Different Fast-gate Regulation by External Cl\(^{-}\) and H\(^{+}\) of the Muscle-type ClC Chloride Channels

MEI-FANG CHEN and TSUNG-YU CHEN

1Center for Neuroscience and 2Department of Neurology, University of California, Davis, CA 95616

ABSTRACT The fast gate of the muscle-type ClC channels (ClC-0 and ClC-1) opens in response to the change of membrane potential (V). This gating process is intimately associated with the binding of external Cl\(^{-}\) to the channel pore in a way that the occupancy of Cl\(^{-}\) on the binding site increases the channel’s open probability (\(P_{o}\)). External H\(^{+}\) also enhances the fast-gate opening in these channels, prompting a hypothesis that protonation of the binding site may increase the Cl\(^{-}\) binding affinity, and this is possibly the underlying mechanism for the H\(^{+}\) modulation. However, Cl\(^{-}\) and H\(^{+}\), modulate the fast-gate \(P_{o}-V\) curve in different ways. Varying the external Cl\(^{-}\) concentrations ([Cl\(^{-}\)]) shifts the \(P_{o}-V\) curve in parallel along the voltage axis, whereas reducing external pH mainly increases the minimal \(P_{o}\) of the curve. Furthermore, H\(^{+}\) modulations at saturating and nonsaturating [Cl\(^{-}\)] are similar. Thus, the H\(^{+}\) effect on the fast gating appears not to be a consequence of an increase in the Cl\(^{-}\) binding affinity. We previously found that a hyperpolarization-favored opening process is important to determine the fast-gate \(P_{o}\) of ClC-0 at very negative voltages. This [Cl\(^{-}\)]-independent mechanism attracted little attention, but it appears to be the opening process that is modulated by external H\(^{+}\).

KEY WORDS: channel gating • ClC-0 • ClC-1 • pH regulation • Cl\(^{-}\) dependence

INTRODUCTION

Ion channels catalyze the transportation of small ions across the cell membrane and, in doing so, maintain normal cellular functions. To achieve functional flexibility, these membrane proteins are regulated by a variety of factors, such as transmembrane voltages, mechanical stretches, extracellular or intracellular ligands, and sometimes the small ions in the aqueous solution (Hille, 1992). In vertebrate skeletal muscles, for instance, external Cl\(^{-}\) and H\(^{+}\) significantly influence the resting membrane conductance and, thus, control the firing pattern of action potentials (Hodgkin and Horowicz, 1959, 1960). Electrophysiological recordings and flux assays have shown that the muscle membrane is more permeable to Cl\(^{-}\) than to K\(^{+}\) (Hodgkin and Horowicz, 1959; Hutter and Pash, 1959; Hutter and Noble, 1960), and if the Cl\(^{-}\) conductance is removed, the membrane current shows little change when the pH is altered (Hutter and Warner, 1967, 1972). These early studies indicated that the chloride conductance on the muscle membrane is regulated by extracellular Cl\(^{-}\} and H\(^{+}\).

Molecular cloning work in the last 10 yr has made significant progress toward understanding the identity of various Cl\(^{-}\) channels. The principal Cl\(^{-}\} channel in the muscle membrane, ClC-1 (Steinmeyer et al., 1991), belongs to the ClC channel family. This muscle channel has a homologue ClC-0 in the Torpedo electric organ (Jentsch et al., 1990; O’Neill et al., 1991), which is derived developmentally from skeletal muscle (Jentsch et al., 1995). The biochemical structure of these “muscle-type” Cl\(^{-}\} channels is made up of two identical subunits (Middleton et al., 1994; Fahlke et al., 1997), and the amino acid sequences of ClC-0 and ClC-1 show ~56% identity. The similarity between these two channels is also shown in their functional properties. The opening of both types of channels display “double-barrel”–like activity (Miller, 1982; Miller and White, 1984; Bauer et al., 1991; Ludewig et al., 1996; Middleton et al., 1996; Saviane et al., 1999; Lin and Chen, 2000), which is controlled by two voltage-dependent gating mechanisms (for review see Miller and Richard, 1990; Pusch and Jentsch, 1994; Jentsch et al., 1999; Maduke et al., 2000). In the case of the gating process with faster kinetics, the channel opens and closes on the millisecond time scale, and the gating is modulated by extracellular Cl\(^{-}\). When [Cl\(^{-}\}] is reduced, the opening curve of this “fast gate” is shifted toward a more depolarized membrane potential (Pusch et al., 1995; Chen and Miller, 1996; Rychkov et al., 1996; 1998). For this and other observations, it has been proposed that the binding of Cl\(^{-}\) to the pores of these channels is required for the depolarization-induced channel opening.

