avoid contamination of voltage-dependent currents. Responses were digitized at a frequency of 10 KHz and analyzed using the pCLAMP 8 system (Axon Instruments, Foster City, CA). All the experiments were carried out at room temperature.

Recording of ATP-evoked Currents: Effect of Trace Metals

ATP-dependent currents were evoked with 5-s ATP pulses applied by superfusion, using a peristaltic pump (Masterflex Pump; Cole Palmer Instruments). ATP concentration-response protocols were performed to derive the EC₅₀ by applying 0.1 to 500 μM pulses of ATP for 5 s; between ATP applications, the cells were washed for 5 min with drug-free buffer. To minimize desensitization because of the larger concentrations of ATP (30 μM or more), these applications were spaced 10 min. To minimize desensitization because of the larger concentrations of ATP (30 μM or more), these applications were spaced 10 min. The ATP EC₅₀, I_max, and n⁰ were obtained from each ATP concentration-response plot by fitting the data using the Hill equation. Full concentration-response curves were derived from single cells; protocols were repeated using several batches of cells.

To study the modulation of the ATP-evoked currents by the metals, separate cell batches expressing the wild-type or the mutant receptors were pre-incubated with 1–300 μM copper or 1–100 μM zinc for 1 min before the addition of a challenge concentration of 10 μM ATP; a value in the order of the nucleotide EC₅₀. ATP applications were regularly spaced at 10-min intervals, the optimal time previously determined to attain full recovery of the ATP-evoked current, an indication of the reversibility of the metal effects. Metal concentration-response plots were fitted using Graph Pad software; the copper IC₅₀ was interpolated from each plot.

Statistical Analysis

Curve fitting was performed using the sigmoidal equations proper of concentration-response curves (GraphPad Prism 3.0; San Diego, CA). ATP and zinc EC₅₀, I_max, and n⁰, as well as the copper IC₅₀ were derived per oocyte in each experimental protocol (13). Maximal amplitudes were also derived. Non-parametric analysis and parametric tests were performed as appropriate (Kruskal-Wallis, Friedman, and Quade (see Ref. 20 for review) and Dunnett’s tests for multiple comparisons with a single control); significance was set at p < 0.05.

RESULTS

Chemical Modification of Histidine Residues with DEPC Impairs the Inhibitory Action of Copper

Copper non-competitively inhibited the currents evoked by ATP in wild-type receptors expressed in oocytes (Fig. 1A). DEPC treatment, which is expected to alkylate-exposed histidine residues, decreased the ATP I_max (44.5 ± 7.8%, p < 0.01) and abolished the inhibitory action of copper (Fig. 1B). In contrast, NEM treatment, which preferentially modifies the SH group of cysteines, significantly increased the ATP I_max (166.3 ± 24.5%, p < 0.001). Moreover, NEM treatment did not abolish the non-competitive copper inhibition (Fig. 1C), suggesting that histidines, rather than cysteines, are primarily involved in the action of copper.

Functional Characteristics of Wild-type and Mutated P2X₄ Receptors Expressed in Oocytes

Both in the wild-type and the receptor mutants, the 15-s ATP pulse application resulted in currents with a distinct rapid peak and a slower component. The P2X₄ receptor mutants resulted in functional channels; their ATP EC₅₀ and I_max values are summarized in Table I. Compared with the wild-type, the ATP EC₅₀ of the H140A and H286A mutants was 6- and 4-fold larger than the wild-type receptor; mutant H241A conserved the wild-type EC₅₀ (Table I). On the other hand, the H286A mutant I_max was reduced by 67% (p < 0.01), whereas that of the H140A and H241A mutants was unaltered. The double and the triple histidine mutants with the H140A and H286A substitution also had a lower I_max and a larger EC₅₀ than the wild-type (Table I).

