Cu(II) Inhibition of the Proton Translocation Machinery of the Influenza A Virus M₂ Protein*

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The homotetrameric M₂ integral membrane protein of influenza virus forms a proton-selective ion channel. An essential histidine residue (His-37) in the M₂ transmembrane domain is believed to play an important role in the conduction mechanism of this channel. Also, this residue is believed to form hydrogen-bonded interactions with the ammonium group of the anti-viral compound, amantadine. A molecular model of this channel suggests that the imidazole side chains of His-37 from symmetry-related monomers of the homotetrameric pore converge to form a coordination site for transition metals. Thus, membrane currents of oocytes of Xenopus laevis expressing the M₂ protein were recorded when the solution bathing the oocytes contained various transition metals. Membrane currents were strongly and reversibly inhibited by Cu²⁺ with biphasic reaction kinetics. The biphasic inhibition curves may be explained by a two-site model involving a fast-binding peripheral site with low specificity for divalent metal ions, as well as a high affinity site (Kᵢₒₑₛ ≈ 2 μM) that lies deep within the pore and shows rather slow-binding kinetics (kₘᵢₒ = 18.6 ± 0.9 μM⁻¹ s⁻¹). The pH dependence of the interaction with the high affinity Cu²⁺-binding site parallels the pH dependence of inhibition by amantadine, which has previously been ascribed to protonation of His-37. The voltage dependence of the inhibition at the high affinity site indicates that the binding site lies within the transmembrane region of the pore. Furthermore, the inhibition by Cu²⁺ could be prevented by prior application of the reversible blocker of M₂ channel activity, BL-1743, providing further support for the location of the site within the pore region of M₂. Finally, substitutions of His-37 by alanine or glycine eliminated the high affinity site and resulted in membrane currents that were only partially inhibited at millimolar concentrations of Cu²⁺. Binding of Cu²⁺ to the high affinity site resulted in an approximately equal inhibition of both inward and outward currents. The wild-type protein showed very high specificity for Cu²⁺ and was only partially inhibited by 1 mM Ni²⁺, Pt²⁺, and Zn²⁺. These data are discussed in terms of the functional role of His-37 in the mechanism of proton translocation through the channel.

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Cu²⁺ Inhibition of Influenza Virus M₂ Ion Channel

FIG. 1. Location of histidine residues and possible mode of interaction of the M₂ transmembrane region with amantadine. A, the previously described model of the transmembrane region of the M₂ protein (18). The fourth helix of the tetramer has been removed from the front of the structure to reveal the pore interior. A large cavity can be seen at Gly-34, followed by an occlusion formed by the His-37 side chains. The backbone atoms and histidine side chains are shown as stick and ball-and-stick representations, respectively. A surface generated using a 1.4 Å radius probe (32) is shown for each helix. The histidine side chains from each of the helices pack together in an arrangement that may greatly retard the flow of most ions through the channel. However, by alternately protonating/deprotonating the N⁺ and N⁰ atoms of the histidine residues, it may be possible to shuttle protons through the channel in a mechanism that resembles the proton shuttle of carbonic anhydrase (23, 24). The central pore in our model reaches its widest diameter (~4 Å) near the center of the bilayer, just above His-37. The widening of the aqueous pore may be important for minimizing the dehydration energy of protons as they pass through the channel; charged species diffusing through an aqueous pore in an otherwise low dielectric environment encounter an unfavorable dehydration energy, which reaches a maximum near the center of the bilayer. This energy barrier may be lowered by widening the pore near the center of the bilayer similar to that found in the K⁺ channel (26).

To test further the role of His-37 in the function and inhibition of M₂, we have examined the ability of Cu(II) to inhibit the channel. Examination of the model (Fig. 1) indicated that this ion could interact with the four histidine imidazole groups similarly to the role proposed for histidine as the chelating ligand for Cu(II) binding to prion protein (27). We demonstrate that Cu(II) indeed binds to a high affinity site within the protein in a slow, time-dependent process.

EXPERIMENTAL PROCEDURES

Site-specific mutagenesis of M₂ cDNA was performed as described previously (15, 19). In vitro synthesis of mRNA was performed using the mMessage mMACHINE T7 Transcription Kit (Ambion, Austin, TX).

