Extracellular Zinc Ion Inhibits ClC-0 Chloride Channels by Facilitating Slow Gating

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ABSTRACT Extracellular Zn$^{2+}$ was found to reversibly inhibit the ClC-0 Cl$^{-}$ channel. The apparent on and off rates of the inhibition were highly temperature sensitive, suggesting an effect of Zn$^{2+}$ on the slow gating (or inactivation) of ClC-0. In the absence of Zn$^{2+}$, the rate of the slow-gating relaxation increased with temperature, with a Q$_{10}$ of $\sim$37. Extracellular Zn$^{2+}$ facilitated the slow-gating process at all temperatures, but the Q$_{10}$ did not change. Further analysis of the rate constants of the slow-gating process indicates that the effect of Zn$^{2+}$ is mostly on the forward rate (the rate of inactivation) rather than the backward rate (the rate of recovery from inactivation) of the slow gating. When ClC-0 is bound with Zn$^{2+}$, the equilibrium constant of the slow-gating process is increased by $\sim$30-fold, reflecting a 30-fold higher Zn$^{2+}$ affinity in the inactivated channel than in the open-state channel. As examined through a wide range of membrane potentials, Zn$^{2+}$ inhibits the opening of the slow gate with equal potency at all voltages, suggesting that a two-state model is inadequate to describe the slow-gating transition. Following a model originally proposed by Pusch and co-workers (Pusch, M., U. Ludewig, and T. Jentsch. 1997. J. Gen. Physiol. 109:105–116), the effect of Zn$^{2+}$ on the activation curve of the slow gate can be well described by adding two constraints: (a) the dissociation constant for Zn$^{2+}$ binding to the open channel is 30 $\mu$M, and (b) the difference in entropy between the open state and the transition state of the slow-gating process is increased by 27 J/mol/K for the Zn$^{2+}$-bound channel. These results together indicate that extracellular Zn$^{2+}$ inhibits ClC-0 by facilitating the slow-gating process.

KEY WORDS: ClC-0 • Zn$^{2+}$ • slow gating • inactivation • temperature dependence

INTRODUCTION

ClC-0, the first cloned member of the ClC channel family, is a voltage-dependent chloride channel that is present abundantly in the Torpedo electric organ. The open–close transition of this channel is known to involve two different gating processes, both of which are voltage dependent (for review, see Miller and Richard, 1990; Pusch and Jentsch, 1994). In one, the channel opens, or activates, when membrane potential is depolarized, and closes, or deactivates, when membrane potential is hyperpolarized. This transition happens in a relatively fast time scale of milliseconds and is thought to be controlled by a “fast” gate (Miller, 1982; Hanke and Miller, 1983; Pusch et al., 1995; Chen and Miller, 1996). The other gating process, which resembles inactivation of other voltage-gated channels, operates in a time scale of tens of seconds. This “slow” gate opens when membrane potential hyperpolarizes and closes when membrane potential depolarizes (White and Miller, 1979; Pusch et al., 1997). Previous studies have suggested that there are two identical “protomers” in a channel, each of which probably contains a pore and an independent fast gate (Miller, 1982; Middleton et al., 1994, 1996; Ludewig et al., 1996). On the other hand, the inactivation (or the close–open transition of the slow gate) occurs at the same time for the two protocannels (Miller and White, 1984; Richard and Miller, 1990; Pusch et al., 1997).

The above two gating processes function in an interesting way in that they both show Cl$^{-}$ as well as voltage dependence. The fast gate decreases its open probability upon reducing the extracellular Cl$^{-}$ concentration (Pusch et al., 1995), suggesting that the permeant ion, Cl$^{-}$, may act as the gating charge for the channel opening (Pusch et al., 1995; Chen and Miller, 1996). The interaction of Cl$^{-}$ with the slow gating is even more intriguing. It was found that Cl$^{-}$ flux through the channel pore was coupled to a nonequilibrium gating among closed, open, and inactivated states of the channel (Richard and Miller, 1990). The underlying mechanism of this phenomenon is still unknown. The slow gating of ClC-0 is difficult to characterize in a quantitative manner at the single channel level because the gate operates at a very slow rate. Recently, by measuring macroscopic current relaxation from Xenopus oocytes expressing ClC-0, Pusch et al. (1997) discovered that the inactivation rate of ClC-0 was highly temperature dependent with a Q$_{10}$ of $\sim$40.

The high temperature dependence of the slow gating of ClC-0 suggests that the process may involve a very
complex conformational change of the channel molecule (Pusch et al., 1997). The mechanism underlying this complex slow-gating transition, however, is not well understood. There have been so far only a few point mutants of the channel whose slow-gating processes are altered (Ludewig et al., 1996, 1997). The amino acid residues that have been identified, however, are scattered throughout the entire channel sequence, making a mechanistic interpretation of the mutation effect rather difficult. Another problem that hinders a better understanding is that there are few useful molecular probes that can interfere with this gating transition. Previously, Cd²⁺, Ni²⁺, and Zn²⁺ have been shown to interact with the permeation and the gating processes in a variety of ion channels (Backx et al., 1992; Karpen et al., 1993; Yellen et al., 1994; Gordon and Zagotta, 1995; Liu et al., 1997). Results from studying the interaction of these divalent cations with channel proteins provide insightful information to understand not only the functional but also the structural aspects of these channel molecules. The interesting issue here is whether these useful metal ions are able to interact with CIC-0.

The modulation of CIC-type Cl⁻ channels by divalent metal ions is not without precedence. Early investigations have revealed that Zn²⁺ inhibits the resting conductance of the frog skeletal muscle (Hutter and Ni, 1987). The molecular mechanism of the slow-gating process of ClC-0, however, was not understood. ClC-0 is similar to ClC-1 not only in the amino acid sequence, but also in gating properties (Jentsch et al., 1990; Steinmeyer et al., 1991). In the present study, we show that Zn²⁺ can also inhibit the ClC-0 channel. Unlike in the case of CIC-1, the inhibition of CIC-0 by Zn²⁺ is reversible. Detailed characterization of this inhibition indicates that the effect appears to be due to a facilitation of the slow gating. This observation thus provides an opportunity of using Zn²⁺ to explore the molecular mechanism of the slow-gating process of ClC-0.