Characterization of the Trace Metal Modulation in Wild-type and Mutated P2X₄ Receptors Expressed in Oocytes

Copper Modulation—Copper decreased in a reversible and concentration-dependent manner the current elicited by a concentration of ATP close to the EC₅₀ of each receptor (Fig. 2). The copper IC₅₀ derived from these experiments was 8.9 ± 1.5 μM (n = 7) for the wild-type receptor, a value that was not significantly different from 8.7 ± 2.4 (n = 4) and 7.1 ± 2.7 μM (n = 6) for the H241A and H286A mutants, respectively. However, the H140A mutant was largely resistant to the modulator action of copper; 600 μM copper inhibited only 33.8 ± 8.5% (n = 4; see Fig. 2), suggesting that His-140 is essential for the copper-induced inhibition.

Zinc Modulation—Zinc facilitated in a biphasic manner the ATP-gated currents; a maximum was reached at 10 μM zinc, which amounted to a 7.1 ± 0.7-fold (n = 7) increase over the
current elicited by an ATP EC$_{50}$, the preferred concentration used to evaluate the zinc modulation. Larger metal concentrations elicited smaller effects, resulting in a bell-shaped curve (recordings and inset in Fig. 3). The H241A and H286A mutants also showed a similar biphasic zinc curve; the maximal potentiation of the currents was 8.9 ± 0.6- and 8.0 ± 2.4-fold for the H241A and H286A mutants, respectively (n = 4; see Fig. 3). In contrast, the zinc curve for the H140A mutant was sigmoid, reaching a maximal potentiation at 30 μM zinc, which resulted in a 27.4 ± 7.7-fold potentiation (p < 0.001, n = 4; see Fig. 3). This potentiation was 4-fold larger than for the wild-type receptor.

### Table I

EC$_{50}$ and $I_{\text{max}}$ values for wild-type and mutated P2X$_{4}$ receptors expressed in X. laevis oocytes and HEK 293 cells

The number of experiments are shown in parentheses. EC$_{50}$ and $n_{H}$ values were derived from the Hill equation. The maximal current ($I_{\text{max}}$) values were obtained applying a saturating concentration of ATP.

|                | Oocytes | HEK cells |
|----------------|---------|-----------|
|                | EC$_{50}$ | $I_{\text{max}}$ | $n_{H}$ | EC$_{50}$ | $I_{\text{max}}$ | $n_{H}$ |
| Wild-type      | 8.9 ± 2.1 (11) | 4676 ± 683 (20) | 1.3 ± 0.2 | 5.2 ± 1.0 (6) | 1.1 ± 0.2 (8) | 1.8 ± 0.4 |
| H140A          | 61.2 ± 9.5$^a$ (8) | 4268 ± 594 (11) | 1.4 ± 0.2 | 5.4 ± 1.2 (3) | 1.1 ± 0.2 (4) | 2.0 ± 0.6 |
| H241A          | 8.4 ± 0.8 (8) | 4939 ± 907 (11) | 1.2 ± 0.3 | 5.1 ± 3.3 (5) | 1.3 ± 0.2 (11) | 1.0 ± 0.2 |
| H286A          | 40.2 ± 8.3$^b$ (11) | 1540 ± 510$^b$ (16) | 1.3 ± 0.4 | 9.3 ± 4.7 (4) | 0.3 ± 0.1$^b$ (5) | 0.6 ± 0.3 |
| H140A/H241A    | 40.8 ± 10.6 (4) | 795 ± 301 (4) | 1.7 ± 0.4 | 5.4 ± 1.7 (4) | 1.1 ± 0.2 (4) | 2.0 ± 0.6 |
| H140A/H286A    | 103.1 ± 22.8$^a$ (5) | 402 ± 125$^a$ (4) | 1.7 ± 0.3 | 5.4 ± 1.1 (3) | 1.1 ± 0.2 (4) | 2.0 ± 0.6 |
| H241A/H286A    | 25.5 ± 6.5 (4) | 1112 ± 600$^a$ (4) | 0.9 ± 0.1 | 9.3 ± 4.7 (4) | 0.3 ± 0.1$^a$ (5) | 0.6 ± 0.3 |
| H140A/H241A/H286A | 114.5 ± 17.2 (4) | 560 ± 135 (5) | 2.5 ± 1.7 | 9.3 ± 4.7 (4) | 0.3 ± 0.1$^a$ (5) | 0.6 ± 0.3 |
| H140T          | 59.6 ± 9.2$^b$ (4) | 3358 ± 724 (5) | 1.7 ± 0.4 | 9.3 ± 4.7 (4) | 0.3 ± 0.1$^a$ (5) | 0.6 ± 0.3 |
| H140R          | 32.2 ± 7.4$^b$ (3) | 43 ± 9$^b$ (5) | 0.5 ± 0.2$^b$ | 9.3 ± 4.7 (4) | 0.3 ± 0.1$^a$ (5) | 0.6 ± 0.3 |

