Differential Modulation by Copper and Zinc of P2X2 and P2X4 Receptor Function

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The modulation by Cu2+ and Zn2+ of P2X2 and P2X4 receptors expressed in Xenopus oocytes was studied with the two-electrode, voltage-clamp technique. In oocytes expressing P2X2 receptors, both Cu2+ and Zn2+, in the concentration range 1–130 μM, reversibly potentiated current activated by submaximal concentrations of ATP. The Cu2+ and Zn2+ concentrations that produced 50% of maximal potentiation (EC50) of current activated by 50 μM ATP were 16.3 ± 0.9 (SE) μM and 19.6 ± 1.5 μM, respectively. Cu2+ and Zn2+ potentiation of ATP-activated current was independent of membrane potential between −80 and +20 mV and did not involve a shift in the reversal potential of the current. Like Zn2+, Cu2+ increased the apparent affinity of the receptor for ATP, as evidenced by a parallel shift of the ATP concentration-response curve to the left. However, Cu2+ did not enhance ATP-activated current in the presence of a maximally effective concentration of Zn2+, suggesting a common site or mechanism of action of Cu2+ and Zn2+ on P2X2 receptors. For the P2X4 receptor, Zn2+, from 0.5 to 20 μM enhanced current activated by 5 μM ATP with an EC50 value of 2.4 ± 0.2 μM. Zn2+ shifted the ATP concentration-response curve to the left in a parallel manner, and potentiation by Zn2+ was voltage independent. By contrast, Cu2+ in a similar concentration range did not affect ATP-activated current in oocytes expressing P2X4 receptors, and Cu2+ did not alter the potentiation of ATP-activated current produced by Zn2+. The results suggest that Cu2+ and Zn2+ differentially modulate the function of P2X2 and P2X4 receptors, perhaps because of differences in a shared site of action on both subunits or the absence of a site for Cu2+ action on the P2X4 receptor.

INTRODUCTION

The P2X receptors are ligand-gated membrane ion channels that are activated by extracellular ATP. These receptor channels received recent attention because of their potential importance in the central and peripheral nervous systems. Activation of P2X receptors elicits excitatory postsynaptic currents or excitatory postsynaptic potentials in both central and peripheral neurons (Bardoni et al. 1997; Edwards et al. 1992, 1997; Evans et al. 1992; Galligan and Bertrand 1994; Gu and MacDermott 1997; Pankratov et al. 1998; Silinsky et al. 1992) and excitatory junction potentials in smooth muscle cells (Sneddon et al. 1982). Activation of P2X receptors also mediates excitatory responses in a variety of central and peripheral neurons (Bean 1990; Sieger and Adams 1991; Khakh et al. 1995; Krishtal et al. 1983; Li et al. 1993, 1997a; Shen and North 1993; Ueno et al. 1992). P2X receptors were found to be widely distributed in the CNS, including cerebral cortex, hippocampus, thalamus, hypothalamus, midbrain, cerebellum, and spinal cord, and in sensory and sympathetic ganglia in the peripheral nervous system (Collo et al. 1996).

Like other neurotransmitter-gated membrane ion channels, P2X receptors in neurons are sensitive to a number of endogenous agents, including Zn2+ (Clauses et al. 1993; Li et al. 1993, 1997a), Cu2+ (Li et al. 1996a), H+ (Li et al. 1996b), Mg2+, and Ca2+ (Krishtal and Marchenko 1984; Li et al. 1997b; Nakazawa and Hess 1993) as well as other neurotransmitters or neuromodulators, such as substance P (Hu and Li 1996; Wildman et al. 1997). Recent studies revealed that these substances can produce differential effects on P2X receptors in neurons. For instance, in rat nodose ganglion neurons, low micromolar concentrations of Zn2+ and Cu2+ enhance ATP-activated current in the majority of neurons but have no effect in a subset of neurons (Li et al. 1993, 1996a). On the other hand, in bullfrog dorsal root ganglion neurons, low micromolar concentrations of Zn2+ inhibit ATP-activated current (Li et al. 1997a). Extracellular protons markedly potentiate ATP-activated current in the majority of neurons from rat nodose ganglion but do not alter ATP-activated current in a subset of these neurons (Li et al. 1993, 1996a). On the other hand, extracellular protons markedly potentiate ATP-activated current in the majority of neurons from rat nodose ganglion but do not alter ATP-activated current in a subset of these neurons (Li et al. 1996a). Similarly, Mg2+ inhibits ATP-activated current in most but not all neurons from rat nodose ganglion (Li et al. 1997b). The molecular mechanisms underlying the diverse effects of these modulators, however, remain to be determined.