**Materials and Methods**

**Molecular Biology and Channel Expression**

All experiments were carried out in *Xenopus* oocytes expressing wild-type ClC-0 originally cloned from *Torpedo* electric organ (Jentsch et al., 1990). The modified version of the cDNA, pRM105, in which most of the 3' and 5' untranslated regions were deleted, was first linearized by restriction enzyme Scal (New England Biolabs Inc., Beverly, MA). Capped cRNA was then transcribed in vitro using the T3 mMessage mMachine kit (Ambion Inc., Austin, TX). *Xenopus* oocytes were surgically removed and dissociated for 1 h in nominally calcium-free OR-2 solution (containing [mM]: 96 NaCl, 2 KCl, 2 MgCl₂, and 5 Na-HEPES, pH 7.6) containing 1 mg/ml of type II collagenase (GIBCO BRL, Gaithersburg, MD). Follicle-free stage V–VI oocytes were selected and injected with 50 nl synthetic mRNA in distilled water (0.01–0.2 mg/ml). Injected oocytes were maintained at 18°C (±3°C) in ND96 solution (containing [mM]: 96 NaCl, 2 KCl, 1.8 CaCl₂, 1 MgCl₂, and 5 Na-HEPES, pH 7.6, plus 0.2 mg/ml gentamicin) and, with daily change of the ND-96 maintaining solution, were usually alive to survive for 7–10 d.

**Electrical Recording and Data Analysis**

Whole oocyte ClC-0 current was recorded with a two-electrode voltage clamp amplifier (725C; Warner Instruments, Hamden, CT) 1–4 d after mRNA injection. Microelectrodes were filled with 3 M KCl and had resistance of 0.2–1.0 MΩ. The current, filtered at 1 kHz by the built-in filter of the amplifier, was digitized at 2–5 kHz using Digidata 1200 data acquisition board and pClamp6 software (Axon Instruments, Foster City, CA). Expression of ClC-0 was checked with a voltage protocol similar to that shown in Fig. 2 A. This protocol elicited a characteristic current crossover in the voltage range from −150 to −80 mV in the injected oocytes (see Fig. 2B), indicating a successful expression of ClC-0.

For a typical experiment, the recording room was set at the desired temperature. Oocytes were then brought out of the incubating chamber to equilibrate the temperature of incubating solution to the room temperature at least 30 min before recording. The bathing (external) solution for assaying the effect of Zn²⁺ was a low Ca²⁺–ND96 solution containing (mM): 96 NaCl, 2 KCl, 0.5 CaCl₂, 1 MgCl₂, and 5 Na-HEPES, pH 7.6. ZnCl₂ (J.T. Baker, Inc., Phillipsburg, NJ) and chloride salts of other transition metal ions were dissolved in water as 100 mM stock solution and then added to the low Ca²⁺–ND96 solution to obtain the indicated concentration.

To examine the kinetics of the slow-gating process, the slow gate was first opened with multiple short hyperpolarized pulses (−120 to −150 mV, 50–200 ms in duration) given at a frequency of 0.5–1 Hz. The membrane potential was then held at −30 mV, a value close to the equilibrium potential of Cl⁻, and a 100-ms voltage pulse to +40 mV was given every 2–6 s to monitor the current relaxation. The frequency of the given +40-mV voltage pulses had no effect on the slow-gating relaxation rate.

To evaluate the effects of Zn²⁺ and other metal ions, the oocyte current was monitored with repetitive voltage pulses at a constant holding potential, usually −30 mV. The metal ions were applied only after the current reached a steady state. EGTA–ND96 solution was used as the control and wash solutions. Its composition was the same as that of the low Ca²⁺–ND96 solution, except that an additional 1 mM CaCl₂ and 1 mM EGTA was added, with the pH adjusted to 7.6 after addition of the extra components. Since EGTA has a higher affinity for Zn²⁺ than for Ca²⁺, the wash solution chelates any leftover Zn²⁺ (Yellen et al., 1994). The switch between EGTA–ND96 solution and low Ca²⁺–ND96 solution causes little change in the current amplitude. For more precise measurements of the on and off rates of Zn²⁺ inhibition (see Fig. 3 A), the flow rate was at 25 ml/min, whereas the volume of the recording chamber was ~1 ml. The time constant of the solution exchange was ~2 s, as judged from the time course of tetraethylammonium (TEA) inhibition of a Shaker K⁺ channel (see Fig. 3B). It therefore took ~5–6 s to exchange the

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1Abbreviation used in this paper: TEA, tetraethylammonium.
solution of the recording chamber to 99%. This delay was relatively small in comparison with the time constants of the slow gating of ClC-0 in most experiments. As the slow gating of ClC-0 and the Zn$^{2+}$ inhibition were highly temperature dependent, all solutions used for recording were incubated at the desired temperature before use. The temperature of the perfusion solution was monitored by a small temperature-sensitive element (Warner Instruments). Typically, its variation was less than ±0.3°C throughout an experiment.

The ClC-0 current amplitude was usually measured near the end of the +40-mV voltage pulse when the opening of the fast gate is at steady state. Because the channel does not close completely even at very negative membrane potential (Chen and Miller, 1996; Ludewig et al., 1997), the nonspecific “leak” current was not subtracted in all experiments. The leak fraction, however, was estimated separately with two approaches. In one set of experiments, oocytes without mRNA injection were recorded in low Ca$^{2+}$-ND96 solution. Alternatively, oocytes expressing ClC-0 were recorded in 0 Cl$^{-}$ solution (containing [mM]: 98 Na-Glutamate, 1.3 MgSO$_4$, and 5 Na-HEPES, pH 7.6), a condition in which the inward Cl$^{-}$ flux is abolished. The measurements in both cases gave leak currents at +40 mV of ~0.1–0.2 μA, which were usually <2–3% of the maximal current in a typical oocyte used in this study. Little current difference between these two ways of leak measurements was discerned, indicating that the endogenous Cl$^{-}$ current of the oocytes did not contribute much to the observed current.

Data analysis was conducted with software programs in pClamp6 (Axon Instruments) and Origin 4.0 (Microcal Software, Inc., Northampton, MA). Curve fittings were performed with an un-weighted least-squares method. Results are presented as mean ± SEM.