$^a$ p < 0.01, as compared with the values obtained with the wild-type P2X$_{4}$ receptor.
$^b$ p < 0.05, as compared with the values obtained with the wild-type P2X$_{4}$ receptor.
Mechanisms of Trace Metal Modulation: ATP Concentration-response Curves—In contrast to the wild-type receptor, 10 μM copper did not modify the ATP concentration-response curve in the H140A mutant, whereas it non-competitively inhibited the H241A and the H286A receptor mutants (see Fig. 4 and Table II). 10 μM zinc displaced the ATP-concentration-response curve of the H140A mutant to the left (Fig. 4) and additionally increased the Imax from 104 ± 8.3% in the wild-type to 205 ± 21.8% (p < 0.001; see Table II). Zinc also increased the ATP Imax of the H286A mutant to 238.2 ± 58.2% (p < 0.001; see Table II and Fig. 4). The effect of zinc in the H241A mutant was identical to that of the wild-type receptor.

Trace Metal Modulation in the Double and Triple Histidine Mutants—The mutants containing the H140A substitution were resistant to the inhibitory action of up to 100 μM copper. In contrast, the mutant containing the other two histidines conserved the inhibition by copper and behaved similar to that of the wild-type receptor (Fig 5A).

Zinc potentiated the magnitude of the ATP current in the H140A/H241A/H286A receptor, reaching a plateau at 100 μM; the maximal potentiation was 32.9 ± 5.5-fold, a value significantly larger than that observed in the wild-type receptor (p < 0.01; see Fig. 5B). In the double receptor mutants H140A/H241A and H140A/H286A, the maximal zinc potentiation amounted to 12.0 ± 6.0% and 11.7 ± 4.7-fold, respectively (p < 0.01 each, n = 5 each), values which are two-three times smaller than those attained with the H140A mutant. In contrast, the H241A/H286A mutant had a biphasic zinc curve, similar to that observed in the wild-type receptor (Fig. 5B); the maximal potentiation reached only 6.2 ± 1.6-fold and served as a negative control, because the maximal potentiation in the wild-type receptor reached 7.1 ± 0.7-fold.

Substitution of His-140 by Other Amino Acids—The copper IC50 in the H140T mutant was 78.5 ± 31.2 μM (n = 4), a value 8-fold higher than in the wild-type receptor (n = 6, p < 0.01; see Fig. 6). The zinc curve was biphasic; its maximal effect reached 17.3 ± 2.9-fold, a value 2.5-fold higher than the wild-type receptor (p < 0.01), but slightly smaller than the maximal potentiation obtained in the H140A mutant (27.4 ± 7.7-fold increase; see Fig. 6).

The currents elicited by the H140R mutant were 100-fold smaller than the wild-type and were long lasting, even after ATP removal (Fig. 6, tracings). The estimated ATP EC50 was 32.2 ± 7.4 μM (n = 3; see Table I); furthermore, the Hill coefficient is about 3-fold less than the wild-type receptor. The weak currents attained with this mutant precluded a detailed
analysis of its interaction with trace metals; in the few successful recordings, the currents were insensitive to the action of up to 100 μM copper (Fig. 6); zinc was not evaluated, because the currents elicited by 1 μM ATP were almost undetectable.

**Patch Clamp Technique Confirms Trace Metal Modulation of the P2X$_4$ Receptor in HEK 293 Cells**

**ATP Potency in Wild-type and Single Histidine Receptor Mutants**—The ATP EC$_{50}$ of the wild-type receptor was similar to that obtained in oocytes (Table I). In contrast to the oocytes, the ATP EC$_{50}$ values of the three single histidine mutants and the wild-type receptor showed no difference in their affinity for ATP (Table I). The H286A mutant had a lowered I$_{\text{max}}$, which reached only 24% of that attained by the wild-type value ($p < 0.001$) as in the oocytes.

