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Zinc Inhibition of Chloride Efflux from Skeletal Muscle of *Rana pipiens* and Its Modification by External pH and Chloride Activity

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Summary. Efflux of $^{36}Cl^-$ from frog sartorius muscles equilibrated in depolarizing solutions was measured. Cl^- efflux consists of a component present at low pH and a pH-dependent component which increases as external pH increases. In depolarized muscles from *Rana pipiens*, the pH-dependent Cl^- efflux has an apparent pK_a near 6.4.

The reduction of Cl^- efflux by external Zn^{2+} was determined at different external pHs and chloride activities. The effect of external chloride activity on the pH-dependent Cl^- efflux was also examined.

At pH 6.5 and a membrane potential of −22 mV, increasing external Cl^- activity from 0.108 to 0.28 M decreased inhibition of the pH-dependent Cl^- efflux at all activities of Zn^{2+}. The Zn^{2+} activity needed to reduce Cl^- efflux by half increased from 0.39×10^{-3} to 2.09×10^{-3} M. By contrast, external Cl^- activity had no measurable effect on the apparent pK_a of the pH-dependent efflux.

At constant Cl^- activity less than 0.21 M, increasing external pH from 6.5 to 7.5 decreased inhibition by low Zn^{2+} activities with either a slight increase or no change in the Zn^{2+} activity producing half-inhibition. In other words, for relatively low Cl^- activities, protection against inhibition of Cl^- efflux by low Zn^{2+} activities was obtained by raising, not lowering, external pH; this is not what is expected if H^+ and Zn^{2+} ions compete at the same site to produce inhibition of Cl^- efflux. We conclude that Zn^{2+} and low pH inhibit Cl^- efflux by separate and distinct mechanisms.

By contrast, the protection against Zn^{2+} inhibition produced by high external Cl^- activity (0.28 M) was partially reversed by raising external pH from 6.5 to 7.5 at all Zn^{2+} activities. The half-inhibition Zn^{2+} activity decreased from 2.09×10^{-3} to 0.68×10^{-3} M.

The results can be simulated quantitatively by a model in which single Cl^- channel elements are in equilibrium with sextets of associated single-channel elements, each sextet having a conductance six times that of a single-channel element. The association into sextets is promoted by OH^- or Cl^- binding to a control site on the single-channel elements. Both the single Cl^- channel element and the sextet of Cl^- channel elements are closed when this same control site instead binds $ZnOH^+$. The sextet has a much higher affinity for $ZnOH^+$ than does the single Cl^- channel element.

Key Words skeletal muscle · Cl^- efflux · Cl^- channel · pH · pOH · zinc · muscle membrane

Introduction

At physiological pH, 65 to 85% of the resting membrane conductance in skeletal muscle is due to Cl^- ions (Hodgkin & Horowicz, 1959; Hutter & Noble, 1960; Palade & Barchi, 1977). The remaining conductance is due to K^+ ions. The Cl^- conductance in frog skeletal muscle falls as pH falls with an apparent pK_a near 7 in *Rana temporaria* (Brooks & Hutter, 1962; Hutter & Warner, 1967*c*). Furthermore, in physiological salt solutions, Zn^{2+} ions, in concentrations up to 0.5 mM, decrease membrane Cl^- conductance with little change in resting K^+ conductance (Mashima & Washio, 1964; Stanfield, 1970). Similar effects of external pH and Zn^{2+} are observed when Cl^- efflux is measured (Hutter & Warner, 1967*a*,*b*). Since Zn^{2+} inhibits Cl^- efflux more at pH 7.4 than at pH 5.0, it has been proposed that Zn^{2+} and H^+ ions compete at a common site that controls Cl^- permeability (Hutter & Warner, 1967*a*).

On the other hand, Cl^- may exit, in part, through a different pathway at pH 5.0 than at pH 7.4 since the fall in Cl^- conductance with decreasing pH is greater than the fall in Cl^- efflux (Hutter & Warner, 1967*b*,*c*). The currently favored interpretation is that at an external pH of 5.0 Cl^- exits mainly through an exchange diffusion pathway (Hutter & Warner, 1967*b*; Skydsgaard, 1987). Since much of the Cl^- efflux at pH 7.4 may be through a different pathway than at pH 5.0, it may be misleading to limit the comparison of the effects of external pH on Zn^{2+} inhibition to pH 5.0 and 7.4.

In the experiments described here, the effects of Zn^{2+} on the Cl^- efflux at pH 6.5 and 7.5 are compared. These comparisons are made in solutions containing different Cl^- concentrations. Although the effects of external pH and $[Cl^-]$ on the dose-response relation for Zn^{2+} inhibition of Cl^- efflux are complex, in most situations we find that

raising pH (rather than lowering it) protects against Zn^{2+} inhibition of Cl^- efflux, particularly at low Zn^{2+} concentrations and relatively low Cl^- concentrations. Raising Cl^- concentration also protects against Zn^{2+} inhibition with substantial protection occurring at high $[Cl^-]$. On the other hand, raising $[Cl^-]$ to the same values has no effect on inhibition by low pH. We conclude that low pH and Zn^{2+} inhibit Cl^- efflux by different mechanisms.

We propose a model in which Cl^- channels exist in two forms, unassociated single-channel elements and associated groups of six channel elements (sextets), which are in equilibrium and have empty control sites with different affinities for the closing ligand, $ZnOH^+$. The empty control site on the unassociated single-channel element may also bind OH^- or Cl^- ion, either of which promotes association of channel elements into sextets with OH^- or Cl^- bound to the control sites. All channel forms with control sites occupied by either OH^- or Cl^- are open and protected from attack by $ZnOH^+$. A preliminary account of these results has been presented at a meeting of the Biophysical Society (Spalding et al., 1989).

Materials and Methods

We measured the efflux of $^{36}Cl^-$ from sartorius muscles isolated from the frog *Rana pipiens*. Both muscles from the same frog were used, one muscle serving as a control for the other. The muscles were attached to stainless steel frames and were placed in an isotonic K_2SO_4 solution for 30 min, during which time contractures were completed. Muscles were then loaded with K^+ and Cl^- by a one- to two-hr soak in one of the hyperosmotic high-KCl solutions described below at a pH of 7.4. After the transient volume changes were over, muscles were placed in high-KCl solution prepared from neutralized $H^{36}Cl$ (New England Nuclear) for another hour. The specific activity of the solutions containing $^{36}Cl^-$ was 4 to 20 μCi/ml.

$^{36}Cl^-$ efflux was measured by suspending the muscles in a series of tubes containing 4 ml of various (inactive) solutions. Muscles were transferred to new tubes after timed intervals (generally 5 min). Washout of the extracellular space was facilitated by rotating the tubes. The fluid of each tube was mixed in a counting vial with a scintillation cocktail mixture, and the radioactivity was measured in a scintillation counter. The radioactivity remaining in the muscle at the end was measured by placing the muscle in distilled water, in one tube for 1 hr and then in a second tube overnight (at least 12 hr), followed by scintillation counting as with the other tubes.

In earlier experiments the scintillation cocktail mixture contained 50% Scintiverse (Fisher Scientific) and 50% Triton-X (Emulsion Engineering). 16 ml of this cocktail was added to each of the 4-ml fluid samples from the muscles. In later experiments the scintillation cocktail was 100% Ecoscint A (National Diagnostics). 12 ml of this cocktail was added to each of the 4-ml fluid samples from the muscles.

After correction for background, $^{36}Cl^-$ efflux was calculated as the fraction of total counts lost from the muscle and expressed as an apparent efflux rate coefficient, k. The average amount of $^{36}Cl^-$ in the muscle during any collection interval was estimated by the sum of the activity found in all the subsequent collection samples, the activity of the distilled water samples, and one-half of the activity leaving the muscle during the given collection period. Throughout, the efflux rate coefficient is referred to simply as "Cl^- efflux" and has the units of min^{-1}. In general, efflux rate coefficients in a given solution were taken as the average of the values for two or three collection intervals (10–15 min). Most results are given in terms of the ratio of two rate coefficients; for example, the fraction of uninhibited Cl^- efflux in any given Zn^{2+} containing solution was obtained by taking the ratio of efflux rate coefficients (or increments) in the presence and absence of Zn^{2+}.

The solutions used in this study are identified by the concentration (in mM) of K^+ and Na^+ (as chloride salts) used in their preparation, for example "150 K^+/120 Na^- solution." In addition, solutions contained (in mM): 5 $MgCl_2$, 1 $CaCl_2$, and 5 or 10 MES, PIPES, HEPES, TAPS, or Tris buffer, depending on the pH of the solution. Solutions used to test the effects of Zn^{2+} were prepared by using $ZnCl_2$ to replace an equimolar amount of $MgCl_2$. For experiments in which Zn^{2+} concentrations greater than 5 mM were to be tested, solutions used for controls were prepared with 120 mM NaCl replaced by 60 mM $MgCl_2$ plus 47.1 mM sucrose to maintain tonicity (total Mg^{2+} concentration now being 65 mM). Solutions with the desired $[Zn^{2+}]$ were made with $ZnCl_2$ replacing an equimolar amount of $MgCl_2$. Hence, comparisons of the effects of zinc were made between solutions having the same total divalent cation concentration.

All experiments were performed at room temperature (near 23°C).

Results

Zn^{2+} Inhibition of Cl^- Efflux and Its Alteration by External pH

The aim of these experiments was to characterize the dose-response relation for Zn^{2+} inhibition of Cl^- efflux in muscles depolarized by high KCl solutions. In addition, the effect of external pH on Zn^{2+} inhibition was examined. We begin with results obtained with muscles equilibrated in solutions containing 150 mM K^+ and 120 mM Na^+ (150 K^+/120 Na^+).

Three experimental protocols are illustrated in Fig. 1. In Fig. 1*A*, the most frequently used protocol is shown in an experiment on a pair of muscles isolated from the same frog. During the first 25 min, the rates of Cl^- loss from both muscles into the high KCl solution at pH 5.0 were closely matched. For the next 20 min, one muscle was exposed to 2.0 mM Zn^{2+} which had no detectable effect on Cl^- efflux. Keeping one muscle exposed to 2.0 mM Zn^{2+}, the pH of the solutions was then raised to 7.5. In response, Cl^- efflux from both muscles increased. The increment of Cl^- efflux from the muscle in 2.0 mM Zn^{2+} was slightly more than half of the increment in the control muscle. This increment is taken as a measure of "pH-dependent Cl^- efflux." Fi-

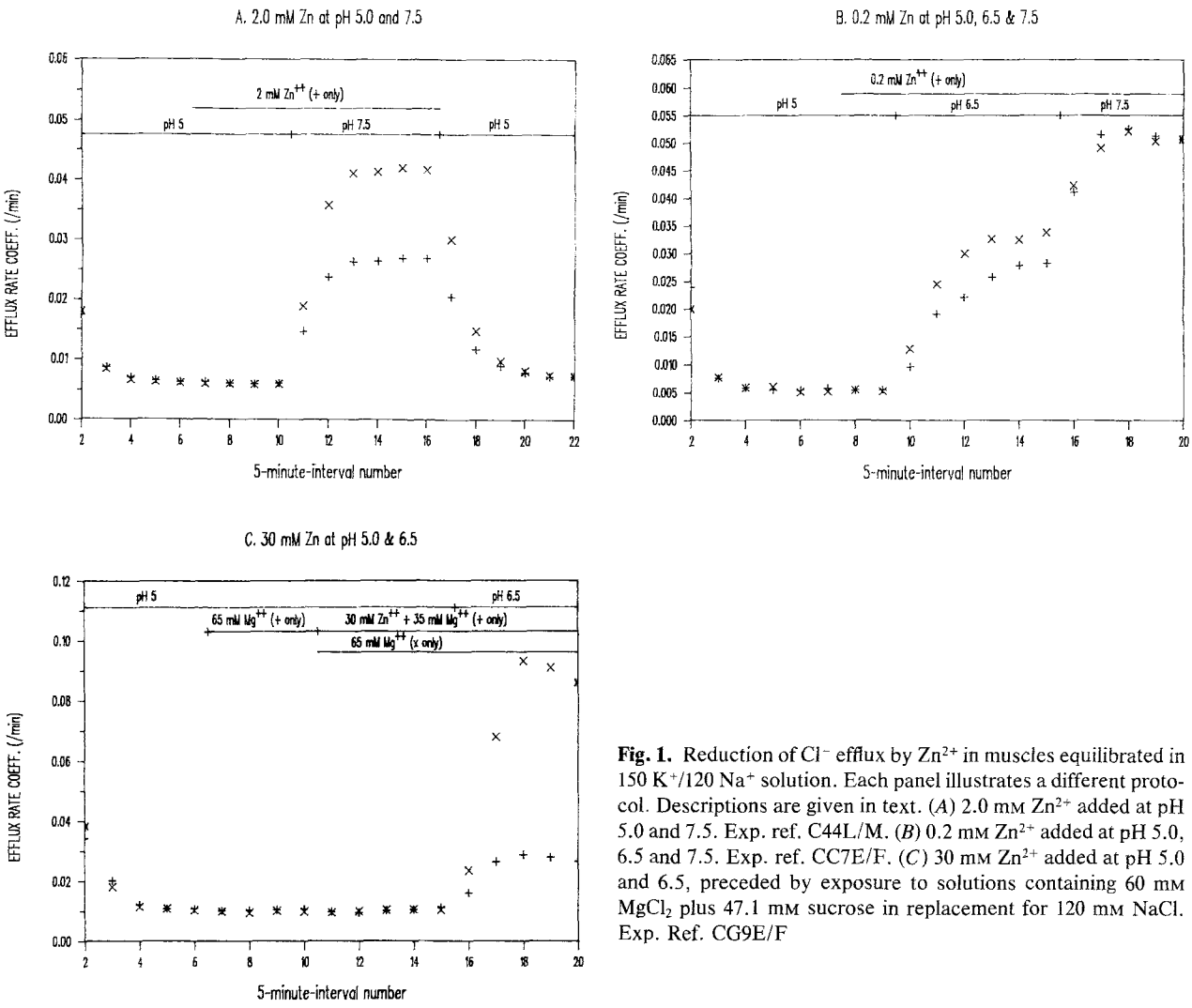

Fig. 1. Reduction of Cl^- efflux by Zn^{2+} in muscles equilibrated in 150 K^+/120 Na^+ solution. Each panel illustrates a different protocol. Descriptions are given in text. (*A*) 2.0 mM Zn^{2+} added at pH 5.0 and 7.5. Exp. ref. C44L/M. (*B*) 0.2 mM Zn^{2+} added at pH 5.0, 6.5 and 7.5. Exp. ref. CC7E/F. (*C*) 30 mM Zn^{2+} added at pH 5.0 and 6.5, preceded by exposure to solutions containing 60 mM $MgCl_2$ plus 47.1 mM sucrose in replacement for 120 mM NaCl. Exp. Ref. CG9E/F