**Results**

**Sensitivity of ClC-0 to Various Divalent Metal Ions**

Fig. 1A shows the effects of several divalent metal ions on the steady state current of the oocyte. These metal ions have different potencies to inhibit the current. The comparison among various ions can usually be carried out on the same oocyte since the inhibition is reversible after the ion is washed out. In these experiments, Zn$^{2+}$ had the strongest effect to inhibit ClC-0 with an apparent IC$_{50}$ of ~1–3 μM (Fig. 1B). Cd$^{2+}$ and Ni$^{2+}$ can also inhibit the current, but with a weaker effect. On the other hand, Co$^{2+}$ and Mn$^{2+}$ had almost no effect at concentrations up to 1–1.3 mM.

**Extracellular Zn$^{2+}$ Has Little Effect on the Fast Gating of ClC-0**

Although Zn$^{2+}$ can reduce the steady state current of ClC-0 with relatively high affinity, its effect on the fast gate of the channel is only minimal. Fig. 2A shows the voltage protocol similar to that used by Pusch et al. (1995) to examine the fast gating of ClC-0. The current families elicited by this voltage protocol in the absence or presence of Zn$^{2+}$ were shown in Fig. 2B. Application of 10 μM Zn$^{2+}$ inhibits the current by about half at all voltages (Fig. 2B, left and middle), indicating a voltage-independent inhibition. The current recovers completely after Zn$^{2+}$ washout (Fig. 2B, right).

The effect of Zn$^{2+}$ on the fast gate of ClC-0 was evaluated in two ways: the steady state open probability ($P_o$) and the kinetics of the fast gate. To determine the $P_o$ of the fast gate from Fig. 2B, the tail current was extrapolated to the onset of period 4. Normalization of these extrapolated values to the maximal value (usually the one following the +80-mV test pulse) yielded the relative $P_o$'s at the corresponding membrane potentials in period 3 (Pusch et al., 1995). Fig. 2C shows that the steady state $P_o$–V curves in the presence and absence of Zn$^{2+}$ are identical, indicating that Zn$^{2+}$ has little effect on the fast gate. The effect of Zn$^{2+}$ on the kinetics of the fast gate was evaluated by examining the fast-gating relaxation. The current deactivation in period 3 was fitted to a single-exponential function (Pusch et al., 1997; Ludewig et al., 1997) and the time constants at various voltages were plotted in Fig. 2D. The effect of Zn$^{2+}$ on

Figure 1. (A) Effects of various divalent metal ions on the steady state current of the ClC-0 Cl$^{-}$ channel. The membrane potential of the oocyte was clamped at −30 or −40 mV. Each circle represents the current amplitude monitored by a 100-ms voltage pulse to +30 or +40 mV given every 5 or 6 s. Dotted lines show zero-current level. Solutions containing desired concentrations of various heavy metal ions were perfused in (downward arrow) and out (upward arrow), as indicated. (B) Concentration-dependent inhibition of the steady state current by Zn$^{2+}$. Holding potential was at −30 mV. Continuous recording at 27.4°C for more than 50 min.

Figure 2A. Voltage protocol similar to that used by Pusch et al. (1995) to examine the fast gating of ClC-0. The current families elicited by this voltage protocol in the absence or presence of Zn$^{2+}$ were shown in Fig. 2B. Application of 10 μM Zn$^{2+}$ inhibits the current by about half at all voltages (Fig. 2B, left and middle), indicating a voltage-independent inhibition. The current recovers completely after Zn$^{2+}$ washout (Fig. 2B, right).

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temperature dependent. To rule out the possibility that
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**Figure 2.** Extracellular Zn$^{2+}$
has little effect on the fast gate of
ClC-0. (A) Voltage protocol used
to evaluate the fast gate of ClC-0.
To focus on the fast gate, multi-
ple short negative voltage pulses
were applied to open the slow
gate before the experiment. A
$-120$mV voltage step was first
given to further ensure maximal
activation of the slow gate (only
partially shown in $I$). The fast
gate was then examined with a
voltage pulse to $+60$mV (2), fol-
lowed by different test voltages
from $-160$ to $+80$mV in 20-mV
steps (3). The tail currents were
measured at $-100$ mV (4). (B)
Families of currents in control
(left), 10 $\mu$M Zn$^{2+}$ (middle) and
after Zn$^{2+}$ washout (right). Dot-
ted line shows level of zero cur-
rent. (C) Steady state $P_o$-V curves
of the fast gate. $\square$, $\blacksquare$, and $\square$ are
control, in 10 $\mu$M Zn$^{2+}$, and
wash, respectively ($n=4$). Solid
curves were drawn according to a
simple voltage equilibrium: $P_o =$
$P_{\text{min}} + (P_{\text{max}} - P_{\text{min}})/[1 + \exp(-z(V - V_m)/RT)]$, with $z = 0.7$–0.83, $P_{\text{max}} = 0.98$–0.99, $P_{\text{min}} = 0.01$–0.16, and $V_m = -73$ to $-85$mV. The
differences among the three $P_o$-V curves were observed only at voltages more negative than $-120$mV where the leak current contributed a
relatively big fraction to the observed current. (D) Time constants of deactivation phase in period 3 plotted against membrane voltages.
Symbols are as those in C.