Consistent with the early findings on the muscle Cl\(^{-}\} conductance, varying the external pH also affects the fast gating of ClC-1. With macroscopic current record-
ings, Rychkov et al. (1996, 1997) showed that the CIC-1 current deactivated to a steady-state level upon membrane hyperpolarization, and the deactivating component diminished as there was an increase of the steady-state component when the external pH was reduced. The open probability ($P_o$) of the channel at a low pH was therefore increased, but the effect was obvious mainly in the hyperpolarized voltage range. It was suggested that the enhanced fast-gate open probability might be due to protonation of the aforementioned Cl$^-$ binding site, and this increased the Cl$^-$ binding affinity. Dissociation of Cl$^-$ from the binding site, therefore, is no longer voltage-dependent, leading to a shallower $P_o$V curve (Rychkov et al., 1996, 1997).

As external Cl$^-$ appears to control the fast gating of CIC-0 and CIC-1 in the same manner, one would expect that H$^+$ would have a similar modulatory effect on the fast gating of CIC-0. However, previous studies on CIC-0 have not clearly shown an effect of external H$^+$ on the fast gating. Miller and White (1980) examined the macroscopic conductance of CIC-0 at a fixed voltage of $-30$ mV, and found that there was no pH effect between pH $\sim 7.4$ and $\sim 6.0$. Another study at the single-channel level reported that although internal pH profoundly affected the fast gating of CIC-0, no effect was discernible when the pH of the trans chamber (corresponding to the external side) was raised from pH 7.0 to 10.0 (Hanke and Miller, 1983). These reports did not show a clear external H$^+$ modulation of the CIC-0 fast gating, nor did they support the hypothesis of protonation of the Cl$^-$ binding site. It is our intention here to examine more rigorously whether external H$^+$ has a modulation on the fast gating of CIC-0 similar to CIC-1. The possibility that external H$^+$ modulates the affinity of the Cl$^-$ binding site is also examined.

**Materials and Methods**

**Expression of CIC-0 and an Inactivation-suppressed Mutant C212S**

The present study focuses on the fast gating of CIC-0. We have isolated the fast gating from the inactivation gating by using an inactivation-suppressed mutant C212S. The construction of the cDNA of the wild-type (WT) channel and the site-directed mutagenesis used to create the C212S mutant have been described previously (Chen, 1998; Lin et al., 1999). For RNA synthesis, the plasmids were linearized by restriction enzyme Scal (New England Biolab). Capped RNAs were synthesized with T3 polymerase (Ambion Inc.). *Xenopus* oocytes were prepared and injected with RNAs as described previously (Chen, 1998).

**Macroscopic Current Recording**

Whole oocyte currents were recorded at room temperatures (20–25°C) using standard two-electrode voltage clamp techniques. Microelectrodes were pulled by a PP-83 puller (Narashige) and, when filled with 3 M KCl, had resistance of 0.2–1.0 MΩ. The ground electrodes were connected to the recording chamber via 3 M KCl salt bridges. The recorded current was filtered by the built-in filter of the amplifier (model 725C, Warner Instruments) and was digitized at 1 or 2 kHz using a Digidata 1200 data acquisition board and pClamp6 software (Axon Instruments). The standard solution for the recording of whole oocyte current contained the following (in mM): 98 NaCl, 2 MgSO$_4$, and 5 HEPES. The pH of the solution was titrated by adding NaOH or glutamic acid. To make solutions with a lower Cl$^-$ concentration, part of the NaCl concentration was replaced by sodium glutamate. When a higher Cl$^-$ concentration such as 300 mM was needed, NaCl was added to the standard solution to obtain the desired concentration. Because the channel is open at the holding potential, a change of [Cl$^-$], inevitably leads to an alteration of the internal Cl$^-$ concentration ([Cl$^-$]). Based on the measured reversal potentials at a steady-state, we estimated that effective [Cl$^-$], were $\sim 60$–70, $\sim 36$–48, and $\sim 15$–20 mM when [Cl$^-$], were 300, 98, and 15 mM, respectively.

