

Chemical Properties of the Divalent Cation Binding Site on Potassium Channels

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ABSTRACT The actions of divalent cations on voltage-gated ion channels suggest that these cations bind to specific sites and directly influence gating kinetics. We have examined some chemical properties of the external divalent cation binding sites on neuronal potassium channels. Patch clamp techniques were used to measure the electrophysiological properties of these channels and Zn ions were used to probe the divalent cation binding site. The channel activation kinetics were greatly (three- to fourfold) slowed by low (2–5 mM) concentrations of Zn; deactivation kinetics were only slightly affected. These effects of Zn were inhibited by low solution pH in a manner consistent with competition between Zn and H ions for a single site. The apparent inhibitory pK for this site was near 7.2. Treatment of the neurons with specific amino acid reagents implicated amino, but not histidyl or sulfhydryl, residues in divalent cation binding.

INTRODUCTION

Calcium and other divalent cations have pronounced effects on the gating properties of voltage-gated ion channels. Elevated concentrations of Ca raise the excitation threshold for nerve and muscle cells; low concentrations have the opposite effect and cause cells to become hyperexcitable. Understanding the mechanisms underlying these effects has been a major puzzle for neurobiologists. The proposed mechanisms fall into two limiting categories originally described by Frankenhaeuser and Hodgkin (1957): (a) Ca ions bind to an external part of the channel pore and so occlude current flow. Membrane depolarization repels Ca from this site and conduction can occur. This extreme form of the “gating-particle theory” is clearly inconsistent with much experimental evidence (see discussion in Hille, 1984). In particular, it cannot account for the steep voltage dependence of channel gating. (b) An alternative mechanism considers that Ca ions interact with negative membrane surface charges that add to the voltage sensed by the channels. That surface charges exist and influence voltage-gated channels is inescapable: changes in solution ionic strength via changes in monovalent cation concentration produce voltage shifts of Na channel

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conductance (Chandler, Hodgkin, and Meves, 1965; Begenisich, 1975; Hille, Woodhull, and Shapiro, 1975). The voltage shifts produced by both divalent and monovalent cations can be quantitatively described by simple surface charge theory.

More recent results, however, are difficult to explain in terms of the usual surface charge models (reviewed in Hille, 1984; Begenisich, 1988). Armstrong and Matteson (1986) found that increasing the external Ca concentration from 20 to 100 mM slows the activation of squid axon K channels by an amount equivalent to an ~25-mV depolarization of the membrane potential. In contrast, channel deactivation was altered by an amount equivalent to only a 4-mV potential change. The usual surface charge models predict equal shifts of these parameters. Other divalent cations (including Zn) produce similar results but often require much lower concentrations even in the presence of a large concentration of Ca which would tend to greatly reduce surface potentials (Gilly and Armstrong, 1982a). A direct, even obligatory, role for Ca is suggested by the fact that complete removal of Ca from the external solution substantially decreases time-dependent K channel gating in squid neurons (Armstrong and Lopez-Barneo, 1987) and in *Shaker* K channels (Armstrong and Miller, 1990) and may remove channel selectivity.

These profound and specific effects on K channels indicate that, in addition to surface charge interactions, divalent cations play an important, direct role in channel gating. Consequently, our knowledge of the structure/function relation of these channels will be advanced by more information on the chemical nature of the group(s) involved in the interaction of divalent cations with K channels. This information may aid in identifying the specific amino acid(s) involved. To this end, we investigated the electrophysiological consequences of the interaction of Zn ions with the divalent cation binding site on squid neuron delayed rectifier K channels. Low concentrations (2–5 mM) of Zn ions were used to probe the divalent cation binding site with a large background of divalent cations (10 mM Ca and 50 mM Mg) to minimize surface charge effects. The channel activation kinetics were greatly (three- to fourfold) slowed by these low concentrations of Zn; deactivation kinetics were only slightly affected. These effects of Zn were inhibited by low solution pH in a manner consistent with competition between Zn and H ions for a single site on the channel protein. The apparent inhibitory pK for this site was near 7.2. Treatment of the neurons with specific amino acid reagents implicated amino, but not histidyl or sulfhydryl, residues in divalent cation binding.