nally, when both muscles were returned to the initial solution (free of Zn^{2+}, pH 5.0), the rates of Cl^- efflux declined to very near their original values. Similar experiments were performed where pH was raised from 5.0 to 6.5 rather than to 7.5. The inhibition by 2.0 mM Zn^{2+} at these two pHs was about equal. A more detailed comparison of the inhibition at different pH levels is given below.

At Zn^{2+} concentrations below 1 mM, another protocol was employed in some experiments, as illustrated in Fig. 1*B*. Again the initial Cl^- efflux at pH 5.0 was nearly the same for both muscles of the pair. For the last 15 min in pH 5.0 solution, one muscle was exposed to 0.2 mM Zn^{2+}, and again there was no effect of Zn^{2+}. For the rest of the experiment, this muscle was kept exposed to 0.2 mM Zn^{2+}. The pH of the solutions was then raised in two steps, first to 6.5 and, after 30 min, to 7.5. An interesting feature of zinc's inhibitory action is that 0.2 mM Zn^{2+} partially inhibits the increment of Cl^- efflux produced by raising pH from 5.0 to 6.5 and that this inhibition is largely reversed by further raising the pH to 7.5. In other words, Cl^- efflux is more sensitive to 0.2 mM Zn^{2+} at pH 6.5 than at pH 7.5.

The effects of [Zn^{2+}] above 5 mM could be tested only at pH 6.5 or lower, since at pH 7.5 Zn^{2+} at these concentrations precipitates as an insoluble hydroxide carbonate. Fig. 1*C* gives the results of an experiment examining the effects of 30 mM Zn^{2+} using a third protocol. After an initial period of equilibration at pH 5.0, the experimental muscle destined to be exposed to Zn^{2+} was placed in an isosmotic solution, in which 120 mM NaCl was replaced by 60 mM $MgCl_2$ and 47.1 mM sucrose, for a period of 20 min. This replacement had no significant effect on Cl^- efflux. Then for the next 25 min, the solutions bathing the experimental muscle had

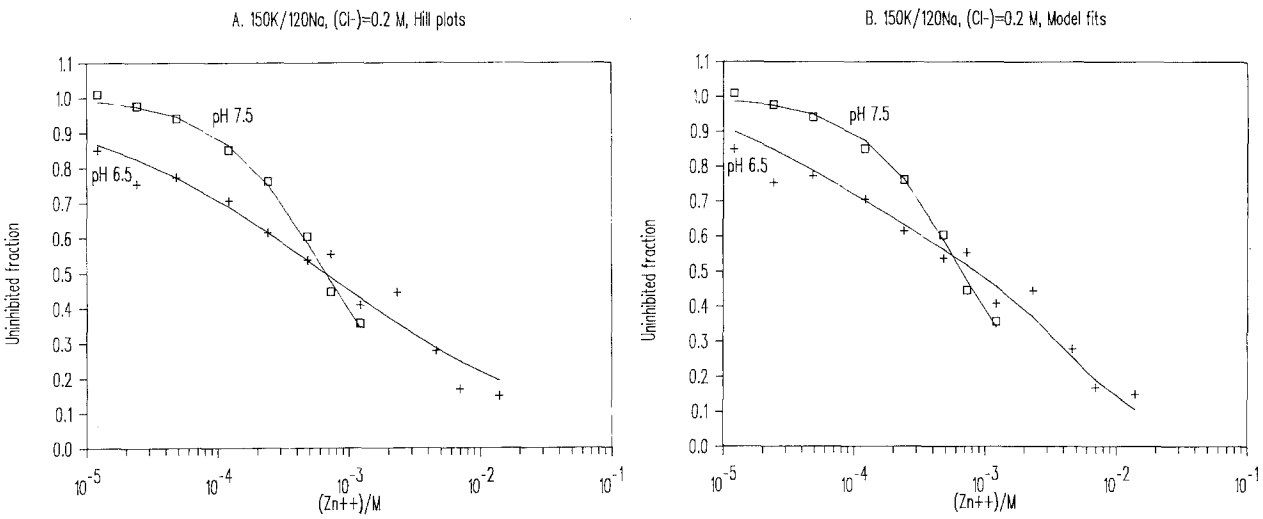

Fig. 2. Zn^{2+} inhibition of Cl^- efflux at pH 6.5 and 7.5 for muscles equilibrated in 150 K^+/120 Na^+ solution ($(Cl^-) = 0.20$ M). Points (same for both panels) plot mean ratio of increment in Cl^- efflux rate coefficient on raising external pH from 5.0 at given Zn^{2+} activity, (Zn^{2+}), to same increment for control muscle in Zn^{2+}-free solution. See equations and table in Appendix B for conversion of $[Zn]_t$ to (Zn^{2+}) and $[Cl^-]$ to (Cl^-). (*A*) Curves from text Eq. (1) where $C^{-1} = 6.54 \times 10^{-4}$ M and $n = 0.47$ for pH = 6.5, and $C^{-1} = 6.65 \times 10^{-4}$ M and $n = 1.09$ for pH = 7.5. (*B*) Curves from fit of model described in Appendix A. Statistics on points are summarized in following table where u is uninhibited fraction of efflux, SEM is standard error of the mean, and n is the number of muscle pairs

$[Zn]_t$ (mM)	(Zn^{2+}) (10^{-4} M)	u (pH = 6.5)	SEM(n) (pH = 6.5)	u (pH = 7.5)	SEM(n) (pH = 7.5)
0.05	0.121	0.850	0.043(4)	1.010	0.042(4)
0.10	0.242	0.753	0.012(4)	0.976	0.017(5)
0.20	0.484	0.773	0.050(6)	0.940	0.040(6)
0.50	1.21	0.705	0.065(6)	0.850	0.094(5)
1.0	2.42	0.614	0.033(8)	0.761	0.049(8)
2.0	4.84	0.536	0.023(6)	0.603	0.022(6)
3.0	7.26	0.553	0.025(6)	0.446	0.026(6)
5.0	12.1	0.408	0.018(14)	0.356	0.020(14)
10.0	23.2	0.445	0.030(4)		
20.0	46.4	0.278	0.030(4)		
30.0	69.6	0.168	0.020(4)		
60.0	139.2	0.149	0.015(4)		

30 mM $MgCl_2$ replaced by 30 mM $ZnCl_2$ while the control muscle was placed in 60 mM $MgCl_2$ plus 47.1 mM sucrose solution. These changes had no measurable effect on Cl^- efflux. For the last 25 min of the experiment with both muscles the external pH was changed from 5.0 to 6.5. The muscle exposed to 30 mM Zn^{2+} plus 30 mM Mg^{2+} exhibited a much smaller Cl^- efflux increment than the control exposed to 60 mM Mg^{2+}; the ratio of the Cl^- efflux increments was 0.21 in this experiment.

Since we shall compare the effects of pH on zinc inhibition in solutions of different Cl^- concentrations and of different ionic strengths, the dose-response relations are plotted as functions of Zn^{2+} activity. Total zinc concentration, $[Zn]_t$, is converted to Zn^{2+} activity, (Zn^{2+}), by allowing for the effects of ionic strength of the solutions and for the formation of chloride complexes with zinc. The details of the calculations are given in Appendix B. The conversion factor that multiplies $[Zn]_t$ to give (Zn^{2+}) varies between 0.30 and 0.22 depending on the solution. For the 150 K^+/120 Na^+ solutions under consideration at this point, the conversion factor is about 0.24. It is useful to note that the magnitude of this factor is mainly due to the small size of the Zn^{2+} activity coefficient at these ionic strengths; the effect of formation of chloride complexes is comparatively small. For example, for the 150 K^+/120 Na^+ solutions, chloride complexes with zinc reduce the Zn^{2+} concentration by only 6%; that is, $[Zn^{2+}] = 0.94 \times [Zn]_t$ (*see* Appendix B). Thus to a first order approximation $[Zn^{2+}]$ and $[Zn]_t$ are nearly equal.

Figure 2 provides semi-logarithmic plots of the

findings for Zn^{2+} activities between 1.21×10^{-5} and 1.21×10^{-3} M at pH 7.5 and between 1.21×10^{-5} and 1.39×10^{-2} M at pH 6.5. The two panels have the same data points, but different curves. In Fig. 2*A*, the equations fitted to the data are of the type

$$u = 1/(1 + (C \cdot (Zn^{2+}))^n) \quad (1)$$

where C and n are constants and u is the fraction of the efflux increment that remains uninhibited by Zn^{2+}. C (in M^{-1}) is the reciprocal of the (Zn^{2+}) which reduces Cl^- efflux by 50%, and n is a measure of the average steepness of the dose-response relation. A nonlinear least-squares procedure was used to obtain the curves in Fig. 2*A*. The curves in Fig. 2*B* result from the model which is described in Discussion and Appendix A.

The average inhibitions plotted in Fig. 2 show that the (Zn^{2+}) required to produce a 50% decrease in the pH-dependent efflux ($1/C$) is about 6.6×10^{-4} M at both pH 6.5 and 7.5. The major difference in the dose-response curves is that for (Zn^{2+}) below 6×10^{-4} M Cl^- efflux is more sensitive to Zn^{2+} at pH 6.5 than at pH 7.5 (*see also* Fig. 1*B*). In terms of Eq. (1), the average steepness of the curve at pH 7.5 is higher ($n = 1.09$) than at pH 6.5 ($n = 0.47$).

There are two possible interpretations for these results at low Zn^{2+} or (Zn^{2+}) less than 6×10^{-4}: either increasing H^+ ion concentration enhances Zn^{2+} inhibition or increasing OH^- ion concentration protects against Zn^{2+} inhibition. If the second alternative is correct, one might expect that, by analogy, increasing Cl^- concentration (at constant transmembrane potential) should also protect against Zn^{2+} inhibition. To test this notion Zn^{2+} inhibition was measured at different external $[Cl^-]$.

Effects of External $[Cl^-]$ on Zn^{2+} Inhibition of Cl^- Efflux

When muscles are equilibrated in either 75 K^+/60 Na^+ solution or in 150 K^+/240 Na^+ solution the internal potential is −22 mV (Spalding, Swift & Horowicz, 1986). The ratio of Cl^- concentrations, $[Cl^-]$, for these two solutions is 2.73 and the ratio of the Cl^- activities, (Cl^-), is 2.59 (*see* Appendix B). Zn^{2+} inhibition of Cl^- efflux in these solutions was measured using experimental protocols similar to those described above.

The average Zn^{2+} inhibitions in muscles equilibrated in 75 K^+/60 Na^+ solutions ($(Cl^-) = 0.108$ M) for pH 6.5 and 7.5 is given in Fig. 3. Both panels plot the same data. The curves in Fig. 3*A* result from fitting Eq. (1) to the points while the curves in Fig. 3*B* come from the model described below. As in the case described before, raising the pH from 6.5 to 7.5 reduces the amount of inhibition produced by Zn^{2+} for (Zn^{2+}) less than 6×10^{-4} M. In terms of Eq. (1), the steepness of the dose-response relation is smaller at pH 6.5 ($n = 0.52$) than at pH 7.5 ($n = 1.11$). There is, in this case, a small increase in $1/C$ (i.e., the (Zn^{2+}) giving 50% inhibition) when the pH is increased from 6.5 ($1/C = 3.88 \times 10^{-4}$ M) to 7.5 ($1/C = 5.7 \times 10^{-4}$ M).