**Trace Metal Modulation**—Copper inhibited in a reversible and concentration-dependent manner the ATP-evoked currents; its IC$_{50}$ was 9.2 ± 4.5 μM ($n = 5$) in the wild-type receptor (see Fig. 7, A and B and Table III). The H140A mutant was resistant to the inhibitory action of up to 300 μM copper; in contrast, this concentration showed a minor, but consistent, current increase (see Fig. 7B and Table III). The copper IC$_{50}$ in the H241A and H286A was 9.3 ± 3.0 and 11.0 ± 4.5 μM, respectively ($n = 4$, each). In the wild-type receptor zinc showed a clear tendency to a biphasic curve, although it was not as marked as that in oocytes (Fig. 7C). In contrast, zinc caused a linear potentiation in the H140A mutant; the potentiation attained with 100 μM zinc was 2.2-fold larger than in the wild-type receptor ($p < 0.05$; see Fig. 7C). Mutation of the other histidines had no effect on the zinc-induced potentiation (see Table III and Fig. 7A).

**DISCUSSION**

The present results identify His-140 as a key residue involved in the inhibitory modulator role of copper in the P2X$_4$ receptor. Consistent with our hypothesis that the rat P2X$_4$ receptor has separate extracellular allosteric copper- and zinc-binding sites, the present results show that the H140A mutation does not eliminate the positive modulator role of zinc; on the contrary, it markedly augments it. The combined use of oocytes and HEK 293 cells allowed us to confirm P2X$_4$ receptor trace metal modulation in two different cell expression model.
The potency of ATP in the wild-type P2X<sub>4</sub> receptor, the n<sub>H</sub> of the ATP concentration-response plots, as well as the modulator characteristics of the trace metals, are similar in both cell expression systems, independent of the recording techniques used.

The potency of ATP in the H140A and/or H286A mutant receptors was lower in oocytes than in HEK 293 cells. This discrepancy does not deter our interpretations, because in both cell expression systems His-140 is essential for the inhibitory copper modulation. Consistently, the His-241 mutation has no effect on the properties of the receptor, including the modulator role of trace metals, confirming the specific contribution of His-140 in the inhibitory action of copper. Additionally, the I<sub>max</sub> of H286A mutant expressed in either system was reduced to a similar extent, suggesting that His-286 could play a role either in the expression or in the assembly of the subunits that compose the channel or modify the ATP binding or the channel conductance, in view of its proximity to the alleged ATP phosphate chain binding site (21).

Histidines are known to form part of consensus copper-binding motifs (16). In view of the lack of a linear consensus copper-binding motif in the primary sequence of the P2X<sub>4</sub> receptor, we hypothesize that the copper coordination site might be formed by amino acids distant in the primary structure of the protein and that come in close proximity upon protein folding. DEPC was used to specifically alkylate the imidazole group of histidines (22); this treatment resulted in the loss of the copper inhibition and a reduction in the P2X<sub>4</sub> ATP-evoked currents of the receptor. We are aware that there are controversial results reported in the literature with respect to the use of DEPC, because some authors have successfully modified histidine residues (23–25), whereas others have failed to detect significant changes (26, 27). Considering that the rat P2X<sub>4</sub> receptor has only three histidine residues, it became challenging to establish which of these imidazole-bearing residues is responsible for the negative modulator role of copper, as well as the decreased channel activity. Site-directed mutagenesis revealed the critical role of His-140 but not His-241 or His-286 in the modulation by copper. Consistent with this interpretation, the copper concentration-response curve of the H241A/H286A mutant could be superimposed to that obtained in the wild-type receptor.

