Model A6 of the original paper contained a typographical error. The corrected model appears below.

\[
\text{Metal occupancy} = \frac{\left(1 + a\frac{H}{K_a}\right)^n}{\left(1 + a\frac{H}{K_a}\right)^n + \frac{K_M}{M}\left(1 + \frac{H}{K_a}\right)^n}
\]

(A6)
pH-dependent Inhibition of Voltage-gated H⁺ Currents in Rat Alveolar Epithelial Cells by Zn²⁺ and Other Divalent Cations

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Abstract: Inhibition by polyvalent cations is a defining characteristic of voltage-gated proton channels. The mechanism of this inhibition was studied in rat alveolar epithelial cells using tight-seal voltage clamp techniques. Metal concentrations were corrected for measured binding to buffers. Externally applied ZnCl₂ reduced the H⁺ current, shifted the voltage-activation curve toward positive potentials, and slowed the turn-on of H⁺ current upon depolarization more than could be accounted for by a simple voltage shift, with minimal effects on the closing rate. The effects of Zn²⁺ were inconsistent with classical voltage-dependent block in which Zn²⁺ binds within the membrane voltage field. Instead, Zn²⁺ binds to superficial sites on the channel and modulates gating. The effects of extracellular Zn²⁺ were strongly pH₉ dependent but were insensitive to pHᵢ, suggesting that protons and Zn²⁺ compete for external sites on H⁺ channels. The apparent potency of Zn²⁺ in slowing activation was ~10⁻² greater at pH₉ 7 than at pH₉ 6, and ~10⁻² greater at pH₉ 6 than at pH₉ 5. The pH₉ dependence suggests that Zn²⁺, not ZnOH⁻, is the active species. Evidently, the Zn²⁺ receptor is formed by multiple groups, protonation of any of which inhibits Zn²⁺ binding. The external receptor bound H⁺ and Zn²⁺ with pKᵢ 6.2–6.6 and pKᵢ 6.5, as described by several models.

Zn²⁺ effects on the proton chord conductance–voltage (gᵢₗ-V) relationship indicated higher affinities, pKᵢ 7 and pKᵢ 8. CdCl₂ had similar effects as ZnCl₂ and competed with H⁺, but had lower affinity. Zn²⁺ applied internally via the pipette solution or to inside-out patches had comparatively small effects, but at high concentrations reduced H⁺ currents and slowed channel closing. Thus, external and internal zinc-binding sites are different. The external Zn²⁺ receptor may be the same modulatory protonation site(s) at which pH₉ regulates H⁺ channel gating.

Key words: metal binding constants • cadmium • pH • hydrogen ion • ion channels

Introduction

Voltage-gated proton channels differ from other voltage-gated ion channels, not only in their extreme selectivity for H⁺, but also in the regulation of their gating by pH₉ and pHᵢ. The mechanism of permeation is believed to differ radically from traditional ion channels, which comprise water-filled pores through which ions diffuse: proton channels appear to conduct H⁺ by a Grotthuss-like mechanism of hopping across a hydrogen-bonded chain spanning the membrane (DeCoursey and Cherny, 1994, 1995, 1997, 1998; Cherny et al., 1995). If ion channels are defined narrowly as water-filled pores, then proton channels are not ion channels, although they conduct protons passively down their electrochemical gradient, and independently of other ionic species. In spite of these fundamental differences, it is remarkable how closely proton channels resemble other voltage-gated channels. H⁺ channels activate upon depolarization with a sigmoidal time course, deactivate exponentially, and exhibit a Cole-Moore effect (DeCoursey and Cherny, 1994) practically indistinguishable from the behavior of delayed rectifier K⁺ channels (Cole and Moore, 1960). Low pH₉ shifts the voltage-activation curves of both H⁺ channels and other ion channels toward positive potentials and slows activation at a given voltage. Voltage-gated proton channels characteristically are inhibited by extracellular polyvalent cations. The transition metals Zn²⁺ and Cd²⁺ have been used most frequently (Thomas and Meech, 1982; Byerly et al., 1984; Barish and Baud, 1984; Mahaut-Smith, 1989; DeCoursey, 1991; Kapus et al., 1993; Demaurex et al., 1993; DeCoursey and Cherny, 1993; Humez et al., 1993; Gordienko et al., 1996; Nordström et al., 1995), but Cu²⁺, Ni²⁺, Co²⁺, Hg²⁺, Be²⁺, Mn²⁺, Al³⁺, and La³⁺ have similar effects (Thomas and Meech, 1982; Meech and Thomas, 1987; Byerly and Suen, 1989; Bernheim et al., 1993; DeCoursey and Cherny, 1994; Eder et al., 1995). To the extent that each has been explored, all of these metal cations shift the voltage dependence of activation (channel opening) to more positive potentials and slow the opening rate (Byerly et al., 1984; Barish and Baud, 1984; Meech and Thomas, 1987; Mahaut-Smith, 1989; DeCoursey, 1991; DeCoursey and Cherny, 1993; Demaurex et al., 1993; Kapus et al., 1993; Nordström et
al., 1995; Gordienko et al., 1996). These effects resemble those of polyvalent cations on other ion channels (e.g., Frankenhaeuser and Hodgkin, 1957; Hille, 1968; Stanfield, 1975; Gilly and Armstrong, 1982; Spires and Begenisich, 1992, 1995; Arcket al., 1994). Closer examination reveals differences, however. The H⁺ channel is much more sensitive to external ZnCl₂ than are voltage-gated K⁺ channels, which require 1,000-fold higher concentrations to produce comparable effects in squid (Spires and Begenisich, 1992), 10–100-fold higher concentrations in frog skeletal muscle (Stanfield, 1975), and 100-fold higher concentrations in Shaker (Spires and Begenisich, 1994). In addition, the effects of ZnCl₂ on squid axon K⁺ channels are similar for addition to either side of the membrane and internally applied ZnCl₂ is quite potent (Begenisich and Lynch, 1974). In contrast, we find that ZnCl₂ has qualitatively different effects on H⁺ channels depending on the side of application and thus binds to distinct external and internal sites.

The effects of metal cations on H⁺ currents have been characterized variously as voltage-dependent block, voltage shifts induced by electrostatic effects on the voltage sensor, and specific binding to the channel. These interpretations invoke different mechanisms. Voltage-dependent block suggests that the metal ion enters the channel and crosses part of the membrane potential field to reach its block site in the pore. Here we explore the effects of ZnCl₂, one of the more potent inhibitors of H⁺ channels, as a prototypical metal inhibitor. We find that voltage-dependent block is not a viable mechanism. Prominent effects of Zn²⁺ reflect specific binding that allosterically alters gating.

A key feature of the inhibition of H⁺ currents by Zn²⁺ is a profound pH dependence, which has not been described previously. Lowering pH decreases the effectiveness of ZnCl₂. Competition between ZnCl₂ and H⁺ has been noted previously for other channels, including Cl⁻ (Hutter and Warner, 1967; Spalding et al., 1990; Ryckov et al., 1997) and K⁺ (Spires and Begenisich, 1992, 1994). We consider whether the pH₀ dependence indicates that (a) the active form is not Zn²⁺ but ZnOH⁺, (b) Zn²⁺ and H⁺ compete for the same binding site, or (c) there is noncompetitive inhibition; i.e., protonated channels have a lower affinity for Zn²⁺. We conclude that the external Zn²⁺ receptor is formed by three or more protonation sites, perhaps comprising His residues, that together coordinate one Zn²⁺.

**MATERIALS AND METHODS**

**Rat Alveolar Epithelial Cells**

Type II alveolar epithelial cells were isolated from adult male Sprague-Dawley rats using enzyme digestion, lectin agglutination, and differential adherence, as described in detail elsewhere (DeCoursey et al., 1988; DeCoursey, 1990), with the exception that we now use elastase without trypsin to dissociate the cells. The rats were anesthetized using sodium pentobarbital. In brief, the lungs were lavaged to remove macrophages, elastase was instilled, and then the tissue was minced and forced through fine gauze. Lectin agglutination and differential adherence further removed contaminating cell types. The preparation at first includes mainly type II alveolar epithelial cells, but after several days in culture the properties of the cells are more like type I cells. H⁺ currents were studied in approximately spherical cells up to several weeks after isolation.

**Solutions**

Solutions contained 100 mM buffer supplemented with tetracyethylammonium (TMA) methanesulfonate (TMAMeSO₄) to bring the osmolarity to ~300 mOsm. One exception was the pH 7.0 solution made with 70 mM PIPES. External solutions contained 2 mM CaCl₂ or 2 mM MgCl₂. Internal solutions contained 2 mM MgCl₂ and 1 mM EGTA. Solutions were titrated to the desired pH with TMA hydroxide (TMAOH) or methanesulfonic acid (solutions using BisTris as a buffer). A stock solution of TMAMeSO₄ was made by neutralizing TMAMeOH with methanesulfonic acid. TPEN (N,N,N′,N′-tetraakis(2-pyridylmethyl)ethylenediamine) was purchased from Sigma Chemical Co.