The average Zn^{2+} inhibition in 150 K^+/240 Na^+ solutions is shown in Fig. 4. Again, fits of Eq. (1) are shown in Fig. 4*A* and curves in Fig. 4*B* are based on the model. With muscles equilibrated in 150 K^+/240 Na^+ solutions ($(Cl^-) = 0.28$ M) the Zn^{2+} inhibition of Cl^- efflux at pH 6.5 was drastically reduced. Furthermore, by contrast with the previous solutions, increasing external pH from 6.5 to 7.5 enhanced Zn^{2+} inhibition at all (Zn^{2+}). In these high Cl^- solutions increasing the external pH from 6.5 to 7.5 decreases $1/C$ from 2.1×10^{-3} M to 6.9×10^{-4} M and decreases the steepness factor n from 1.70 to 0.96.

Figure 5 shows a comparison of Zn^{2+} inhibition at pH 6.5 in 150 K^+/240 Na^+ solutions and 75 K^+/60 Na^+ solutions. It is clear from the figure that the reduction in Zn^{2+} inhibition produced by raising (Cl^-) from 0.108 to 0.28 M is due to both a steepening in the dose-response curve and an increase in $1/C$; in terms of Eq. (1), n increases from 0.52 to 1.70 while $1/C$ increases from 3.9×10^{-4} M to 2.1×10^{-3} M.

The finding that increasing (Cl^-) protects against Zn^{2+} inhibition appears to support the notion that the protective effect produced by increasing external pH may be due to the increase in $[OH^-]$; that is, a channel site that confers protection against Zn^{2+} by binding an anion might bind either OH^- or Cl^-. Nevertheless, the results are complex. At any given pH, as (Cl^-) is increased from 0.108 M (75 K^+/60 Na^+) to 0.20 M (150 K^+/120 Na^+) to 0.28 M (150 K^+/240 Na^+), $1/C$ increases progressively. Increasing (Cl^-) from 0.108 to 0.28 M produces a smaller increase in $1/C$ at pH 7.5 than at pH 6.5: $1/C$ increases by a factor of 1.2 at pH 7.5, but by a factor of 5.4 at pH 6.5. On the other hand, at Zn^{2+} activities below those producing 50% inhibition, increasing external pH from 6.5 to 7.5 (and hence increasing external $[OH^-]$) decreases Zn^{2+} inhibition when (Cl^-) is 0.108 and 0.20 M but increases Zn^{2+} inhibition when (Cl^-) is 0.28 M (in the latter situation Cl^- strongly protects against Zn^{2+} inhibition). A mechanism that simulates these findings is given below.

Zn^{2+} Action on Cl^- Efflux at pH 5.0

Zn^{2+} is not without effect on Cl^- efflux when the external pH is 5.0. Cl^- ion activity in the equilibrat-

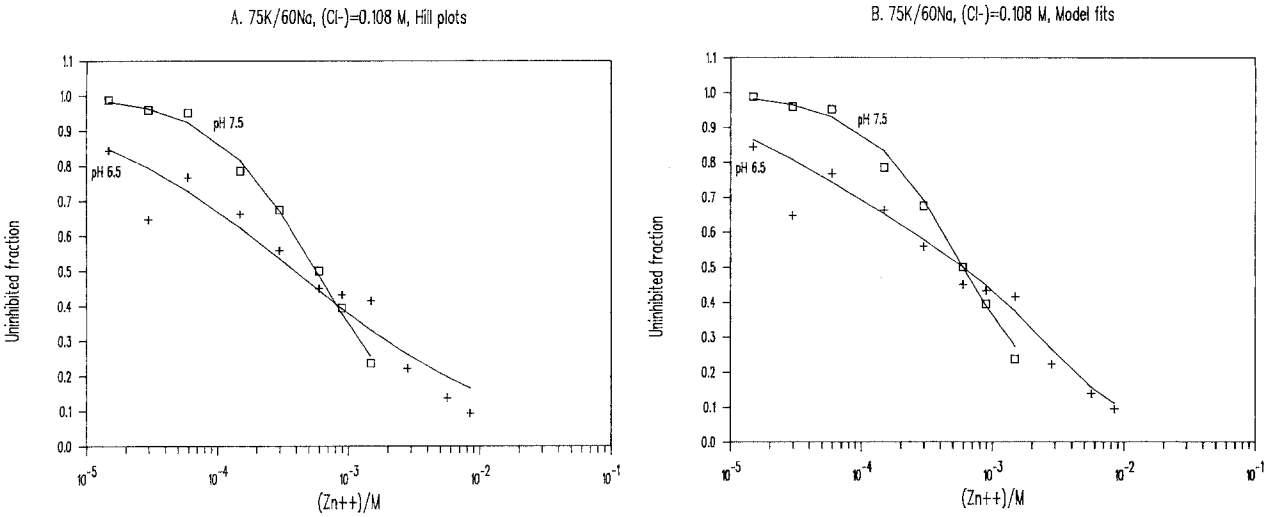

Fig. 3. Zn^{2+} inhibition of Cl^- efflux at pH 6.5 and 7.5 for muscles equilibrated in 75 K^+/60 Na^+ solution ((Cl^-) = 0.108 M). Format is same as in Fig. 2. (*A*) Curves from text Eq. (1) with $C^{-1} = 3.88 \times 10^{-4}$ M and $n = 0.52$ for pH = 6.5, and $C^{-1} = 5.69 \times 10^{-4}$ M and $n = 1.11$ for pH = 7.5. (*B*) Curves from fit of model in Appendix A. Statistics on points are summarized in the following table

$[Zn]_t$ (mM)	(Zn^{2+}) (10^{-4} M)	u (pH = 6.5)	SEM(n) (pH = 6.5)	u (pH = 7.5)	SEM(n) (pH = 7.5)
0.05	0.149	0.844	0.020(5)	0.988	0.072(6)
0.10	0.297	0.647	0.017(5)	0.959	0.048(6)
0.20	0.594	0.767	0.041(5)	0.951	0.053(9)
0.50	1.485	0.662	0.054(5)	0.785	0.052(5)
1.0	2.97	0.558	0.050(4)	0.674	0.051(6)
2.0	5.94	0.450	0.024(4)	0.500	0.053(6)
3.0	8.91	0.432	0.106(4)	0.395	0.055(6)
5.0	14.85	0.415	0.073(8)	0.236	0.018(6)
10.0	28.2	0.221	0.032(4)		
20.0	56.4	0.137	0.011(4)		
30.0	84.6	0.093	0.018(4)		

ing solutions also influences the action of Zn^{2+} at this pH.

Figure 6*A* compares the normalized Cl^- efflux at external Cl^- activities of 0.108 and 0.28 M. At the low Cl^- activity, Cl^- efflux declines with (Zn^{2+}) to a minimum of 70% normal at 2.8×10^{-3} M. When (Zn^{2+}) is 5.6×10^{-3} and 8.5×10^{-3} M, there is less net inhibition. At the high Cl^- activity, there is no statistically significant inhibition at any concentration, but rather a net stimulation of Cl^- efflux at 6.5×10^{-3} and 1.3×10^{-2} M (Zn^{2+}). Thus, high Cl^- activities protect against Zn^{2+} inhibition even at an external pH of 5.0. In addition, a stimulatory action of Zn^{2+} becomes apparent.

Figure 6*B* gives the normalized Cl^- efflux when the equilibrating solution was 150 K^+/120 Na^+ ((Cl^-) = 0.20 M). The curve has a broad minimum. The inhibitions for (Zn^{2+}) at 7.3×10^{-4} and 1.2×10^{-3} M are about 10% and are statistically significant. Again, there is net stimulation at high Zn^{2+} activities. In general, the curve in this solution is intermediate between the curves for the two solutions in Fig. 6*A*.

Effects of $[Cl^-]$ on the External pH Dependence of Cl^- Efflux

It is a well-established fact that decreasing external pH reduces Cl^- efflux and conductance from frog skeletal muscles. If this inhibition were due to increasing H^+ ion concentration and were produced by the same mechanism as the inhibition produced by Zn^{2+}, one would expect that high external Cl^- concentrations may protect against external H^+ ion inhibition as well as Zn^{2+} inhibition. The Cl^- efflux *vs.* pH curve would be shifted to lower pHs. The magnitude of these changes will depend on the na-

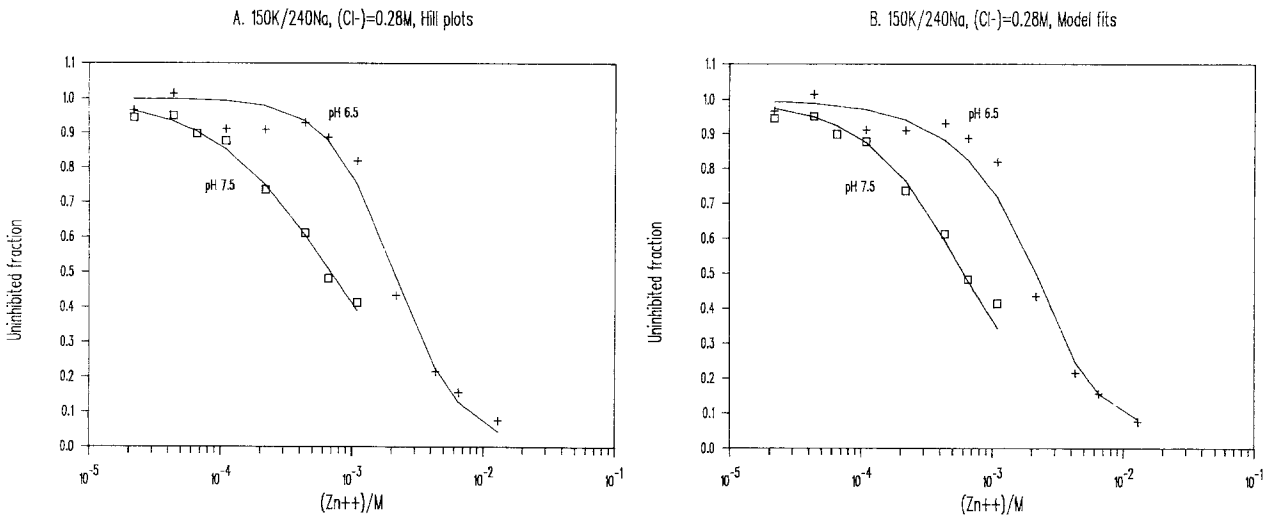

Fig. 4. Zn^{2+} inhibition of Cl^- efflux at pH 6.5 and 7.5 for muscles equilibrated in 150 K^+/240 Na^+ solution ($(Cl^-) = 0.28$ M). Format is same as in Fig. 2. (*A*) Curves from text Eq. (1) with $C^{-1} = 2.09 \times 10^{-3}$ M and $n = 1.70$ for pH = 6.5, and $C^{-1} = 6.84 \times 10^{-4}$ M and $n = 0.96$ for pH = 7.5. (*B*) Curves from fit of model in Appendix A. Statistics on points are summarized in the following table

$[Zn]_t$ (mM)	(Zn^{2+}) (10^{-4} M)	u (pH = 6.5)	SEM(n) (pH = 6.5)	u (pH = 7.5)	SEM(n) (pH = 7.5)
0.10	0.219	0.967	0.076(6)	0.944	0.056(6)
0.20	0.438	1.014	0.069(4)	0.950	0.088(6)
0.50	1.095	0.910	0.115(4)	0.877	0.056(5)
1.0	2.19	0.909	0.040(10)	0.735	0.069(5)
2.0	4.38	0.930	0.051(8)	0.611	0.040(6)
3.0	6.57	0.886	0.038(8)	0.481	0.039(6)
5.0	10.95	0.818	0.080(8)	0.412	0.019(6)
10.0	21.6	0.433	0.050(4)		
20.0	43.2	0.214	0.031(4)		
30.0	64.8	0.155	0.025(4)		
60.0	129.6	0.075	0.010(3)		

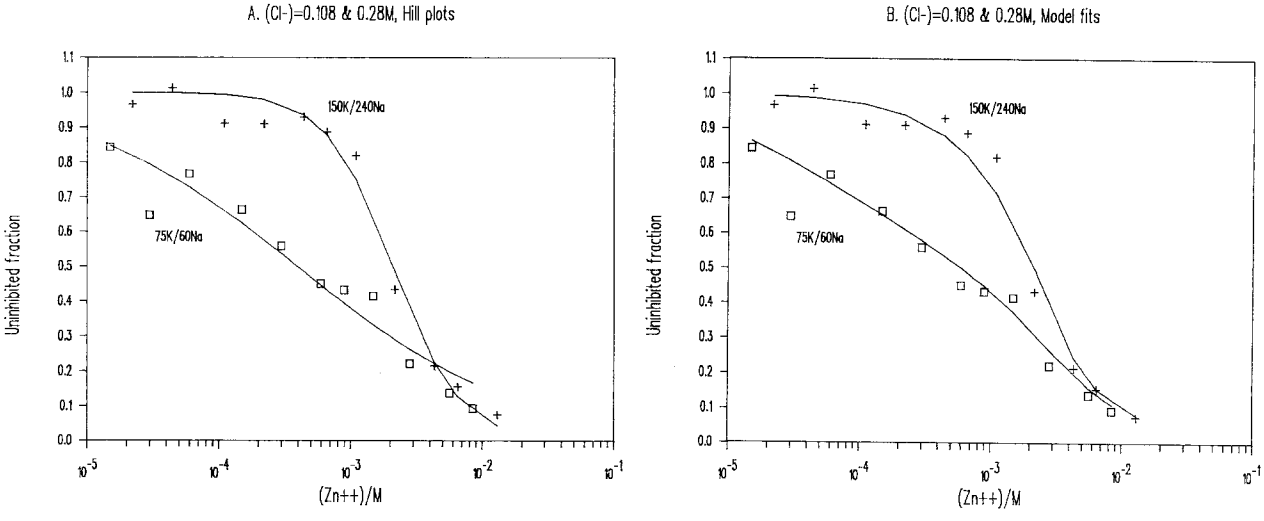

Fig. 5. Comparison of Zn^{2+} inhibition of Cl^- efflux at pH = 6.5 between muscles equilibrated in 75 K^+/60 Na^+ solution and muscles equilibrated in 150 K^+/240 Na^+ solution. The relevant data points and curves are taken from Figs. 3 and 4