To examine the quasi steady-state inactivation curve of the channel (see Fig. 1), a protocol modified from a previously described method (Pusch et al., 1997; Chen, 1998) was used. The membrane potential (V) was held at $-30$ mV, and a 3.5-s prepulse from 0 to $-140$ mV in $-10$-mV steps was followed by a 0.4-s test pulse at $+60$ mV. The current was measured at the end of the $+60$-mV test pulse, and was normalized to the maximal current obtained at pH 7.6.

The fast-gating properties of the channel were evaluated with a voltage protocol described before (Chen, 1998; Lin et al., 1999; Lin and Chen, 2000). In brief, multiple short negative voltages were first applied to open the inactivation gate before the experiment. A $-120$-mV voltage step was applied for 300 ms to ensure the opening of the inactivation gate. The voltage was then clamped at $+80$ mV for 100 ms, followed by 200-ms test voltages from $+120$ (or $+80$) to $-160$ mV in $-20$-mV steps. The voltage protocol was completed by a tail voltage step at $-100$ mV. The recorded current trace was shown only from 80 ms before the start of the test pulse to 200 ms after the end of the test voltage (see Fig. 2A and Fig. 3). In Fig. 2A, the tail current traces were enlarged from 20 ms before until 50 ms after the end of the test pulse. For deriving the fast-gate $P_o$, the tail currents were fitted to a single-exponential function, and their initial values were obtained by extrapolation. These initial tail currents were normalized to the maximal one obtained following the most depolarized test potential ($+80$ or $+120$ mV). To estimate the opening and closing rates of the fast gate, the relaxation time constant (τ) was first determined by fitting the current deactivation process to a single-exponential function. The opening rate (α) and closing rate (β), thus, were calculated according to:

\[ \alpha = P_o / \tau, \]  
\[ \beta = (1 - P_o) / \tau. \]

However, this method was limited by the degree of the current deactivation. When the membrane potential is more depolarized than $-40$ mV, or when the external pH is <5, the current deactivation component is so small that the time constant cannot be reliably estimated. The opening and closing rates, thus, cannot be obtained under these conditions.

**Single-channel Recordings**

The single-channel behavior of the channel was examined at room temperatures (19–23°C) using excised inside-out patch recording...
techniques (Hamill et al., 1981). Detailed procedures were similar to those described previously (Lin et al., 1999; Lin and Chen, 2000). Briefly, the pipet (external) solution contained the following (in mM): 110 NaCl, 5 MgCl₂, 1 CaCl₂, and 5 HEPES, pH 5.6–9.6. The bath (internal) solution contained the following (in mM): 110 NMDG-Cl, 5 MgCl₂, 1 EGTA, and 5 HEPES, pH 7.6. The pH of the internal and external solutions were titrated with NaOH and NMDG, respectively. The current was filtered at 200 Hz (4 pole Bessel) and was digitized by an acquisition board (model DAP 800; Microstar Laboratories, Inc.) at 1 kHz with homemade software (Chen and Miller, 1996; Lin et al., 1999; Lin and Chen, 2000). For the C212S mutant, the dwell-time distribution of the nonconducting events always followed a single-exponential function, indicating that the inactivation was suppressed in this point mutant (Lin et al., 1999). Although the single-channel recording trace shows three equidistant current levels, it is still possible to derive fast-gating parameters assuming the presence of double barrels and independent gating of the two protopores. Accordingly, the $P_o$ of the fast gate is determined from Eq. 2:

$$P_o = f_1/2 + f_2,$$

where $f_1$ and $f_2$ represent the probabilities of the intermediate and the fully open levels, respectively. Intuitively, the opening rate ($\alpha$) and closing rate ($\beta$) are as follows:

$$\alpha = 1/(2\tau_1),$$  \hspace{1cm} (3a)

$$\beta = 1/(2\tau_2),$$  \hspace{1cm} (3b)

where $\tau_1$ and $\tau_2$ are the time constants of the distributions of nonconducting and fully open events, respectively. However, in the very acidic condition (pH = 5.6), $\tau_1$ was difficult to obtain because $P_o$ became so large that the closed events were rare. We exploited the fact that events at the middle level occurred more frequently and also had a longer dwell time than those at the zero current level. The time constant of the dwell-time distribution of the middle level $\tau_1$, therefore, was determined and the opening and closing rates were calculated according to a method described in Chen and Miller (1996):

$$\alpha = P_o/\tau_1,$$  \hspace{1cm} (3c)

$$\beta = (1-P_o)/\tau_1,$$  \hspace{1cm} (3d)

Under experimental conditions where both $\tau_1$ and $\tau_2$ can be reliably obtained, $\alpha$ and $\beta$ were calculated using both these methods (Eqs. 3a and 3b versus Eqs. 3c and 3d) and agreed within 10% (for the comparison of these two methods also see Chen and Miller, 1996).