A preliminary report of these findings has been presented (Begenisich and Spires, 1991).

METHODS

The data in this report were obtained with stellate ganglion giant fiber lobe (GFL) cells from the squid *Loligo pealei* obtained at the Marine Biological Laboratory, Woods Hole, MA. These GFL cells are the cell bodies whose axons fuse to form the third-order giant axons (Young, 1939). The cells were dissociated using techniques similar to those of Llano and Bookman (1986). The dissociated cells were plated on glass coverslips coated with CellTak (Collaborative Research, Bedford, MA) and cultured for several hours to five days before use. As described by Brismar and Gilly (1987), the dissociated cells initially lack Na channel currents but develop these currents after several days.

Similar to squid giant axons (Llano, Webb, and Bezanilla, 1988; Perozo, Vandenberg, Jong, and Bezanilla, 1991a; Perozo, Jong, and Bezanilla, 1991b), these GFL cells express two types of K channels distinguishable by their unitary current levels (equivalent to ~10- and 20-pS slope conductances) and kinetics (Nealey, T., S. Spires, R. A. Eatock, and T. Begenisich, manuscript in preparation). However, the cell macroscopic current is dominated by the large conductance channel. This is due to (a) the higher expression levels of the large conductance channel; (b) the larger current of this channel type; and (c) the fact that the large conductance channel activates more quickly upon depolarization.

The whole cell configuration of the patch clamp technique was used. The potassium channel currents from these cells are often large (~100 nA) and so present problems for conventional patch clamp amplifiers. Consequently, we used a circuit of our own design with a wide bandwidth amplifier (model 3554; Burr-Brown/Intelligent Instrumentation Inc., Tucson, AZ) and a 10-M Ω feedback resistance. This circuit allowed compensation for 92–95% of the measured series resistance. This high level of compensation was possible because of the quality of the cancellation of stray (fast) capacitance (Sigworth, 1983). Cancellation of cell (slow) capacitance was not used. The complete duration of the cell capacity current (with series resistance compensation) was always <50 μ s and often near 35 μ s. The resistance of the electrodes (filled with internal solution) was <1 M Ω and often <0.5 M Ω . The membrane voltages have been corrected for the liquid junction potential between the pipette and bath solutions.

Membrane currents were acquired with a 12-bit analog/digital converter controlled by a laboratory personal computer. The voltage clamp pulses were generated by a 12-bit digital/analog converter controlled by the computer system. Linear capacitative and leakage currents were subtracted from most records using a -P/4 procedure (Bezanilla and Armstrong, 1977). Currents were filtered at 10 or 20 kHz with a four-pole Bessel filter.

The bath solution consisted of (mM): 390 NaCl, 50 KCl, 50 MgCl₂, and 10 CaCl₂. Unless otherwise indicated, this solution was buffered with 15 mM HEPES (for solution pH values of 7.0 and 7.5) or 15 mM MES (for pH 6.0, 6.25, and 6.5 solutions). The internal (pipette) solution consisted of (mM): 250 K-glutamate, 30 KF, 20 KCl, 20 KOH, 15 K₂HPO₄, 10 EGTA, and 350 glycine, pH 7.4. All experiments were done at 15–16°C.

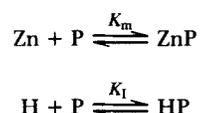
Na channel currents were usually small or absent in our experiments, consistent with earlier reports (Llano and Bookman, 1986; Brismar and Gilly, 1987). In the few cases where these currents were present, they were blocked by 300 nM tetrodotoxin. It was not necessary to block Ca channel currents since these were negligible compared with the very large K channel currents (Llano and Bookman, 1986; Chow, 1991).