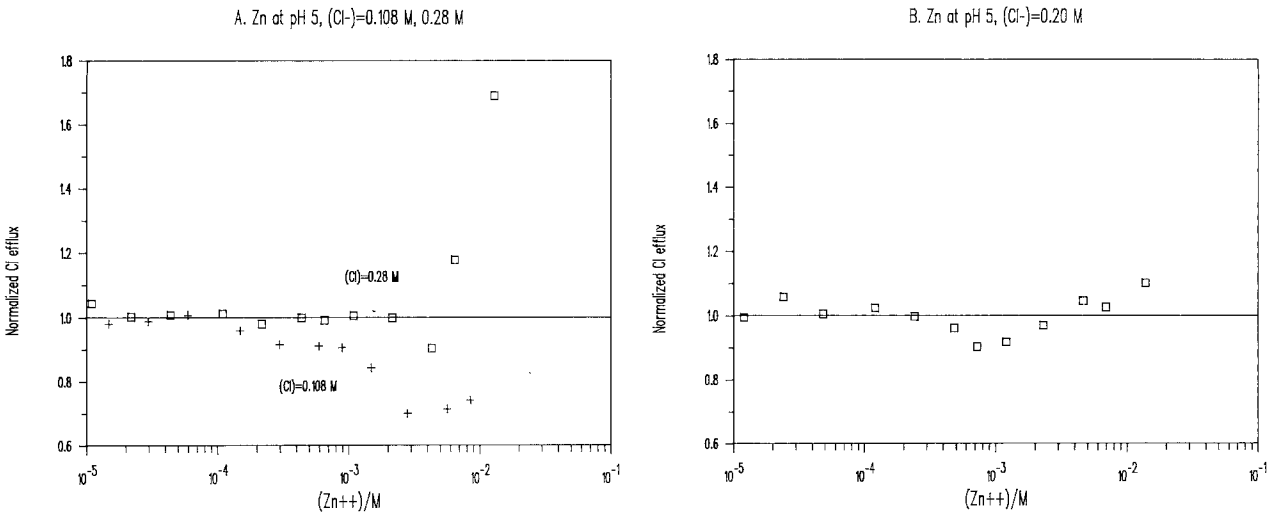

Fig. 6. Zn^{2+} action on Cl^- efflux at pH 5.0 for muscles equilibrated in 75 K^+/60 Na^+, 150 K^+/240 Na^+, and 150 K^+/120 Na^+ solutions. Points are average Cl^- efflux in Zn^{2+}-containing solutions normalized to efflux in Zn^{2+}-free solutions. (*A*) Squares show means for 75 K^+/60 Na^+; crosses show means for 150 K^+/240 Na^+. (*B*) Points are means for 150 K^+/120 Na^+. The statistics on the points are summarized in the following table. See tables in Figs. 2, 3 and 4 for values of (Zn^{2+}) corresponding to $[Zn]_i$ for each solution. Details of conversion are given in Appendix B.

Solution	150 K^+/120 Na^+		150 K^+/240 Na^+		75 K^+/60 Na^+	
$[Zn]_i$/mM	u	SEM(n)	u	SEM(n)	u	SEM(n)
0.05	0.995	0.045(4)	1.044	0.009(6)	0.981	0.014(11)
0.10	1.058	0.020(7)	1.002	0.009(10)	0.988	0.011(11)
0.20	1.005	0.033(8)	1.008	0.017(10)	1.008	0.011(11)
0.50	1.024	0.031(8)	1.012	0.015(10)	0.958	0.014(11)
1.00	0.997	0.026(16)	0.980	0.016(16)	0.915	0.032(8)
2.00	0.961	0.022(12)	1.000	0.048(16)	0.910	0.031(8)
3.00	0.902	0.019(12)	0.992	0.029(16)	0.905	0.023(8)
5.00	0.917	0.031(12)	1.006	0.037(16)	0.842	0.027(12)
10.00	0.970	0.048(4)	0.999	0.025(4)	0.699	0.090(4)
20.00	1.046	0.055(4)	0.903	0.065(4)	0.714	0.037(4)
30.00	1.026	0.036(4)	1.178	0.162(4)	0.742	0.051(4)
60.00	1.101	0.082(4)	1.690	0.162(4)		

The following gives the average Cl^- efflux rate coefficient, k, and the standard deviation, SD, at pH 5.0 for the various solutions; n denotes the number of muscles in the sample

150 K^+/120 Na^+		150 K^+/240 Na^+		75 K^+/60 Na^+	
k/min^{-1}	SD(n)	k/min^{-1}	SD(n)	k/min^{-1}	SD(n)
0.0072	0.0021(120)	0.0115	0.0030(208)	0.0113	0.0040(152)

ture of the mechanism involved. At one extreme, increasing (Cl^-) by a factor of 2.6 might increase the external $[H^+]$ required to produce a 50% reduction in Cl^- efflux by a factor of 5.4 (*see above*) as it does in the case of Zn^{2+}. This amounts to an acid shift of the apparent pK_a of 0.73 pH units. On the other hand, due to the difference in charge between Zn^{2+} and H^+ ions, increasing (Cl^-) by a factor of 2.6 might increase the external $[H^+]$ required to produce a 50% reduction in Cl^- efflux by only a factor of 2.6, an acid shift of the apparent pK_a of 0.41 pH units. With this in mind, the external pH dependence of Cl^- efflux was determined in muscles equilibrated in 75 K^+/60 Na^+ solutions and in 150 K^+/240 Na^+ solutions.

The effect of raising external pH from 5.0 to 6.0

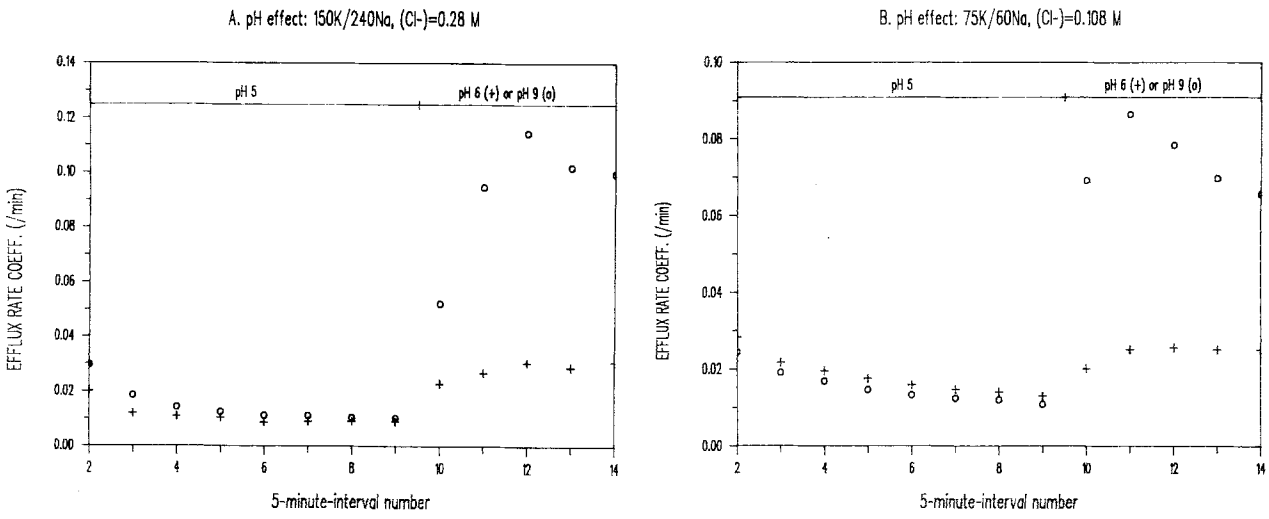

Fig. 7. Stimulation of Cl^- efflux by increasing external pH from 5.0 (5 mM MES) to 6.0 (10 mM MES) as compared to increasing external pH from 5.0 to 9.0 (10 mM TAPS) in paired muscles. (*A*) Muscles equilibrated in 150 K^+/240 Na^+. Exp. ref. CL0A/B. (*B*) Muscles equilibrated in 75 K^+/60 Na^+. Exp. ref. CL7J/K

as compared with 9.0 is shown in Fig. 7 in pairs of muscles equilibrated in $(Cl^-) = 0.28$ M (Fig. 7*A*) or $(Cl^-) = 0.108$ M (Fig. 7*B*). The ratio of the Cl^- efflux increment produced by increasing pH to 6.0 to the increment produced by increasing pH to 9.0 (referred to as "normalized flux increment") is 0.18 in 0.108 M (Cl^-) and 0.21 in 0.28 M (Cl^-). If high Cl^- activity protects against H^+ ion inhibition, then in the high Cl^- solutions the normalized flux increment should be substantially greater than in the low Cl^- solution. Judging by these experiments, there does not seem to be more than a marginal effect at pH 6.0.

The average results of all such experiments for various external pHs are given in Fig. 8. There is no measurable shift in the apparent pK_a of the pH-sensitive Cl^- efflux. Apart from a small decrease in the normalized flux increment between pH 7.0 and 8.0 for high Cl^- solutions, there is little difference in the pH dose-response curves.

Our conclusion, therefore, is that inhibition of Cl^- efflux by decreasing external pH is mediated through a different mechanism than inhibition by external Zn^{2+}.

Lack of Significant Effect on V_m of Altering pH and $[Zn^{2+}]$

One purpose for using high-KCl equilibrating solutions is that the internal potential of the fibers equals both the chloride and potassium equilibrium potential (Hodgkin & Horowicz, 1959; Spalding et al., 1986). Using standard 3 M KCl-filled glass microelectrode impalement techniques we measured the internal potential of sartorius muscle fibers in high-KCl solutions. The muscles were exposed to each pH for periods of 30 to 35 min; i.e., periods comparable to those employed for efflux measurements. We found no statistically significant change of internal potential when the external pH was varied between 5.0 and 9.0 in any of the high-KCl solutions. Similarly, there was no statistically significant change of internal potentials when 5 mM total zinc was added to solutions for periods of comparable duration.

At external total zinc concentrations of 30 and 60 mM, the standard impalement procedures fail to give accurate results. When microelectrode tips are first exposed to external solutions containing high Zn^{2+} concentrations and then are inserted into fibers they develop very high tip resistances and substantial tip potentials of variable magnitude. To circumvent this problem, we took a different approach to avoid the exposure of the internal microelectrode to Zn^{2+}.

Small bundles containing 10 to 15 single fibers were isolated from semitendinosus muscles and were equilibrated in solutions of the same composition used in the efflux studies on sartorius muscles. The bundles were mounted in a small flow chamber of the type used by Hodgkin and Horowicz (1959). With the preparation in flowing Zn^{2+}-free solution, a microelectrode was inserted into a single fiber. The external reference electrode was a low-resistance KCl-filled microelectrode placed downstream from the muscle fibers. Solutions could then be changed rapidly and subsequent slow flows could be

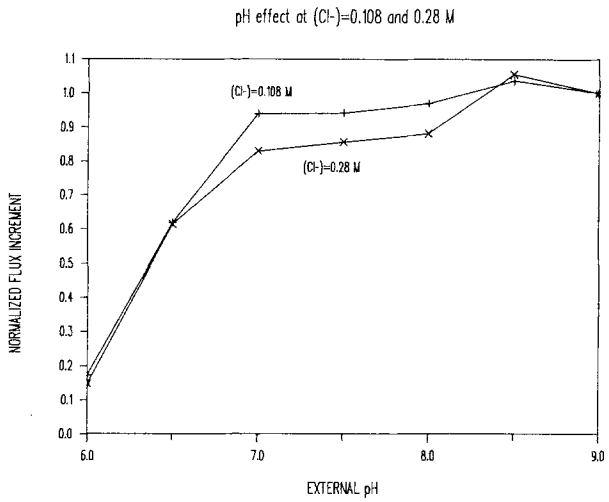

Fig. 8. Comparison of the effect of raising external pH on Cl^- efflux between muscles equilibrated in 75 K^+/60 Na^+ solution and muscles equilibrated in 150 K^+/240 Na^+ solution. For each muscle pair, the normalized Cl^- efflux increment, *dkn*, is calculated as the ratio of the increment of Cl^- efflux rate coefficient on raising pH in one muscle from 5.0 (5 mM MES) to the test pH to the increment in rate coefficient on raising pH in the other muscle from 5.0 to 9.0 (10 mM TAPS). Statistics on points are given in the following table.