**Buffers and Their Metal Binding Properties**

The following buffers were used near their negative logarithm of the acid dissociation constant (pKᵢ) (20°C) for measurements at the following pH: pH 5.0, Homopipes (homopiperazine-N,N'-bis-2-(ethanesulfonic acid), pKₐ 4.61); pH 5.5–6.0 Mes (pKₐ 6.15); pH 6.5 BisTris (bis[2-hydroxyethyl]iminotris[hydroxymethyl]methane, pKₐ 6.50); pH 7.0 PIPES (pKₐ 6.80); pH 7.5–8.0 HEPES (pKₐ 7.55). Buffers were purchased from Sigma Chemical Co., except for Homopipes (Research Organics). Buffers such as Tricine and BES that reportedly complex strongly with transition metals (Good et al., 1966) were avoided. We could not find information in the literature on the Zn²⁺ or Cd²⁺ binding properties of the buffers used. Therefore, we measured the binding constants for a number of buffers, according to the method described by Good et al. (1966). This consisted of titrating the buffer alone, and then together with an equimolar amount of the metal salt (usually 10 mmol in a 100-ml vol). The binding constant was calculated from the relationship (Eq. 1):

\[
K'_{M} = \frac{Z^{2} [H_{M}]^{a} - 1}{[B]} \frac{[K_{A}^{a} + 1]}{[H_{M}]^{a}},
\]

where K'ₘ is the metal binding constant, Kₐ is the proton dissociation constant defined in Scheme III (−pKᵢ value), [H⁺] is the H⁺ concentration at the midpoint of the titration curve in the presence of the metal being tested and [B] is the total buffer concentration. The higher the affinity of the buffer for metal, the greater the shift in the titration curve. Table I gives the results.

Good et al. (1966) reported that the affinity of several buffers for Ca²⁺ was generally about five log units weaker than that for Cu²⁺. A notable exception is Mes, which binds Ca²⁺ weakly but Cu²⁺ negligibly (Good et al., 1966). We find that Zn²⁺ is bound roughly two log units more weakly than Cu²⁺, consistent with the lower affinity binding of Zn²⁺ than Cu²⁺ to various ionizable groups on proteins (Bredow, 1973). One exception to this rule is that PIPES did bind Zn²⁺ weakly, whereas Ca²⁺ was bound negligibly (Good et al., 1966). All buffers bound Cd²⁺ detectably and to roughly the same extent that they bound Zn²⁺. It should be noted...
Solubility of Zn(OH)\textsubscript{2} and Other Metal Dihydroxides

An upper limit to the concentration of ZnCl\textsubscript{2} is set by the limited solubility of Zn(OH)\textsubscript{2} (K\textsubscript{sp} ≈ 4 × 10\textsuperscript{-17}; Lide, 1995). The maximal soluble concentrations: ~40 μM at pH 8, ~4 mM at pH 7, and ~400 mM at pH 6, were not approached during experiments. We encountered solubility problems when titrating the buffers to test for metal binding (above). For this purpose, we usually used 10 mM ZnCl\textsubscript{2}, and in fact the solutions began to precipitate just 1 h after addition of ZnCl\textsubscript{2}. For this reason, we used a Flaming Brown automatic pipette puller (Sutter Instruments, Co.) from EG-6 glass (Garner Glass Co.), coated with Sylgard 184 (Dow Corning Corp.), and heated polished to a tip resistance ranging typically from 3 to 10 MΩ. Electrical contact with the pipette solution was achieved by a thin silver wire connected to the bath through an agar bridge made with Ringer’s solution. The current signal from the patch clamp (List Electronic) was recorded and analyzed using a Laboratory Data Acquisition and Display System (Indec Corp.). Seals were formed with Ringer’s solution (mM: 160 NaCl, 4.5 KCl, 2 CaCl\textsubscript{2}, 1 MgCl\textsubscript{2}, 5 HEPES, pH 7.4) in the bath, and the zero current potential established after the pipette was in contact with the cell. Bath temperature was controlled by Peltier devices and monitored by a resistance temperature detector element (Omega Scientific) in the bath.

Because the voltage dependence of H\textsuperscript{+} channel gating depends strongly on ΔpH, the threshold for activation ranging from ~80 to +80 mV at ΔpH 2.5 and ~1.5, respectively (Cherny et al., 1995), the holding potential, V\textsubscript{hold}, must be adjusted appropriately. V\textsubscript{hold} was set such that the membrane potential is ~0 mV. In the inside-out patch configuration, the solution in the pipette sets pH\textsubscript{o}, defined as the pH of the solution bathing the original extracellular surface of the membrane, and the bath solution sets pH\textsubscript{i}. Currents and voltages are presented in the normal sense: that is, upward currents represent current flowing outward through the membrane from the original extracellular surface, and potentials are expressed by defining the original bath solution as 0 mV. Current records are presented without correction for leak current or liquid junction potentials.

### Data Analysis

The time constant of H\textsuperscript{+} current activation, τ\textsubscript{act}, was obtained by fitting the current record by eye with a single exponential after a brief delay (DeCoursey and Cherny, 1995) (Eq. 2):

\[
I(t) = I_0 - I_{\text{act}} \exp \left(\frac{-(t - t_{\text{delay}})}{\tau_{\text{act}}}\right).
\]

where \(I_0\) is the initial amplitude of the current after the voltage step, \(I_{\text{act}}\) is the steady state current amplitude, \(t\) is the time after the voltage step, and \(t_{\text{delay}}\) is the delay. The H\textsuperscript{+} current amplitude is \(I_0 - I_{\text{act}}\). No other time-dependent conductances were observed consistently under the ionic conditions employed. Tail current time constants, \(\tau_{\text{tail}}\), were fitted to a single exponential (Eq. 3):

\[
I(t) = I_0 \exp \left(\frac{-(t - t_{\text{delay}})}{\tau_{\text{tail}}}\right) + I_{\text{tail}}.
\]

where \(I_0\) is the amplitude of the decaying part of the tail current.
RESULTS

Effects of Extracellular ZnCl₂ on H⁺ Currents

The inhibition of H⁺ currents by external ZnCl₂ is illustrated in Fig. 1. The H⁺ current elicited by a pulse to +10 mV is reduced in a concentration-dependent manner by ZnCl₂. The rate the current turns on during a depolarizing voltage pulse is slower, as seen more clearly in Fig. 1 B, where the currents are scaled to the same value at the end of the pulse. Another effect (explored below) is to shift the voltage dependence of H⁺ channel gating to more positive voltages. To some extent, the reduced H⁺ current amplitude and slower activation can be attributed to this voltage shift. One implication is that any attempt to quantitate the apparent "block" of H⁺ currents by ZnCl₂ by measuring the current at the end of a pulse will be arbitrary because the result depends strongly on the length of the pulse and the voltage selected for the measurement. The apparent extent of block at the end of the pulses in Fig. 1 would be greatly reduced if longer test pulses were applied and especially if a more positive test potential were selected.

Zn²⁺ Block Is Not Voltage Dependent

If ZnCl₂ binds with rapid kinetics to a site in the H⁺ channel within the membrane electrical field, this should manifest itself in the instantaneous current-voltage relationship. The control instantaneous current-voltage (I-V) relationship in Fig. 2 A (∙) exhibits

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Figure 1. (A) Effects of ZnCl₂ on the H⁺ current elicited by a 4-s pulse to +10 mV in a cell studied at pH 7.0/5.5. The inset shows the pH of the pipette and bath solutions. (B) The same currents scaled to the same value at the start and end of the 4-s pulse, illustrating the slowing of the activation time course. The steps in the 10 μM record are due to the resolution of the A-D converter.

Figure 2. (A) Instantaneous current-voltage relationships in a cell studied at pH 7.0/6.5 before (∙) and after (■) addition of 10 μM ZnCl₂ to the bath. A prepulse to +40 mV for control and +100 mV in the presence of ZnCl₂ was applied to open H⁺ channels, followed by a test pulse to the voltage on the abscissae. The current at the start of the test pulse, after the capacitive transient, is plotted. (B) Data from A after correction for the current at the end of the prepulse, and normalized to be equal at +100 mV. Dividing the test current by that at the end of the prepulse corrects for variation in the activation of the g₀ during different prepulses. The symbols have the same meaning as in A.
Cherny and DeCoursey

moderate outward rectification, consistent with previous studies (Byerly et al., 1984; Kapus et al., 1993; Bernheim et al., 1993; Cherny et al., 1995; DeCoursey and Cherny, 1996). The instantaneous I-V relationship in the presence of 10 μM ZnCl₂ is also plotted. The currents are reduced even though the prepulse was 40 mV more positive. After both sets of currents are scaled to match at +100 mV (Fig. 2B), the currents superimpose, indicating that there is no rapid voltage-dependent block. In some experiments with CdCl₂, there was a suggestion that the inward currents were reduced preferentially, but this effect was too small to be sure of, even with data spanning 200 mV. Thus, metals have negligible effects on the instantaneous I-V relation of H⁺ channels.