We used several amino acid reagents that were relatively specific for histidyl, sulfhydryl, or amino groups. All reagents were applied in the bath solution under conditions similar to those described previously (Spires and Begenisich, 1990, 1992). Diethylpyrocarbonate (DEP) and rose bengal modify histidine residues in many model peptides and native proteins (Means and Feeney, 1971; Lundblad and Noyes, 1984). 0.35 mM DEP was applied at pH 6.0 for 4.5 min. A 1- μ M concentration of rose bengal was used at pH 6.5 for 10 min. Mercuric ions and many mercury-containing compounds react with sulfhydryl groups with high specificity (Means and Feeney, 1971). We treated cells with 0.25–0.75 mM *p*-chloromercuriphenyl sulfonic acid (*p*-CMPSA) for 6–14 min in solutions of pH 6.5–7.5. Modification of amino groups was done with trinitrobenzene sulfonic acid (TNBS). Because TNBS reacts with the neutral form of amino groups, treatment was done with solutions of elevated pH (8.2 or 9.0 buffered with 15 mM CHES). These treatments produced large effects on the squid neuron K channel gating kinetics, consistent with our results on axon K channels (Spires and Begenisich, 1990, 1992).

The quantitative analysis of our data included the fitting of exponential time functions to ionic current records. As shown by White and Bezanilla (1985), the final approach to

steady-state K channel current can be fit by a single exponential time function. Therefore, as described previously (Spires and Begenisich, 1989), we obtained an estimate of the K channel ionic current activation time constant by fitting an exponential function between 50–65% and 90–95% of the current maximum. Ionic tail currents were also fit with a single exponential time function from 90–95% of maximum to ~20% or less. Time constants obtained in this way are called deactivation time constants. All fits of theoretical functions used the “simplex” algorithm (Caceci and Cacheris, 1984).

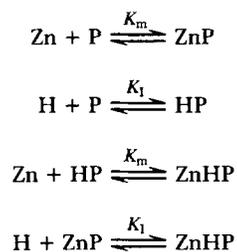
We considered two simple models for the interaction of Zn^{2+} and H^+ ions with the K channel protein. In the *competitive* model, both ions compete for the same site on the potassium channel protein (P):



where K_m and K_1 are the binding affinities of Zn and H for a channel site. In this model, the protein can exist with a bound H^+ ion (HP), a Zn^{2+} ion bound (ZnP), or no ions bound (P). We are interested in the Zn effect, which is assumed to be proportional to the fraction of sites occupied by Zn at a given pH (or H^+ ion concentration, [H]) and Zn concentration ([Zn]):

$$Zn \text{ effect} \equiv \frac{ZnP}{P + ZnP + HP} = \frac{1}{1 + \frac{K_m}{[Zn]} \left(1 + \frac{[H]}{K_1} \right)} \quad (1)$$

In the *noncompetitive* model, Zn^{2+} and H^+ ions do not compete for the same channel site:



For this model, the Zn effect is taken to be:

$$Zn \text{ effect} \equiv \frac{ZnP}{P + ZnP + HP + ZnHP} = \frac{1}{\left(1 + \frac{K_m}{[Zn]} \right) \left(1 + \frac{[H]}{K_1} \right)} \quad (2)$$

These equations were fit to the appropriate data (e.g., Fig 4) with the “simplex” algorithm.

RESULTS

External Zn ions have simple and reversible actions on K channels in GFL cells as illustrated in Fig. 1. The left panel of this figure includes superimposed K channel currents elicited by several depolarizing membrane voltages. In the presence of 2 mM external Zn (middle panel) (a) the currents activated much more slowly; (b) the deactivation kinetics were faster; and (c) channel activation was shifted to more

depolarized potentials. These actions of external Zn on GFL K channels were readily reversible (right panel) and identical to those on the squid giant axon K channel currents (Gilly and Armstrong, 1982a). A quantitative analysis of Zn effects on K channel kinetics is illustrated in Fig. 2. In part *A* of this figure activation and deactivation time constants (determined as described in Methods) at several membrane potentials are shown. Symbols at potentials more negative than -30 mV represent deactivation time constants and those for more positive potentials represent activation time constants. Zn decreased the deactivation and increased the activation time constants. As noted by Gilly and Armstrong (1982a), these Zn effects on the time constants cannot be described by a simple shift of membrane voltage. The effects on the deactivation time constant were small and equivalent to a shift of a few millivolts. However, Zn slowed the activation time constants by such large amounts that, for example, the activation time constant at ~ 30 mV in the presence of Zn was about the same as the control value at -28 mV: an effective 58-mV shift.