Microinjection and Culture of Oocytes—Ovarian lobules from individually identified Xenopus laevis females (Nasco, Fort Atkinson, WI) were surgically removed and treated with collagenase B (2 mg/ml; Boehringer Mannheim) in Ca²⁺-free OR-2 solution (82.5 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 5 mM HEPES-NaOH, pH 7.5) at 24 °C for 30–45 min to liberate oocytes from follicle cells. Defolliculated oocytes were washed in OR-2 and maintained in ND-96 solution (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 2.5 mM sodium pyruvate, 5 mM Glucose).
Cu²⁺ Inhibition of Influenza Virus M₂ Ion Channel

**Table I**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>0.1 mM (●)</th>
<th>0.5 mM (●)</th>
<th>1.0 mM (●)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(I_{\text{lim}})</td>
<td>0.11 ± 0.01</td>
<td>0.14 ± 0.01</td>
<td>0.14 ± 0.01</td>
</tr>
<tr>
<td>(\alpha_f)</td>
<td>0.48 ± 0.02</td>
<td>0.46 ± 0.02</td>
<td>0.25 ± 0.0</td>
</tr>
<tr>
<td>(K_{\text{fast}}) (Global)</td>
<td>93 ± 5 M s⁻¹</td>
<td>93 ± 5 M s⁻¹</td>
<td>93 ± 5 M s⁻¹</td>
</tr>
<tr>
<td>(K_{\text{slow}}) (Global)</td>
<td>18.6 ± 0.9 M s⁻¹</td>
<td>18.6 ± 0.9 M s⁻¹</td>
<td>18.6 ± 0.9 M s⁻¹</td>
</tr>
</tbody>
</table>

**Fig. 2. Time course of inhibition of the wild-type M₂ ion channel protein by Cu²⁺.** The symbols in this and the following figures show the recorded currents (mean ± S.E. for 4 cells), and the lines show the currents fit with a double exponential function. See Equation 1.

\[
I(t) = I_{\text{lim}} + (1 - \lim)(\alpha_fexp(-\text{Cu}^{2+}t) + (1 - \alpha_f)exp(-\text{Cu}^{2+}t))
\] (Eq. 1)

See Table I for parameters that were fitted to these data.

HEPES-NaOH, pH 7.5) until injection of ~40 ng of mRNA. After this time they were maintained in ND-96, pH 8.5, in order to minimize the proton flux due to the action of the M₂ ion channel. All solutions were equilibrated with room air except for solutions containing Cu(I), which were equilibrated with N₂. In control experiments we found that the currents of oocytes expressing the M₂ protein incubated in Barth’s solution equilibrated with N₂ (pH 7.5 or pH 6.2) for up to 20 min were indistinguishable from those of oocytes incubated at the same pH in Barth’s solution equilibrated with room air. Metabolic labeling of oocytes and analysis of proteins by SDS-polyacrylamide gel electrophoresis was carried out as described previously (15).

Electrophysiological Recordings—24–48 h after RNA injection, whole-cell currents were recorded with a two-electrode voltage-clamp apparatus consisting of a differential amplipipette (Nihon Kohden CEZ-1100) that recorded the voltage difference between a pipette (filled with 3 M KCl) located in the cell and another in the surrounding bath. A voltage-clamp amplifier (Nihon Kohden CEZ-1100) provided feedback current to the oocyte through a second intracellular pipette. Oocyte voltage clamp records were recorded in standard Barth’s solution (0.3 M NaCl, 0.71 M CaCl₂, 0.82 M MgSO₄, 1.0 mM KCl, 2.4 M NaHCO₃, 88 mM NaCl, 15.0 mM MES, pH 6.2, or 15.0 mM HEPES, pH 7.5) or a modified Barth’s solution as indicated. Amantadine hydrochloride (Sigma) (10 mM stock in Barth’s solution), CuCl₂, and BL-1743 (kindly provided by Dr. Mark Krystal, Squibb, Wallingford, CT) were diluted as indicated. To check that the oocytes did not develop nonspecific leakage currents during the recordings, we applied amantadine to the bathing solution (pH out 5.0–7.5) or a modified Barth’s solution as indicated. Amantadine hydrochloride (100 μM) for 2–5 min at the end of the measurements from each oocyte. Data from an oocyte were only used if the current in the presence of amantadine was less than 10% of the initial current.

Reagents—CdCl₂, MgCl₂, PtCl₃(NH₃)₂, and ZnCl₂ were purchased from Sigma; CuCl₂ and Cu(I) tetrakis(acetonitrile)hexafluorophosphate were purchased from Aldrich; MnCl₂ was purchased from J. T. Baker Inc., and AgNO₃ was purchased from Fisher.