To clarify the specific requirement of His-140 on the binding of copper, we replaced this residue by threonine and arginine. The results obtained with these mutants confirmed the requirement of this particular imidazole at the copper-binding site to maintain the maximal copper effect. These results further stress the stringent spatial and chemical requirements of His-140 for the action of copper but not zinc. The finding that the substitution of His-140 by a threonine residue did not abrogate the effect of copper suggests that other amino acids participate in the full inhibitory effect of copper. Computerized models of the copper-binding site of the P2X<sub>4</sub> receptor revealed that Thr-123, Ser-124, Ser-141, and Thr-146 are found adjacent to His-140 in the receptor three-dimensional structure. It is therefore plausible that these amino acids may participate in the binding of copper, as threonine has been reported to form part of the copper-binding site in plastocyanin (28). The H140R mutant dramatically altered the receptor conformation and properties precluding its further evaluation. The mechanism of the copper coordination and the identification of adjacent amino acid residues that participate in the copper coordination complex remain as open issues.

The H140A mutant shows a dramatic change in the zinc concentration-response curve from a bell-shaped curve to a sigmoid. Two possible mechanisms may account for this novel finding. Either the trace metal-binding sites are allosterically coupled in such a way that interactions among them might occur, or the receptor has two allosteric metal-binding sites. One of the sites binds copper or zinc and causes inhibition (with higher copper affinity than zinc affinity), and the other site binds zinc but not copper and causes potentiation. Large zinc concentrations interact at the copper-prefering inhibitory metal-binding site. The lack of the inhibitory copper-binding site in the H140A mutant resulted in an augmented and sigmoid zinc concentration-response curve. Altogether, the present results are consonant with our working hypothesis that the copper-inhibitory and zinc-facilitator binding sites must be distinct.

The biphasic nature of the zinc modulation has also been observed in other ligand-gated ion channels. For example, the neuronal nicotinic receptors can be either facilitated or inhibited by zinc, depending on the combination of receptor subunits (24). Recombinant homeric glycine receptors expressed in HEK cells also show a biphasic action of zinc, an effect postulated to occur by metal binding at two separate sites on the α subunit and involving two histidine residues for its inhibitory

---

![Fig. 5. Metal effects on double and triple histidine mutants of the P2X<sub>4</sub> receptor.](image)

**A**. 1–100 μM copper (1 min pre-incubated) had no effect on ATP-evoked currents on H140A/H241A (closed squares), H140A/H286A (open squares), or H140A/H241A/H286A (closed triangles) but inhibited the ATP currents of the H241A/H286A mutant (open triangles). B. Zinc-induced potentiation of H140A/H241A/H286A mutant (open triangles) and H241A/H286A (open triangles). Each metal was pre-applied for 1 min before the ATP pulse in separate oocytes. The dashed line represents the zinc potentiation in the H140A receptor mutant. Curves represent the mean of four–five separate oocytes studied in each case. Symbols represent the mean values, and bars represent the S.E.

---

<sup>2</sup> P. Bull, personal communication.
FIG. 6. Substitution of histidines by threonine and arginine. A, concentration-response curves for copper (left panel) and zinc (right panel) in the H140T mutant (open squares). The fine dashed line represents the curves obtained in the H140A mutant; dashed lines represent the results obtained in the wild-type receptor (wt). B, representative tracings show that the H140R mutant is resistant to copper applications, as summarized in the right panel. Symbols and columns represent the mean values, and bars represent the S.E.

FIG. 7. Metal modulation of ATP-evoked currents in P2X, receptors expressed in HEK 293 cells. A, representative tracings from single HEK cells each expressing the wild-type or the H140A, H241A, or H286A receptor mutants. The effects of 10 μM copper and zinc were tested pre-incubating independently each metal for 1 min before a 10 μM ATP-pulse. Copper had no effect on the H140A mutant. The experiments were repeated in at least four separate cells in each case. The metal modulation is reversible; care was taken to recover the original ATP-evoked current prior to further testing. Copper (B) and zinc (C) concentration-response curves for the wild-type (closed circles) and the H140A mutant (open squares). *, p < 0.05; **, p < 0.01, as compared with the values obtained in the wild-type receptor. Symbols indicate the mean values, and bars indicate the S.E.
modulation but not for its potentiating effect (23). The P2X_2 receptor, another member of the P2X receptor family, is poten-
tiated by zinc (14, 15); two histidines have been proposed to
coordinate zinc in its extracellular domain (29). Because copper
also positively modulates the P2X_2 receptor, it is not unlikely
that the P2X_2 receptor possesses only a non-selective facilitator
trace metal-binding site or a very low affinity inhibitory metal
binding-site. In most ionotropic receptors zinc modulates via
a facilitator action; however, the N-methyl-D-aspartate receptor
is inhibited by zinc. Interestingly, the inhibitory action of zinc
depends on the subunit conformation and apparently on critical
histidine (30) and/or cysteine residues (31). In this particular
glutamate receptor, zinc fails to show a biphasic nature, stress-
ing that the modulator role of this metal varies between recep-
tors and is not an intrinsic metal property.