The data of Fig. 2 showed that to account for the actions of Zn on K channel gating kinetics with a voltage shift mechanism would require a small (a few millivolts) shift of

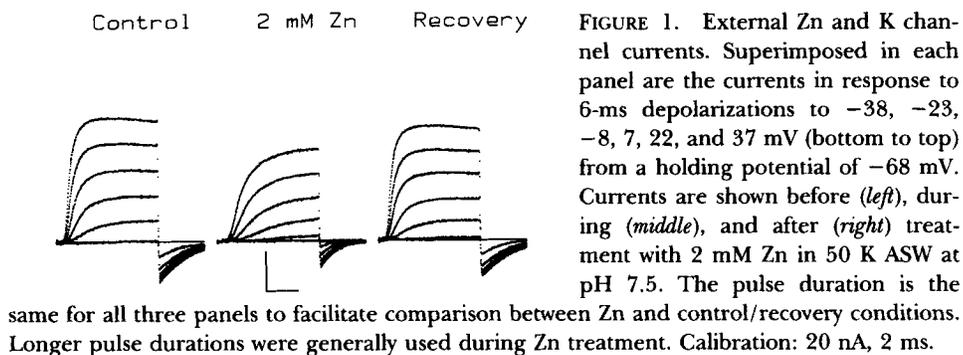


FIGURE 1. External Zn and K channel currents. Superimposed in each panel are the currents in response to 6-ms depolarizations to -38 , -23 , -8 , 7 , 22 , and 37 mV (bottom to top) from a holding potential of -68 mV. Currents are shown before (*left*), during (*middle*), and after (*right*) treatment with 2 mM Zn in 50 K ASW at pH 7.5. The pulse duration is the

same for all three panels to facilitate comparison between Zn and control/recovery conditions. Longer pulse durations were generally used during Zn treatment. Calibration: 20 nA, 2 ms.

deactivation simultaneously with a large (perhaps 58 mV) shift of activation. A much simpler description of Zn action is suggested by a plot of the ratio of the time constant in Zn to the control value against membrane potential (Fig. 2 *B*). Thus, the effects of Zn can be viewed as a constant ($\sim 20\%$) increase in the deactivation kinetics and a constant (approximately threefold) slowing of channel activation kinetics.

That Zn actions cannot be described by a voltage shift but rather as specific, constant modification of deactivation and activation kinetics suggests that Zn may interact with a specific binding site on the K channel protein. As a first step in identifying the chemical group(s) involved, we attempted to determine the pK of the binding site by examining the effects of Zn in solutions of different pH. An example of such an experiment on a GFL cell is illustrated in Fig. 3.

The upper half of this figure contains data obtained at pH 7.5 and is similar to Fig. 1, except that 5 mM Zn was used. The large slowing of activation is apparent, as is the increased rate of deactivation. The effects of Zn were much smaller when the experiment was repeated on this cell, but at an external pH of 6.0 (lower half).

The results of a quantitative analysis of such experiments are illustrated in Fig. 4. The ordinate in this figure is a measure of the Zn effect on activation kinetics and is defined as:

$$\text{Relative Zn effect} = \tau_{\text{Zn}}/\tau_{\text{control}} - 1 \quad (3)$$

where τ_{control} is the activation time constant in the absence and τ_{Zn} is the activation time constant in the presence of Zn. Since the effect of Zn on the activation time constant was independent of voltage (Fig. 2 *B*), any potential could have been used