**Data Analysis**

Data analysis of the macroscopic current was conducted using the software programs, pClamp6 (Axon Instruments) and Origin 4.0 (Microcal Software, Inc.). Analysis of the single-channel recording trace was performed using a homemade program (Chen and Miller, 1996; Lin et al., 1999; Lin and Chen, 2000). Because single-channel current amplitudes at voltages close to the reversal potential (0 mV) are small, the single-channel traces were further digitally filtered at 200 Hz, leading to a final filter frequency of ~140 Hz (~3dB). We did not correct the missed events since the change in opening rate by H⁺ was much greater than the error that resulted from filtering the recording traces. A previous single-channel recording study using the same digitizing rate and a similar cutoff frequency was shown to have an estimated error for the opening rates of ~5–6% (Chen and Miller, 1996).

Single-channel current amplitudes were determined from all-points amplitude histograms and the time constants of the three current levels were estimated from the dwell-time distribution. All nonconducting events of C212S were included in the analysis because the inactivation of this mutant is suppressed (Lin et al., 1999). All results shown in the figures are presented as mean ± SEM. Curve fitting was performed with an unweighted least-squares method. In fitting the opening rates with Eq. 4 (see Fig. 8, B and C), multiple datasets obtained at different pHs were fitted at the same time, with a shared $\gamma(0)$ in each dataset. See Fig. 8 legend for a detailed description.

**RESULTS**

**Inactivation of C212S Mutant Is Suppressed in Nonphysiological Conditions**

In the attempt to isolate the H⁺ and Cl⁻ effects on the fast gating of ClC-0, we face a problem due to the inactivation of the channel. In ClC-0, the inactivation mech-

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**Figure 1.** Quasi steady-state inactivation curves of the WT ClC-0 and the C212S mutant at different pH values. (A) Whole oocyte currents of the WT and C212S at three external pH. See MATERIALS AND METHODS for the voltage protocol to examine the slow-gate inactivation. Dotted lines represent zero-current level. (B) Quasi steady-state inactivation curve of the WT (left, $n = 3$) and the C212S mutant (right, $n = 4$) at pH 7.6 (circles), 9.6 (triangles), and 5.6 (squares).
External pH Effects on ClC Fast Gating

An organism operates at a time scale of tens to hundreds of seconds at room temperatures (Pusch et al., 1997; Chen, 1998), therefore, it is not difficult to separate the two gating processes under physiological conditions because of a 100- or 1,000-fold difference in their kinetics. However, when the experimental conditions were changed to, for example, low [Cl\(^-\)]\(_o\) and a nonphysiological pH, ascribing the observed phenomena solely to the effects on the fast gating can become questionable. To ensure that the observed effect was on the fast gating, we simultaneously examined the WT channel and an inactivation-suppressed mutant, C212S. This point mutant has been shown to have a nondetectable inactivation probability. More importantly, its fast-gating properties and the single-channel conductance are not discernibly different from those of the WT channel (Lin et al., 1999). Fig. 1 A shows a comparison of the whole cell current from oocytes expressing the WT channel and the C212S mutant in three external pH conditions. The quasi-steady-state inactivation curves are shown in Fig. 1 B. Although the inactivation of the WT channel is prominent and is subjected to alteration by the external pH (Fig. 1 B, left), the inactivation of C212S, whether in acidic, neutral, or alkaline pH, appears to be suppressed (Fig. 1 B, right).

**Fast Gating of C212S Is Identical with that of the WT Channel**

To ensure that C212S can be used to study the fast gating of ClC-0, we further compared the responses of the fast gating of WT and C212S to different [Cl\(^-\)]\(_o\). Fig. 2 A shows macroscopic currents in the presence of 98 and 4 mM [Cl\(^-\)]\(_o\). For the WT channel, the maximal tail current at 4 mM [Cl\(^-\)]\(_o\) is smaller than that at 98 mM [Cl\(^-\)]\(_o\). In C212S, the situation is reversed; the maximal tail current at 4 mM [Cl\(^-\)]\(_o\) is larger. The inactivation of C212S is also suppressed at low [Cl\(^-\)]\(_o\) (data not shown). Thus, the bigger inward tail current at 4 mM than at 98 mM [Cl\(^-\)]\(_o\), results from a larger electrochem-
ical driving force that pushes Cl$^{-}$ out of the cell at $-100$ mV. On the other hand, a smaller tail current at 4 mM [Cl$^{-}$]$_{o}$ in the WT channel reflects a higher inactivation probability at low [Cl$^{-}$]$_{o}$ (Chen and Miller, 1996).

