Inhibitory Effects of Metals on ATP-Induced Current Through P2X<sub>7</sub> Receptor in NG108-15 Cells

Tomokazu Watano, Isao Matsuoka and Junko Kimura*

Department of Pharmacology, Fukushima Medical University, School of Medicine, 1 Hikarigaoka, Fukushima 960-1295, Japan

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Abstract—We investigated the effects of heavy metal ions on the ATP-induced nonselective cation current through P2X<sub>7</sub>-receptor (I<sub>Ns-P2X<sub>7</sub></sub>) in NG108-15 cells using the whole-cell patch-clamp technique. Cu<sup>2+</sup> inhibited the I<sub>Ns-P2X<sub>7</sub></sub> most potently among the metal ions investigated. Other metals such as Ni<sup>2+</sup>, Cd<sup>2+</sup>, Zn<sup>2+</sup> and Co<sup>2+</sup> also inhibited the I<sub>Ns-P2X<sub>7</sub></sub> in concentration-dependent manners. The order of potency was Cu<sup>2+</sup> > Ni<sup>2+</sup> > Cd<sup>2+</sup> > Zn<sup>2+</sup> > Co<sup>2+</sup> with IC<sub>50</sub> values of 16 nM, 0.79 μM, 1.2 μM, 3.0 μM and 4.6 μM, respectively. Fe<sup>2+</sup> (10 and 100 μM) and Mn<sup>2+</sup> (10 μM) did not affect the I<sub>Ns-P2X<sub>7</sub></sub>. A high concentration of Mn<sup>2+</sup> (100 μM) slightly inhibited the I<sub>Ns-P2X<sub>7</sub></sub>. When the concentration-response curve of ATP was obtained in the presence of 3 and 10 nM Cu<sup>2+</sup>, the maximal response but not the EC<sub>50</sub> value appeared to be reduced, suggesting that the inhibition is not competitive. These results suggest that under physiological and toxicological conditions, metal ions, such as Cu<sup>2+</sup>, Ni<sup>2+</sup>, Cd<sup>2+</sup>, Zn<sup>2+</sup> and Co<sup>2+</sup>, may modulate P2X<sub>7</sub>-receptor channels as inhibitors.

Keywords: ATP, P2X<sub>7</sub>-receptor, Nonselective cation current, Metal ion, NG108-15 cell

The P2X<sub>7</sub>-receptor is one of seven subtypes (P2X<sub>1</sub>–P2X<sub>7</sub>) of P2X receptors, which are ligand-gated nonselective cation channels (1, 2). It has unique properties compared to the other P2X receptors (3, 4). The P2X<sub>7</sub>-receptor requires concentrations of ATP (>0.3 mM) much higher than the other P2X (P2X<sub>1</sub>–P2X<sub>6</sub>) receptors for activation (5). The effective form of ATP for the P2X<sub>7</sub>-receptor is the free base (ATP<sup>4+</sup>) (6), and benzoylbenzoic ATP (BzATP) is a more potent agonist than ATP (7–9).

NG108-15 cells are hybrids of mouse neuroblastoma N18TG-2 and rat glioma C6Bu-1 cells and possess the mouse P2X<sub>7</sub>-receptor originating from N18TG-2 cells (10). The cDNA sequence of P2X<sub>7</sub>-receptor in NG108-15 cells was almost identical (ref. 10; I. Matsuoka, unpublished result) of full length sequence) to that of mouse P2X<sub>7</sub>-receptor first cloned by Chessell et al. (11) in NTW8 mouse microglial cells. A high concentration (>0.3 mM), but not a low concentration, of ATP activates a nonselective cation current, indicating that the only functional P2X receptor is the P2X<sub>7</sub>-receptor in NG108-15 cells (7, 12, 13).

Rat and human P2X<sub>7</sub>-receptors transform themselves from channels to large pores by prolonged application of ATP (8, 14), but this does not occur in mouse P2X<sub>7</sub>-receptors in NG108-15 cells (15). Therefore, the P2X<sub>7</sub>-receptors in NG108-15 cells are different from those in rat and human macrophages and glial cells.