**Apparent On and Off Rates of Zn$^{2+}$ Inhibition Are
Temperature Dependent**

To further explore the mechanism of the Zn$^{2+}$ effect, the kinetics of the inhibition was studied at a holding potential of $-30$mV. As shown in Fig. 3 A, the amplitude of the whole oocyte current spontaneously
decreases due to the closing of the slow gate, with the
time to reach a steady state depending on the temperature.
After the relaxation was close to a steady state, 10 $\mu$M Zn$^{2+}$ was perfused in and out of the bath solution.
The apparent on and off rates of Zn$^{2+}$ inhibition were
surprisingly slow. In addition, both rates were highly
temperature dependent. To rule out the possibility that
the slow kinetics and the temperature dependence of
the Zn$^{2+}$ effect were due to slow solution exchange, this
exchange rate was measured using TEA to inhibit a
Shaker K$^+$ channel with the inactivation ball removed
(Fig. 3 B). Since TEA is known to block the Shaker K$^+$
channel with on and off rates in the micro- to milli-
second time range (Hille, 1992), the time constants ($\sim$1–3 s)
measured from the TEA inhibition mostly reflect the
delay of the solution exchange. Fig. 3 C compares the
time constants of the Zn$^{2+}$ inhibition of ClC-0 with
those of the TEA inhibition of the Shaker K$^+$ channel.
The apparent on and off rates of the Zn$^{2+}$ inhibition of
ClC-0 are clearly temperature dependent, whereas the
solution exchange rate is not. Furthermore, the kinetics
of the Zn$^{2+}$ inhibition is much slower than that of the
TEA inhibition. Except for high temperature condi-
tions that gave relatively fast inhibitory kinetics, the
rate of the solution exchange was usually 10-fold faster
than that of the Zn$^{2+}$ inhibition, indicating that the
slow kinetics of the Zn$^{2+}$ inhibition on ClC-0 do not
arise from the solution exchange.

**Extracellular Zn$^{2+}$ Facilitates the Inactivation Relaxation**

The strong temperature dependence of the inhibition
kinetics suggests that a complicated process may be in-
volved in Zn$^{2+}$-induced inhibition. With the inhibition
showing such slow kinetics and high temperature de-
pendence, it seems unlikely that Zn$^{2+}$ acts as an open
channel blocker to reduce the single-channel conduc-
tance of ClC-0. On the other hand, Zn$^{2+}$ is more likely

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718 Inhibition of ClC-0 by Zn$^{2+}$
to alter the slow gating of ClC-0, which intrinsically has a high Q10 (Pusch et al., 1997). To examine this possibility, the slow-gating relaxation of ClC-0 was studied at various temperatures with or without extracellular Zn2+ (Fig. 4). Consistent with a previous study (Pusch et al., 1997), the slow-gating relaxation follows a single-exponential decay. In the absence of Zn2+, the time constant of the current relaxation is smaller with a higher temperature. At a fixed temperature, Zn2+ also speeds up the inactivation relaxation process; the relaxation time constant decreases with increasing Zn2+ concentration. From records like these, an Arrhenius plot of the time constants of the slow-gating relaxation at different Zn2+ concentrations was obtained (Fig. 5). The time constant, τ, depends on both temperature and Zn2+ concentration. In the absence of Zn2+, the slope of the fit-
The inhibition of ClC-0 by Zn\(^{2+}\) shown in Fig. 4 (gating relaxation was evaluated from experiments similar to those shown in Fig. 4 (n = 7–8). Solid lines were the best fit to Y = A + BX, where Y is log10(τ) and X is the inverse of temperature. External \([\text{Zn}^{2+}]\) (μM) and the Q10s of the fitted lines were: (□) 0, 36.5; (△) 10, 46.2; (○) 100, 29.7, × and • are the time constants of \text{Zn}^{2+}\text{inhibition} and recovery shown in Fig. 3 C.

**Figure 5.** Arrhenius plot of the slow-gating relaxation time constants in various external \([\text{Zn}^{2+}]\). The time constant τ of the slow-gating relaxation was evaluated from experiments similar to those shown in Fig. 4 (n = 7–8). Solid lines were the best fit to Y = A + BX, where Y is log10(τ) and X is the inverse of temperature. External \([\text{Zn}^{2+}]\) (μM) and the Q10s of the fitted lines were: (□) 0, 36.5; (△) 10, 46.2; (○) 100, 29.7, × and • are the time constants of \text{Zn}^{2+}\text{inhibition} and recovery shown in Fig. 3 C.

A Four-State Scheme Describes the Inhibitory Effect of \text{Zn}^{2+}\n
The inhibition of ClC-0 by the extracellular \text{Zn}^{2+} shown above can be explained by a simple four-state model (Scheme I). Here, z represents \text{Zn}^{2+}, and O and I are the open and inactivated states of the channel. \(K_1\) and \(K_2\) are gating equilibrium constants between O and I and between Oz and Iz, whereas \(K_1\) and \(K_2\) are apparent dissociation constants of \text{Zn}^{2+} binding to the open and inactivated channels, respectively. In this scheme, the closed state of the channel is ignored because most of the noninactivated channels are in the open state (versus close state) at a holding potential of −30 mV (Pusch et al. 1995; Chen and Miller, 1996). The binding and unbinding of \text{Zn}^{2+} to the channel are probably several orders of magnitude faster than the slow-gating transition because the latter operates at a time scale of tens to hundreds of seconds (Fig. 4). Thus, the rate-limiting steps of the steady state current inhibition by \text{Zn}^{2+} and the current recovery after \text{Zn}^{2+} washout must reside between Oz and Iz, and between O and I, respectively. This consideration predicts that the observed on rate of \text{Zn}^{2+} inhibition should be identical to the inactivation rate at the same \text{Zn}^{2+} concentration. Furthermore, the observed off rate of \text{Zn}^{2+} inhibition must also equal the inactivation relaxation rate in the absence of \text{Zn}^{2+}. Fig. 5 compares, at various temperatures, the apparent on and off rates of \text{Zn}^{2+} inhibition with the inactivation relaxation rates with or without 10 μM \text{Zn}^{2+}. Except for the comparisons of the on rate of \text{Zn}^{2+} inhibition with the inactivation rate at high temperatures, the above two predictions are fulfilled. At the highest two temperatures, 28.3°C and 30.0°C, the time constants of the inhibition by 10 μM \text{Zn}^{2+} were 19.0 ± 5.1 (n = 4) and 9.4 ± 1.4 (n = 5) s, respectively, whereas the inactivation relaxation time constants at the same \text{Zn}^{2+} concentration were 11.5 ± 3.1 (n = 8) and 6.5 ± 0.9 (n = 8) s. The difference between these two cases is ~3–8 s, a value comparable to the time for the solution exchange. The 3–8-s delay, however, is relatively small in comparison with slow-gating relaxation rates in other experimental conditions. Thus, these results show that the apparent on rate for \text{Zn}^{2+} to inhibit the steady state current is indeed equal to the inactiva-