Solution	75 K^+/60 Na^+		150 K^+/240 Na^+	
(pH)	*dkn*	SEM(*n*)	*dkn*	SEM(*n*)
6.0	0.173	0.022(8)	0.148	0.034(8)
6.5	0.618	0.033(8)	0.614	0.043(7)
7.0	0.940	0.044(12)	0.830	0.034(10)
7.5	0.942	0.030(19)	0.858	0.039(18)
8.0	0.970	0.020(10)	0.882	0.048(12)
8.5	1.038	0.079(6)	1.056	0.051(8)
9.0	1.000		1.000	

The following gives the average Cl^- efflux rate coefficient, *k*, and the standard deviation, SD, at pH 5.0 and 9.0 for the two equilibrating solutions. *n* gives the number of muscles in the sample

Solution	pH = 5.0		pH = 9.0	
	k/min^{-1}	SD(*n*)	k/min^{-1}	SD(*n*)
75 K^+/60 Na^+	0.0131	0.0045(97)	0.0698	0.0214(97)
150 K^+/240 Na^+	0.0110	0.0025(58)	0.0819	0.0281(58)

maintained for periods of over an hour with stable membrane potentials and stable microelectrode resistances.

With this technique, 60 mM total zinc produced a depolarization of about 1 to 1.5 mV during a 25- to 30-min exposure in solutions of the two types of 150 mM K^+ series at pH 5.0 and 6.5. We conclude that the effects of pH and Zn^{2+} in the high-KCl solutions used in these experiments are not ascribable to changes in membrane potential.

Discussion

Cl^- Efflux at External pH 5.0

Apart from Zn^{2+} activities greater than 6.5×10^{-3} M, when $(Cl^-) = 0.28$ M, Zn^{2+} either has no measurable effect or modestly inhibits Cl^- efflux at pH 5.0 (Fig. 6). In the past, the interpretation has been that the pathways for Cl^- efflux at pH 5.0 differ from the pH-dependent Cl^- channel system. More than one pathway may be involved in acid solution. There is evidence for a substantial contribution of exchange diffusion to Cl^- efflux (Hutter & Warner, 1967*b*; Skydsgaard, 1987). Further, salicylate and other aromatic anions appear to stimulate exchange diffusion of Cl^- in frog muscle (Venosa, Ruarte & Horowicz, 1972). On the other hand, there is also evidence for a measurable, though small, Cl^- conductance in acid solutions (Hutter & Warner, 1967*c*; Vaughan & Fong, 1978; Loo, McLarnon & Vaughan, 1981). The proportion of Cl^- efflux through each of these pathways is not well determined. Whatever the proportion, our results indicate at least one is sensitive to external Zn^{2+}.

At high Cl^- activities application of very high external Zn^{2+} concentrations ($(Zn^{2+}) > 6.5 \times 10^{-3}$ M) increases Cl^- efflux in acid solutions. A possible interpretation for this finding is that, for these circumstances, Zn^{2+} enters the myoplasm and either activates some latent Cl^- pathway or potentiates one of the operative pathways. The mechanism and/or pathway involved is an open question.

Despite the complexity of the situation, for purposes of data analysis we have taken the Cl^- efflux at pH 5.0 in the presence and absence of Zn^{2+} as a pH-independent baseline to subtract from the efflux obtained on raising external pH ("pH-dependent Cl^- efflux"). For external pHs of 6.5 and 7.5, the total Cl^- efflux is larger than the efflux at pH 5.0 by factors of five to ten (for example, *see* Fig. 1).

Relation between External pH and Cl^- Efflux

Compared to the findings for *R. temporaria* (Hutter & Warner, 1967*b*), the dependence of Cl^- efflux on external pH below 9.0 for *R. pipiens* is steeper and the midpoint occurs at lower pH. As shown in Fig. 8, the apparent pK_a for *R. pipiens* is 6.4; the midpoint for *R. temporaria* is near pH 7.0 (Skydsgaard, 1987). If a relation of the type efflux = 1/(1 +

$10^{n(6.4-pH)}$) is used to describe the data in Fig. 8 when external (Cl^-) = 0.108 M then the steepness factor, n, is about 1.7.

Difference between the Inhibitions Produced by Zn^{2+} and by pH

In this study, two kinds of experimental findings have led us to conclude that different mechanisms are involved when Cl^- efflux is inhibited by Zn^{2+} and by low pH. First, for (Cl^-) less than 0.2 M and for relatively low (Zn^{2+}), lowering pH enhances Zn^{2+} inhibition rather than reducing it as would be expected on the hypothesis that Zn^{2+} and H^+ inhibit Cl^- efflux by competitive binding to the same site. Second, equilibration of muscles at high Cl^- activities confers substantial protection against Zn^{2+} inhibition while not measurably altering the inhibition produced by lowering pH.

Other reports lead to a similar conclusion. In patch-clamp studies of chloride channels derived from *R. pipiens* and *Rana esculenta*, Zn^{2+} ions block Cl^- currents through open channels while H^+ ions do not alter channel conductances but rather alter the probability of open conductance states (Woll et al., 1987; Woll & Neumcke, 1987). A similar alteration of open conductance substate probabilities by pH without alteration in the substrate conductances has been observed in Cl^- channels derived from *Torpedo* electroplax (Hanke & Miller, 1983).

Influence of pOH and Cl^- Concentration on Zn^{2+} Inhibition

Our results show that altering pOH has dual effects on Zn^{2+} inhibition that depend on Cl^- activity.

For external Cl^- activities equal to or less than 0.20 M (Figs. 2 and 3), raising OH^- activity diminishes Zn^{2+} inhibition for Zn^{2+} activities producing less than a 50% reduction in Cl^- efflux while having little or no effect on the Zn^{2+} activity needed to reduce Cl^- efflux by 50%.

By contrast, when (Cl^-) is 0.28 M, raising (OH^-) not only enhances Zn^{2+} inhibition for Zn^{2+} activities producing less than a 50% reduction in Cl^- efflux (Fig. 4) but also lowers the half-inhibition Zn^{2+} activity. At low OH^- activity (pH 6.5), (Cl^-) at 0.28 M markedly protects against Zn^{2+} inhibition, producing a substantial shift in the dose-response curve to higher (Zn^{2+}) relative to the curves with low (Cl^-). It appears that, under conditions of significant protection by high (Cl^-), raising external (OH^-) shifts the Zn^{2+} dose-response curve back to lower Zn^{2+} activities.

Any model proposed to explain the experimental results has to account for both the reduction of Zn^{2+} inhibition by raising either external OH^- or Cl^- activity and the enhancement of Zn^{2+} inhibition by raising OH^- activity when (Cl^-) is initially high enough to protect against Zn^{2+} inhibition.

Zinc Complexes in Aqueous Salt Solutions

Zinc forms various complexes with anions in aqueous solutions. The principal anions of concern in the solutions used for these studies are Cl^-, OH^- and CO_3^{2-}.

The mononuclear zinc complexes that can be formed with Cl^- and OH^- are $ZnCl^+$, $ZnCl_2$, $ZnCl_3^-$, $ZnCl_4^{2-}$, $ZnOH^+$, $Zn(OH)_2$, $Zn(OH)_3^-$, and $Zn(OH)_4^{2-}$. The only species present in significant amounts in the solutions used are $ZnOH^+$, $ZnCl^+$, $ZnCl_2$, and possibly $ZnCl_3^-$. As will be seen below, a case can be made for $ZnOH^+$ being the species involved in inhibiting Cl^- efflux by blocking Cl^- channels. As far as blocking action is concerned, the involvement of $ZnCl_2$ and $ZnCl_3^-$ can be ruled out by the observations of Woll et al. (1987) that the potential dependence of blockade with Zn^{2+} present is such as would be expected with a cation blocker. Blockade by $ZnCl^+$ is ruled out by the observations in this report that increasing Cl^- activity protects against, rather than potentiates, Zn^{2+} inhibition of Cl^- efflux. If $ZnCl^+$ were the blocking species, for any given Zn^{2+} activity more $ZnCl^+$ would be present when the Cl^- activity is higher and the inhibition of Cl^- efflux should show an increase rather than the observed decrease.

Furthermore, none of the chloride complexes of zinc can explain the protective effect of increased external Cl^- activities. When the external Cl^- activity is raised from 0.108 to 0.28 M, the protection against zinc inhibition is greatest at low zinc activities and least at high zinc activities (*see* Fig. 5). If one of the zinc chloride complexes provided protection against inhibition, then, for a given increment in (Cl^-), at low zinc activities protection should be small and should increase with zinc activity as the protective zinc chloride complex builds up (for the range of Cl^- activities used in the various solutions all the zinc chloride complexes increase monotonically in activity as either Cl^- or Zn^{2+} is raised). As noted, this is not what is observed. In the modeling, therefore, Cl^- is treated as a weak binding ligand of Zn^{2+} which produces inactive complexes (*see* Appendix B).

As regards $ZnCO_3$ and hydrozincite, $Zn_5(OH)_6(CO_3)_2$, the measured equilibrium constants are adequately summarized in terms only of solubility

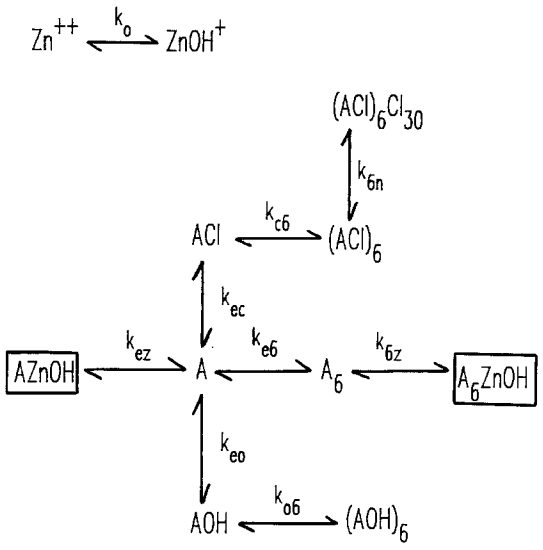

Fig. 9. Reaction network for the model used to simulate Zn^{2+} inhibition of Cl^- efflux. The individual reactions are described in the Discussion and the mathematical details are given in Appendix A. Channel forms closed to the chloride movements are shown in rectangular boxes

products (Stumm & Morgan, 1981). This implies that the concentration of soluble intermediates are negligible. Therefore, we assume these do not play a role in the effects produced on Cl^- efflux by the solutions employed since zinc activity was kept low enough that zinc did not come out of solution as an insoluble solid.

Properties of Cl^- Channels Revealed by Patch-Clamp Studies

Before describing the model developed to simulate Zn^{2+} inhibition at different pOHs, it will be useful to highlight some relevant features of Cl^- channel properties as examined by patch-clamp methods. In many cell membranes, including those of frog skeletal muscle, Cl^- channels often appear to have more than one open substate (Coronado & Latorre, 1982; Miller, 1982; Geletyuk & Kazachenko, 1985; Krouse, Schneider & Gage, 1986; Woll et al., 1987). Depending on the preparation, the number of detectable substates varies between 2 and 16. In some cases, the substate conductances are integral multiples of the unit conductance of one substate; in other cases, adjacent substates are separated by nearly equal conductance steps. At physiological Cl^- concentrations these conductance steps tend to be either about 10–15 pS or about 60–70 pS. In most cases, Cl^- channels of this type tend, when open, to reside in only a few of the conductance levels available.

Model for Zn^{2+} Inhibition of Cl^- Efflux

Although Eq. (1), which involves only two adjustable parameters, gives not unreasonable fits to the data, it is a purely empirical describer. When n does not equal 1 and varies with conditions, no unique interpretation comes from the approximate applicability of this equation. Some mechanistic explanation is desirable and therefore we explore in detail one possible model. The complete reaction network for the model is shown in Fig. 9 and the equations resulting from this reaction network are developed in Appendix A. The methods of calculation and the estimated values of the various apparent equilibrium constants are also described in Appendix A.

In view of the above behavior of chloride channels, we assume for the purpose of modeling that active pH-dependent Cl^- channels exist either as independent single elements, A, or as associated groups composed of six elements, A_6. The single elements and the associated group ("sextet") are in a dynamic equilibrium that can be represented as a reaction of the form

$$6\,A \longleftrightarrow A_6 \tag{2}$$

in which the conductance and Cl^- efflux of the sextet is the sum of the conductances and Cl^- efflux through its six constituent elements. The Cl^- efflux through each element is assumed unchanged by association into a sextet.

On each Cl^- channel element there is a binding site for OH^- ions and the binding reaction can be represented as

$$A + OH^- \longleftrightarrow AOH^-. \tag{3}$$

This binding of OH^- ions is assumed not to change the Cl^- efflux through the channel element. Channel elements with OH^- bound, AOH^-, also associate into sextets, $(AOH^-)_6$:

$$6\,AOH^- \longleftrightarrow (AOH^-)_6. \tag{4}$$

Again, it is assumed that the Cl^- efflux of the sextet is the sum of the efflux through the six elements and that efflux through each element is unchanged by association. As will be seen below, the association equilibrium constant for reaction (Eq. (2)) is much smaller than for reaction (Eq. (4)). Thus, high external pH favors conversion of A to AOH^- and $(AOH^-)_6$ rather than to A_6. It is to be remembered that we assume that the Cl^- channel system is activated by an independent pH-dependent mechanism.