Although this investigation focused on the modulator role of
copper and zinc, we have not ignored that the selectivity of the
P2X receptor metal-binding sites might not be exclusive for
these trace metals and may even encompass pH modulation.
In the human P2X_1 receptor, the pH sensor is related to His-286,
because the replacement of this residue by an alanine abolishes
the pH-associated effect (27). The H286A mutant studied here
showed a lower channel conductance, making it likely that this
amino acid could be acting as a pH sensor in both rat and human
receptors. We also do not exclude that these trace metal-
binding sites are the sites of action of other metals, including
lead, cadmium, and mercury, known to produce brain
toxicity. In this regard, Acun˜ a-Castillo et al. (12) showed that
mercury inhibited whereas cadmium facilitated the ATP-
whether the neuromodulator role of trace metals is operant
separate and distinct trace metal binding-sites, explaining the
differential modulation exerted by copper and zinc. As to
whether the neuromodulator role of trace metals is operant in
vivo remains a challenging question. The proposal that trace
metals are novel atypical brain transmitters (1) underscores
the notion that trace metals may have important consequences
in synaptic activity that may be related to profound changes in
brain functioning.

Acknowledgments—We appreciate the CONICYT-CNRS bilateral
research program, which allowed collaborating with F. Rassendren.
The editorial assistance of Brenda Watt is greatly appreciated.