**RESULTS**

Inhibition of Currents by Cu²⁺—Cu²⁺ is a potent, time-dependent inhibitor of the channel activity of oocytes that express the M₂ protein (Fig. 2 and Table I). In the absence of M₂ protein, Xenopus oocytes have very small currents in the range of pH 4.0–pH 9.0. Oocytes that express M₂ protein have currents that are dependent on pH of the bathing medium (14, 25). For pH 7.5 the current is double the background current found in control oocytes. However, at pH 6.2 the current is about 8-fold higher than the current at pH 7.5 (14, 25). Bathing oocytes that express wild-type M₂ protein in a solution that contains 100 μM amantadine causes the currents to decrease to background levels within 2–5 min (14, 16). Prolonged exposure of oocytes to Cu²⁺ can be toxic, so we took a number of precautions to ensure that the observed inhibition was specific to the M₂ ion channel. Before applying media containing transition metals, we first confirmed that the whole-cell current of each oocyte was activated by low pH out. We also tested each cell for the inhibition of current by amantadine by applying the drug after washing out the transition metal. The data reported here are from oocytes whose currents were increased 4–6-fold by decreasing pH out from pH 7.5 to pH 6.2 and whose currents were reduced to 100–200 nA by amantadine (100 μM for 2 min). These values for activation by low pH and inhibition by amantadine are within the range for M₂-expressing oocytes in the absence of transition metals and thus provide assurance that the inhibition reported is specific to the M₂ ion channel and is not confounded with nonspecific leakage currents.

Addition of Cu²⁺ to the bathing solution (pH out = 6.2) gives rise to a time-dependent decrease in the amantadine-sensitive channel activity, approaching full inhibition at long times (Fig. 2 and Table I). The time course of inhibition differs from that for amantadine or other hydrophobic drugs, which generally show simple, pseudo-first order decay kinetics under conditions where the drugs are in large excess. Instead, the data for inhibition by Cu²⁺ is biphasic, and the data can be fit by a sum of two exponentials (Fig. 2 and Table I), representing a fast and a slow process with both relaxation times linearly proportional to the concentration of Cu²⁺ between 100 and 1000 μM. Indeed, we show below that the initial rapid rate (93 ± 5 M⁻¹ s⁻¹) is associated with a nonspecific, partial block of the channel that is not dependent on His-37, whereas the slower process (18.6 ± 0.9 M⁻¹ s⁻¹) requires the presence of histidine at position 37. The second order rate constant associated with this latter process is nearly 2 orders of magnitude slower than the value observed for inhibition of the M₂ channel activity (A/Udorn subtype) by amantadine.

Fig. 3 and Table II illustrate the recovery of the amantadine-sensitive channel activity following removal of Cu²⁺. Again, the data can be fit by two exponentials. Interestingly, the curves show a small initial burst, possibly reflecting dissociation from the low affinity site, followed by a slow recovery of channel activity representing dissociation from the high affinity site. Indeed, the fraction of the fast component of the recovery rate constants, assuming that these are kinetically independent (mechanistic analysis from Appendix A yields 400 μM or 250 μM, depending on the mechanism chosen). The corresponding dissociation constant for the high affinity site (using

1 The abbreviation used is: MES, 4-morpholineethanesulfonic acid.
the ratio of slow off to fast on rates (constant) would be about 2 µM (Appendix A yields 1.9 or 1.6 µM), significantly tighter.

PH Dependence of the Cu²⁺-Binding Site—To help identify the nature of the binding sites, we first determined if they could be titrated within the range of pH we were able to test. This titration was done by measuring the time course of inhibition and recovery from inhibition in solutions of various pH values. These experiments paralleled previous experiments in which we showed that the pH dependence of the interaction required the presence of His-37. Similarly, the time course of inhibition was slower at acidic pH (Fig. 4), and the recovery was faster for the lower values of pH. We were able to test only the effects of pH in the range pH 5.2–6.2. Below pH 5.2 irreversible effects due to large proton currents flowing for many seconds were seen, and above pH 6.2 the current amplitude was too small to measure inhibition directly.