At least seven P2X receptor subunits, designated P2X1–P2X7, were cloned to date (Buell et al. 1996a). Each of these subunits can form ATP-selective homomeric cation channels when expressed in Xenopus oocytes or cell lines. Characterization of the properties of recombinant P2X receptor subunits should prove to be a useful first step in resolving the disparate effects of modulators on P2X receptors in neurons. In this regard, results of recent studies revealed a differential modulation of P2X receptor subunits by endogenous agents. For instance, extracellular Ca2+ strongly inhibits the P2X2 subunit but not the P2X1 subunit (Evans et al. 1996). In addition, low micromolar concentrations of Zn2+ potentiate P2X2 and P2X4 subunits (Brake et al. 1994; Garcia-Guzman et al. 1997; Seguela et al. 1996; Wildman et al. 1998) but inhibit the P2X2 subunit (Virginio et al. 1997). Moreover, extracellular protons...
inhibit P2X₁, P2X₃, P2X₄, and P2X₇ subunits but potentiate the P2X₂ subunit as well as the P2X₆ and P2X₃ heteromeric receptor (Stoop et al. 1997). To characterize further the physiological regulation of P2X receptor subunits, we investigated the effects of Cu²⁺ and Zn²⁺ and their possible interactions on recombinant P2X₂ and P2X₄ receptors.

METHODS

Preparation of cRNA and expression of receptors

cRNA was synthesized in vitro from a linearized cDNA template with T7 RNA polymerase in the presence of the cap analogue 7 mGpppG and was injected into Xenopus oocytes with a pressurized microinjection device (PV 800 Pneumatic PicoPump, World Precision Instruments; Sarasota, FL). Mature X. laevis frogs were anesthetized by immersion in water containing 3-aminobenzoic acid ethyl ester (2 g/l). Oocytes were excised, mechanically isolated into clusters of four to five oocytes, and water containing 3-aminobenzoic acid ethyl ester (2 g/l). Oocytes were

with two sharp electrodes filled with 3 M KCl. Electrode tip resistances were in the range 0.5–1.5 MΩ. Oocytes were excised, mechanically isolated into clusters of four to five oocytes, and shaken in a water bath in two changes of 0.2% collagenase A in a solution containing (in mM) 83 NaCl, 2 KCl, 1 MgCl₂, and 5 HEPES, pH 7.4, for 1 h each. Each oocyte was injected with a total of 10 ng of RNA in 50 nl of diethylpyrocarbonate-treated water and was incubated at 17°C for 2–5 days in modified Barth’s saline containing sodium pyruvate (2 mM), penicillin (10,000 UI), streptomycin (10 mg/l), gentamycin (50 mg/l), and theophylline (0.5 mM).

The care and use of animals in this study was approved by the Animal Care and Use Committee of the National Institute on Alcohol Abuse and Alcoholism (protocol no. LMCN-SP-05) in accordance with National Institutes of Health guidelines.

Electrophysiological recording

Two-electrode, voltage-clamp recording was performed at room temperature with a Geneclamp (Axon Instruments; Foster City, CA) amplifier. Oocytes were placed in a recording chamber and impaled with two sharp electrodes filled with 3 M KCl. Electrode tip resistances were in the range 0.5–1.5 MΩ. Oocytes were usually voltage clamped at −70 mV, except as indicated. Currents were recorded on a pen recorder (Model RS3400, Gould; Valley View, OH). Oocytes were constantly superfused at the rate of ∼2.5 ml/min with bathing medium containing (in mM) 95 NaCl, 2 KCl, 2 CaCl₂, and 5 HEPES, pH 7.4. Solutions of ATP (as the sodium salt) and Cu²⁺ (as CuCl₂) or Zn²⁺ (as ZnCl₂) were prepared daily in extracellular medium. Solutions of ATP and Cu²⁺ or Zn²⁺ were administered either via the bathing solution, which was applied by gravity flow from a 0.5-mm silica tube connected to a seven-barrel manifold. Solutions were changed via manually switched solenoid valves. At least 5 min was allowed to elapse between agonist applications.