The effects of ZnCl₂ and other metals might reflect voltage-independent interaction of the metal with the channel or nearby membrane. By binding to or screening negative charges near the external side of the H⁺ channel, metals could bias the membrane potential sensed by the channel’s voltage sensor (Frankenhaeuser and Hodgkin, 1957). In the simplest scenario, the voltage-dependent properties of the channel will simply shift along the voltage axis. Fig. 3A illustrates proton chord conductance (gₚ)–V relationships in one cell in the absence (dashed lines) or presence of 100 μM

Figure 3. Effects of divalent metals on the gₚ-V relationship are incompatible with the idea of voltage-dependent block. (A) The gₚ-V relationships in a cell studied in the presence of several metals: controls (dashed lines), 0.1 mM CdCl₂ (●), 1 mM CdCl₂ (▼), 10 mM CdCl₂ (■), 10 mM NiCl₂ (▲), and 0.1 mM ZnCl₂ (○). The sequence was control, all CdCl₂ concentrations, control, NiCl₂, ZnCl₂, and control. (B) The same gₚ-V relationships in A shifted along the voltage axis so that they superimpose at small gₚ. Other than small differences in the limiting gₚ_max, the shape of the voltage dependence appears similar. The voltage shifts applied were: 0, +8, and +34 mV for 0.1, 1.0, and 10 mM CdCl₂, respectively, +15 mV for NiCl₂ and +18 mV for ZnCl₂. (C) The same gₚ-V relationships plotted on linear axes and scaled to have similar gₚ_max appear to simply shift along the voltage axis. The scale factor was determined by taking the ratio of gₚ in the presence of metal to that at +80 mV in the first control measurement. To compensate for the apparent voltage shift (compare A and B), the gₚ value used for this purpose for the metal data was shifted by 10 mV (1 mM CdCl₂, 10 mM NiCl₂), 20 mV (ZnCl₂), or 30 mV (10 mM CdCl₂). All scale factors were <2.5. (D) The steepness of the apparent voltage dependence of "block" by divalent cations is similar to that of the gₚ-V relationship itself. The data in C are plotted as a ratio of the gₚ in the presence of metal to that in its absence, at each voltage, using the same symbols as other parts of this figure. There is no block at any voltage at 0.1 mM CdCl₂ (●). The control gₚ-V relationship (C, dashed line) was fitted to a simple Boltzmann distribution and normalized to its fitted maximum. The slope factors of Boltzmann fits were 12.5 mV for control, and for metal ranged from 8 to 13 mV in fits constrained to limit at 1.0.
ZnCl₂ (●), 10 mM NiCl₂ (▲), or several concentrations of CdCl₂ (solid symbols). When shifted along the voltage axis, the \( g_H \) vs. \( V \) relationships appear quite similar (Fig. 3 B), consistent with this mechanism. These metals may reduce the limiting \( g_H \) (\( g_{H,max} \)) slightly, although for the data shown here this effect was smaller than the variability in the control measurements. At higher metal concentrations, some reduction in \( g_{H,max} \) usually became evident, but was difficult to measure accurately. In Fig. 3 C, the \( g_H \) vs. \( V \) relationships are plotted on linear axes, scaled to the same \( g_{H,max} \) to illustrate their similar shape and slope. The predominant effect is a simple voltage shift.

Even though there is no rapid voltage-dependent block (Fig. 2), the apparent voltage shift might conceivably reflect a slow block/unblock process. If we estimate the steady state voltage dependence of this apparent ZnCl₂ block in the usual manner by plotting the ratio \( I_{H}(\text{ZnCl}_2)/I_{H}(\text{control}) \), the apparent steepness of the "voltage-dependent block" will be identical to the steepness of the Boltzmann relationship in the absence of Zn²⁺. This being the case, the data in Fig. 3 D strongly suggest that metals shift the voltage sensed by the channel rather than binding to the channel in a voltage-dependent manner.

**ZnCl₂ Slows H⁺ Channel Opening**

A prominent effect of ZnCl₂ is to slow the activation of H⁺ currents. We quantified this effect by fitting the turn-on of current during depolarizing pulses to a single exponential, after a delay. This procedure provides a reasonable fit under most conditions. In the presence of ZnCl₂, both the delay and \( \tau_{act} \) were increased by roughly the same factor. We focussed mainly on metal effects on \( \tau_{act} \), which are illustrated in Fig. 4 for the same cell shown in Fig. 3. Because the \( \tau_{act} \) vs. \( V \) relationship is nearly exponential (linear on semi-log axes), it is not possible to distinguish whether \( \tau_{act} \) is slowed or its voltage dependence is shifted, or both. In the simplest case of a Huxley-Frankenhaeuser-Hodgkin voltage shift, all kinetic parameters should be shifted equally along the voltage axis. To explore the extent to which this model might apply, the \( \tau_{act} \) data in Fig. 4 B were "corrected" by the voltage shift determined for the \( g_H \) vs. \( V \) relationship (Fig. 3 B). To a rough approximation, the \( \tau_{act} \) effect in CdCl₂ and NiCl₂ appears to be explainable by this simple voltage shift. Closer examination of Fig. 4 B and other data (not shown) at high CdCl₂ concentrations indicates that CdCl₂ slows activation somewhat more than is accounted for by the shift of the \( g_H \) vs. \( V \) relationship, consistent with a previous study of CdCl₂ on H⁺ currents (Byerly et al., 1984). In contrast, ZnCl₂ slows channel opening dramatically, and far beyond its shift of the \( g_H \) vs. \( V \) relationship. The effects of ZnCl₂ are dominated by an interaction with the H⁺ channel that results in \( \tau_{act} \) slowing, beyond a simple voltage shift of all parameters.

**ZnCl₂ and CdCl₂ Have Minor Effects on H⁺ Channel Closing**

The tail current decay seemed faster in the presence of external ZnCl₂ or CdCl₂. However, attempts to evaluate metal effects on H⁺ channel closing were hampered by
the tendency of metals to reduce \( \text{H}^+ \) currents and by the weak voltage dependence of the closing rate (Cherny et al., 1995). The latter property (\( \tau_{\text{tail}} \) changes e-fold in \( \sim 50 \text{ mV} \)) means that a 35-mV shift of the \( \tau_{\text{tail}}-V \) relationship would change \( \tau_{\text{tail}} \) at a given voltage by a factor of only two. Examination of data on \( \text{ZnCl}_2 \) and \( \text{CdCl}_2 \) in a number of cells under different conditions gave the impression that the \( \tau_{\text{tail}}-V \) relationship may have been shifted in the positive direction at most by roughly the amount that the \( g_{\text{H}}-V \) relationship was shifted, but little effect was seen in some experiments.

**pH Dependence of Metal Effects**

Fig. 5 illustrates the effects of \( \text{ZnCl}_2 \) on \( \text{H}^+ \) currents at three \( \text{pH}_o \). \( \text{ZnCl}_2 \) reduces the \( \text{H}^+ \) current at each voltage, slows activation, and shifts the voltage dependence of activation to more positive voltages. At each \( \text{pH}_o \), the effects are similar, but the concentration of \( \text{ZnCl}_2 \) required to produce these effects is much greater at low \( \text{pH}_o \). In this sense, lowering \( \text{pH}_o \) decreases the efficacy of \( \text{ZnCl}_2 \). To quantitate the effects of \( \text{ZnCl}_2 \), we measured \( \tau_{\text{act}} \) and calculated the ratio of \( \tau_{\text{act}} \) in the presence of \( \text{ZnCl}_2 \) to that in its absence in the same cell at the same voltage. In most cells, this ratio was the same at all voltages, thus the effect of \( \text{ZnCl}_2 \) is a uniform voltage-independent slowing. Average ratios at several \( \text{pH}_o \) are plotted in Fig. 6 and can be thought of as reflecting the “apparent potency” of \( \text{ZnCl}_2 \) at various \( \text{pH}_o \). The concentration required to slow \( \tau_{\text{act}} \) twofold is (\( \mu \text{M} \)) 0.22 at \( \text{pH}_o \) 8, 0.46 at \( \text{pH}_o \) 7, 5.4 at \( \text{pH}_o \) 6, 89 at \( \text{pH}_o \) 5.5, and 1,000 at \( \text{pH}_o \) 5. The apparent potency of \( \text{ZnCl}_2 \) (estimated for a fourfold slowing of \( \tau_{\text{act}} \) where the curves are parallel) decreased only 2.3-fold between \( \text{pH}_o \) 8 and 7, 10-fold between \( \text{pH}_o \) 7 and 6, and 103-fold between \( \text{pH}_o \) 6 and 5.

Most of the buffers used bind \( \text{Zn}^{2+} \) detectably (Table I). In Fig. 6 B, the data from Fig. 6 A are replotted after correcting the metal concentrations for binding by buffer. The correction factors are given in the legend. The main effect is to reduce the shift in apparent potency between \( \text{pH}_o \) 7 and 8. After correction, the concentration required to slow \( \tau_{\text{act}} \) twofold is (\( \mu \text{M} \)) 0.22 at \( \text{pH}_o \) 8, 0.27 at \( \text{pH}_o \) 7, 4.3 at \( \text{pH}_o \) 6, 80 at \( \text{pH}_o \) 5.5, and 1,000 at \( \text{pH}_o \) 5. The apparent potency of \( \text{ZnCl}_2 \) (again estimated for a fourfold slowing of \( \tau_{\text{act}} \) where the curves are parallel) decreased 1.3-fold between \( \text{pH}_o \) 8 and 7, 14-fold between \( \text{pH}_o \) 7 and 6, and 129-fold between \( \text{pH}_o \) 6 and 5.