Metals such as vanadium (V), chromium (Cr), manganese (Mn), iron (Fe), cobalt (Co), nickel (Ni), copper (Cu), zinc (Zn) and molybdenum (Mo) are essential trace elements utilized as cofactors of various enzymes (16). Metal ions are known to affect various ion channels (17) including P2X receptor channels. Rat P2X<sub>7</sub>-receptor currents were inhibited by various divalent cations such as Ca<sup>2+</sup>, Mg<sup>2+</sup> (8, 14), Cu<sup>2+</sup>, Cd<sup>2+</sup>, Zn<sup>2+</sup>, Ni<sup>2+</sup>, Co<sup>2+</sup>, Mn<sup>2+</sup>, Ba<sup>2+</sup> and Sr<sup>2+</sup> (18) in HEK293 cells that stably express the receptor. The effects of metal ions on the mouse P2X<sub>7</sub>-receptor current have not been investigated in NG108-15 cells except for Mg<sup>2+</sup> (7). Therefore, we examined the effects of metal ions such as Cu<sup>2+</sup>, Ni<sup>2+</sup>, Cd<sup>2+</sup>, Zn<sup>2+</sup>, Co<sup>2+</sup>, Mn<sup>2+</sup> and Fe<sup>2+</sup> on the mouse P2X<sub>7</sub>-receptor-mediated current in NG108-15 cells using the whole-cell voltage clamp.

Materials and Methods

Cell culture

NG108-15 cells were grown in high-glucose Dulbecco’s modified Eagle’s medium supplemented with 7% fetal bovine serum, 100 μM hypoxanthine, 0.4 μM aminopterin and 16 μM thymidine (19). Cells were seeded in 12-well tissue culture plates and incubated at 37°C in a humidified atmosphere of 10% CO<sub>2</sub> and 90% air.
Whole-cell clamp

$I_{\text{NS-P2X7}}$ was measured using the whole-cell configuration of the patch-clamp technique (20) as described previously (7, 10, 21). Patch pipettes had a tip resistance of about 2 MΩ when filled with an intracellular solution containing 110 mM CsOH, 30 mM CsCl, 50 mM DL-aspartic acid, 5 mM MgATP, 3 mM MgCl$_2$, 5 mM potassium creatine phosphate, 10 mM EGTA and 20 mM HEPES (pH 7.2 with DL-aspartic acid). The cells were dispersed and their currents were recorded in a small chamber (2 mm $\times$ 10 mm) which was perfused with Tyrode solution containing 140 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl$_2$, 1 mM MgCl$_2$, 0.33 mM NaH$_2$PO$_4$, 5.5 mM glucose and 5 mM HEPES (pH 7.4 with NaOH). The temperature of the external solution was maintained at approximately 37°C by a silicon tube water jacket.

Membrane potentials were controlled by a model CEZ-2300 patch-clamp amplifier (Nihon Kohden, Tokyo). Membrane currents were acquired on-line and subsequently analyzed by computer (DimensionV333C; Dell, Round Rock, TX, USA) using pCLAMP7 software (Axon, Foster City, CA, USA). The current-voltage (I-V) relation was obtained by ramp pulses from a holding potential of $-100$ mV, initially depolarized to 60 mV, then hyperpolarized to $-120$ mV, and depolarized back to the holding potential at a speed of 1.0 V/s. The ramp pulse was applied every 3 s.

To induce $I_{\text{NS-P2X7}}$, ATP (1 mM) was applied in the superfusate repetitively for 10–20 s each time with 1-min intervals. Although $I_{\text{NS-P2X7}}$ did not run-down significantly, we induced the $I_{\text{NS-P2X7}}$ at least twice to confirm that the current magnitudes were similar, and then a metal ion (Cu$^{2+}$, Ni$^{2+}$, Cd$^{2+}$, Zn$^{2+}$, Co$^{2+}$, Mn$^{2+}$ or Fe$^{3+}$) was applied. After perfusing the metal for 30 s, ATP was added. The effect of a metal is expressed as percent inhibition, which was calculated by dividing the peak $I_{\text{NS-P2X7}}$ magnitude at $-100$ mV in the presence of a metal by that just before introducing it.