Drugs and chemicals

All of the drugs and chemicals used in these experiments were purchased from Sigma Chemical (St. Louis, MO), except CuCl₂, which was purchased from Aldrich Chemical (Milwaukee, WI), and the salts, which were purchased from Mallinckrodt (Paris, KY).

Estimation of Zn²⁺ concentration

Concentrations of free Zn²⁺ were estimated with the program “Bound and Determined” (Brooks and Storey 1992), which compensates for variation in temperature, pH, and ionic strength. Values for Mn²⁺ were used as estimates of Zn²⁺ concentrations because ATP has similar affinities for Mn²⁺ and Zn²⁺ (16 vs. 14 μM) (Sillen and Martell 1964), and the software does not directly calculate Zn²⁺ concentration. All concentrations of ATP, Zn²⁺, and Cu²⁺ given are total concentrations unless stated otherwise.

RESULTS

Modulation of P2X₂ receptors by Cu²⁺ and Zn²⁺

ATP, at concentrations of ≤500 μM, did not evoke detectable ion current in uninjected oocytes (n = 6, data not shown). Figure 1 illustrates the ATP-activated inward current in oocytes expressing P2X₂ receptors and the potentiation of that
current by extracellular Cu$^{2+}$ and Zn$^{2+}$. As shown in Fig. 1A, the amplitude of inward current activated by 50 μM ATP was greatly enhanced by the application of 10 μM Cu$^{2+}$. To compare the effect of Cu$^{2+}$ with that of Zn$^{2+}$ (Brake et al. 1994; Wildman et al. 1998), potentiation of ATP-activated current by Zn$^{2+}$ was also tested. At the same concentration, Zn$^{2+}$ produced enhancement of ATP-activated current that was comparable with that of Cu$^{2+}$ in the same oocyte. On average, in the same oocytes, 10 μM Cu$^{2+}$ or 10 μM Zn$^{2+}$ increased the amplitude of current activated by 50 μM ATP by 240 ± 32% (n = 12) or 167 ± 24% (n = 14), respectively. The enhancement by both divalent cations was concentration dependent between 1 and 130 μM (Fig. 1B). The EC$_{50}$ values for Cu$^{2+}$ and Zn$^{2+}$ enhancement of current activated by 50 μM ATP were 16.3 ± 0.9 μM and 19.6 ± 1.5 μM, the slope factors were 1.5 and 1.6, and the maximal effects were 845 ± 16% and 837 ± 26% of control, respectively. The EC$_{50}$, slope factor, and $E_{\text{max}}$ values obtained for Cu$^{2+}$ did not differ significantly from those for Zn$^{2+}$ (ANOVA, $P > 0.1$). Cu$^{2+}$ or Zn$^{2+}$ alone (1–130 μM) did not activate ion current in any oocytes tested (data not shown, n = 5).

Experiments performed to elucidate the mechanism by which Cu$^{2+}$ augments ATP-activated current are shown in Fig. 2. As shown in Fig. 2A, the magnitude of Cu$^{2+}$ potentiation decreased with increasing ATP concentration. On average, 5 μM Cu$^{2+}$ increased the amplitude of the current activated by 10 and 100 μM ATP by 383 ± 28% (n = 6) and 15.3 ± 4% (n = 5), respectively. The graph in Fig. 2B shows the concentration-response curves for ATP-activated currents in the absence and presence of 5 μM Cu$^{2+}$. As can be seen, Cu$^{2+}$ shifted the ATP concentration-response curve to the left, reducing the EC$_{50}$ for ATP from 51.7 ± 1.9 μM in the absence of Cu$^{2+}$ to 15.5 ± 0.3 μM in the presence of 5 μM Cu$^{2+}$ (ANOVA, $P < 0.01$) without significantly changing the slope or maximal value (ANOVA, $P > 0.1$). The lack of effect of Cu$^{2+}$ on the maximal value of the ATP concentration-response curve was apparently not because of chelation of Cu$^{2+}$ by high concentrations of ATP, as increasing the Cu$^{2+}$ concentration threefold, which would yield a calculated concentration of free Cu$^{2+}$ greater than that required to produce potentiation (results for Zn$^{2+}$ potentiation of P2X$_4$ receptors are described subsequently), did not potentiate current activated by 100 μM ATP (results not shown).