Measurements made in the same external solutions with different pipette pH gave no indication that \( \text{pH}_i \) affects the interaction between externally applied \( \text{ZnCl}_2 \) and \( \tau_{\text{act}} \). As illustrated in Fig. 6, there was no obvious difference in the effects of \( \text{ZnCl}_2 \) at constant \( \text{pH}_o \) in cells studied with \( \text{pH}_i \) 5.5 (solid symbols and continuous lines) or at \( \text{pH}_i \) 6.5 (open symbols and dashed lines). This result is consistent with externally applied \( \text{ZnCl}_2 \) exerting its effect at the external side of the membrane.

Besides slowing activation, metals also shift channel opening to more positive voltages. This voltage shift was estimated from graphs of the \( g_{\text{H}}-V \) relationships in the absence or presence of metal and is plotted in Fig. 7. This parameter was somewhat arbitrary and less well defined than \( \tau_{\text{act}} \), because it required extrapolating the fitted time course of \( \text{H}^+ \) current and measuring \( V_{\text{rev}} \) in each solution (whenever \( \text{pH}_o \) was changed). Nevertheless, the \( \text{pH}_o \) sensitivity of the \( g_{\text{H}}-V \) relationship to \( \text{ZnCl}_2 \) (solid symbols) qualitatively resembles that of \( \tau_{\text{act}} \). In fact, the interaction between \( \text{ZnCl}_2 \) and \( \text{pH}_o \) manifested in the \( g_{\text{H}}-V \) relationship appears to be somewhat stronger than that for the \( \tau_{\text{act}}-V \) relationship.
Zinc Effects on Proton Channels

The concentration of ZnCl₂ required to produce a 20-mV depolarizing shift of the $g_H$-V relationship was 0.13 mM at pH₀ 8.0, 0.77 mM at pH₀ 7.0, 54 mM at pH₀ 6.0, 470 mM at pH₀ 5.5, and 12.4 mM (by extrapolation) at pH₀ 5.0. The apparent potency of ZnCl₂ thus decreased sixfold between pH₀ 8 and 7, 70-fold between pH₀ 7 and 6, and 230-fold between pH₀ 6 and 5. The larger difference between the effective potency of ZnCl₂ between pH 7 and pH₀ 8 requires a higher $pK_a$ for the steady state conductance measurement than for the kinetic $t_{act}$ measurement (see discussion).

Effects of Intracellular ZnCl₂ on H⁺ Currents

Effects of internally applied ZnCl₂ were studied in the whole-cell configuration and in inside-out patches. Figure 8 illustrates families of H⁺ currents in cells studied at pH 6.5/6.5 without (A) and with (B) 2.5 mM ZnCl₂ added to the pipette solution. The H⁺ currents appear generally similar, although closer inspection reveals that the tail currents decayed more slowly in the cell with internal ZnCl₂. The pipette solution contained 1 mM EGTA and BisTris buffer (which will bind ~90% of the Zn²⁺ under these conditions, Table I), so the addi-
tion of 2.5 mM ZnCl₂ results in a free [Zn²⁺] ~ 170 μM. The families of H⁺ currents at pH 6.5/6.5 were recorded in a control cell (A) and with 2.5 mM ZnCl₂ in the pipette solution (B), and are scaled according to membrane capacity. The measured V₉₀ was +4 mV in A and +1 mV in B. Note the slower tail current decay with ZnCl₂ in the pipette. Considering binding of ZnCl₂ to 1 mM EGTA and 100 mM BisTris in the pipette solution, the free [Zn²⁺] was ~ 170 μM. (C) Identical 8-s pulses to +50 mV were applied before and after addition of the pipette solution used in B to the bath, in the same cell shown in A, demonstrating the dramatic effects of this solution applied externally. The threshold for activating outward H⁺ current shifted from +30 to +60 mV after adding ZnCl₂ to the bath (determined using high gain and 5-mV increments). (D) Average (mean ± SEM) H⁺ current-voltage relationships normalized according to membrane capacity, in 6–10 control cells (●), 9–14 cells studied with 2.5 mM ZnCl₂ added to the pipette solution (■), and 3 cells studied with 2.5 mM CdCl₂ in the pipette solution (○), all at pH 6.5/6.5. *Values for ZnCl₂ at +80 and +100 mV differ significantly from control (P < 0.05).

Our impression was that there was nothing unusual in the behavior of the g₉₀ in these experiments. H⁺ currents were studied after allowing at least 5–10 min equilibration of the ZnCl₂-containing pipette solution. The amplitude of I₉₀ normalized to the input capacity (Fig. 8 D) was reduced significantly (P < 0.005) at +80 and +100 mV in cells studied with 2.5 mM ZnCl₂ in the pipette, on average after 29 min in whole-cell configuration. Internal ZnCl₂ at high concentrations reduces I₉₀, but this effect is not very pronounced.

Fig. 9 illustrates mean τₗₚ values in cells studied at pH 6.5/6.5 with (■) and without (○) 2.5 mM ZnCl₂ in the pipette solution. No difference in the kinetics of H⁺ current activation was detected. However, channel closing was significantly slower in cells studied with internal ZnCl₂. Fig. 9 shows mean values of τₗₚ in cells studied with internal ZnCl₂ (●) and in control cells (○). The deactivation rate on average was 3.1-fold slower with internal ZnCl₂ (measured between −50 and +10 mV). In three cells studied with 2.5 mM CdCl₂ added to the pipette solutions, the average slowing of τₗₚ was 1.8-fold at 10 voltages from −80 to +20 mV (P < 0.05 at each voltage) (not shown). Applied internally, ZnCl₂ thus slows closing without affecting activation. In contrast, externally applied ZnCl₂ slowed activation and, if anything, accelerated deactivation. Clearly the internal and external sites of action of ZnCl₂ are functionally quite different.

A concern during these experiments was the extent to which ZnCl₂ in the pipette solution actually diffused into the cell. ZnCl₂ diffusion into the cell will be slowed by binding to cytoplasmic proteins, acting as fixed buffers. That measurable effects on τₗₚ were seen is evidence that the ZnCl₂ diffused into the cells to a significant extent. The mobility of ZnCl₂ is not unusually small (Robinson and Stokes, 1959). V₉₀ values were consistent with the applied ΔpH (pH 6.5/6.5, Nernst potential = 0 mV), suggesting that buffer from the pipette solution diffused into the cell. In 11 cells studied...
Zinc Effects on Proton Channels

Figure 9. Effects of intracellular ZnCl₂ on the mean (±SEM) activation time constant, $\tau_{\text{act}}$, and deactivation time constant, $\tau_{\text{tail}}$, in cells studied at pH 6.5/6.5. Tail current decay was fitted with a single exponential in three to nine control cells (○) and in four to eight cells studied with 2.5 mM ZnCl₂ added to the pipette solution (●). All $\tau_{\text{tail}}$ values with ZnCl₂ in the pipette differ significantly from control ($P < 0.01$). Values of $\tau_{\text{act}}$ obtained by fitting a single exponential after a delay are plotted from 10–12 control cells (□) and from four to nine cells studied with 2.5 mM ZnCl₂ added to the pipette solution (■).

Inside-out patches were studied at pH₀ 7.5 or 6.5 (pipette pH) and pH₁ 6.5 (bath pH). Addition of 2.5 mM ZnCl₂ to the bath (∼170 μM free Zn²⁺) reduced the $H^+$ current amplitude (Fig. 10 B). This effect of ZnCl₂ was reversible upon washout (Fig. 10 C). The reduction of $H^+$ currents was similar to that observed in whole-cells dialyzed with ZnCl₂ containing pipette solutions (Fig. 8 D), suggesting that similar concentrations were reached in the whole-cell experiments. There was no clear shift of the voltage dependence of gating. If anything, there was sometimes a small shift to more negative voltages. A small hyperpolarizing shift might be explainable by the slight lowering of pH after addition of ZnCl₂ to the solution (0.023 U calculated, 0.05 U measured), due to displacement of protons from buffer. In some inside-out patches, the $H^+$ currents decreased progressively and gradually after addition of ZnCl₂. Spontaneous rundown may account for this largely irreversible loss of $H^+$ current. In summary, the inside-out patch data support the conclusion that effects of internally applied ZnCl₂ differ qualitatively as well as quantitatively from those of externally applied ZnCl₂. Internal application of high concentrations of ZnCl₂ produces only modest effects.