To analyze the inhibition mode of Cu$^{2+}$, we compared the current densities at different concentrations of ATP between the cells treated with and those not treated with Cu$^{2+}$. The current density was obtained by dividing the current by the membrane capacitance (Cm). Cm was obtained using the test protocol in pCLAMP7 software with the following equation:

$$Cm = \frac{\Delta i}{\Delta V}$$

where $i_x$ is the capacitative current. Data are expressed as means ± S.E.M. IC$_{50}$ values are expressed as geometric means with 95% confidence intervals. Since the effective form of ATP is ATP$^4$ for the P2X$_7$ receptor, we plotted the concentration-response curve between the current density and [ATP$^4$]. [ATP$^4$] was calculated from [ATP] according to Feden et al. (22) by the following equation:

$$[\text{ATP}^4] = [\text{ATP}]_{\text{total}} / (10^{3.94} [\text{Ca}]_{\text{total}} + 10^{4.28} [\text{Mg}]_{\text{total}})$$

Values of the maximum current density ($I_{\text{max}}$) and the apparent EC$_{50}$ values of [ATP$^4$] were obtained by directly fitting the data with the computer program ‘Origin’ (Origin Lab Corporation, Northampton, MA, USA) using a Logistic dose-response model.

Chemicals

ATP (adenosine 5′-triphosphate disodium salt; Wako, Osaka), CuCl$_2$, NiCl$_2$, CdCl$_2$, ZnCl$_2$, CoCl$_2$, MnCl$_2$ and FeCl$_3$ (all from Sigma, St. Louis, MO, USA) were dissolved in water as stock solutions and diluted with Tyrode solution to the desired final concentration. The pH was adjusted to 7.4 with NaOH.

RESULTS

Effects of metal ions on $I_{\text{NS-P2X7}}$

Figure 1A shows typical chart recordings (upper panels) and current-voltage (I-V) relationships (lower panels) of the control current before ATP application and the $I_{\text{NS-P2X7}}$ in response to 1 mM ATP in the absence (left), presence of (middle), and after washing out (right) Cu$^{2+}$. After a brief application of ATP for 15 s, Cu$^{2+}$ was superfused for 30 s before another application of ATP in the presence of Cu$^{2+}$. Cu$^{2+}$ at 10 nM inhibited the $I_{\text{NS-P2X7}}$ to 30% of the control at $-100$ mV in this cell.

Using the same protocol, we investigated the effects of Ni$^{2+}$, Cd$^{2+}$, Zn$^{2+}$ and Co$^{2+}$ on the $I_{\text{NS-P2X7}}$. The metal ions were applied 30 s before ATP application. In the presence of each of these metal ions, the $I_{\text{NS-P2X7}}$ was reduced. The inhibitions were reversible and were completely reversed after washing out the metals for 1 min. The effects of Mn$^{2+}$ and Fe$^{3+}$ were also investigated. Mn$^{2+}$ at 100 µM slightly inhibited the $I_{\text{NS-P2X7}}$, but Fe$^{3+}$ at 10 and 100 µM did not affect the current (Fig. 2).

Concentration-inhibition curves for Cu$^{2+}$, Ni$^{2+}$, Cd$^{2+}$, Zn$^{2+}$ and Co$^{2+}$ are shown in Fig. 2. The percent inhibition of the $I_{\text{NS-P2X7}}$ induced by 1 mM ATP was measured at $-100$ mV at each concentration of the metals. The inhibitory effects of the metal ions were concentration-dependent. By fitting the curves, the IC$_{50}$ values of Cu$^{2+}$, Ni$^{2+}$, Cd$^{2+}$, Zn$^{2+}$ and Co$^{2+}$ obtained were 16 (4.7–59) nM, 0.79 (0.18–2.2), 1.2 (0.41–3.7), 3.0 (0.68–12) and 4.6 (0.71–25) µM (geometric means with 95% confidence intervals), respectively, with Hill coefficients of 0.48, 0.71, 0.48 and 0.61, respectively.
We next investigated the inhibitory pattern of Cu$^{2+}$ to see whether or not it is competitive at two different concentrations of Cu$^{2+}$ (3 and 10 nM). We employed ATP$^{-}$ instead of ATP in the ligand axis, because ATP$^{-}$ is an effective form of ATP for the P2X$_7$ receptor (6). Figure 3 shows the concentration-response curves for ATP-induced $I_{NS\cdot P2X7}$. Cu$^{2+}$ at 3 and 10 nM inhibited the $I_{NS\cdot P2X7}$ by 20 – 25% and 42 – 45%, respectively, at each ATP concentration tested. This suggests that Cu$^{2+}$ is not a competitive inhibitor with respect to ATP. When the $I_{\text{max}}$ and EC$_{50}$ values of ATP$^{-}$ were obtained by directly fitting the data (see Materials and Methods), the estimated $I_{\text{max}}$ value was 46.1 ± 8.3 pA/pF (n = 9) in the control and was significantly reduced...
by Cu$^{2+}$ in a concentration-dependent manner to $24.9 \pm 1.4$ pA/pF ($n=4$) and $20.6 \pm 0.1$ pA/pF ($n=7$, $P<0.01$) with 3 and 10 nM Cu$^{2+}$, respectively. In contrast, the estimated EC$_{50}$ value of ATP$^{4-}$ was $88.4 \pm 22.8$ mM ($n=9$) in the control and were not significantly affected by Cu$^{2+}$, because those were $60.7 \pm 3.5$ mM ($n=4$) and $70.9 \pm 0.3$ mM ($n=9$) with 3 and 10 nM Cu$^{2+}$, respectively. These data are consistent with idea that the inhibitory effect of Cu$^{2+}$ on ATP-induced I$_{NS-P2X7}$ is not competitive.