To examine the fast gating of the WT and C212S, we systematically varied [Cl$^{-}$]$_{o}$ and performed a series of experiments similar to those shown in Fig. 2 A. The $P_{o}$-V curves at various [Cl$^{-}$]$_{o}$ are plotted in Fig. 2 B, in which the curve shifts to more depolarized voltages when [Cl$^{-}$]$_{o}$ is reduced. For the WT channel, the $V_{1/2}$ of the fitted $P_{o}$-V curve changes by $\sim$20–30 mV with a fourfold reduction in [Cl$^{-}$]$_{o}$. In C212S, the degree of the shift is not significantly different. These results indicate that WT and C212S have similar [Cl$^{-}$]$_{o}$-dependent fast-gating properties. The responsible Cl$^{-}$ binding sites are likely to be intact in the inactivation-suppressed mutant, C212S.

Effects of External pH on the Fast Gating: Macroscopic Current Recordings

The influences of external pH on the fast gating of WT and C212S are compared in Fig. 3. At neutral pH, the macroscopic current deactivates according to a single-exponential function in response to membrane hyperpolarization. The deactivated currents at various negative voltages usually cross with each other, a hallmark of ClC-0 current. When the pH is increased from 7.6 to 9.6, the time constant and the steady-state component of the current deactivation are not significantly changed. On the other hand, the proportion of the steady-state component increases significantly as the pH is decreased from 7.6 to 5.6. Consequently, the deactivating currents do not cross with each other in this acidic condition. Fig. 4 A shows the $P_{o}$-V curves derived from the experiments similar to those shown in Fig. 3. The effect of pH is prominent only when the pH is changed from neutral to acidic conditions. Reducing the external pH elevates the open probability of the channel mostly at the hyperpolarized voltages. This behavior is similar to the pH effect on the fast-gate $P_{o}$-V curve of ClC-1 (Rychkov et al., 1996). To obtain a mechanistic insight into this H$^{+}$ modulation, we derived the opening rate, $\alpha$, and the closing rate, $\beta$, of the fast gate from the current deactivation time constants and the
steady-state open probabilities (see MATERIALS AND METHODS). Fig. 4 (B and C) indicates that external H\(^+\) profoundly increases the opening rate of the fast gate, whereas the closing rate is almost not affected. The effect of H\(^+\) on the opening rate, again, is more prominent as the membranes become more hyperpolarized.

Effects of External pH on the Fast Gating:
Single-channel Recordings

To directly observe the channel behavior in response to external H\(^+\), we performed single-channel recordings on excised inside-out patches. Representative single-channel recording traces of C212S are shown in Fig. 5 A. It is obvious by observing the recording traces or by comparing the all-points amplitude histograms (Fig. 5 B) that the channel conductances at various external pH are all the same. The areas of the all-points amplitude histograms or the measured state probabilities of the three current levels (see Fig. 5 B legend) also confirm the conclusion that the \(P_o\) of the fast gate is increased mainly as the condition moves from neutral to acidic pH. Dwell-time distributions of the events at the three current levels. Same traces as in B. Δ, ■, and ○ represent the closed (level 0), intermediate (level 1), and fully open (level 2) current levels, respectively. The fitted time constants (\(\tau_0\), \(\tau_1\), and \(\tau_2\)) are as follows (in ms): for pH 9.6, 17.5, 18.7; for pH 7.6, 6.6, 10.9, and 19.8; and for pH 5.6, 1.8, 4.3, and 20.3. As discussed in MATERIALS AND METHODS, because there are only 140 closed events at pH 5.6 in this analysis and their durations are heavily affected by the cutoff frequency, the estimate of \(\tau_0\) at this acidic condition may have a relatively large error.
proximation, the results from the single-channel experiments recapitulate the macroscopic current recordings except a larger minimal $P_\text{o}$. This difference between the macroscopic and single-channel recordings is probably due to a different internal Cl$^-$/H$^+$ concentration in these two recording conditions (Chen and Miller, 1996; Ludewig et al., 1997). However, the key observations with respect to the external H$^+$ modulation are similar in the macroscopic and single-channel recordings; that is, the closing rate is not affected, whereas the opening rate increases with a lower pH at hyperpolarized voltages. This results in a higher minimal $P_\text{o}$ of the $P_\text{o}$-V curve. At depolarized potentials, the opening rates from different pH tend to converge to the same value. This external H$^+$ effect is fundamentally different from external Cl$^-$/H$^+$ modulation because varying [Cl$^-$/H$^+$]o shifts the $P_\text{o}$-V curve in parallel along the voltage axis (Pusch et al., 1995; Chen and Miller, 1996).