Voltage Dependence of Cu²⁺ Binding—If the Cu²⁺-binding sites lie within the transmembrane region, then the rate of association of the positively charged copper ion may be accelerated at negative applied potentials. To test this possibility we measured the time course of inhibition by 250 µM Cu²⁺ at various holding voltages. We found that the rate of onset of inhibition was greater for more negative holding voltages (Fig. 5 and Table III), consistent with the presence of at least one Cu²⁺-binding site that is located at least partially inside the electric field of the membrane.

Directionality of Cu²⁺ Inhibition—Cationic, open-pore blocker molecules usually attenuate only the current originating from the side of the membrane to which they are applied. An example of this is the block of the outward, but not inward, K⁺ current of the squid axon by internally applied tetraethylammonium⁺ cation (28). We tested the directionality of the inhibition by Cu²⁺ by measuring the current–voltage relationship of the oocyte (pH_ext = 6.2) with slowly varying ramps of current before and at various times after the application of Cu²⁺. For these experiments, the leakage current in the presence of amantadine was subtracted in order to obtain an accurate estimate of the reversal potential, and the membrane voltage was restricted to +50 mV to avoid activating endoge-
nous channels. We found (in six cells) that inward and outward currents were attenuated equally (see Fig. 6) and that the reversal voltage remained constant with increasing percentage inhibition by Cu$_{2}^{+}$. Thus, inhibition by Cu$_{2}^{+}$, like inhibition by amantadine (see Fig. 6, inset), is bi-directional.

Binding of BL-1743 Prevents Binding of Cu$_{2}^{+}$—If Cu$_{2}^{+}$ binds to an internal site, it should be possible to block binding by prior application of a compound that either competes for the same site or occupies the outer regions of the pore. The compound BL-1743 has been shown to be a reversible inhibitor of the currents of the M$_{2}$ ion channel (15). Mutations conferring resistance to BL-1743 map to the pore region immediately above His-37 (as viewed in Fig. 1), suggesting that BL-1743 penetrates deeply into the pore.

We tested the ability of BL-1743 to prevent inhibition by Cu$_{2}^{+}$. The test depended on the fact that recovery from inhibition by BL-1743 (Fig. 7 and Ref. 15) is considerably faster than that from 0.5 mM Cu$_{2}^{+}$ (4 min versus 4 h, respectively). To determine whether BL-1743 prevents inhibition by Cu$_{2}^{+}$, we performed the following steps: (i) inhibited the channel completely with BL-1743; (ii) while maintaining the concentration of BL-1743 constant, added 0.5 mM Cu$_{2}^{+}$ to the solution for 5 min (such that the Cu$_{2}^{+}$ would have completely inhibited the currents had it been applied alone); (iii) washed out free Cu$_{2}^{+}$ from the recording chamber briefly (2 min) while BL-1743 was still maintained in the solution; (iv) washed out BL-1743 and measured the time course of recovery. Our earlier results demonstrated that the time course of recovery from inhibition by Cu$_{2}^{+}$ alone is much slower than that from BL-1743. Thus, if Cu$_{2}^{+}$ had gained access to an internal binding site in the presence of BL-1743, then during washout of BL-1743 the recovery would have been slow and incomplete. However, we found that the time course of the recovery during washout of BL-1743 did not differ from the time course that would have been measured had Cu$_{2}^{+}$ not been applied (Fig. 7). Thus, BL-1743 prevented the binding of Cu$_{2}^{+}$ to a presumably internal site.

Mutation of His-37 Eliminates the High Affinity Cu$_{2}^{+}$-binding Site—The above results strongly suggest that the high affinity Cu$_{2}^{+}$-binding site lies within the transmembrane pore of M$_{2}$. To confirm these observations, we examined the ability of Cu$_{2}^{+}$ to inhibit a number of variants of M$_{2}$ in which potential chelating groups were altered.

![Fig. 6. Cu$^{2+}$ inhibits both inward and outward amantadine-sensitive currents.](image_url)