### DISCUSSION

We demonstrated that metal ions such as Cu$^{2+}$, Ni$^{2+}$, Cd$^{2+}$, Zn$^{2+}$ and Co$^{2+}$ inhibit ATP induced nonselective-cation currents via the mouse P2X$_7$ receptors in NG108-15 cells. The IC$_{50}$ values for Cu$^{2+}$, Ni$^{2+}$, Cd$^{2+}$, Zn$^{2+}$ and Co$^{2+}$ were 0.016, 0.79, 1.2, 3.0 and 4.6 $\mu$M, respectively, with Hill coefficients of 0.48, 0.71, 0.48, 0.71 and 0.61, respectively. Mn$^{2+}$ only slightly inhibited the I$_{NS-P2X7}$ at 0.1 mM, but Fe$^{2+}$ was ineffective up to 0.1 mM. The Hill coefficients of these metals are mostly less than 1, possibly indicating that multiple binding sites with different affinities contribute to the inhibition or the binding of a metal ion reduces the binding of another metal ion (negative cooperativity).

Virginio et al. (18) studied IC$_{50}$ values of divalent cations in rat P2X$_7$ receptors using whole cell recordings of currents and dye uptake, using BzATP as an agonist. Their results and the present results are qualitatively the same, especially in rank order of potency, with Cu$^{2+}$ by far the most effective inhibitor. They observed I-V relations and the relations they found are quite similar to those shown in this study. There is only one interesting difference; they found a Cu$^{2+}$ IC$_{50}$ of 500 nM, while we found an IC$_{50}$ of 16 nM, although Ni$^{2+}$, Cd$^{2+}$ and Zn$^{2+}$ IC$_{50}$ values were approximately the same in both studies. In human P2X$_7$ receptor, 1 $\mu$M Cu$^{2+}$ inhibited BzATP-induced inward current by 59.4 $\pm$ 6.8%, suggesting that the IC$_{50}$ value is larger than 1 $\mu$M (18). The difference between IC$_{50}$ of Cu$^{2+}$ in this study and those reported by Virginio et al. (18) or Chessel et al. (23) could be due to a difference in species or agonists used. Virginio et al. (18) or Chessel et al. (23) used BzATP as an agonist to study the effects of Cu$^{2+}$ on rat or human P2X$_7$ receptor, respectively, while we used ATP to study those on mouse P2X$_7$ receptors. Rat and human P2X$_7$ receptor could form a large pore (8, 14), whereas mouse P2X$_7$ receptor could not (15). The mechanism(s) underlying formation of the permeabilizing pore is not known but the C-terminal domain is required (8). The amino acid sequence of the C-terminal domain of the P2X$_7$ receptor expressed in NG108-15 cells is 80% and 82% identical to those of human and rat P2X$_7$ receptors, respectively. Recently, some amino acid residues that are critical for P2X$_7$ receptor pore-forming activity are identified. Those include tyrosine at position 343 (24) and glutamic acid at position 496 (25). The tyrosine at position 343 is located at the end of the second transmembrane region, and its phosphorylation seems to be important for the full receptor function (24). Those amino acids are conserved in the P2X$_7$ receptor expressed in NG108-15 cells. Recent studies also revealed that the P2X$_7$ receptor interacts with several proteins through the C-terminal of the receptor protein, and such assembly is critical for the P2X$_7$ receptor pore-forming activity (24). Kim et al. (26) demonstrated that such assembly was pronounced in peripheral macrophage and bone marrow cells, but absent in cells derived from the brain. Since NG108-15 cells are of central nervous system origin and the inhibitory effects of Cu$^{2+}$ were previously examined in peripheral cell lines (18, 23), different sensitivities to Cu$^{2+}$ may be related to such different assemblies of the P2X$_7$ receptors.