REFERENCES
1. Bara˜ nano, D. E., Ferris, C. D., and Snyder, S. H. (2001) Trends Neurosci.
24, 99–106
2. Assaf, S., and Chung, S. H. (1984) Nature 308, 734–736
3. Howell, G. A., Welch, M. G., and Fredericksson, C. J. (1984) Nature 308,
737–738
4. Kardos, J., Kovacs, I., Hajos, F., Kalman, M., and Simonyi, M. (1989) Neuro-
sci. Lett. 103, 139–144
5. Bloomenthal, A. B., Goldwater, E., Pritchett, D. B., and Harrison, N. L. (1994)
Mol. Pharmacol. 46, 1156–1159
6. Soto, F., Garcia-Guzman, M., Gomez-Hernandez, J. M., Hoffmann, M., Kar-
schin, C., and Stuhmer, W. (1996) Proc. Natl. Acad. Sci. U. S. A. 93,
3684–3688
7. Trombley, P. Q., and Shepherd, G. M. (1996) J. Neurophysiol. 76, 2536–2546
8. Yan Ma, J., and Narahashi, T. (1993) Brain Res. 607, 222–232
9. Palma, E., Maggi, L., Miledi, R., and Eusebi, F. (1998) Proc. Natl. Acad.
Sci. U. S. A. 95, 10246–10250
10. North, R. A. (2002) Physiol. Rev. 82, 1013–1067
11. Rubin, M. E., and Soto, F. (2001) J. Neurosci. 21, 641–663
12. Acun˜ a-Castillo, C., Morales, B., and Huidobro-Toro, J. P. (2000) J. Neurochem.
74, 1529–1537
13. Coddou, C., Villalobos, C., Gonzalez, J., Acun˜ a-Castillo, C., Loeb, B., and
Huidobro-Toro, J. P. (2002) J. Neurochem. 80, 626–633
14. Xiong, K., Peoples, R. W., Montgomery, J. P., Chiang, Y., Stewart, R. R.,
Weight, F. F., and Li, C. (1999) J. Neurophysiol. 81, 2088–2094
15. Lorca, R., Gonzalez, J., and Huidobro-Toro, J. P. (2001) Biol. Res. 34, 105
(Abstr. R-921)
16. Aitken, A. (1999) Mol. Biotechnol. 12, 241–253
17. Richardson, J., Thomas, K. A., Rubin, B. H., and Richardson, D. C. (1975)
Proc. Natl. Acad. Sci. U. S. A. 72, 1349–1353
18. Vallee, B. L., and Auld, D. S. (1990) Biochemistry 29, 5647–5659
19. Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., and Pease, L. R. (1989)
Gene 71, 51–59
20. Theodorsson-Norheim, E., (1987) Comput. Biol. Med. 17, 85–99
21. Hansen, M. A., Barden, J. A., Balcar, V. J., Keay, K. A., and Bennett, M. R.
(1997) Biochem. Biophys. Res.Comm. 236, 670–675
22. Eyzaquirre, J. (1996) Biol. Res. 29, 1–11
23. Harvey, R. J., Thomas, P., James, C. H., Wilderspin, A., and Smart, T. G.
(1999) J. Physiol. 520, 83–84
24. Hsiao, B., Dweck, D., and Lautie, C. W. (2001) J. Neurosci. 21, 1848–1856
25. Wilkins, M. E., Hosie, A. M., and Smart, T. G. (2002) J. Neurosci. 22,
5328–5333
26. Stoop, R., Surprenant, A., and North, A. (1997) J. Neurophysiol. 78, 1837–1840
27. Clarke, C. E., Benham, C. D., Bridger, A., George, A. R., and Meadows, H. J.
(2000) J. Physiol. 523, 697–703
28. Adman, E. T. (1991) Adv. Protein Chem. 42, 145–197
29. Clyne, J. D., LaPoint, L. D., and Hume, R. I. (2002) J. Physiol. 539, 347–359
30. Low, C. M., Zheng, F., Lyuboslavsky, P., and Traynelis, S. F. (2000) Proc. Natl.
Acad. Sci. U. S. A. 97, 11062–11067
31. Choi, Y., Chen, H. Y., and Lipton, S. A. (2001) J. Neurosci. 21, 392–400
32. North, R. A., and Surprenant, A. (2000) Annu. Rev. Pharmacol. Toxicol. 40,
563–580
33. Hornung, M. S., and Trombley, P. Q. (2001) J. Neurophysiol. 86, 1652–1660

TABLE III
Trace metal modulation of wild-type and mutant P2X_4 receptors expressed in HEK 293 cells

| + Cu^{2+} | + Zn^{2+} |
|-----------|-----------|
| Control 10 100 | Control 10 30 100 |
| Wild-type 100 ± 3.8 52.7 ± 5.0a 9.8 ± 4.3a | 100 ± 4.6 197.1 ± 20.4a 167.4 ± 19.4a |
| H140A 100 ± 4.7 95.6 ± 5.2 148.2 ± 24.2a | 100 ± 6.4 243.8 ± 15.3a 308.3 ± 15.9a |
| H241A 100 ± 2.3 56.9 ± 4.6a 14.3 ± 5.4a | 100 ± 4.9 200.4 ± 44.7a 183.8 ± 37.6a |
| H286A 100 ± 2.3 63.2 ± 9.4a 10.5 ± 9.2a | 100 ± 3.5 217.5 ± 20.1a 194.6 ± 26.1b 167.3 ± 22.0b |

a p < 0.001, as compared with control conditions.
b p < 0.05, as compared with control conditions.
Histidine 140 Plays a Key Role in the Inhibitory Modulation of the P2X₄ Nucleotide Receptor by Copper but Not Zinc
Claudio Coddou, Bernardo Morales, Jorge González, Marta Grauso, Felipe Gordillo, Paulina Bull, Francois Rassendren and J. Pablo Huidobro-Toro

J. Biol. Chem. 2003, 278:36777-36785.
doi: 10.1074/jbc.M305177200 originally published online June 20, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M305177200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 32 references, 9 of which can be accessed free at http://www.jbc.org/content/278/38/36777.full.html#ref-list-1