$H^+$ Modulations at Saturating and Nonsaturating [Cl$^-$/H$^+$]o

The binding site for the [Cl$^-$/H$^+$]-dependent fast-gate opening has an apparent Cl$^-$/H$^+$ binding affinity of $\sim$50 mM (Chen and Miller, 1996). With [Cl$^-$/H$^+$]o of 150–300 mM, the effect of Cl$^-$ on the fast gating is nearly saturated (Pusch et al., 1995; Chen and Miller, 1996; Lin and Chen, 2000). If external H$^+$ modulates the fast gating through increasing the Cl$^-$ binding affinity, the effects should not be equal under different [Cl$^-$/H$^+$]o. Specifically, one would expect a bigger H$^+$ effect at nonsaturating than at saturating [Cl$^-$/H$^+$]o. Fig. 7 (A and B) shows such experiments in which the fast-gate $P_\text{o}$-V curves are compared at 15 or 300 mM [Cl$^-$/H$^+$]o. (A) Comparison of the $P_\text{o}$-V curves under different pH at 15 mM [Cl$^-$/H$^+$]o ($n = 3$). (B) Comparison as performed in A at 300 mM [Cl$^-$/H$^+$]o ($n = 3–4$). Data points were connected with short straight lines.

![Figure 6. Fast gating properties of single C212S channels at different external pH. (A) Steady-state open probability. (B) Opening rate of the fast gate. (C) Closing rate of the fast gate. All rate constants were calculated according to Eqs. 3c and 3d. External pHs were as follows: 5.6; 6.6; 7.6; 8.6; and 9.6.](image1)

![Figure 7. External H$^+$ modulation of the fast gate $P_\text{o}$-V curve of C212S at saturating and nonsaturating [Cl$^-$/H$^+$]o. Data were derived from macroscopic current recordings at pH 7.6 (○) and 5.6 (□). (A) Comparison of the $P_\text{o}$-V curves under different pH at 15 mM [Cl$^-$/H$^+$]o ($n = 3$). (B) Comparison as performed in A at 300 mM [Cl$^-$/H$^+$]o ($n = 3–4$). Data points were connected with short straight lines.](image2)
We have shown that C212S mutant can be used to study the fast gating of ClC-0. The mutation suppresses the inactivation of ClC-0, but the mutant channel retains normal channel conductance and fast-gating properties, including voltage dependence and modulations by external Cl\(^-\) (Fig. 2) and pH (Figs. 3 and 4). Therefore, we are confident that the conclusions drawn with respect to H\(^+\) modulation on the fast gating of C212S can be applied to the WT channel.

The effect of external H\(^+\) on the fast gating is mainly on the opening rate, whereas the closing rate is insensitive to the pH change. Higher \([\text{H}^+]_{\circ}\) increases the opening rate, but the modulation is larger at hyperpolarized voltages. In effect, the \(P_o-V\) curve shows a larger minimal \(P_o\) when pH is reduced. This phenomenon is different from the effect obtained by varying \([\text{Cl}^-]\)\(_\circ\), which shifts the \(P_o-V\) curve along the voltage axis (Fig. 2). With such a difference even at the level of a qualitative description of the \(P_o-V\) curve, it is already difficult to believe that external H\(^+\) modulates the fast gating through protonation of the Cl\(^-\) binding site. To examine this problem more rigorously, we compared the H\(^+\) effect at a nonsaturating (15 mM) and at a nearly saturating \([\text{Cl}^-]\)\(_\circ\) (300 mM). Experimental results show that H\(^+\) does not have a smaller modulatory effect in a saturating \([\text{Cl}^-]\)\(_\circ\) condition (Fig. 7). This result strongly supports the idea that H\(^+\) modulation of the fast gating is unlikely to be due to an increase in the affinity of the Cl\(^-\) binding site responsible for the \([\text{Cl}^-]\)\(_\circ\)-dependent channel opening.