**Table III**

Parameters fitted to the time course of onset of inhibition and recovery from inhibition by 0.25 mM Cu$^{2+}$ for membrane voltages between -40 and +40 mV (see Fig. 5)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>(○) -40 mV</th>
<th>(■) -20 mV</th>
<th>(▲) 0 mV</th>
<th>(▼) +20 mV</th>
<th>(●) +40 mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_{ip}$ (M s$^{-1}$)</td>
<td>112.3</td>
<td>94.9</td>
<td>37.6</td>
<td>201.0</td>
<td>43.0</td>
</tr>
<tr>
<td>$k_{op}$ (M s$^{-1}$)</td>
<td>16.7</td>
<td>11.3</td>
<td>14.2</td>
<td>9.37</td>
<td>11.9</td>
</tr>
<tr>
<td>$10^{2}k_{ip}$ (s$^{-1}$)</td>
<td>51.8</td>
<td>0.388</td>
<td>0.71</td>
<td>2.8</td>
<td>Unmeas.*</td>
</tr>
<tr>
<td>$10^{2}k_{op}$ (s$^{-1}$)</td>
<td>0.31</td>
<td>0.386</td>
<td>0.084</td>
<td>0.076</td>
<td>Unmeas.</td>
</tr>
</tbody>
</table>

*Unmeasured.
region is His-37, and this residue has been shown to be important for amantadine inhibition. Thus, we compared the ability of Cu$^{2+}$ to inhibit the mutant proteins M$_2$-H37A and M$_2$-H37G with its ability to inhibit the wild-type M$_2$ protein. In addition, we examined the role of the Asp-24, which lies near the N terminus of the a-helix, and Ser-31, which lines a portion of the predicted pore. Finally, wild-type M$_2$ protein has two cysteine residues at positions 17 and 19 (29), the charged aspartate residue at position 24, or the polar serine residue at position 31. Perhaps this difference was due to the reduced charge compensating for the lower ionic strength of the solution.

We also tested the possibility that the cysteine residues located at position 17 and 19 (29), the charged aspartate residue at position 24, or the polar serine residue at position 31 might provide a low affinity binding site for Cu$^{2+}$ that may be partly responsible for the rapid recovery from inhibition by 1 mM Cu$^{2+}$ (see Fig. 3, Table II, and “Discussion”). We applied Cu$^{2+}$ to oocytes expressing the cysteineless (29) mutant protein and the mutant proteins M$_2$-D24A, M$_2$-S31A, and M$_2$-S31G. The only departure from the behavior of wild-type protein for any of these proteins in response to application of Cu$^{2+}$ (0.1 and 1.0 mM) was that the rate of onset of inhibition was slightly slower for the M$_2$-D24A mutant protein than for the wild-type protein. Perhaps this difference was due to the reduced charge of the M$_2$-D24A protein at the mouth of the presumed pore region. These results suggest that neither Cys-17, Cys-19, Asp-24, nor Ser-31 participate directly in the inhibition of the channel by Cu$^{2+}$.

**Metal Ion Specificity—**To characterize further the ion specificity of the low and high affinity binding sites in M$_2$, we evaluated a series of transition metal ions, including Cd$^{2+}$, Mg$^{2+}$, Mn$^{2+}$, Ni$^{2+}$, Pt$^{2+}$, and Zn$^{2+}$. As was expected for the wild-type protein (Fig. 5 and Table III). Moreover, we found that the inhibition of neither mutant protein was strongly dependent on pH (see Fig. 9 for M$_2$-H37G), in contrast to the greater pH dependence found for the wild-type protein (Fig. 4). These results suggest that replacement of His-37 with a residue incapable of coordinating Cu$^{2+}$ leaves the ion channel with a low affinity binding site that is located near the outside of the electric field.

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**TABLE IV**

Parameters fitted to the inhibition of the current of M$_2$-H37G mutant protein by 0.1 mM Cu$^{2+}$ for voltages between −40 and +20 mV