The influence of membrane potential on the potentiation by Cu$^{2+}$ and Zn$^{2+}$ of ATP-activated current was evaluated by constructing current-voltage relationships for ATP-activated current. Figure 3A shows the current-voltage relationship for current activated by 50 μM ATP in the absence and presence of 5 μM Cu$^{2+}$. Cu$^{2+}$ produced a similar percentage enhancement of amplitude of current activated by ATP at membrane voltages between −80 and +20 mV and did not alter the reversal potential of ATP-activated current. In five of five cells tested, Cu$^{2+}$ enhanced ATP-activated current in a voltage-independent manner (ANOVA, $P > 0.25$) and did not significantly change the reversal potential of ATP-activated current (Student’s t-test, $P > 0.25$). Similarly, as shown in Fig. 3B, Zn$^{2+}$ potentiation of ATP-activated current was voltage independent (ANOVA, $P > 0.25$, n = 4), and Zn$^{2+}$ did not significantly change the reversal potential of ATP-activated current (Student’s t-test, $P > 0.25$, n = 4).

Because Cu$^{2+}$ and Zn$^{2+}$ are closely related metals and have similar augmenting effects on ATP-activated current mediated by P2X$_{3}$ receptors, we hypothesized that they might act at a common binding site. Results of an experiment designed to test this hypothesis are shown in Fig. 4. A near-threshold concentration of ATP was used to obtain a current in the presence of a maximally effective concentration of Zn$^{2+}$ that was lower in amplitude than the maximal ATP-activated current. In the cell shown in Fig. 4A, a maximally effective concentration of Zn$^{2+}$ (130 μM) potentiated current activated by 4 μM ATP by 2,867%, and 10 μM Cu$^{2+}$ increased the ATP-activated current by 1,467%. However, concomitant application of 130 μM Zn$^{2+}$ and 10 μM Cu$^{2+}$ failed to produce enhancement of ATP-activated current greater than that produced by Zn$^{2+}$ alone. On average, the potentiation of ATP-activated current produced by Cu$^{2+}$ and Zn$^{2+}$ applied together was not different from that produced by Zn$^{2+}$ applied alone (Student’s t-test, $P > 0.25$, n = 5; Fig. 4B).
Cu²⁺ and Zn²⁺ Modulation of P2X Receptors

Cu²⁺ and Zn²⁺ modulation of P2X receptors by Zn²⁺ and Cu²⁺

The ATP-activated inward current in oocytes expressing P2X₄ receptors and the modulation of that current by extracellular Zn²⁺ and Cu²⁺ are illustrated in Fig. 5. As shown in Fig. 5A, 10 µM Zn²⁺ markedly increased the amplitude of current activated by 5 µM ATP. By contrast, the same concentration of Cu²⁺ did not affect current activated by the same concentration of ATP. Zn²⁺ potentiation of ATP-activated current was concentration dependent between 0.5 and 20 µM. The EC₅₀ value for Zn²⁺ potentiation of current activated by 5 µM ATP was 2.4 ± 0.2 µM, the slope factor was 1.8, and the maximal effect was 214 ± 12% of control (Fig. 5B). Zn²⁺ alone (0.5–20 µM) did not activate ion current in any oocytes tested (data not shown, n = 5). In contrast to the potentiation of ATP-activated current by Zn²⁺, Cu²⁺, in the same concentration range, did not significantly affect ATP-activated current (ANOVA, P > 0.25; Fig. 5B). In addition, Cu²⁺ at a concentration of 50 µM did not potentiate ATP-activated current in oocytes expressing P2X₄ receptors (Student’s t-test, P > 0.5, n = 7).

As the maximal potentiation by Cu²⁺ of ATP-activated current in P2X₂ receptors occurred at the lowest ATP concentration, we tested whether Cu²⁺ would potentiate the current activated by a near-threshold concentration of ATP in P2X₄ receptors. Results from one such experiment are illustrated in Fig. 6. In this experiment, 5 and 20 µM Cu²⁺ did not appreciably affect the current activated by 1.5 µM ATP. By contrast, 5 µM Zn²⁺ markedly enhanced ATP-activated current in the same cell. Similar results were obtained in five other experiments.