In a previous single-channel recording study, the fast gate of ClC-0 was found to consist of two opening processes with opposite voltage dependence (Chen and Miller, 1996). In one, the opening is favored by membrane depolarization and is intimately associated with the binding of Cl\(^-\) from the external solution. It was hypothesized that when the Cl\(^-\) binding site is occupied, the bound Cl\(^-\) was further transferred through the membrane electric field to an inner binding site to open the channel. Because Cl\(^-\) carries a negative charge, this inward movement of the permeant ion was thought to be the source of the voltage dependence of the opening process (Chen and Miller, 1996). As a result, the channel tends to open more when the membrane is depolarized. The other mechanism contributing to the opening of the fast gate does not involve external Cl\(^-\). The rate of this type of channel opening process, in contrast to the aforementioned \([\text{Cl}^-]\)\(_\circ\)-dependent mechanism, is faster when the membrane is hyperpolarized. This opening process is obscure, but it is the mechanism that maintains the fast-gate \(P_o\) at a nonzero value even at extremely negative voltages (Chen and Miller, 1996; Ludewig et al., 1997).

As the effect of external H\(^+\) modulation is more prominent at more negative voltages, we suspect that H\(^+\) is acting on this \([\text{Cl}^-]\)\(_\circ\)-independent opening process. To explore this possibility, we adopt a five-state scheme to describe the summation of the above two fast-gate opening processes:

\[
\begin{align*}
\text{C} & \xrightarrow{\alpha_1} \text{O} \\
\text{Cl}^- & \xrightarrow{K} \\
\text{C} & \xrightarrow{\alpha_1} \text{O} \\
\gamma & \\
\text{O} & 
\end{align*}
\]

(SCHEME I)

This five-state scheme simplifies the described previously six-state model (Chen and Miller, 1996) by lumping together the inward movement of the bound Cl\(^-\) and the subsequent channel opening step. As the Cl\(^-\) movement is the rate limiting step, \(\gamma\) determines the opening rate in this \([\text{Cl}^-]\)\(_\circ\)-dependent process. The rate constant \(\alpha_1\) represents the rate of the hyperpolarization-favored opening process. The observed opening rate \(\alpha\), thus, is the sum of \(\alpha_1\) and \(\gamma\) weighted by a factor determined by \([\text{Cl}^-]\)\(_\circ\) and the Cl\(^-\) dissociation constant \(K\) (Chen and Miller, 1996):

\[
\alpha(V) = \alpha_1(V) + \gamma(V)([\text{Cl}^-]_\circ/K)/(1+[\text{Cl}^-]_\circ/K),
\]

where the rate parameters vary with voltage according to Eqs. 5a and 5b:

\[
\begin{align*}
\alpha_1(V) &= \alpha_1(0) \exp(z_{\alpha_1} F V / R T), \\
\gamma(V) &= \gamma(0) \exp(z_{\gamma} F V / R T).
\end{align*}
\]

If H\(^+\) modulation is solely acting on \(\alpha_1\), we should be able to describe the effect of pH on the fast-gate opening rate by varying the value of \(\alpha_1\) but keeping all the other parameters unchanged. In modeling this condition, we applied the values of all parameters shown in Table II of Chen and Miller (1996); i.e., \(K = 50\) mM, \(\alpha_1(0) = 2.4\) s\(^{-1}\) (pH = 7.6), \(\gamma(0) = 446\) s\(^{-1}\), \(z_{\alpha_1} = -0.29\), and \(z_{\gamma} = 0.7\). [Cl\(^-\)]\(_\circ\) is 98 mM in this study. Fig. 8 shows that such a model indeed provides a good approximation. When the value of \(\alpha_1(0)\) is increased presumably by protonation, the observed opening rates are enhanced mostly at hyperpolarized voltages. At depolarized voltages, in contrast, the opening rates at different external pH converge to the same value.

To gain a structural insight into this H\(^+\) modulation...
site, we fitted the observed opening rates to Eq. 4 (Fig. 8, A and B). Curve fitting for data at different pH was performed at the same time with a shared value of $\gamma(0)$ for each dataset. The fitted values of $\gamma(0)$, either from the macroscopic current recordings or from the single-channel experiments, vary only within a factor of 2 from that reported in Chen and Miller (1996). More importantly, these curve fittings show that the change in the observed opening rate can be well described by varying only one parameter, $\alpha_1(0)$, indicating that the modulation of external H$^+$ can be explained by an effect on the hyperpolarization-favored opening process. The fitted $\alpha_1(0)$'s in these analyses were plotted in Fig. 8 D as a function of external pH. Curve fitting by a logistic function gives a pK$_a$ of $\sim$5.3 for the protonation site. However, this number can only be viewed as an approximated pK$_a$ value of the titratable site because the titration curve is not saturated even at the most acidic pH in which reliable data can be obtained.