<table>
<thead>
<tr>
<th>Voltage (mV)</th>
<th>$k_f$ (s$^{-1}$)</th>
<th>$k_i$ (s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>−40</td>
<td>561</td>
<td>0.0396</td>
</tr>
<tr>
<td>−20</td>
<td>660</td>
<td>0.0320</td>
</tr>
<tr>
<td>0</td>
<td>590</td>
<td>0.031329</td>
</tr>
<tr>
<td>+20</td>
<td>475</td>
<td>0.0086490</td>
</tr>
</tbody>
</table>
replacement of a relatively soft for a hard metal ion, the replacement of Cu$^{2+}$ with Mg$^{2+}$ led to essentially no inhibition, indicating that Mg$^{2+}$ does not interact with either site. Similarly, Mn$^{2+}$, which has ligand preferences similar to Mg$^{2+}$, but also shows some "soft" character, inhibited the channel by less than 10% at 1 mM concentration. We next examined Cu$^{2+}$ and Zn$^{2+}$, which have ligand preferences similar to Cu$^{2+}$ but have a preference for octahedral or tetrahedral complexes and would be less likely to assume the distorted square pyramidal complex hypothesized for Cu$^{2+}$. These metal ions showed partial inhibition of the channel (Table V), and recovery from inhibition by these metal ions was nearly complete within 2–5 min. We therefore tentatively assign their effects to interactions with the low affinity site. In a similar manner, we examined Ni$^{2+}$ and Pt$^{2+}$, which have a preference for forming square planar complexes; again these ions gave rise to only partial, rapidly reversible inhibition of the channel (Table V). All of these data suggest that the high affinity metal ion-binding site is quite specific for Cu$^{2+}$, whereas the remaining, low affinity site is less specific and able to interact with a variety of metal ions. To confirm this suggestion, we tested the ability of 1 mM Cd$^{2+}$, Ni$^{2+}$, and Zn$^{2+}$ to inhibit the currents of the M2-H37A and M2-H37G mutant proteins. Indeed, all three metal ions inhibited these mutants in a manner similar to that observed for the wild-type protein (data not shown).

**DISCUSSION**

The experiments described here demonstrate that Cu$^{2+}$ and other transition metals are capable of inhibiting the M2 ion channel of influenza A virus. Inhibition and recovery from inhibition can be explained by a model with two binding sites, each capable of inhibiting the current upon binding a ligand. One site, of low affinity and low specificity for Cu$^{2+}$, is located near the outside of the electric field of the membrane and only partially impeds ionic current through the channel. The second site has higher affinity for Cu$^{2+}$, is located inside the applied electric field, and more completely blocks current flow. The high affinity site is probably formed by the association of imidazole side chains of His-37 from the transmembrane helices of the M2 tetramer.

The strong voltage dependence of the level of inhibition of the current of the wild-type channel by Cu$^{2+}$ (Fig. 5 and Table III) suggests that the imidazole of His-37 is tightly coupled to the high affinity binding site for Cu$^{2+}$. This is consistent with models showing His-37 to be located well inside the presumed transmembrane domain (18, 22), which begins with Pro-25. Also, inhibition by low [Cu$^{2+}$] is dependent on pH$_{out}$.

<table>
<thead>
<tr>
<th>Metal</th>
<th>Concentration</th>
<th>Steady state fractional inhibition (Mean ± S.E.)</th>
<th>Time</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cu$^{2+}$</td>
<td>500 μM</td>
<td>0.95 ± 0.01</td>
<td>600</td>
<td>4</td>
</tr>
<tr>
<td>Cu$^{2+}$</td>
<td>50 μM</td>
<td>0.71 ± 0.05</td>
<td>60</td>
<td>4</td>
</tr>
<tr>
<td>Zn$^{2+}$</td>
<td>1 mM</td>
<td>0.28 ± 0.02</td>
<td>300</td>
<td>4</td>
</tr>
<tr>
<td>Ni$^{2+}$</td>
<td>1 mM</td>
<td>0.13 ± 0.01</td>
<td>300</td>
<td>4</td>
</tr>
<tr>
<td>Pt$^{2+}$</td>
<td>1 mM</td>
<td>0.35 ± 0.00</td>
<td>120</td>
<td>4</td>
</tr>
<tr>
<td>Mg$^{2+}$</td>
<td>2 mM</td>
<td>0.04 ± 0.01</td>
<td>300</td>
<td>4</td>
</tr>
<tr>
<td>Mn$^{2+}$</td>
<td>1 mM</td>
<td>0.07 ± 0.03</td>
<td>300</td>
<td>4</td>
</tr>
</tbody>
</table>

*Currents measured at −120 mV.*
effects on other ion channels. Among the best-studied of these channels is the Na⁺ channel; however, the inhibition of the Na⁺ channel by transition metals differs greatly from the inhibition of the M₂ ion channel by Cu²⁺. 1) Cu²⁺ inhibits both inward and outward currents of the M₂ ion channel, but transition metals inhibit the inward current of the cardiac Na⁺ channel. 2) The Cu²⁺-binding site in M₂ is highly specific for Cu²⁺, whereas Cd²⁺ and Mn²⁺ inhibit the cardiac Na⁺ channel but do not inhibit the M₂ ion channel. 3) The high affinity binding site differs; the imidazole of His-37 of the transmembrane domain of the M₂ ion channel is probably the high affinity binding site for Cu²⁺, but Cys-401 of the pore region of the cardiac Na⁺ channel is thought to be the high affinity binding site for Cd²⁺ and Zn²⁺ (30, 31). Such differences in metal binding indicate that these ion channel proteins have stable, but different, structures that present coordinating ligands of different affinities to permeating metal ions.