**Figure 6.** Slow-gating relaxation rate as a function of external \([\text{Zn}^{2+}]\). Data points were fitted to \(Y = Y_0 + X \cdot Y_o/(X + K_{1/2})\). The fitted \(K_{1/2}\) (μM) and \(Y_o/\text{Yo}\) were: (21.3°C, □) 33.2, 29.2; (23.1°C, ○) 40.4, 35.9; (24.8°C, △) 39.0, 26.3; (26.5°C, •) 26.6, 27.5; (28.3°C, ●) 22.0, 18.6; (30.0°C, ▲) 21.5, 18.7. The increase of the inactivation rate by \text{Zn}^{2+}, if all comes from the activation entropy, corresponds to an increase of \(\Delta S\) of 24.3–29.8 J/mol per °K (see text for detailed discussion).
tion relaxation rate at the same Zn$^{2+}$ concentration, indicating that Zn$^{2+}$ is very likely acting on the inactivation process of the channel.

$$\begin{align*}
O & \overset{K_1}{\rightleftharpoons} I \\
Z & \overset{K_d}{\rightleftharpoons} Z
\end{align*}$$

(Scheme 1)

The four-state scheme given above requires that the Zn$^{2+}$ binding affinity of the inactivated channel be higher than that of the open channel; that is, $K_d > K_{d*}$. This is because the ratio $K_d/K_{d*}$ must equal $K_o/K_i$, which is >1. Let transition rates $\alpha_1$ and $\beta_1$ be the forward rate (the rate of inactivation) and the backward rate (the rate of recovery from inactivation) of the slow gating of the unliganded ClC-0, respectively, and $\alpha_2$ and $\beta_2$ be those of the Zn$^{2+}$-bound channel. It can be shown that if Zn$^{2+}$ increases only the forward rate (that is, $\beta_1 = \beta_2$), the $K_{1/2}$ obtained from Fig. 6 would be $K_i$. On the other hand, if the effect of Zn$^{2+}$ is solely on $\beta$ (that is, $\alpha_1 = \alpha_2$), $K_{1/2}$ would be $K_{d*}$. The latter possibility can be ruled out even at this stage since the increase of $\beta$ alone by Zn$^{2+}$ would have led to a potentiation instead of an inhibition of the steady state current. There is, however, a possibility that Zn$^{2+}$ may affect both the forward and backward rates of the slow-gating process. To address this question, it will be informative to evaluate the equilibrium between O and I by constructing the activation curve of the slow gate.

**Effect of Zn$^{2+}$ on the Activation Curve of the Slow Gate**

The voltage-dependent activation of the slow gate was examined with a protocol shown on top of Fig. 7 A, in which a 7-s prepulse from 0 to $-140$ or $-150$ mV in $-10$-mV steps was followed by a 0.8-s test pulse at $+40$ mV. This voltage protocol had been used previously to examine the activation curve of the slow-gating process, and it was found that the 7-s prepulse is long enough to maximally activate the slow gate (Pusch et al., 1997). As shown in Fig. 7 A, Zn$^{2+}$ inhibits the activation of the slow gate in a dose-dependent manner. Fig. 7 B plots the current measured at the test pulse as a function of the prepulse voltage. This quasi–steady state activation curve shows a biphasic response for the open probability of the slow gate. The peak of the activation curves is around $-120$ mV in this experiment carried out at 23.8°C. The activation curves at 19.8° and 28.0°C reveal a similar pattern of voltage dependence (Fig. 7 C). In all three temperatures, 10 and 100 µM Zn$^{2+}$ do not change the biphasic behavior of the activation curve, suggesting that Zn$^{2+}$ has little effect on the current-decreasing phase at very negative potentials. The nonmonotonic behavior of the activation curve has been observed previously (Ludewig et al., 1997; Fong et al., 1998). The current reduction at the hyperpolarized end of the curve may be due to an additional unknown mechanism that closes the channel with strong negative potential. Therefore, I have restricted my attention to the voltage range more depolarized than $-120$ mV to simplify the analysis. In all situations, Zn$^{2+}$ inhibits currents at all voltages with approximately the same degree, suggesting that the inhibition has little voltage dependence. The fractional inhibition ranges from $\sim50$ to 70% at 10 µM Zn$^{2+}$ and $\sim80$ to 95% at 100 µM Zn$^{2+}$.

**Effect of Zn$^{2+}$ Is Mostly on the Forward Rate of Inactivation**

With information from kinetics as well as quasi–steady state equilibrium, it is possible to evaluate the rate constants of the slow-gating process. In the quasi–steady state activation curve, the current does not further decrease when the membrane potential is more depolarized than $-30$ mV (Fig. 7, B and C; also see Pusch et al., 1997). The noninactivated current fraction, obtained by normalizing the current amplitude at $-30$ mV (I) to the maximal current in the activation curve ($I_{\text{max}}$), can be expressed as a function of the forward ($\alpha$) and backward ($\beta$) rate constants of the slow-gating process:

$$I/I_{\text{max}} = \beta/(\alpha + \beta).$$

(1)

In addition, the observed time constant $\tau$ of the slow-gating relaxation shown in Fig. 4 is also a function of $\alpha$ and $\beta$, with the following relation:

$$\tau = 1/(\alpha + \beta).$$

(2)

Table I lists, at three temperatures, the relaxation time constant $\tau$ and the steady state fraction of the noninactivated current, $I/I_{\text{max}}$. The calculated forward rate constant $\alpha$ and backward rate constant $\beta$ are also shown. Without considering the leak current (numbers outside parentheses in Table I), both $\alpha$ and $\beta$ increase with Zn$^{2+}$. The ratio between $\beta$ at 100 µM Zn$^{2+}$ ($\beta_{100}$) and in the absence of Zn$^{2+}$ ($\beta_0$) ranges from $\sim2$ to 5, whereas the ratio between $\alpha$ at 100 µM Zn$^{2+}$ ($\alpha_{100}$) and $\alpha$ in the absence of Zn$^{2+}$ ($\alpha_0$) ranges from $\sim13$ to 18. Even under such situations where the analysis is contaminated with the leak current, most of the Zn$^{2+}$ effect is on the forward rate $\alpha$. When 1, 2, or 3% of the total current is considered as the leak, the ratio $\alpha_{100}/\alpha_0$ is not sensitive to variable leak fractions, whereas the ratio $\beta_{100}/\beta_0$ becomes $\sim2–4$, 1–3, and 0.6–1.5, respectively. Clearly, as more of the current is subtracted, the effect of Zn$^{2+}$ on $\beta$ gets smaller. It is difficult to give an exact number for the leak fraction from these experi-
Inhibition of ClC-0 by Zn$^{2+}$

M. Chen and G. Miller (1996; Ludewig et al., 1997). However, the leak currents observed from recordings of uninjected oocytes were usually ~0.1–0.2 μA at +40 mV, and the maximal currents of the oocytes for the experiments in Fig. 7C were ~3–8 μA. Therefore, it seems reasonable to give a leak fraction of at least 2–3%. This number can also be independently checked by recording oocytes in 0 Cl$^{-}$ solution (see materials and methods).