As was considered before, Zn^{2+} itself combines

with OH^- ions to form a series of hydroxide complexes. For the pH range and Zn^{2+} ion concentrations dealt with in this study the only soluble complex that exists in significant amounts is formed by the reaction

$$Zn^{2+} + H_2O \longleftrightarrow ZnOH^+ + H^+. \quad (5)$$

The pK_a of this reaction is 9.0 (Baes & Mesmer, 1976). In the model it is assumed that the site on the Cl^- channel element that binds OH^- ions may instead bind $ZnOH^+$. This binding can be represented by the reaction

$$A + ZnOH^+ \longleftrightarrow AZnOH^+. \quad (6)$$

When $ZnOH^+$ is bound, the channel element closes to Cl^- movement and can no longer enter into the association reaction. In addition, it is assumed that in a sextet, A_6, the binding site of each element remains accessible to $ZnOH^+$ as represented by the reaction

$$A_6 + ZnOH^+ \longleftrightarrow A_6ZnOH^+. \quad (7)$$

When any one channel element of a sextet binds $ZnOH^+$, all six elements close to Cl^- movement.

It is further assumed that Cl^- ion can bind to the OH^- binding site on each channel element and that when Cl^- is bound the elements can form sextets. These steps can be represented by the reactions

$$A + Cl^- \longleftrightarrow ACl^- \quad (8)$$

and

$$6\,ACl^- \longleftrightarrow (ACl^-)_6. \quad (9)$$

Since the results show only a modest protection against Zn^{2+} inhibition when external (Cl^-) is raised from 0.108 to 0.20 M the affinity of this site for Cl^- ions is low. Nevertheless, when external (Cl^-) is raised to 0.28 M there is considerable protection to Zn^{2+} inhibition, at least at pH 6.5. To accommodate this observation, additional binding of Cl^- ions must be revealed in the (Cl^-) range between 0.20 and 0.28 M. The binding function must be steep in this concentration range at pH 6.5 and yet not manifest itself to the same degree at pH 7.5. In the model these requirements are met by the specific binding of 5 Cl^- ions to additional sites on each of the channel elements in the sextet $(ACl^-)_6$. We assume these additional sites are exposed only in the $(ACl^-)_6$ form. This stabilization can be represented by the reaction

$$(ACl^-)_6 + 30Cl^- \longleftrightarrow (ACl^-)_6Cl_{30}^{-}. \quad (10)$$

It is assumed that this stabilization does not alter the Cl^- efflux through the individual Cl^- channel elements. This has the effect of stabilizing the $(ACl^-)_6$ group at external (Cl^-) above 0.21 M at low pH.

Application of Model to Zn^{2+} Inhibition Results

We give first an illustration of how the scheme works for the case when there is little binding of OH^- or Cl^- to channel elements (i.e., at relatively low (Cl^-) and low pH). In the absence of external Zn^{2+}, any channel element can be in one of seven forms: three unassociated forms and four associated forms. Only A and A_6 are liable to attack by $ZnOH^+$. Because the association constant for Eq. (2) is small, a channel element is always more likely to be in the A form than in the A_6 form. However, A_6 has a much higher affinity for $ZnOH^+$ than A. At low concentrations $ZnOH^+$ attacks mainly A_6 because of the high affinity of A_6 for $ZnOH^+$. For each $ZnOH^+$ bound to A_6 six channel elements are closed. As one raises external (Zn^{2+}) the $ZnOH^+$ concentration increases, and the A form increasingly binds $ZnOH^+$. Ultimately, at high $ZnOH^+$ concentrations binding by A dominates, promoting the dissociation of A_6ZnOH^+. This behavior produces a broad dose-response curve with $n < 1$ in the Hill plots at pH 6.5 and external (Cl^-) < 0.21 M.

This behavior is illustrated in Fig. 10 for Zn^{2+} inhibition in 75 K^+/60 Na^+ at pH 6.5 and 7.5. Fig. 10*A* shows the external (Zn^{2+}) dependence at pH 6.5 of the inhibited forms, $[AZnOH^+]$ and $6 \cdot [A_6ZnOH^+]$, and the sum of all the uninhibited forms, U which gives the total Cl^- efflux. The dependence on external (Zn^{2+}) of the two forms open to attack by $ZnOH^+$, $[A]$ and $6 \cdot [A_6]$, is given in Fig. 10*B* as a log-log plot. Although $[A_6]$ is less than $[A]$ by over three orders of magnitude, its affinity for $ZnOH^+$ is over four orders of magnitude higher. Consequently, for external (Zn^{2+}) up to slightly over 4×10^{-4} M a greater fraction of channel elements is closed by the binding of $ZnOH^+$ to A_6 than by binding to A (Fig. 10*A*). Above this value of (Zn^{2+}), the fraction of closed-channel elements in the $AZnOH^+$ form rises sharply and $[A_6ZnOH^+]$ falls with increasing (Zn^{2+}). Near $(Zn^{2+}) = 3 \times 10^{-3}$ M, the fraction of open-channel elements, U, drops somewhat more steeply as a function of increasing (Zn^{2+}). At high (Zn^{2+}), the system is largely in the $AZnOH^+$ form. This results in a slightly better fit to the data in this concentration range than is obtained from fitting Eq. (1)—*compare* Fig. 2A and *B*.

Figure 10*C* and *D* give the analogous plots for

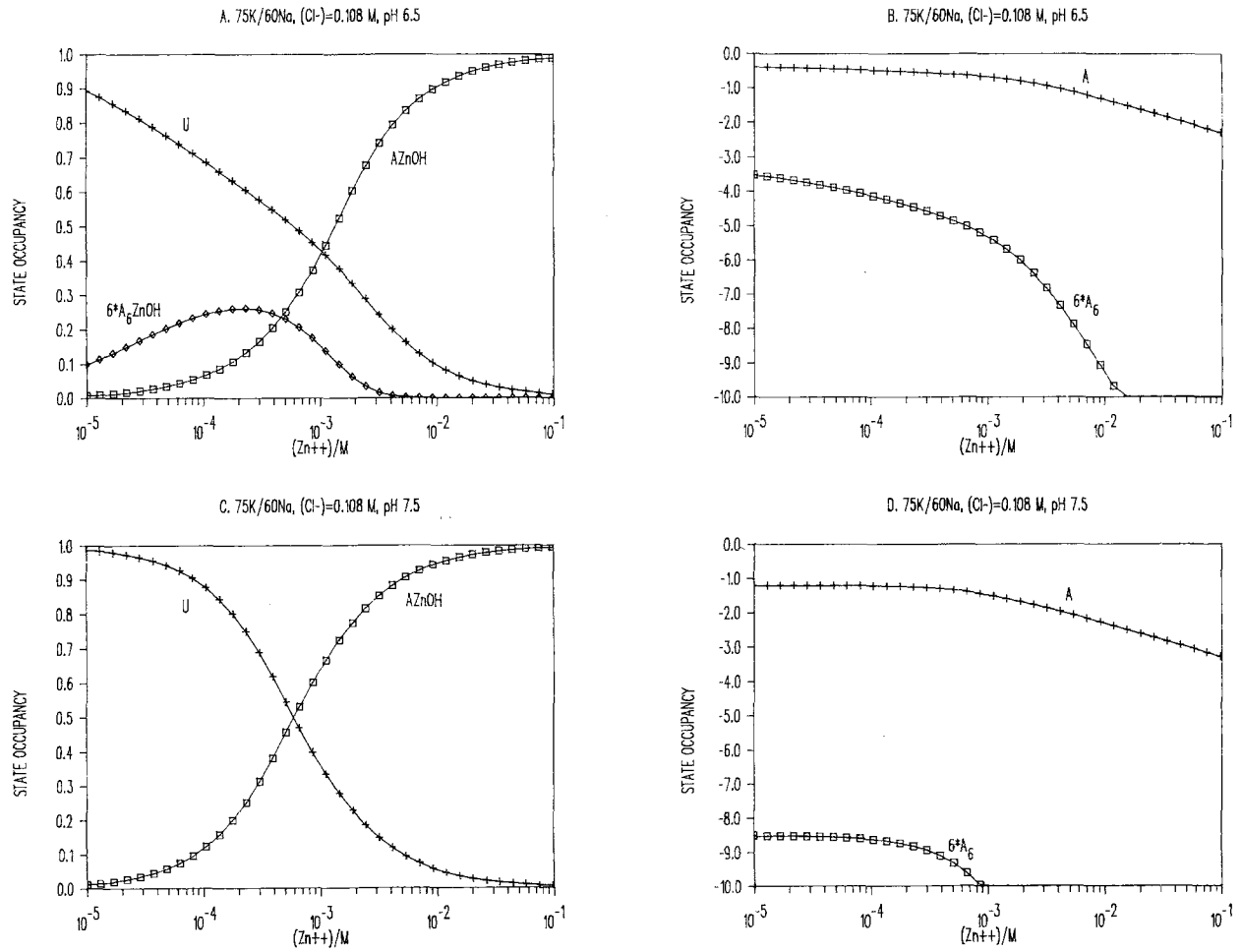

Fig. 10. Calculations using reaction network model of Fig. 9 for Zn^{2+} inhibition in 75 K^+/60 Na^+ solution at pH 6.5 and 7.5. Mathematical details and parameter values are given in Appendix A. *See* Discussion for full description. (*A*) Calculations for pH 6.5. Results presented on a semi-log plot. Crosses give fraction of uninhibited channel elements in all open forms (i.e., *U*, *see* Eqs. (A28) and (A29)). Diamonds give fraction of channel elements closed in the form A_6ZnOH^+ (i.e., $6 \cdot [A_6ZnOH^+]$, *see* Eq. (A17)). Squares give fraction of channel elements closed in the form $AZnOH^+$ (i.e., $[AZnOH^+]$, *see* Eq. (A14)). (*B*) Calculations for pH 6.5. Results presented on a log-log plot. Crosses give fraction of open channel elements in the unassociated, uncomplexed form *A* (i.e., [*A*], *see* Eq. (A27)). Squares give fraction of open-channel elements in the associated, uncomplexed form A_6 (i.e., $6 \cdot [A_6]$, *see* Eq. (A5)). (*C*) Calculations for pH 7.5. Format and symbols as in *A*. (*D*) Calculations for 7.5. Format and symbols as in *B*

pH 7.5. Compared to pH 6.5, at any given (Zn^{2+}) the ($ZnOH^+$) is increased by a factor of nearly ten. If there were no effect of pH on the Cl^- channel elements, the apparent sensitivity to Zn^{2+} would increase. However, the proposed binding of OH^- to *A* at high pH tends to protect channels against attack by $ZnOH^+$. When the external pH is increased from 6.5 to 7.5, the total fraction of channel elements with bound OH^- ($[AOH^-] + 6 \cdot [(AOH^-)_6]$) increases from 0.50 to 0.93 and this reduces both [*A*] and [A_6]. [A_6] drops by about five orders of magnitude (*compare* Fig. 10*B* with *D*); this more than compensates for the 10-fold increase in ($ZnOH^+$) so that $[A_6ZnOH^+]$ is negligible for all (Zn^{2+}) at pH 7.5. On the other hand, the fraction of channel elements in the *A* form at low (Zn^{2+}) drops from 0.45 at pH = 6.5 to 0.061 at pH 7.5 (i.e., by a factor of 7.4; *compare* Fig. 10*B* with *D*). This drop does not compensate completely for the 10-fold increase in ($ZnOH^+$) and consequently the rise in $[AZnOH^+]$ occurs at lower Zn^{2+} activities (*compare* Fig. 10*A* with *C*). The external (Zn^{2+}) producing 50% inhibition remains close to 6×10^{-4} M because at this concentration the shift in the $[AZnOH^+]$ curve just about compensates for the reduction in $[A_6ZnOH^+]$ (*compare* Fig. 10*A* with *C*). Below this Zn^{2+} activity, raising pH produces an apparent protection against inhibition due to depletion of A_6 and of A_6ZnOH^+, the major closed form at low (Zn^{2+}). Above this Zn^{2+} activity, inhibition is greater owing to the left-