Figure 7A shows that Zn²⁺ shifted the ATP concentration-response curve to the left, reducing the EC₅₀ value for ATP-activated current from 6.7 ± 1.3 µM in the absence of Zn²⁺ to 2.8 ± 0.2 µM in the presence of 5 µM Zn²⁺ (ANOVA, P < 0.01) without changing the slope or maximal value (ANOVA, P > 0.1). The lack of effect of Zn²⁺ on the maximal value of the ATP concentration-response curve did not appear to be due to chelation of Zn²⁺ by high concentrations of ATP, as addition of 10 µM Zn²⁺ yielded a calculated free Zn²⁺ concentration of 6.9 µM, but did not potentiate current activated by 100 µM ATP (results not shown). This calculated concentration of free Zn²⁺ is substantially greater than that produced by 5 µM Zn²⁺ in the presence of ATP-activated current produced by Cu²⁺ and Zn²⁺ applied together. The average potentiation of 4 µM ATP by Cu²⁺ and Zn²⁺ applied together was not different from that produced by Zn²⁺ applied alone (Student’s t-test, P > 0.25, n = 5).
5 μM ATP (4.4 μM), which produces marked potentiation of ATP-activated current. As shown in Fig. 7B, there was no difference in the percent potentiation by 5 μM Zn²⁺ of 5 μM ATP-activated current at membrane holding potentials between −80 and +20 mV (ANOVA, P > 0.1, n = 4). Furthermore, Zn²⁺ did not change the reversal potential of ATP-activated current (Student’s t-test, P > 0.1, n = 4).

To evaluate a possible interaction of Cu²⁺ with the Zn²⁺ site on the P2X₄ subunit, we examined whether Cu²⁺ could affect Zn²⁺ potentiation of ATP-activated current. As shown in Fig. 8A, Cu²⁺ did not alter either ATP-activated current or Zn²⁺ potentiation of ATP-activated current. The average potentiation of ATP-activated current produced by 10 μM Zn²⁺ was 211 ± 8% of control in the absence of Cu²⁺ and 212 ± 9% of control in the presence of 10 μM Cu²⁺; these values are not significantly different (Student’s t-test, P > 0.5, n = 5; Fig. 8B).

**DISCUSSION**

Both Cu²⁺ and Zn²⁺ may be involved in the modulation of CNS function, as both ions were demonstrated to be widely
Cu²⁺ AND Zn²⁺ MODULATION OF P2X RECEPTORS

FIG. 8. Effect of Cu²⁺ on Zn²⁺ potentiation of ATP-activated current in P2X₂ receptors. A: records illustrating similar enhancement by 10 µM Zn²⁺ of currents activated by 5 µM ATP in the absence and the presence of 10 µM Cu²⁺. Records are sequential current traces (from left to right) obtained from a single oocyte. B: bar graph illustrating the average potentiation of 5 µM ATP-activated current by 10 µM Zn²⁺ in the absence and the presence of 10 µM Cu²⁺, Cu²⁺ did not alter the average potentiation of ATP-activated current produced by Zn²⁺. (Student’s t-test, P > 0.5; n = 5).

distributed in brain (Barden 1971; Frederickson 1989; Kozma et al. 1981; Szerdahelyi and Kásá 1986) and can be released on stimulation (Assaf and Chung 1984; Kardos et al. 1989). Previous studies revealed that these ions could produce similar modulation of the function of P2X receptors. For instance, micromolar concentrations of Cu²⁺ and Zn²⁺ potentiate ATP-activated current in rat nodose ganglion neurons (Li et al. 1993, 1996a). By contrast, micromolar concentrations of Cu²⁺ and Zn²⁺ inhibit ATP-activated current mediated by P2X₇ receptors (Virgino et al. 1997). However, this study provides evidence that Cu²⁺ and Zn²⁺ differentially regulate the function of P2X₂ and P2X₄ receptors.