Effects of CdCl₂ on $H^+$ Currents

Although we intended to study ZnCl₂ as a prototype for the effects of all polyvalent cations on $H^+$ currents, there were subtle differences between the effects of ZnCl₂ and CdCl₂. Both metals slowed activation and shifted the $g_H$-V relationship to more positive voltages. However, to a first approximation, the effects of CdCl₂ could be viewed as a simple shift of all parameters to more positive voltages. “Correction” of the $\tau_{\text{act}}$-V relationships in Fig. 4 according to the shift observed in the $g_H$-V relationship in Fig. 3 normalized the data for CdCl₂, but not for ZnCl₂. In other words, ZnCl₂ has a pronounced additional slowing effect. Examination of $\tau_{\text{act}}$ data in individual cells revealed that ZnCl₂ effects

Figure 10. Effects of intracellular ZnCl₂ on $H^+$ currents in an inside-out patch studied at pH 6.5/6.5. The first family (A) was recorded within 5 min after forming the inside-out patch. The family in B was recorded starting 2.5 min after addition of 2.5 mM ZnCl₂, and the family in C was recorded starting 1.5 min after washout. In all parts, the cell was held at −40 mV, and 16-s pulses were applied in 20-mV increments. Calibration bars in A apply to all families.
could usually be approximated as uniform slowing at all voltages, whereas the relative slowing by CdCl₂ sometimes decreased for larger depolarizations. As a result of this subtle difference, there was not a unique “slowing factor” for CdCl₂, and we did not try to plot CdCl₂ data in Fig. 6. The slowing of \( \tau_{\text{act}} \) by CdCl₂ was strongly \( \text{pH}_0 \) dependent, however. To a first approximation, the \( \text{pH}_0 \) dependence of CdCl₂ was similar to that of ZnCl₂.

Another difference between metals is evident in Fig. 7. The shifts of the \( g_{\text{th}}-V \) relationships indicate that CdCl₂ is \( \sim 30 \times \) less potent at either \( \text{pH}_0 \) 7 or 6. In contrast, the slowing of \( \tau_{\text{act}} \) by 100 \( \mu \text{M} \) ZnCl₂ exceeded that by 10 mM CdCl₂ over most voltages (Fig. 4 A), and thus there is a \( > 100 \)-fold difference in potency for this effect. Thus the relative potency of the two metals for slowing \( \tau_{\text{act}} \) and shifting the \( g_{\text{th}}-V \) relationship differs. Perhaps distinct binding sites are involved in these effects, and the relative affinities of the metals for the sites differ. ZnCl₂ has a high affinity for the site that slows activation, whereas most of the effects of CdCl₂ are consistent with binding to a “nonspecific” site that shifts the apparent membrane potential sensed by the \( \text{H}^+ \) channel.

D I S C U S S I O N

Polyvalent cations and protons have similar effects on many ion channels (Hille, 1968; Woodhull, 1973; Kwan and Kass, 1993; Arkett et al., 1994), perhaps because they bind to similar sites. It has been postulated that the function of voltage-gated proton channels requires at least two distinct types of protonation sites. Conduction likely occurs via a hydrogen-bonded chain (Nagle and Morowitz, 1978; DeCoursey and Cherny, 1994, 1995, 1997, 1998, 1999a,b), in which case the entryway of the “channel” is a protonation site, where \( \text{H}^+ \) must bind to initiate permeation. The second type of protonation sites are allosteric regulatory sites (Byerly et al., 1984) that govern the strong \( \Delta \text{pH} = \Delta \text{pH}_{\text{gr}} \) dependence of gating; i.e., the 40 \( \text{mV} / \text{U} \) shift in the voltage-activation curve with changes in either \( \text{pH}_0 \) or \( \text{pH}_i \) (Cherny et al., 1995). The \( \Delta \text{pH} \)-dependent gating mechanism was explained economically by assuming identical internally and externally accessible regulatory protonation sites (Cherny et al., 1995). More recent evidence suggests the internal and external sites have distinct chemical properties (DeCoursey and Cherny, 1997).

Given this background, \( \text{H}^+ \) channels might be affected by \( \text{Zn}^{2+} \) in several ways. (a) Binding at or near the entry to the channel should inhibit \( \text{H}^+ \) current by preventing \( \text{H}^+ \) binding or reducing the local [\( \text{H}^+ \)] available to enter the channel. The attenuation of \( g_{\text{th,max}} \) at high metal concentrations might reflect local \( \text{H}^+ \) depletion by this mechanism. However, most of the effects of metals are not compatible with metal binding to and occluding the channel entry. (b) Binding to a site remote from the entry but which is sensed by the voltage sensor of the channel could shift the position of the voltage dependency of gating, the most simple mechanism of which would result in all voltage-dependent parameters shifting equally along the voltage axis. This mechanism is consistent with most of the effects of \( \text{Cd}^{2+} \) and \( \text{Ni}^{2+} \). (c) Binding near the allosteric sites on either side of the membrane might reduce the local [\( \text{H}^+ \)] electrostatically, and hence affect gating in the same manner as an increase in \( \text{pH} \). The effects of metals are in the wrong direction for this mechanism to apply. (d) Finally, metal binding to the allosteric protonation sites might have a similar effect on gating as protonation of these sites, and might thus mimic the effects of low \( \text{pH} \) near the site. The details of the effects in this case are hard to predict, because due to differences in binding kinetics and steric factors, \( \text{Zn}^{2+} \) can hardly be expected to mimic a single \( \text{H}^+ \), or even two \( \text{H}^+ \). Nevertheless, most of the effects of \( \text{Zn}^{2+} \) can be explained by assuming that it binds to the same regulatory sites as protons, and has the same effects as protons in our model (Cherny et al., 1995). Thus, \( \text{Zn}^{2+} \) (or \( \text{H}^+ \)) binding at the external site prevents channel opening, and \( \text{Zn}^{2+} \) (or \( \text{H}^+ \)) binding at the internal site prevents channel closing.

Zn\(^{2+}\) Is Not a Voltage-dependent Blocker of H\(^+\) Channels

Although polyvalent cation effects on \( \text{H}^+ \) currents in various cells are quite similar, some authors have characterized these effects as modification of the voltage dependence of gating (Byerly et al., 1984; Barish and Baud, 1984; DeCoursey, 1991; Kapus et al., 1993; DeCoursey and Cherny, 1993, 1994; 1996; Demaurex et al., 1993), whereas others describe the effects as voltage-dependent block (Bernheim et al., 1993; Gordienko et al., 1996). These views are not equivalent. The voltage dependence of ionic block is generally assumed to arise from the entry of the blocker into the channel pore partway across the membrane potential field, where it gets stuck, physically occluding the pore. Interpreted in terms of voltage-dependent block, metal binding affinity depends strongly on voltage (Bernheim et al., 1993; Gordienko et al., 1996), whereas effects due to binding to a modulatory site can be explained with a fixed \( K_M \). Because the instantaneous I-V relation was simply scaled down by ZnCl₂ with no detectable voltage dependence (Fig. 2), we ruled out the possibility of rapidly reversible binding of \( \text{Zn}^{2+} \) to a site within the membrane potential field.

Even though there is no rapidly reversible block, the more obvious effects of ZnCl₂ could be due to a slow time-dependent block/unblock. Five arguments oppose the idea that the slow activation of \( \text{H}^+ \) current in the presence of \( \text{Zn}^{2+} \) reflects voltage-dependent un-
binding of Zn\(^{2+}\) from the channel. (a) If \(\tau_{\text{act}}\) in the presence of metals (several seconds) reflects the unblock rate, then block must have very slow kinetics. If we assume that \(pK_{\text{M}} = 6.5\) (Fig. 11) and that the binding rate of \(Zn^{2+}\) is \(3 \times 10^7\) M\(^{-1}\) s\(^{-1}\), a characteristic rate of complex formation between \(Zn^{2+}\) and proteins (Eigen and Hammes, 1963), then the unbinding rate is 9.5 s\(^{-1}\). Thus, \(Zn^{2+}\) probably binds and unbinds in a fraction of a second. If the kinetics are rapid, effects should have been manifested in the instantaneous I-V relation. (b) In normal drug-receptor reactions, the unblock rate is independent of concentration. However, increasing the concentration of \(ZnCl_2\) slowed H\(^{+}\) current activation progressively. There was no indication that two populations of gating behavior resulted, as would be predicted if \(ZnCl_2\) modified a fraction of channels that then opened slowly with the remaining channels opening at the normal rate. A single exponential (after a delay) continued to fit the data at all \([ZnCl_2]\). Thus it appears that \(ZnCl_2\) binds and unbinds the channel repeatedly during a single pulse, with the slowing effect related to the fraction of time \(ZnCl_2\) is bound to the channel. (c) The steady state voltage dependence of this apparent \(Zn^{2+}\) block, defined as the ratio \(I_{\text{H}}(Zn^{2+})/I_{\text{H}}(\text{control})\), is quite steep: a simple Boltzmann fit gives slope factors 8-13 mV (Fig. 3 D). In terms of traditional voltage-dependent block mechanisms (Woodhull, 1973), if \(z\) is the charge on the blocking ion and \(\delta\) is the fraction of the membrane potential sensed by the ion at the block site, then \(z\delta = 2.0\), which implies that \(Zn^{2+}\), \(Cd^{2+}\), and \(Ni^{2+}\) traverse \(\geq 100\%\) of the membrane field to reach the block site. Several examples of \(\delta > 1.0\) for ionic blockade exist in the \(K^{+}\) channel literature and are traditionally explained by interaction between permeant ions in a multiply occupied channel (e.g., Hille and Schwarz, 1978). Because it is unlikely for a hydrogen-bonded-chain conduction mechanism to support multiple protons simultaneously, especially at physiological pH (DeCoursey and Cherny, 1999a), explaining the high \(z\delta\) observed for divalent cation "blockade" is problematic. (d) If \(ZnCl_2\) simply shifted the \(g_{\text{H}}\)-V relationship along the voltage axis, then the apparent steepness of the block, defined as the ratio \(I_{\text{total}}(Zn^{2+})/I_{\text{H}}(\text{control})\), will be precisely identical to the steepness of the \(g_{\text{H}}\)-V relationship in the absence of \(Zn^{2+}\). The slopes of the fractional block curves, 8-13 mV (Fig. 3D), and control \(g_{\text{H}}\)-V relationships, 8-10 mV (DeCoursey and Cherny, 1994; Cherny et al., 1995), are the same, consistent with a simple voltage shift. (e) Finally, any part of the \(H^{+}\) channel conductance pathway comprised of hydrogen-bonded chain would not allow \(Zn^{2+}\) passage; thus the possibility for voltage-dependent block by \(Zn^{2+}\) could exist only in an aqueous vestibule. We conclude that polyvalent cations do not exert their effects by entering into the pore, but instead bind to sites on the channel.