The results of our experiments have important implications for future work. First, Cu²⁺ has an unpaired electron and thus could serve as a probe for structural studies of the M₂ molecule using either electron paramagnetic resonance or ENDOR methods. Second, the inhibition of the M₂ ion channel by low [Cu²⁺] shares several important properties with the inhibition by amantadine; both are slowed by low pH, both inhibit currents in both inward and outward directions, and both depend on the presence of His-37. If Cu²⁺ and amantadine interact with the imidazole of His-37 in a similar fashion, then information gained using Cu²⁺ may be helpful in the design of inhibitors that are more useful than amantadine.

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APPENDIX

This Appendix derives kinetic models for two-state binding of Cu²⁺ to the M₂ ion channel protein. We envision an outer site located in the pore of the channel close to the outside of the membrane electric field and an inner site located near the inside and well within the transmembrane electric field. Binding of Cu²⁺ to the outer site is assumed to be capable of inhibiting the current to a fraction of its initial value, whereas binding to the inner site inhibits completely. States are defined by their location and Cu²⁺ occupancy: Sa is the state defined by unoccupied inner and outer sites, Sb by occupancy of only the outer site, Sc by only the inner site, and Sd by both sites occupied. Different pseudo-equivalent kinetic schemes can be devised to connect the states. We used two as follows: the first (Scheme 1) prohibiting Sa to Sc but allowing a direct, reversible Sa to Sd transition; and the second (Scheme 2) allowing Sa to Sc and prohibiting Sa to Sd. Note that no distinction is made in this mechanism between the two sides of the membrane so it is possible that the Sd to Sa transition involves Cu²⁺ entering the cytoplasm of the oocyte.

The forward and reverse reaction rate constants for each transition are defined in the following equations of Schemes 1 and 2.

\[
\frac{dS_a}{dt} = -(k_{a1}[Cu^{2+}] + k_{a2}[Cu^{2+}]^2)S_a + k_{b1}S_b + k_{d1}S_d
\]

\[
\frac{dS_b}{dt} = -(k_{a1} + k_{a2})S_b + k_{b1}[Cu^{2+}]S_a + k_{c2}S_c
\]

\[
\frac{dS_c}{dt} = -(k_{a1}[Cu^{2+}] + k_{b1}S_b + k_{b1}S_c + k_{d1}S_d
\]

\[
\frac{dS_d}{dt} = -(k_{b1} + k_{d1})S_d + k_{a1}[Cu^{2+}]S_a + k_{a1}[Cu^{2+}]S_c
\]

SCHEME 1

SCHEME 2

These equations, with the initial condition that Sa = 1 and all other states are empty, were used (by the program MLAB; Civilized Software, Inc., Bethesda, which has a built-in differential equation solving routine (33)) to generate current-time records for the three different Cu²⁺ concentrations (100, 500, and 1000 μM). Copper concentrations were taken to be step functions with transitions corresponding to those applied in each experiment, and the rate constants were estimated by curve-fitting. The parameters fitted to the data using each of these model schemes are shown in Table IA.

Standard errors, as reported by the MLAB program, are for qualitative comparison only because they depend on the un-supportable assumption that the curve-fitting error function varies linearly with the fitting parameters. The number of parameters in the models discourages quantitative interpretation of these results.

To compare the site affinities for Cu²⁺, the reactions Sa + Cu²⁺ → Sc and Sa + Cu²⁺ → Sd are the relevant equilibria. The “operational” site dissociation constants \(K_{outer}\) and \(K_{inner}\) corresponding to the above reactions can be calculated from the rate constant ratios. For Scheme 1, \(K_{outer} = k_{a1}/k_{a1} = 0.4 \text{ mM}\) and \(K_{inner} = K_{outer} k_{b1}/k_{d1} = 1.9 \mu M\). For Scheme 2, \(K_{outer} = k_{b1}/k_{d1} = 0.25 \text{ mM}\) and \(K_{inner} = k_{b1}/k_{d1} = 1.6 \mu M\).
REFERENCES