Low micromolar concentrations of Cu²⁺ were previously reported to potentiate ATP-activated current mediated by P2X₂ receptors by increasing the apparent agonist affinity (Brake et al. 1994; Wildman et al. 1998). In the current study on P2X₂ receptors, Zn²⁺ potentiated the current activated by 50 µM ATP with an EC₅₀ value of 19.6 µM. Similarly, Cu²⁺ markedly potentiated current activated by 50 µM ATP in P2X₂ receptors, with an EC₅₀ value of 16.3 µM, which did not differ significantly from the EC₅₀ value for Zn²⁺. Cu²⁺ shifted the ATP concentration-response curve to the left in a parallel manner, decreasing the EC₅₀ value for ATP, as was found for Zn²⁺. These results suggest that Cu²⁺ and Zn²⁺ may facilitate the function of P2X₂ receptors via a common mechanism, perhaps through a common binding site. If this is the case, when this site is saturated by Zn²⁺, Cu²⁺ should not be able to further enhance the function of the receptor, as was found in this study. The amplitude of ATP-activated current in the presence of a maximally effective concentration of Zn²⁺ was not increased further by addition of Cu²⁺. This was not due to a “ceiling effect,” that is, the ATP-gated receptors were not already maximally activated in the presence of Zn²⁺ because 300 µM ATP activated a current of substantially greater amplitude. Thus Cu²⁺ and Zn²⁺ most probably act on a common site on the ATP-gated, receptor-channel complex. The location of the Cu²⁺–Zn²⁺ site cannot be precisely identified at present, but results of current-voltage experiments suggest that this site is beyond the influence of the membrane electrical field (Woodhull 1973) because the effects of both ions were not voltage dependent between −80 and +20 mV.

To date, P2X₄ receptors were cloned from rat brain (Bo et al. 1995; Séguela et al. 1996; Soto et al. 1996), rat superior cervical ganglion (Buell et al. 1996b), and human brain (Garcia-Guzman et al. 1997), and Zn²⁺ potentiation of ATP-activated current was reported for P2X₄ receptors isolated from rat brain (Séguela et al. 1996; Soto et al. 1996) and human brain (Garcia-Guzman et al. 1997). In this study, in oocytes expressing P2X₄ receptors cloned from rat superior cervical ganglion (Buell et al. 1996b), low micromolar concentrations of Zn²⁺ potentiated ATP-activated current. Like P2X₂ receptors, Zn²⁺ enhanced ATP receptor function by producing a parallel leftward shift in the ATP concentration-response curve. These results are consistent with previous studies in which Zn²⁺ was shown to induce a leftward shift of the concentration-response curve for ATP in P2X₄ receptors isolated from human brain (Garcia-Guzman et al. 1997). In addition, the effect of Zn²⁺ on P2X₄ receptors was not voltage dependent, suggesting that its site of action is not influenced by the membrane electrical field. In contrast to the effect of Zn²⁺ on P2X₄ receptors, 0.5–50 µM Cu²⁺ did not significantly affect ATP-activated current in oocytes expressing P2X₄ receptors. Furthermore, Cu²⁺ did not alter Zn²⁺ potentiation of ATP-activated current, suggesting that it does not interact with the Zn²⁺ site over this concentration range.

Cu²⁺ and Zn²⁺ at low micromolar concentrations were previously reported to differentially modulate glycine receptors in rat olfactory bulb neurons (Trombley and Shepherd 1996). The effects of Cu²⁺ and Zn²⁺ on glycine receptors are dependent on the state of the receptor; both Cu²⁺ and Zn²⁺ had no effect on the desensitized component of the current evoked by high concentrations of glycine, but Zn²⁺ dramatically potentiated Cu²⁺ inhibited the current activated by nondesensitizing concentrations of glycine. Similarly, the results of this study suggest important differences in the modulatory sites of Cu²⁺ and Zn²⁺ on the P2X₂ and P2X₄ subunits. The observation that Cu²⁺ and Zn²⁺ interact with the site on the P2X₂ receptor with similar affinity may indicate that the dimensions of this site, or the dimensions of the path of access to the site, are sufficient to accommodate both ions. The lack of effect of Cu²⁺ on the P2X₄ subunit may thus indicate that the dimensions or path of access to the site are not sufficiently large to accommodate the larger Cu²⁺ ion. An alternative possibility is that on the P2X₄ subunit there are separate sites for Cu²⁺ and Zn²⁺ but that both sites affect receptor function via a common mechanism (e.g., binding of either ion to its site produces the same conformational change in the receptor, increasing its affinity for ATP). If this is the case, then the inability of Cu²⁺
to enhance ATP-activated current mediated by P2X4 receptors may be due to the absence of the Cu\(^{2+}\) site on this subunit. Future studies may be able to distinguish between these two alternatives.

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