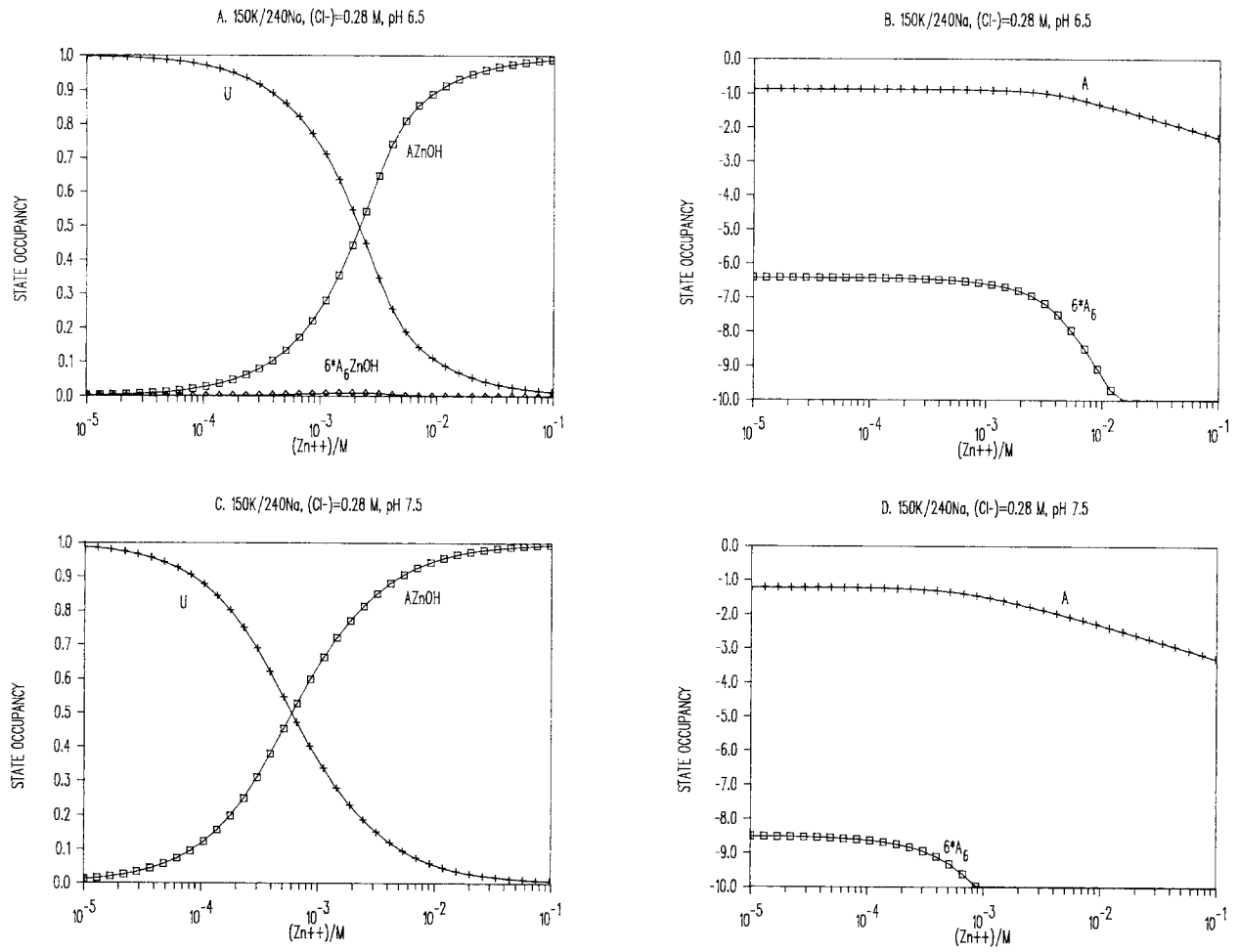

Fig. 11. Calculations using reaction network model of Fig. 9 for Zn^{2+} inhibition in 150 K^+/240 Na^+ solution at pH 6.5 and 7.5. Panel layout, format and symbols as in Fig. 10. Full description given in the Discussion with mathematical details and parameter values given in Appendix A

ward shift of the [AZnOH$^+$] curve. Experiments at Zn^{2+} activities of 6×10^{-4} M and 9×10^{-4} M show too much variability to convincingly demonstrate the predicted small increase in inhibition, but the average inhibition at a Zn^{2+} activity of 1.5×10^{-3} M is significantly larger at pH 7.5 than at pH 6.5 (*see* Fig. 3). For higher activities, Zn^{2+} comes out of solution at pH 7.5.

Figure 11 presents calculations for Zn^{2+} inhibition in 150 K^+/240 Na^+ ((Cl^-) = 0.28 M) at pH 6.5 and 7.5. At pH 6.5 the main difference for muscles equilibrated in the higher (Cl^-) is that, in the absence of external Zn^{2+}, 69% of the channel elements are protected from attack in the stabilized $(ACl^-)_6Cl_{30}^-$ form. With another 3% in the other forms with bound Cl^- and 14% with bound OH^-, only 14% remain in the vulnerable A form, and the fraction in the A_6 form is about three orders of magnitude less than in the solution with low (Cl^-) (*compare* Figs. 10*B* and 11*B*). Consequently, the rises in [AZnOH$^+$] and [A_6ZnOH$^+$] occur at higher Zn^{2+} activities and, more significantly, $6 \cdot$ [A_6ZnOH$^+$] is greatly reduced, reaching a maximum of barely over 0.01 (*compare* Figs. 10*A* and 11*A*). The net effect of increasing (Cl^-) to 0.28 M at external pH 6.5 is to steepen the Zn^{2+} inhibition curve and to shift the 50% inhibition point to a higher (Zn^{2+}).

In terms of the reaction scheme, it is interesting to note how raising external pH from 6.5 to 7.5 increases the inhibition by any given (Zn^{2+}) when external (Cl^-) = 0.28 M. Raising the pH by one unit increases ($ZnOH^+$) by a factor of 10, while [A] drops by only a factor of 2.2, from 0.136 to 0.061, and [A_6] drops by about two orders of magnitudes (*compare* Fig. 11*B* and *D*). Consequently, at pH 7.5 [AZnOH$^+$] is formed at lower external Zn^{2+} activities and $6 \cdot$ [A_6ZnOH$^+$] is again insignificant. Since $6 \cdot$ [A_6ZnOH$^+$] is also minimal at pH 6.5, the deple-

tion of this state produced when pH is raised to 7.5 results in only an insignificant degree of apparent protection at low Zn^{2+} concentrations when external (Cl^-) = 0.28 M, as contrasted to when external (Cl^-) = 0.108 M.

Comments on Model

For simplicity the model is developed in terms of two kinds of substate, an unassociated channel element and an associated, coupled group of six elements. A less restrictive and perhaps more realistic model would include associated, coupled groups of multiple elements other than six. This would be equivalent to adding more substates. Since additional groups introduce more adjustable parameters and since one associated group with a high affinity for $ZnOH^+$ suffices to give a reasonable fit, it appears unnecessary to expand the model in this fashion.

One of our goals was a model and set of parameter values which fit not only the Zn^{2+} inhibition results from muscles equilibrated in 75 K^+/60 Na^+ and 150 K^+/240 Na^+ solutions in which the internal potential of the fibers was −22 mV but also the results from muscles equilibrated in 150 K^+/120 Na^+ solution in which the internal potential was −16 mV. We have assumed that this 6-mV difference does not alter the network parameters to any large extent. If one allows steeply potential-dependent parameters, then other model networks could be made to fit the results.

Another model that we tried involved a reaction network in which not one but two Cl^- ions associate with single-channel elements which then can associate into groups of six, the reactions not involving Cl^- binding remaining the same. With this alternative model one obtains good fits without adding the extra stabilizing step which requires the binding of five additional Cl^- ions to each channel element in the associated state. However, with this alternate model the set of parameters required for muscles equilibrated in 150 K^+/120 Na^+ solution differs substantially from those required for muscles equilibrated in 75 K^+/60 Na^+ and 150 K^+/240 Na^+ solutions. In other words, the alternate model requires a larger set of parameter values to adequately fit the data in hand.

In general, the above points bring out the well-recognized fact that if there is no unique descriptive model then the parameter values required to produce a good fit are also not unique. Nevertheless, the alternate models that we were able to fit to the results shared several fundamental features. First, there are at least two forms of the Cl^- channel system which have different affinities for the closing ligand, $ZnOH^+$. Second, when Cl^- concentration is low, raising OH^- ion concentration protects against Zn^{2+} inhibition as OH^- ions compete for the sites that bind $ZnOH^+$. Third, Cl^- ions can also bind to these sites and confer protection against $ZnOH^+$. And finally, when the control sites are substantially protected by high Cl^- activity then increasing (OH^-) enhances Zn^{2+} inhibition by raising $ZnOH^+$ activity.

That cations play a role in anion movements through anion channels has been suggested by Franciolini and Nonner (1987). They have proposed a permeation mechanism for chloride channels which involves an activated complex of a negatively charged site in the channel with both a cation and an anion coming from either of the adjacent phases. If such a mechanism should apply to the Cl^- channels in frog skeletal muscle, then $ZnOH^+$ could block Cl^- movements by preventing the formation of the activated complex.

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Appendix A

Introduction

This section develops the equations for the reaction network given in Fig. 9 and gives the procedures for estimating the constants used to fit the data.

The plan of the development involves the derivation of explicit formulas for the concentrations of each of the nine channel forms in terms of the concentration of the single-channel element, $[A]$, the external activities (OH^-), (Cl^-), and (Zn^{2+}), and the various equilibrium constants. These are substituted into a conservation equation for the total number of channel elements. This yields a polynomial of the 6th degree in $[A]$. The solution of the polynomial is obtained using a numerical procedure. Once $[A]$ is found, the fraction of each of the nine forms or any combination of them is calculated. The fitting procedure consists of adjusting the set of equilibrium constants to fit six experimental Zn^{2+} inhibition data sets.

Reaction and Equation for $ZnOH^+$

To make explicit the role of OH^- ions, all equations are developed on a pOH scale. We assume that pOH = 14 − pH. The main reaction between Zn^{2+} and OH^- ions can be written as

$$Zn^{2+} + OH^- \longleftrightarrow ZnOH^+. \tag{A1}$$

$(ZnOH^+)$, is given by

$$(ZnOH^+) = K_0^{-1} \cdot (Zn^{2+}) \cdot (OH^-) \tag{A2}$$

where K_0 is the equilibrium constant for the reaction (Eq. (A1)). Equation (A2) can be rewritten as

$$(ZnOH^+) = (Zn^{2+}) \cdot 10^{(pk-pOH)} \tag{A3}$$

where $pk = -\log(K_0)$ and $pOH = -\log((OH^-))$. Since the pK_a of this reaction on the pH scale is 9.0 (Baes & Mesmer, 1976), pk = 5.0 (on the pOH scale) is used in all the calculations.

Reaction and Equation for A_6

The reaction for the association of single Cl^- channel elements, A, into sextets, A_6, can be written as

$$6\,A \longleftrightarrow A_6. \tag{A4}$$

$[A_6]$ is given by

$$[A_6] = K_{e6} \cdot [A]^6 \tag{A5}$$

where K_{e6} is the equilibrium constant for the reaction.

Reactions and Equations for AOH^- and $(AOH^-)_6$

The binding reaction for OH^- ions to Cl^- channel elements can be written as

$$A + OH \longleftrightarrow AOH^-. \tag{A6}$$

$[AOH^-]$ is given by the relation

$$[AOH^-] = K_{eo}^{-1} \cdot (OH^-) \cdot [A] \quad \text{(A7)}$$

or

$$[AOH^-] = [A] \cdot 10^{(pK-pOH)} \quad \text{(A8)}$$

where K_{eo} is an equilibrium constant and $pK = -\log(K_{eo})$.

Association of single-channel elements with bound OH^-, AOH^-, into sextets, $(AOH^-)_6$, is represented by the reaction

$$6\ AOH^- \longleftrightarrow (AOH^-)_6. \quad \text{(A9)}$$

$[(AOH^-)_6]$ is given by

$$[(AOH^-)_6] = K_{o6} \cdot [AOH^-]^6 \quad \text{(A10)}$$

or, using Eq. (A8),

$$[(AOH^-)_6] = K_{o6} \cdot [A]^6 \cdot 10^{6(pK-pOH)} \quad \text{(A11)}$$

where K_{o6} is the equilibrium constant for the reaction (Eq. (A9)).

Reactions and Equations for $AZnOH^+$ and A_6ZnOH^+

The site that binds OH^- ions on each channel element may instead bind $ZnOH^+$ which closes the channel. This reaction can be expressed as

$$A + ZnOH^+ \longleftrightarrow AZnOH^+. \quad \text{(A12)}$$

The concentration of single-channel elements with bound $ZnOH^+$, $[AZnOH^+]$, is given by

$$[AZnOH^+] = K_{ez}^{-1} \cdot (ZnOH^+) \cdot [A] \quad \text{(A13)}$$

or, using Eq. (A3),

$$[AZnOH^+] = K_{ez}^{-1} \cdot (Zn^{2+}) \cdot [A] \cdot 10^{(pk-pOH)} \quad \text{(A14)}$$

where K_{ez} is the equilibrium constant for the reaction (Eq. (A12)).

A_6 can also bind $ZnOH^+$. It is assumed that the binding site on each channel element in the associated group remains accessible to $ZnOH^+$, and binding of $ZnOH^+$ to any one of the sites closes all six channel elements in the group. This reaction can be written as

$$A_6 + ZnOH^+ \longleftrightarrow A_6ZnOH^+. \quad \text{(A15)}$$

The concentration of A_6 with bound $ZnOH^+$ is given by

$$[A_6ZnOH^+] = 6 \cdot K_{6z}^{-1} \cdot (ZnOH^-) \cdot [A_6] \quad \text{(A16)}$$

or, using Eqs. (A3) and (A5),

$$[A_6ZnOH^+] = 6 \cdot K_{e6} \cdot [A]^6 \cdot K_{6z}^{-1} \cdot (Zn^{2+}) \cdot 10^{(pk-pOH)} \quad \text{(A17)}$$

where K_{6z} is the equilibrium constant for reaction (Eq. A15)).