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![Figure 11](image-url)  
**Figure 11.** Comparison of the \(\tau_{\text{act}}\) data replotted from Fig. 6 with the slowing predicted by Eq. A6, assuming that the \(H^{+}\) channel cannot open while \(Zn^{2+}\) is bound to its receptor (see text for details). The meaning of the symbols is the same as in Fig. 6, and all curves are the predictions for \(pK_{\text{a}} 6.3, pK_{\text{M}} 6.5, \) and cooperativity factor \(a = 0.03.\)**

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**Zn\(^{2+}\) is the Active Species of Zinc**

In solution, zinc exists as several chemical species, whose relative proportions depend strongly on pH.

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830  
**Zinc Effects on Proton Channels**
One plausible explanation for the increased apparent potency of ZnCl$_2$ at higher pH is that ZnOH$^-$, rather than the divalent form, is the species acting on H$^+$ channels. As pH is increased, the proportion of ZnCl$_2$ in monohydroxide form, ZnOH$^-$, increases 10-fold per unit increase in pH, and the pH dependence should saturate around the pK$_a$ of ZnCl$_2$. We show here that the apparent potency of both ZnCl$_2$ and CdCl$_2$ increase at higher pH, and the pH$_a$ dependence saturates for ZnCl$_2$. However, saturation occurs at a pH that is too low by $\sim$10, and the change at low pH$_a$ is at least 100-fold per unit increase in pH (Figs. 5-7), both inconsistent with the hypothesis that the monohydroxide form is active. If ZnOH$^-$ were the active form, an additional mechanism (e.g., competition with H$^+$) would be required to enhance the pH sensitivity of its effects. Several polyhydroxide forms of zinc (with net negative charge) also are increasingly represented at high pH, but we rule these out as candidates for interaction with the H$^+$ channel because (a) it seems unlikely that anions and protons would compete for the same sites, and (b) the fraction of all of these forms combined at pH 5 is $<10^{-15}$ of the total ZnCl$_2$ present (Baes and Mesmer, 1976). In conclusion, the most probable form of ZnCl$_2$ active on H$^+$ channels is the divalent form.

Model of Interaction between Zn$^{2+}$ and H$^+$ that Slows Activation

The appendix explores the predictions of several possible mechanisms of competition between Zn$^{2+}$ and H$^+$ for hypothetical binding sites on H$^+$ channels. The pH$_a$ dependence of Zn$^{2+}$ effects on $\tau_{\text{act}}$ are reasonably compatible with Models 4, 5, or 6 (see Fig. 13 for all models). These models assume that the external Zn$^{2+}$ receptor on proton channels is formed by multiple protonation sites that are accessible to the external solution and that coordinate the binding of a single Zn$^{2+}$. If H$^+$ and Zn$^{2+}$ compete directly for the same site(s), then at least two to three protonation sites must exist. If H$^+$ and Zn$^{2+}$ bind to different sites, then there must be substantial interaction between them, and the range of the pH dependence indicates that protonation of one site lowers the affinity of the remaining site(s) for Zn$^{2+}$ by a factor $\sim$30. Similar binding constants reproduce the pH dependence of Zn$^{2+}$ effects using any of several models: pK$_a$ is 6.5 and pK$_a$ is 6.2-6.6 and is somewhat model dependent.

To apply the model equations in the appendix to real data, it is necessary to define the effect that metal binding has on channel behavior. By making one assumption, we can define the entire body of $\tau_{\text{act}}$ data in Fig. 6. We assume that when Zn$^{2+}$ is bound to its receptor on the H$^+$ channel, the channel cannot open. For the simplest case of a two-state channel, with Scheme I:

\begin{equation}
\text{Closed} \xrightarrow{\alpha} \text{Open}
\end{equation}

where $\alpha$ is the opening rate and $\beta$ is the closing rate, the time constant is $(\alpha + \beta)^{-1}$. Because the slowing of $\tau_{\text{act}}$ was voltage independent, $\beta$ evidently is negligibly small in the voltage range measured, hence $\tau_{\text{act}} \approx \alpha^{-1}$. The opening rate will be slowed by the factor $(1 - P_{Zn})$, where $P_{Zn}$ is the probability that the receptor is occupied by Zn$^{2+}$ (the occupancy plotted in Fig. 13). Thus the observed time constant will be $\sim[\alpha (1 - P_{Zn})]^{-1}$, and the ratio of $\tau_{\text{act}}$ in the presence of ZnCl$_2$ to that in its absence will be simply $(1 - P_{Zn})^{-1}$. Given these assumptions, $\tau_{\text{act}}$ is slowed by a factor of 2.0 at the K$_M$ of Zn$^{2+}$.

In Fig. 11, the $\tau_{\text{act}}$ data from Fig. 6 are replotted with smooth curves superimposed that assume Model 6, in which the Zn$^{2+}$ receptor is formed by three protonation sites and protonation of each site reduces the affinity of the receptor for Zn$^{2+}$ by a factor a. We selected Model 6 because it comes closest to embodying the pH dependence observed. The entire set of theoretical curves is determined by the assumption that a Zn$^{2+}$-bound channel cannot open, and by pK$_M$ = 6.5, pK$_a$ = 6.3, and cooperativity factor a = 0.03. Setting a to 0.03 produces an $\sim$100-fold change in apparent Zn$^{2+}$ potency between pH 5 and 6 that resembles the $\tau_{\text{act}}$ data. With a = 0.01, the shift was too large, and at a = 0.1 the shift was too small. We could not collect data at pH 4, which might have revealed whether the saturation of the effect at very low pH predicted by this model (appendix) occurs. The agreement is generally excellent, although the slope of the data appears shallower than that defined by the theory. Expressed in terms of Zn$^{2+}$ activity rather than concentration, calculated with to the Davies equation (Stumm and Morgan, 1981) at the ionic strength of all solutions used, $\sim$0.13 M, the pK$_M$ is 7.0.
The mechanistic interpretation is that protonation of the response relationship at high pH₀, where binding is unaffected by pH. pKₐ is somewhat model dependent, and is defined by the pH₀ at which the interaction between metal and H⁺ saturates. For a given model, this is set by the size of the shift in the high pH₀ region. Thus, in Fig. 12, pKₐ is 7.0 because this produces a sixfold shift between pH₀ 8 and 7, as observed in the data. Finally, it was necessary to assume some interaction between binding sites, because pure competition in a three-site model predicts too large a shift at low pH₀. The value of the interaction factor, a, is established by the entire shift over the pH₀ range from 8 to 5. This shift was 10⁵ in the data, and a = 0.01 matched this value. Setting a = 0.02 reduced the range to 3 × 10⁴ and at a = 0 (pure competition) the range was too large, 4 × 10⁵. The mechanistic interpretation is that protonation of one of the sites lowers the affinity of the Zn²⁺ receptor 100-fold. Assuming the same model, the affinity of Cd²⁺ for the external metal receptor is lower than that of Zn²⁺ by ~2 U (roughly pKₐ 6).

A depolarizing shift in the g₁₉-V relationship at low P_open can be approximated as a reduction of P_open by a constant fraction. Because the slope factor of the g₁₉-V relationship fit by a simple Boltzmann function is ~10 mV (DeCoursey and Cherny, 1994; Cherny et al., 1995), an e-fold reduction in P_open should produce a 10-mV depolarizing shift. In Fig. 12, we use Model 6 under the assumption that the channel cannot open when Zn²⁺ is bound. The voltage shift is given by ln(1 - P(Zn²⁺) × 10 mV). The g₁₉-V data were best described by pKₐ = 8.0, pK₈ = 7.0, and a = 0.01. Expressed in terms of Zn²⁺ activity rather than concentration, pKₐ is 8.5.