The data corrected with leak subtraction are shown within parentheses in Table I. The leak fraction is cho-

![Figure 7. Effects of Zn$^{2+}$ on the quasi–steady state activation curve of the slow gate. (A) Family of current traces elicited at 0, 10, and 100 μM Zn$^{2+}$ with a voltage protocol shown on top. All traces were from the same oocyte at 24.0°C. The membrane potential was first held at −30 mV for the slow gating to reach steady state before each experiment started. Horizontal dotted lines indicate level of zero current. (B) Quasi–steady state activation curves at various Zn$^{2+}$ concentrations from the experiment shown in A. The current amplitudes were measured at the vertical dotted line shown in B. (C) Averaged quasi–steady state activation curves at 19.8, 24.0, and 28.3°C. Symbols are as those in B (n = 3–5).

### Table I

| [Zn$^{2+}$] (μM) | τ (s) | 1/$I_{\text{max}}$ | β (× 10$^{-3}$ s$^{-1}$) | α (× 10$^{-3}$ s$^{-1}$) | α$_{10}$ | α$_{100}$ | α$_{100/α_0}$ |
|-----------------|-------|------------------|------------------------|-------------------------|---------|----------|-------------|
|                 | 0     | 10               | 100                    | 0                       | 10      | 100      | 100         |
| 19.8°C          | 1093.3| 344.7            | 75.5                   | 0.2508                  | 0.1271  | 0.0516   | 2.98        |
|                 |       |                  |                        | (0.2208)                | (0.0971)| (0.0216) |            |
| 24.0°C          | 235.4 | 67.1             | 17.8                   | 0.2213                  | 0.0891  | 0.0391   | 1.42        |
|                 |       |                  |                        | (0.1913)                | (0.0591)| (0.0091) |            |
| 28.3°C          | 51.1  | 13.2             | 4.2                    | 0.0908                  | 0.0561  | 0.0378   | 5.06        |
|                 |       |                  |                        | (0.0608)                | (0.0261)| (0.0078) |            |

The table lists the slow-gating relaxation time constant (τ) calculated from Fig. 5, the noninactivated current fraction (1/$I_{\text{max}}$) obtained from Fig. 7C, the forward (α) and the backward (β) rates of inactivation at three different temperatures, and in 0, 10, and 100 μM Zn$^{2+}$. β was calculated by dividing 1/$I_{\text{max}}$ by τ (Eq. 1), whereas α was obtained by subtracting β from 1/τ (Eq. 2). The numbers outside the parentheses were derived from original measured current without leak subtraction. The 1/$I_{\text{max}}$ values in parentheses were obtained by subtracting 0.03 from the original values outside parentheses and the α’s and β’s in parentheses were calculated accordingly.

722 Inhibition of ClC-0 by Zn$^{2+}$
seen to be 3% of the maximal current. Under this condition, the α’s at 100 μM Zn²⁺ are ~13–18-fold larger than those in the absence of Zn²⁺. On the other hand, Zn²⁺ has almost no effect on the backward rate β at all three temperatures. This argues that K₂/K₁ should be equal to α₂/α₁ if Scheme I is considered. The ratio Yₐ/Yₑ from Fig. 6 can be used to approximate α₂/α₁ since β is small in comparison with α (compare βₒ and β₁₀₀ with α₀ and α₁₀₀ in Table I). The dissociation constant for Zn²⁺ binding to the open channel (Kₒ) is ~30 μM, whereas Yₑ/Yₒ ranges from 18.6 to 35.9 (Fig. 6).

To a first degree of approximation, the dissociation constant for Zn²⁺ binding to the inactivated channel (Kᵢ*) should be ~1 μM. It is interesting to point out that Kᵢ* should be close to the apparent Kᵢ/₂ of Zn²⁺ inhibition on the steady state current at ~30 mV, where the O ↔ I transition favors the I state. Furthermore, the more the I state is favored (that is, the higher the Kᵢ value as the temperature is raised), the closer the observed affinity to Kᵢ*. In Fig. 3 A, the fractional inhibition of the steady state current by 10 μM Zn²⁺ is >50%, consistent with the Kᵢ* value inferred from the above analysis. The dose-dependent inhibition of the steady state current by Zn²⁺ performed at temperatures around 25–30°C usually gave an apparent Kᵢ/₂ of ~1–3 μM (Fig. 1 B).

**Discussion**

Transition metal ions have long been used to explore the structure and function of ion channels. In the present study, I have shown that Zn²⁺ can reversibly inhibit CIC-0. The effect of Zn²⁺ on the fast gate is only minimal and cannot explain the magnitude of Zn²⁺ inhibition. Rather, Zn²⁺ appears to inhibit the channel by facilitating the slow-gating process. Such a coupled equilibrium system, in which the binding of a divalent metal ion is coupled to the inactivation of an ion channel, has been characterized on the Shaker K⁺ channel (Yellen et al., 1994). The binding of Cd²⁺ to the external mouth of the Shaker channel via an engineered cysteine was found to modulate the C-type inactivation of the channel, with the Cd²⁺ affinity increased by 45,000-fold for the inactivated channel. This effect is due to a destabilization of the open state as well as a stabilization of the inactivated state of the Shaker channel. In the present study, although the Zn²⁺ affinity of the inactivated CIC-0 is only 30-fold higher than that of the open channel, the same principle of coupled binding still applies. The effect of Zn²⁺ on the slow-gating equilibrium of CIC-0 appears to come mostly from an increase of the forward rate constant of the inactivation.