Reactions and Equations for ACl^- and $(ACl^-)_6$

The OH^- binding site can bind Cl^- instead and when Cl^- is bound the channel elements can associate in a group of six. These reactions can be represented by

$$A + Cl^- \longleftrightarrow ACl^- \quad \text{(A18)}$$

$$6\ ACl^- \longleftrightarrow (ACl^-)_6. \quad \text{(A19)}$$

The relevant concentrations are given by

$$[ACl^-] = K_{ec}^{-1} \cdot (Cl^-) \cdot [A] \quad \text{(A20)}$$

where K_{ec} is the equilibrium constant for the reaction (Eq. (A18)), and

$$[(ACl^-)_6] = K_{c6} \cdot [ACl^-]^6 \quad \text{(A21)}$$

or, using Eq. (A20),

$$[(ACl^-)_6] = K_{c6} \cdot [A]^6 \cdot (K_{ec}^{-1} \cdot (Cl^-))^6 \quad \text{(A22)}$$

where K_{c6} is the equilibrium constant for the reaction (Eq. (A19)).

When $(ACl^-)_6$ is formed, five additional sites on each channel element that specifically bind Cl^- become exposed. The additional binding of Cl^- stabilizes the sextet with bound Cl^-. The experimental basis for assuming this step is given in the Discussion. This stabilization reaction can be represented by

$$(ACl^-)_6 + 30\ Cl^- \longleftrightarrow (ACl^-)_6Cl_{30}^-. \quad \text{(A23)}$$

The concentration of this stabilized form is given by

$$[(ACl^-)_6Cl_{30}^-] = (K_{6n}^{-1} \cdot (Cl^-))^{30} \cdot [(ACl^-)_6] \quad \text{(A24)}$$

or, using Eq. (A22),

$$[(ACl^-)_6Cl_{30}^-] = K_{c6} \cdot [A]^6 \cdot (K_{ec}^{-1} \cdot (Cl^-))^6 \cdot (K_{6n}^{-1} \cdot (Cl^-))^{30} \quad \text{(A25)}$$

where K_{6n} is the equilibrium constant for the reaction (Eq. (A23)).

Conservation of Channel Elements and the Equation for A

The conservation equation of single Cl^- channel elements is

$$[A] + [AOH^-] + [ACl^-] + [AZnOH^+] + 6 \cdot \{[A_6] + [(AOH^-)_6] + [(ACl^-)_6] + [(ACl^-)_6Cl_{30}^-] + [A_6ZnOH^+]\} = 1. \quad \text{(A26)}$$

The following are substituted in Eq. (A26): for $[AOH^-]$ Eq. (A8), for $[ACl^-]$ Eq. (A20), for $[AZnOH^+]$ Eq. (A14), for $[A_6]$ Eq. (A5), for $[(AOH^-)_6]$ Eq. (A11), for $[(ACl^-)_6]$ Eq. (A22), for $[(ACl^-)_6Cl_{30}^-]$ Eq. (A25), and for $[A_6ZnOH^+]$ Eq. (A17). After rearrangement one obtains

$$[A] \cdot \{1 + 10^{(pK-pOH)} + K_{ec}^{-1} \cdot (Cl^-) + K_{cz}^{-1} \cdot (Zn^{2+}) \cdot 10^{(pk-pOH)}\} + 6 \cdot [A]^6 \cdot \{K_{c6} \cdot (1 + K_{6z}^{-1} \cdot (Zn^{2+}) \cdot 10^{(pk-pOH)}) + K_{o6} \cdot 10^{6(pK-pOH)} + K_{c6} \cdot (K_{ec}^{-1} \cdot (Cl^-))^6 \cdot (1 + (K_{6n}^{-1} \cdot (Cl^-))^{30})\} - 1 = 0. \quad \text{(A27)}$$

Upon assigning values to the external concentrations of OH^-, Cl^-, and Zn^{2+} ions and the various equilibrium constants as described below, Eq. (A27) was solved numerically for $[A]$ using the secant method as implemented by the MathCAD software program.

Equations for Uninhibited and Inhibited Fractions of Cl^- Efflux

U, the uninhibited fraction of Cl^- efflux, is the sum of single Cl^- channel elements in all forms not closed by $ZnOH^+$:

$$U = [A] + [AOH^-] + [ACl^-] + 6 \cdot \{[A_6] + [(AOH^-)_6] + [(ACl^-)_6] + [(ACl^-)_6Cl_{30}^-]\}. \quad \text{(A28)}$$

Using the substitutions above,

$$U = [A] \cdot \{1 + 10^{(pK-pOH)} + K_{ec}^{-1} \cdot (Cl^-)\} + 6 \cdot [A]^6 \cdot \{K_{e6} + K_{o6} \cdot 10^{6(pK-pOH)} + K_{c6} \cdot (K_{ec}^{-1} \cdot (Cl^-))^6 \cdot (1 + (K_{6n}^{-1} \cdot (Cl^-))^{30})\}. \quad \text{(A29)}$$

The inhibited fraction of Cl^- efflux, B, is the sum of single-channel elements in the two forms closed by $ZnOH^+$ and is given by

$$B = [AZnOH^+] + 6 \cdot [A_6ZnOH^+] \quad \text{(A30)}$$

or, using the above substitutions,

$$B = \{[A] \cdot K_{ez}^{-1} \cdot (Zn^{2+}) + 6 \cdot [A]^6 \cdot K_{e6} \cdot K_{6z}^{-1} \cdot (Zn^{2+})\} \cdot 10^{(pk-pOH)}. \quad \text{(A31)}$$

Procedures for Adjustment of Parameters in Reaction Network

In the initial stage, a network was considered containing only A, A_n, $AZnOH^+$, and A_nZnOH^+ where n was allowed to take on integral values from 3 to 6. The parameters in this reduced network were adjusted to the data obtained in the 75 K^+/60 Na^+ solution at pH 6.5. The rationale was the assumption that for this solution the degree of interaction between the control site and OH^- and Cl^- would be minimal. The values of n and K_{e6} which gave a good approximation to the data set were $n = 6$ and $K_{e6} = 0.01$. These two parameters were fixed at these values for subsequent adjustments.

In succeeding steps the equations for the whole network were employed. In the second step the data to be fitted was expanded to include results at both pH 6.5 and 7.5 in 75 K^+/60 Na^+ solution. The parameters relating to the forms binding Cl^- were assigned values that made the Cl^--bound forms negligible. The remaining parameters were adjusted to give a reasonable fit to the data sets for the two pHs. The values obtained for pK (or K_{eo}) and K_{o6} in this step needed only minor readjustments in subsequent steps as more data sets to be fit were added.

In the third step the data set to be fitted was enlarged to include results at pH 6.5 and 7.5 in both 75 K^+/60 Na^+ and 150 K^+/120 Na^+ solutions. In this step the value of K_{6n} was assigned a value that kept the stabilized form, $(ACl^-)_6Cl_{30}^-$, small. The remaining parameters were again adjusted to give a good fit to the four data sets.

Finally, the data to be fitted was enlarged to include all of the Zn^{2+} inhibition results at pH 6.5 and 7.5 in 75 K^+/60 Na^+, 150 K^+/120 Na^+, and 150 K^+/240 Na^+ solutions. Except for $n = 6$ and $K_{e6} = 0.01$ all parameters were free to be adjusted to give a reasonable fit. The values used in the calculations presented above were as follows: $pK = -\log(K_{eo}) = 7.5$, $K_{e6} = 0.01$, $K_{o6} = 1.0$, $K_{ec} = 1.14$ M, $K_{c6} = 15{,}000$, $K_{6n} = 0.21$ M, $K_{6z} = 9.5 \cdot 10^{-11}$ M, and $K_{ez} = 1.6 \cdot 10^{-6}$ M. For the 150 K^+/120 Na^+ solution at pH 6.5, $K_{ez} = 2.4 \cdot 10^{-6}$ M.

Appendix B

The purpose of this section is to estimate the free Zn^{2+} ion concentration and its activity in solutions of different Cl^- concentrations taking into account the formation of zinc chloride complexes and the ionic strength of the solutions.

The reactions for the known mononuclear zinc chloride complexes can be written as

$$Zn^{2+} + nCl^- \longleftrightarrow ZnCl_n^{(2-n)}$$

where n is an integer between 1 and 4; i.e., the complexes are $ZnCl^+$, $ZnCl_2$, $ZnCl_3^-$, and $ZnCl_4^{2}$. The activity of the various complexes expressed in terms of the thermodynamic equilibrium constants, β_n, are given by

$$a_{ZnCl_n} = \beta_n a_{Zn}(a_{Cl})^n \quad \text{(B1)}$$

where a_{Zn} *and* a_{Cl} are the activities of Zn^{2+} and Cl^- ions. In most contexts we denote a_{Zn} as (Zn^{2+}) and a_{Cl} as (Cl^-). The activity for any species x in solution is given by the relation

$$a_x = f_x[x] \quad \text{(B2)}$$

where f_x is the activity coefficient and $[x]$ is the concentration of x.

Since the solutions used have ionic strengths less than 0.5, we assume that the Davies Equation can be used to calculate the activity coefficient for any species x in this study (Butler, 1964; Stumm & Morgan, 1981). For a species x with charge z_x, the activity coefficient given by this equation is

$$\log f_x = -0.5z_x^2 \left[\frac{I^{0.5}}{1 + I^{0.5}} - 0.2I\right]\left[\frac{298}{t + 273}\right]^{(2/3)} \quad \text{(B3)}$$

where I is the ionic strength of the solution as usually defined and t is temperature in degrees centrigrade.

The concentrations of the various complexes can be obtained by transforming Eq. (B1) to

$$[ZnCl_n] = k_n[Zn^{2+}][Cl^-]^n \quad \text{(B4)}$$

where k_n is given by

$$k_n = \frac{\beta_n f_{Zn}(f_{Cl})^n}{f_{ZnCl_n}}. \quad \text{(B5)}$$

For Eq. (B5) the activity coefficients are calculated from Eq. (B3) using the ionic strength of the solution being employed. The value of β_n used in the calculations ($\beta_1 = 0.65$, $\beta_2 = 1.51$, $\beta_3 = 0.04$, and $\beta_4 = 0.03$) were obtained from the work of Scibona, Orlandini and Danesi (1966).

Table B1

Solution (mM)	$[Zn]_t$ (mM)	I (M)	$[Cl^-]$ (M)	f_{Cl}	a_{Cl} (M)	Q	f_{Zn}	Qf_{Zn}
75 K^+/60 Na^+	≦5	0.163	0.147	0.745	0.109	0.966	0.307	0.297
75 K^+/60 Na^+	>5	0.193	0.147	0.735	0.108	0.968	0.291	0.282
150 K^+/120 Na^+	≦5	0.298	0.282	0.712	0.201	0.941	0.257	0.242
150 K^+/120 Na^+	>5	0.358	0.282	0.705	0.199	0.943	0.246	0.232
150 K^+/240 Na^+	≦5	0.418	0.402	0.699	0.281	0.916	0.239	0.219
150 K^+/240 Na^+	>5	0.478	0.402	0.696	0.280	0.918	0.235	0.216

The conservation equation for the total concentration of zinc, $[Zn]_t$, is given by

$$[Zn^{2+}] + [ZnCl^+] + [ZnCl_2] + [ZnCl_3^-] + [ZnCl_4^{2-}] = [Zn]_t. \tag{B6}$$

Substituting Eqs. (B4) in Eq. (B6) one obtains the relation

$$[Zn^{2+}] = Q[Zn]_t \tag{B7}$$

where

$$Q = \frac{1}{1 + k_1[Cl^-] + k_2[Cl^-]^2 + k_3[Cl^-]^3 + k_4[Cl^-]^4}. \tag{B8}$$

The activity of Zn^{2+} is given by the relation (using Eqs. (B2) and (B9))

$$a_{Zn} = Qf_{Zn}[Zn]_t = (Zn^{2+}). \tag{B9}$$

Table B1 lists the calculated values of various parameters for the solutions used in this study.

With regard to the values of a_{Cl} in the model calculations we have used 0.108 M for 75 K^+/60 Na^+ solutions, 0.20 M for the 150 K^+/120 Na^+ solutions, and 0.28 M for the 150 K^+/240 Na^+ solutions (*see* a_{Cl} values).

A few comments can be made about the values given in Table B1. The amount of zinc complexed as chlorides in solutions with the largest $[Cl^-]$ is a bit less than 9% of the total amount added; for lower $[Cl^-]$ the amount complexed is less (*see* Q values). Consequently, we have neglected the slight reduction in the free Cl^- ion concentrations as compared to the total amounts added, since $[Cl^-]$ is generally substantially larger than $[Zn^{2+}]$. The major factor reducing the activities of both Zn^{2+} and Cl^- ions is the ionic strength dependence of the activity coefficients (*see* f_{Zn} and f_{Cl} values). Note that by Eq. (B3) $f_{Zn} = (f_{Cl})^4$.