The g₁₉-V data were best described by pKₐ = 8.0, pK₈ = 7.0, and a = 0.01 (Fig. 12), whereas the optimal values for the same model of τ act data were pKₐ = 6.5, pK₈ = 6.3, and a = 0.03 (Fig. 11). The different parameter values describing the interactions observed for the g₁₉-V relationship and τ act may reflect that the former is a steady state parameter and the latter a kinetic one. Alternatively, distinct metal binding sites may be involved in slowing τ act and shifting the g₁₉-V relationship, as suggested by the greater relative potency of ZnCl₂ (pared with other metals) for the slowing effect. If so, the "nonspecific" site at which polyvalent metals shift all voltage-dependent parameters simply has a higher pKₐ than the site that regulates τ act. Another possibility is that the Zn²⁺ receptor has a higher affinity for both protons and Zn²⁺ when the channel is open. This idea is incompatible with the external Zn²⁺ receptor being comprised of the regulatory protonation sites that govern gating in our model, because these sites become inaccessible to the external solution when the channel is open (Cherny et al., 1995). We saw no evidence of decay of H⁺ current in the presence of metals, which would be expected if metals bound (with resolvable kinetics) preferentially to open channels. A final possibility is that fitting the τ act and g₁₉-V data simply provides two ways to estimate the binding parameters of the Zn²⁺ receptor.

**Evidence that Metals Bind to the External Site that Regulates pH-dependent Gating**

The modeling exercise indicates that protons and polyvalent cations (at least Zn²⁺ and Cd²⁺) compete for a common site at the external surface of the H⁺ channel. Furthermore, the metal receptor can also bind two or more H⁺, and protonation inhibits metal binding. The best fit was achieved with the assumption that three protonation sites coordinate one Zn²⁺. We propose that metal binds to the same external modulatory sites at which extracellular protons regulate the gating of H⁺ channels. Extracellular metals and protons have
qualitatively similar effects on channel gating. Both slow activation (increase \( \tau_{act} \)), shift the voltage-activation curve (\( g_{m}-V \) relation) to more positive potentials, and have relatively small effects on the channel closing rate. In our model (Cherny et al., 1995), the \( \Delta p \) dependence of gating arises from the requirement that three externally accessible sites must be deprotonated for the channel to open. The agreement between the numbers of protonation sites involved in gating and \( Zn^{2+} \) binding may be serendipitous, but lends support to both models.

**Internal Metal Binding Site**

Although metals produce dramatic effects on \( H^{+} \) currents at quite low concentrations for external application, internally applied \( ZnCl_{2} \) or \( CdCl_{2} \) also altered \( H^{+} \) currents. Deactivation was slowed with no effect on \( \tau_{act}, \) and \( H^{+} \) current amplitude was reduced. Because internally applied \( ZnCl_{2} \) had relatively weak effects, we could not study them in as much detail, and could not determine whether \( Zn^{2+} \) and \( H^{+} \) compete for internal sites. Nevertheless, in our model (Cherny et al., 1995), the first step in channel closing is deprotonation at internally accessible sites. Thus, the slowing of deactivation by internal \( ZnCl_{2} \) with no effect on the opening rate is qualitatively consistent with the idea that \( Zn^{2+} \) binds to the same internal protonation sites that help regulate gating.

Because the effects of internal and external addition were qualitatively different, distinct metal binding sites must exist at the inner and outer surfaces of the channel. In contrast, \( ZnCl_{2} \) has similar effects whether applied externally or internally to \( K^{+} \) channels, on ionic currents (Begenisich and Lynch, 1974; Spires and Begenisich, 1992, 1994) as well as on gating currents (Spires and Begenisich, 1995), leading Spires and Begenisich (1995) to conclude that \( Zn^{2+} \) can reach its binding site in the channel from either side of the membrane. The dissimilarity of effects on \( H^{+} \) channels leads us to conclude that there are distinct internal and external sites and, furthermore, that negligible quantities of these metals applied internally reach the external binding site. Not only is there no evidence that \( ZnCl_{2} \) can cross the membrane, the lack of effects of \( pH_{o} \) on external \( ZnCl_{2} \) effects (Fig. 6) indicates that intracellular protons do not affect the local \( pH \) near the external \( Zn^{2+} \) receptor. Thus, \( H^{+} \) channels are less promiscuous than are \( K^{+} \) channels. In turn, this conclusion supports the concept that voltage-gated proton channels are not water-filled pores that might conduct detectable amounts of \( Zn^{2+} \) or \( Cd^{2+} \) (or perhaps \( ZnOH^{+} \) or \( CdOH^{+} \)), but instead comprise a hydrogen-bonded chain. The extremely high selectivity of \( H^{+} \) channels is another argument for this conduction mechanism (DeCoursey and Cherny, 1994, 1998, 1999a,b).

**The Chemical Nature of the Protonation Sites on \( H^{+} \) Channels**

To account for the \( \Delta p \) dependence of the voltage activation curve of the \( H^{+} \) channel, we originally proposed identical external and internal protonation sites with \( pK_{a} \) 8.5 (Cherny et al., 1995). Deprotonation at the external site was the first step in channel opening, and deprotonation at the internal site was the first step in channel closing. That deuterium substitution slowed activation threefold with negligible effects on closing suggested that the external and internal sites were chemically different, with the external site likely composed of His, Lys, or Tyr residues and the internal site possibly a sulphydryl group, presumably Cys (DeCoursey and Cherny, 1997). A classical example of a His forming a \( Zn^{2+} \)-binding site is carbonic anhydrase, in which zinc is coordinated between three His residues (and one \( OH^{-} \)) to form the catalytic site of this metalloenzyme (Silverman and Vincent, 1983). Chelators can remove this zinc and it can then be replaced by various other ligands, which bind with a relative potency \( Hg >> Cu > Zn > Cd, Ni > Co > Mn \) (Silverman and Vincent, 1983), a sequence similar to that reported for metal inhibition of \( H^{+} \) currents (see above). The data presented here are compatible with the idea that the \( Zn^{2+} \) binding site is the same site at which external protons regulate gating. In this regard, it is intriguing that \( pH_{o} \) acting on extracellular His residues shifts the voltage dependence of the gating of a plant \( K^{+} \) channel (Hoth et al., 1997). Protonation of this stomatal guard cell channel shifts the activation curve toward more positive voltages, just as the \( g_{m}-V \) relation of voltage-gated proton channels is shifted to the right at lower \( pH_{o} \). Because this \( K^{+} \) channel is activated by hyperpolarization, it is activated by low \( pH_{o} \), whereas the voltage-gated proton channel is activated by depolarization and thus is inhibited by low \( pH_{o} \). Zinc binding sites have been created in \( \alpha \)-hemolysin channels by introducing His residues (Walker et al., 1994; Kasianowicz et al., 1999). The external \( Zn^{2+} \) receptor on \( H^{+} \) channels binds \( Zn^{2+} \) with a substantially higher affinity, \( pK_{M} \) \( \sim 6.5 \), than the "normal" association constant for 1:1 binding of \( Zn(II) \) to His, \( pK_{a} \) 2.5 (Breslow, 1973). The higher affinity is compatible with our conclusion that multiple His (or other ionizable groups) coordinate the binding of a single \( Zn^{2+} \). The \( Zn^{2+} \) dissociation constant for carbonic anhydrase, in which three His coordinate one \( Zn^{2+} \), is 4 pM (Kiefer et al., 1993). The typical \( pK_{a} \) of His in proteins ranges from 6.4 to 7.2 (Breslow, 1973), encompassing the \( pK_{a} \) values derived from most of the models tested here. Thus, many types of evidence point to His as a likely candidate for forming the external \( Zn^{2+} \) receptor.

Henderson (1998) demonstrated recently that mutation of any of three His residues to Leu in a putative
transmembrane domain abolished the H\(^+\) conductance associated with NADPH oxidase in neutrophils. This intriguing result may support the identity of the external modulatory site as His. However, epithelial and phagocyte H\(^+\) channels differ significantly (DeCoursey, 1998), and some phagocyte H\(^+\) channels have a higher sensitivity to ZnCl\(_2\) (Bánfi et al., 1999). Furthermore, the role of one or more of the His might be in conduction, forming part of the hydrogen-bonded chain, rather than in regulation of gating.

The much weaker deuterium isotope effect on H\(^+\) channel closing than on opening led to the suggestion of Cys as a candidate for the internal regulatory protonation site because sulfhydryl groups typically have smaller pK\(_a\) shifts in D\(_2\)O (DeCoursey and Cherny, 1997). The weak effects of internal ZnCl\(_2\) reported here, however, must be reconciled with the typically high affinity binding of Zn\(^{2+}\) to Cys (Breslow, 1973). If Cys does help form the internal site, steric constraints may allow proton or deuteron binding, but disfavor close approach by Zn\(^{2+}\).

Pathophysiological Significance

Because they are more sensitive to polyvalent metal cations than most other ion channels, H\(^+\) channels would be among the first to register effects of metal poisoning. Human plasma zinc levels are maintained at 10–75 μM (Cornelis and Versieck, 1980), most of which is complexed with plasma proteins or phosphates. However, the g\(_m\)-V relationship is quite sensitive to Zn\(^{2+}\) at physiological pH with a distinct shift at <0.1 μM ZnCl\(_2\) (Fig. 7). ZnCl\(_2\) or CdCl\(_2\) suppress the respiratory burst—the release of bactericidal reactive oxygen species—in human neutrophils in vitro, presumably by inhibiting H\(^+\) currents (Henderson et al., 1988). Inhalation of zinc oxide produces metal fume fever, apparently by elevating plasma interleukin-6 (a pyrogen produced by granulocytes) levels (Fine et al., 1997). Voltage-gated proton channels in alveolar epithelium may contribute to CO\(_2\) extrusion by the lung (DeCoursey, 2000). Volume regulation of alveolar epithelial cells is inhibited by high concentrations of ZnCl\(_2\) (Jones et al., 1982). This evidence is circumstantial, but worth worrying about.