The inhibition of CIC channels by transition metal ions was also found in ClC-1 and ClC-2. However, the apparent affinity of Zn²⁺ inhibition of ClC-0 is very different from those of ClC-1 and ClC-2. For the Torpedo channel, the apparent Kᵢ/₂ for Zn²⁺ to inhibit the steady state current is ~1–3 μM (Fig. 1 B). In contrast, Zn²⁺ and Cd²⁺ inhibit ClC-1 and ClC-2 with apparent Kᵢ/₂’s at least 50–100-fold higher (Kürz et al., 1997; Rychkov et al., 1997; Clark et al., 1998), suggesting a different inhibition mechanism. As the inhibition of CIC-0 by Zn²⁺ is through the facilitation of the slow gating, the lack of a high-affinity block in ClC-1 and ClC-2 by Zn²⁺ or Cd²⁺ may suggest the absence of a similar Zn²⁺-sensitive, highly temperature-dependent slow-gating process in these two mammalian channels.

Although the four-state scheme (Scheme I) appears to explain well the effect of Zn²⁺ at a constant holding potential of ~30 mV, problems with this simple scheme are obvious when the membrane potential is changed. In Scheme I, the same voltage and temperature dependence are assigned at the transitions O ↔ I and O₂ ↔ I₂. Under this condition, hyperpolarization, which opens the slow gate and shifts the channel (Zn²⁺ bound or unbound) mostly to the O state, would reduce the Zn²⁺ inhibition since the Zn²⁺-bound O state is conducting. At an extremely negative membrane potential, the current should be the same whether in the absence or presence of Zn²⁺. In other words, Zn²⁺ must shift the activation curve to the left along the voltage axis without changing the maximal and minimal current levels (see modeling in Fig. 8 A). The experimental results in Fig. 7, however, show that this appears not to be the case.

The problem of Scheme I discussed above comes from naively assigning the slow gating as a simple process between only two states, O and I. Pusch et al. (1997) pointed out that any Markov-type model to describe the slow gating itself requires at least four states. Based on the quasi–steady state activation curves at different temperatures, they suggested the model shown in Scheme II. Here, O₁ and O₂ represent the two states in which the slow gate is open (that is, the noninactivated states), whereas I₁ and I₂ are the two states in which the slow gate is closed (the inactivated states). Kₒₘₖ is an equilibrium constant between states A and B. The peculiar feature of this model is that the transitions between O and I (O₁ ↔ I₁ and O₂ ↔ I₂) are temperature, but not voltage, dependent. The voltage dependence instead resides in the transitions between states 1 and 2 (O₁ ↔ O₂ and I₁ ↔ I₂; Pusch et al., 1997). Thus, as long as the free energy of the slow-gating transition of channel population 1 differs from that of channel population 2 (that is, Kₒ₁₁₁ ≠ Kₒ₂₂₂), the open probability of the slow gate would reveal voltage dependence. At a fixed voltage (e.g., ~30 mV), the total free energy of the slow-gating transition for all channels is constant at the same temperature, so Scheme II would automati-
Inhibition of ClC-0 by Zn\textsuperscript{2+}

In the absence of Zn\textsuperscript{2+}, the assignments 10 kJ/mol for $\Delta H$ and 0.29 kJ/mol/K for $\Delta S$ were used for the transition $A \leftrightarrow B$. The values of the above parameters in the absence of Zn\textsuperscript{2+} were assumed to be of the form: $1/\kappa_{AB} = P_n/P_h = \exp(-\Delta G_{AB}/RT) = \exp(\Delta S_{AB}/R - \Delta H_{AB}/RT + \Delta H_{AB}/R + \Delta S_{AB}/R)\cdot\Delta T_{AB}/RT$, where $\kappa_{AB}$ is the equilibrium constant, and $\Delta G_{AB}$, $\Delta S_{AB}$, and $\Delta H_{AB}$ are the differences in Gibbs free energy, entropy, and enthalpy between the two states, respectively. $R$, $T$, and $V$ have their usual meanings. $V$ is the membrane voltage and $\Delta H_{AB}$ and $\Delta S_{AB}$ are the "gating valences" describing the voltage dependence of the transition $A \leftrightarrow B$. The values of the above parameters in the absence of Zn\textsuperscript{2+} were the same as those in a previous paper (Pusch et al., 1997) except for $\Delta H_{O1O2}$. They are shown as follows ($\Delta H$ in kJ/mol, $\Delta S$ in kJ/mol K):

(A) $\Delta H_{O1} = -80, \Delta S_{O1} = -0.29, \Delta H_{A} = -77, \Delta S_{A} = -0.26, \Delta H_{O1O2} = 10, \Delta S_{O1O2} = 0, \Delta H_{O2I2} = -2, \Delta S_{O2I2} = -28$. Assigning 10 kJ/mol for $\Delta H_{O1O2}$ makes all curves in B shift to the left by ~40 mV so that the curves from modeling are more similar to the experimental data with respect to their positions along the voltage axis. It does not affect the effect of Zn\textsuperscript{2+}, which shifts the curve from top to bottom. Even with this adjustment, the assigned values in B do not provide a superb prediction for the plateau at positive voltages as they give a much larger noninactivated fractional current than the data shown in Fig. 7. This difference is not important since the pattern of Zn\textsuperscript{2+} inhibition is basically the same as that in the experimental data. The numbers 0, 10, and 100 indicate Zn\textsuperscript{2+} concentrations.