Cu$^{2+}$ and Mg$^{2+}$ inhibit the P2X$_7$ receptor and the possibility that these divalent cations bind to ATP and reduce the concentration of ATP$^{4-}$, an effective form of ATP as a ligand, has been suggested (8, 14). However, the inhibitory effects of metals in the present study cannot be due to their binding to ATP, because the IC$_{50}$ values of all the metals tested were less than 5 $\mu$M, while ATP was at 1 mM. ATP$^{4-}$ concentrations calculated by the equation described by Fedan et al. (22) ranged between 14 and 144 $\mu$M for the concentration range of ATP between 0.5 and 5 mM. The IC$_{50}$ value of the most potent inhibitor, Cu$^{2+}$, was as low as 16 nM, which is 1000 times less than the calculated ATP$^{4-}$ values. Therefore it is likely that these metals interact with the P2X$_7$ receptors directly and allosterically. The concentration-response curves for ATP-induced I$_{NS-P2X7}$ in the presence of 3 and 10 nM Cu$^{2+}$ (Fig. 3) showed that the inhibition rate of Cu$^{2+}$ on I$_{NS-P2X7}$ was constant at different concentrations of ATP. This also supports our view that Cu$^{2+}$ does not reduce the concentration of ATP$^{4-}$.

There have been various reports on the effects of metals on P2X receptor channels. Zn$^{2+}$ has been investigated most thoroughly. Zn$^{2+}$ potentiated P2X$_2$ and P2X$_3$ channels at 1–100 $\mu$M (27). Cu$^{2+}$ at 1–130 $\mu$M also enhanced cloned P2X$_2$ channels, but not P2X$_3$ channels (28). In human P2X$_4$ channels expressed in Xenopus oocytes, low concentrations (1–10 $\mu$M) of Zn$^{2+}$ enhanced the current, while it was inhibited by a high concentration (1 mM) (29). In rat P2X$_4$ channels expressed in Xenopus oocytes, low concentrations (1–30 $\mu$M) of Zn$^{2+}$ enhanced the current, while it was inhibited by high concentrations (0.3–1 mM) of Zn$^{2+}$ and Cu$^{2+}$ (3–30 $\mu$M) (30). In guinea pig hepatocytes, Cd$^{2+}$ (2 mM) and Ni$^{2+}$ (2 mM) inhibited ATP (100 $\mu$M)-induced current (31). In the rat P2X$_7$ receptor, only inhibitions, but not potentiations, by Cu$^{2+}$ and Zn$^{2+}$ have been reported (18). Taken together, the unique inhibitory effects of metals, especially the high potency of...
Cu²⁺, on the mouse P2X₇ receptors may become a useful tool for distinguishing the mouse P2X₇ receptor function from that of other ATP receptors.

The metals we investigated, Cu²⁺, Zn²⁺, Ni²⁺, Co²⁺, Fe³⁺ and Mn²⁺ are essential trace elements that are cofactors of various enzymes (32). In addition, it has been suggested that Cu²⁺ acts as a neurotransmitter (32–34). Similar findings have also been reported for Zn²⁺ (32, 35, 36) and Fe²⁺ (32). These metals are released from nerve terminals via exocytotic mechanisms, possibly along with other neurotransmitters, and modulate the effects of the co-released neurotransmitters. Further experiments are necessary to elucidate the precise mechanisms of interaction between metals, ATP, and ATP receptors.

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