Appendix

Competition between Zn\(^{2+}\) and H\(^+\)

If Zn\(^{2+}\) is the sole form of ZnCl\(_2\) active on H\(^+\) channels, then the strong pH\(_o\) sensitivity of its effects (Figs. 5–6) could be explained in two ways. Zn\(^{2+}\) and H\(^+\) might compete for the same site on the channel, or protonation of the channel might lower its affinity for Zn\(^{2+}\). Several classes of mechanisms encompassing both possibilities can be envisioned. Here we will explore the extent to which the stoichiometry of the apparent competition between metal and protons defines the nature of the metal receptor. To make this analysis as model-independent as possible, we will simply consider the probability that Zn\(^{2+}\) is bound to its receptor. In the discussion we assumed specific mechanisms by which metals slow H\(^+\) channel activation (Fig. 11) or shift the g\(_m\)-V relationship (Fig. 12), and determine the model parameters given those assumptions.

If one Zn\(^{2+}\) (M) competes with one H\(^+\) (H) for the same binding site or receptor (R), each will have a dissociation constant, defined in Schemes II and III, respectively (the brackets indicating concentration have been omitted):

\[
\frac{M + R}{k_f} \xrightarrow{k_b} \frac{RM}{k_f}
\]  
(Scheme II)

and

\[
\frac{H + R}{k_f} \xrightarrow{k_b} \frac{RH}{k_f}
\]  
(Scheme III)

as K\(_M\) = k\(_b\)/k\(_f\) and K\(_H\) = k\(_f\)/k\(_b\).

We define pK\(_M\) the way pK\(_a\) is defined, as the negative logarithm of K\(_M\). Note that K\(_M\) as defined here is the inverse of the metal-buffer binding constant defined by Good et al. (1966), thus we indicate their parameter as K\(_M\) (Table I). The fraction of channels with metal bound to them is given by a "simple competition" model:

\[
\frac{RM}{R + RM + RH} = \frac{1}{1 + \frac{K_M}{K_H}}
\]  
(A1)

where the number of free, metal-bound, or protonated receptors is R, RM, or RH, respectively (see Spires and Begenisich, 1992). Eq. A1, illustrated in Fig. 13 A, predicts that, well below the pK\(_a\), the apparent potency of metal will decrease 10-fold per unit decrease in pH (Clark, 1926). An example in which such a relationship holds is the affinity of zinc for the bc\(_2\) complex, which decreases 10-fold per unit decrease in pH between pH\(_o\) 7 and 5 (Link and von Jagow, 1995). Eq. A1 agrees with the ~10-fold reduction in potency seen between pH\(_o\) 7 and 6, but the decrease in apparent potency of Zn\(^{2+}\) between pH\(_o\) 6 and 5 is closer to 100-fold (Fig. 6). Therefore, this model must be abandoned.

We next consider noncompetitive inhibition, in which Zn\(^{2+}\) and H\(^+\) bind independently to different sites, and the effect is observed only when Zn\(^{2+}\) and not H\(^+\) is bound. This "noncompetitive inhibition" model is:

\[
\frac{RM}{R + RM + RH + RHM} = \frac{1}{\left(1 + \frac{K_M}{K_H}\right)}
\]  
(A2)
In Eq. A2, metal simply becomes ineffective at low pH (Fig. 13 B). Furthermore, the “threshold” concentration of metal is the same at all pH. In contrast, the data exhibit large effects of Zn\(^{2+}\) at low pH\(_0\), where the concentration–response relationship appears simply to shift to higher [Zn\(^{2+}\)] (Figs. 5 and 6). Furthermore, the threshold concentration that produces detectable effects changes radically with pH. Therefore, we rule out purely noncompetitive models.

The simple competition model could be altered to reflect that Zn\(^{2+}\) and H\(^+\) have qualitatively similar effects when bound to H\(^+\) channels. Both cations slow activation at a given voltage, and shift the voltage-activation curve to more positive potentials. The formulation of this “both Zn\(^{2+}\) and H\(^+\) effective” model is (Eq. A3):

\[
\frac{RM + RH}{R + RM + RH} = \frac{M}{1 + \frac{H}{K_M} + \frac{H}{K_a}}.
\]

As is apparent from Fig. 13 C, if there is competitive binding and Zn\(^{2+}\) and H\(^+\) have the same effects, then at low pH\(_0\) there will be little further effect of adding metal because all the sites are already protonated. There is a small shift of the threshold [Zn\(^{2+}\)], but this occurs at the expense of reducing the maximal effect. At least this simple form of the “both effective” class of models is incompatible with the data.

A more complicated model is necessary to explain the \(\sim100\)-fold shift in apparent potency of Zn\(^{2+}\) between pH\(_0\), 6 and 5. One possibility is that each channel has multiple protonation sites near enough to each other that two can coordinate a single Zn\(^{2+}\). The divalency of Zn\(^{2+}\) suggests this idea naturally. A channel that coordinates Zn\(^{2+}\) between His and Asp side groups has been described (Kasianowicz et al., 1999). We model this possibility by assuming that the Zn\(^{2+}\) receptor can bind two protons. Protonation of either site prevents Zn\(^{2+}\) binding. The metal occupancy will be given by Eq. A4 (2 H\(^+\) and 1 Zn\(^{2+}\) compete):

\[
\frac{RM}{R + RM + RH} = \frac{1}{1 + \frac{H}{K_M} \left[1 + \frac{2}{K_a}ight]}
\]
assuming that the two protonation sites are identical and independent. Here \( k_a = k_f / k_l \), with \( k_f \) and \( k_l \) defined in the two partial reactions of the degenerate two-step system (Bernasconi, 1976) (Schemes IV and V):

\[
H + R \xrightarrow{k_l} RH
\]

(Scheme IV)

and

\[
H + RH \xrightarrow{k_f} RH_2
\]

(Scheme V)

Fig. 13D shows the prediction of Eq. A4, assuming that \( pK_a = 6.6 \) and \( pK_M = 6.5 \). The values of \( pK_a \) and \( pK_M \) are established from the pH range at which the pH sensitivity of Zn\(^{2+}\) diminishes, and from the position of the concentration–response curve at high pH\(_o\), respectively. In Eq. A4, the apparent efficacy of Zn\(^{2+}\) decreases 100-fold/U at low pH\(_o\), consistent with the shift observed between pH 6 and 5. The equation describes the \( T_{1000} \) data (Fig. 6) fairly well, although the calculated shift between pH 6 and 5 is only 70 rather than 100, because the low pH\(_o\) behavior is not fully manifest at these relatively high pH\(_o\). It might be expected that protonation of the first site would lower the proton affinity of the second site. We modeled this by lowering the \( pK_a \) of the second protonation reaction, but there were only subtle differences in the predicted behavior for two sites with identical \( pK_a \) compared with two sites with 1 U different \( pK_a \) having the same average value.

If the H\(^+\) channel were a trimer or tetramer, there might reasonably be three or four equivalent protonation sites. Assuming that protonation of any site prevents metal binding, this purely competitive model is described by (see Perez-Cornejo et al., 1998):

Metal occupancy = \[
\frac{1}{1 + \frac{K_M}{M} \left(1 + \frac{H}{K_a}\right)^n}
\]

(A5)

In Eq. A5, the shift in apparent Zn\(^{2+}\) potency at low pH\(_o\) is 10\(^3\) for \( n = 3 \) and 10\(^4\) for \( n = 4 \). Although the data do not exhibit a shift that large, the three-site Model 5 (Fig. 13F, “H\(^+\) and 1 Zn\(^{2+}\) compete”) can simulate the data tolerably well using \( pK_a \) 6.2 and \( pK_M \) 6.5 because the 1,000-fold/U shift is attained only at lower pH than the range of the data.

One final variant will be considered, in which the channel has multiple protonation sites that interfere with Zn\(^{2+}\) binding, either allosterically or electrostatically. Protonation of each successive site lowers the affinity of the metal binding site for Zn\(^{2+}\), but protonation and Zn\(^{2+}\) binding are not mutually exclusive. The general “indirect interaction, multimeric site” model is (Perez-Cornejo et al., 1998):

Metal occupancy = \[
\frac{\left(1 + a \frac{H}{K_a}\right)^n}{\left(1 + a \frac{H}{K_a}\right)^n + \frac{K_M}{M} \left(1 + a \frac{H}{K_a}\right)^n}
\]

(A6)

where factor \( a \) is a cooperativity factor that is unity for completely independent binding of H\(^+\) and Zn\(^{2+}\), decreases as the metal affinity is reduced, and becomes 0 when there is pure competition (in which case Eq. A6 reduces to Eq. A5). This model behaves as though, in the limit of high and low pH, the affinity of the site for Zn\(^{2+}\) is high or low, respectively. Thus, the shift in apparent metal potency saturates at both high and low pH in contrast with purely competitive Models 1, 4, and 5 (Fig. 13), which saturate at high pH only.

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R E F E R E N C E S


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