![Diagram](image)

Figure 8. Simulation for the effect of Zn\textsuperscript{2+} on the quasi-steady-state activation curve of the slow gate. Activation curves were generated based on Scheme I (A) and Scheme III (B) in the presence of 0, 10, and 100 μM Zn\textsuperscript{2+} at 20°C (left), 24°C (middle), and 28°C (right). The ratio of the occupation probability of any two states, A and B, is assumed to be of the form: $1/\kappa_{AB} = P_n/P_h = \exp(-\Delta G_{AB}/RT) = \exp(\Delta S_{AB}/R - \Delta H_{AB}/RT + \Delta H_{AB}/R + \Delta S_{AB}/R)\cdot\Delta T_{AB}/RT$, where $\kappa_{AB}$ is the equilibrium constant, and $\Delta G_{AB}$, $\Delta S_{AB}$, and $\Delta H_{AB}$ are the differences in Gibbs free energy, entropy, and enthalpy between the two states, respectively. $R$, $T$, and $V$ have their usual meanings. $V$ is the membrane voltage and $\Delta H_{AB}$ and $\Delta S_{AB}$ are the "gating valences" describing the voltage dependence of the transition $A \leftrightarrow B$. The values of the above parameters in the absence of Zn\textsuperscript{2+} were the same as those in a previous paper (Pusch et al., 1997) except for $\Delta H_{O1O2}$. They are shown as follows ($\Delta H$ in kJ/mol, $\Delta S$ in kJ/mol K):

(A) $\Delta H_{O1} = -80, \Delta S_{O1} = -0.29, \Delta H_{A} = -77, \Delta S_{A} = -0.26, \Delta H_{O1O2} = 10, \Delta S_{O1O2} = 0, \Delta H_{O2I2} = -2, \Delta S_{O2I2} = -28$. Assigning 10 kJ/mol for $\Delta H_{O1O2}$ makes all curves in B shift to the left by ~40 mV so that the curves from modeling are more similar to the experimental data with respect to their positions along the voltage axis. It does not affect the effect of Zn\textsuperscript{2+}, which shifts the curve from top to bottom. Even with this adjustment, the assigned values in B do not provide a superb prediction for the plateau at positive voltages as they give a much larger noninactivated fractional current than the data shown in Fig. 7. This difference is not important since the pattern of Zn\textsuperscript{2+} inhibition is basically the same as that in the experimental data. The numbers 0, 10, and 100 indicate Zn\textsuperscript{2+} concentrations.

![Diagram](image)

As Zn\textsuperscript{2+} apparently exerts its effect on the slow-gating process, one can examine whether Scheme II is valid by studying the effect of Zn\textsuperscript{2+} on the quasi–steady state activation curve. The examination can be illustrated in Scheme III. Here, the four states in front represent Scheme II and each of the four species is then connected to a Zn\textsuperscript{2+}-bound state. The dissociation constants for Zn\textsuperscript{2+} binding to the channels ($K_d$ and $K_d^*$) and the equilibrium gating constants among the Zn\textsuperscript{2+}-unbound states have the same meanings as those in Schemes I and II, respectively. To model the effects of Zn\textsuperscript{2+} on activation curves, the enthalpy and entropy of the transitions among Zn\textsuperscript{2+}-unbound states were taken from Pusch et al. (1997). In addition, two pieces of information from Fig. 6 are also used: the dissociation constant of Zn\textsuperscript{2+} binding to the open-state channel ($K_d$) is 30 μM, and the entropy difference of the slow-gating transition in the Zn\textsuperscript{2+}-bound channel is increased by 27 J/mol K. The latter is calculated from the ratio $Y_{en}/Y_{ov}$ according to transition state theory and assuming that the backward rate of the inactivation process is not affected by Zn\textsuperscript{2+} (see results). Fig. 8A shows that Zn\textsuperscript{2+} shifts the activation curve along the voltage axis when only two states, O and I, are used to describe the slow gating (Scheme I). On the other hand, the activation curves derived from Scheme III show a Zn\textsuperscript{2+} inhibition pattern similar to the experimental results (Fig. 8B). The curve shifts along the longitudinal axis by raising extracellular Zn\textsuperscript{2+}, a pattern similar to that made by increasing temperature (Pusch et al., 1997). This similarity is not surprising since a change in temperature is equivalent to a change in entropy with respect to their contributions to the free energy of the slow-gating process.
Scheme III thus describes the effect of Zn\(^{2+}\) on the quasi–steady state activation curve well, but only at voltages more depolarized than \(-120\) mV. As the membrane potential is more negative than \(-120\) mV, the open probability of the slow gate becomes paradoxically smaller. There is very limited information about the slow gating at such hyperpolarized membrane potentials, so the underlying mechanism is difficult to address at the present time. However, the facilitation of inactivation upon depleting permeant ions has been observed in other channels (Baukrowitz and Yellen, 1995, 1996; Townsend and Horn, 1997). This possibility cannot be ruled out here since reduction of the intracellular Cl\(^{-}\) concentration, at least near the internal mouth of the channel, is very likely to occur in such a hyperpolarized condition. The slow gating of ClC-0 has long been known to couple to the Cl\(^{-}\) flux across the channel pore (Richard and Miller, 1990). Thus, the slow gating of ClC-0 could be more complicated than the above gating scheme if the Cl\(^{-}\) effect is considered.

The high activation energy of the inactivation of ClC-0 is consistent with a complex conformational change thought to involve subunit interaction between two protomers (Pusch et al., 1997). In the Arrhenius plot, Zn\(^{2+}\) simply shifts the line describing temperature dependence without altering the slope of the line (Fig. 5). This suggests that Zn\(^{2+}\) binding does not change the activation enthalpy of the slow-gating process, but results in a bigger entropy difference between the open and transition states. Assuming the transition state with or without Zn\(^{2+}\) has the same entropy, the open state must have a lower entropy in the presence than in the absence of Zn\(^{2+}\). One can imagine that the complex slow-gating process may involve a step that requires some kind of order in the channel protein (or part of the channel protein, for example, a less movable loop), which renders the inactivation process relatively unfavorable. Zn\(^{2+}\), in binding to the channel, may facilitate this process by providing the order that would otherwise be more difficult to reach in the absence of Zn\(^{2+}\). This suggestion, however, is only one of the many interpretations based on thermodynamic arguments. The structural evidence to support this implication awaits more characterization of the channel molecule.

In summary, I have shown that Zn\(^{2+}\) can reversibly inhibit the ClC-0 Cl\(^{-}\) channel from Torpedo electroplax. This effect is likely due to an increase of the inactivation rate of the channel with little changes on the rate of recovery from the inactivation state. The molecular structure of the slow gate and how it functions are largely unknown. Zn\(^{2+}\), due to its effect on the inactivation process of ClC-0, may be used to further explore the slow gating of ClC-0 in the future.

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