The results presented in this study also raise an interesting possibility that internal Cl$^-$ may interact with external H$^+$ to modulate the hyperpolarization-activated fast gating. A comparison of the external H$^+$ modulations between the macroscopic and single-channel experiments shows that the effect is bigger with a higher [Cl$^-$]$_i$ (compare Fig. 4 with Fig. 6). This can be better seen in Fig. 8 D, in which the fitted $\alpha_1(0)$ in single-channel experiments ([Cl$^-$]$_i$ = 120 mM) is more than fivefold larger than that obtained from the macroscopic current recordings ([Cl$^-$]$_i$ $\sim$40 mM) at an external pH of 5.6. An increase of [Cl$^-$]$_i$ is known to raise the minimal $P_0$ of the fast-gate $P_o$V curve (Chen and Miller, 1996; Ludewig et al., 1997; also see Fig. 7). Although this effect was shown to be largely due to a reduction of the closing rate, a slight increase of the opening rate at the hyperpolarized voltage range was also reported at a neutral pH (see Fig. 10 of Chen and Miller, 1996). In the present study, the internal Cl$^-$ effect on the opening rate is also small at a neutral or alkaline external pH as can be seen from a similar $\alpha_1(0)$ between macroscopic and single-channel experiments (Fig. 8 D). However, the difference in the fitted $\alpha_1(0)$ between these two types of experiments becomes larger when the external pH is more acidic. Because [Cl$^-$]$_i$ in the whole oocyte recordings are not precisely known, these observations remain only qualitative. It will require a better control of [Cl$^-$]$_i$ to explore the exact functional role of internal Cl$^-$ in the hyperpolarization-favored fast gating and to examine the possible coupling between the actions of H$^+$ and Cl$^-$.

In summary, external H$^+$ modulates the fast gate of CIC-0 in a way similar to that observed in CIC-1. The effect is to enhance the opening rate, leading to an increase in the fast-gate $P_0$ mostly at the hyperpolarized voltages. This modulation of the fast-gate opening, however, is different from that produced by the external Cl$^-$. External Cl$^-$ increases the depolarization-favored opening rate, whereas H$^+$ modulates the hyperpolarization-activated fast-gating. These two opening processes, which not only have opposite voltage dependence, but also show characteristic modulations by different external small ions, should be considered together to properly describe the fast-gate openings of the muscle-type CIC channels.

Figure 8. External H$^+$ activates the hyperpolarization-favored opening process. (A) Modeling the fast-gate opening rate according to Scheme I. The solid curve was generated according to Eq. 4, using the values listed in Table II of Chen and Miller (1996). See Discussion for the value of each parameter. Dotted curves were synthesized by increasing the value of $\alpha_1(0)$ as shown on the left of each curve. (B and C) Examination of the fast-gate by the sum of two opening processes. Opening rate data were taken from macroscopic (B) and single-channel recordings (C) as those shown in Figs. 4 B and 6 B, respectively. The dataset at each external pH was fitted to Eq. 4 constrained with a shared value of $\gamma(0)$. Only $\alpha_1(0)$ and $\gamma(0)$ were allowed to vary in the simultaneous multiple-curve fitting process. All the other parameters were the same as those in A. The fitted $\gamma(0)$ were 590 s$^{-1}$ and 340 s$^{-1}$ in B and C, respectively. (D) The fitted $\alpha_1(0)$ from B (□) and C (X) as a function of external pH. Data points from macroscopic recordings were further fitted to a logistic function, $\alpha_1 + (\alpha_1 - \alpha_1)/[1 + [H^+]_o/K]$$_H$, where $K$ is the dissociation constant of the protonation site for H$^+$. The minimal ($\alpha_1$) and maximal ($\alpha_1$) $\alpha_1(0)$ were 0.85 s$^{-1}$ and 31.6 s$^{-1}$, respectively. The fitted $K$ is 4.7 $\times$ 10$^{-5}$ M, corresponding to pK$_a$ 5.3.
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