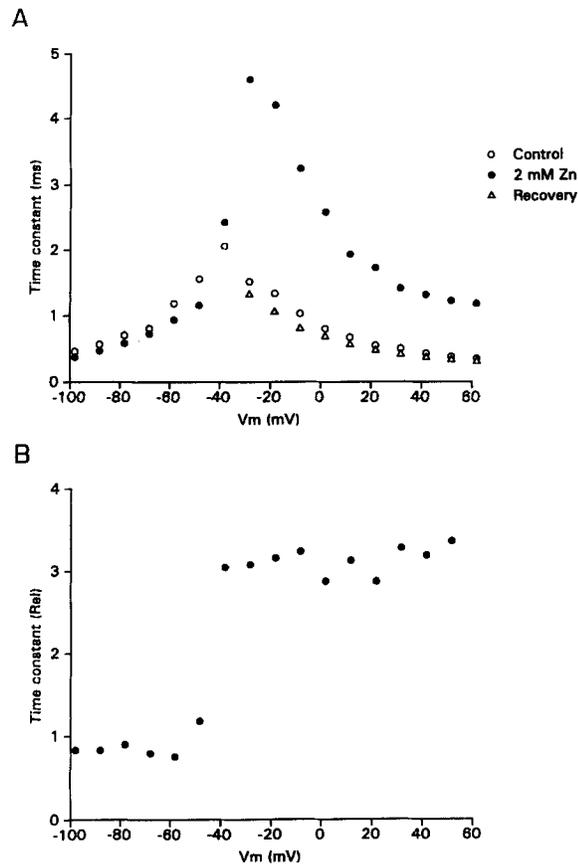


FIGURE 2. External Zn and K channel activation and deactivation time constants. (*A*) Time constants obtained from exponential fits as described in Methods. Ordinate values for potentials more positive than -30 mV are activation time constants. Values for potentials more negative than -30 mV are deactivation time constants obtained from "tail" currents after a 10-ms depolarization to $+2$ mV. (*B*) Relative time constants and Zn. The data are replotted as the ratios of the time constants in Zn to control values.

for this analysis. We chose to use 22 mV, as this was approximately in the middle of the activation voltage range examined. With this normalization, an abscissa value of 0 represents no Zn effect and, for example, a value of 1.0 represents a factor of two increase in the activation time constant.

The data of Fig. 4 show that the Zn effect at both 2 and 5 mM Zn was inhibited at low solution pH. This result was not due to pH-dependent binding of Zn to the H ion buffers used, as the measured effects of Zn were independent of buffer concentration.

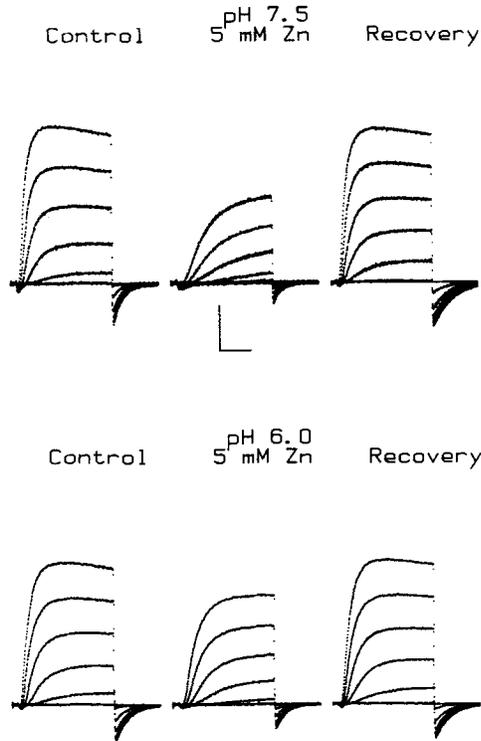


FIGURE 3. Zn action on ionic currents at pH 7.5 and 6.0. Superimposed in each panel are the currents in response to 6-ms depolarizations to -38, -23, -8, 7, 22, and 37 mV (bottom to top) from a holding potential of -68 mV. (*Upper panel*) Currents are shown before (*left*), during (*middle*), and after (*right*) treatment with 5 mM Zn in pH 7.5 50 K ASW. (*Lower panel*) Data from the same cell treated with 5 mM Zn but at pH 6.0. Calibration: 10 nA, 2 ms. Small inward (probably Na channel) currents were apparent in this cell.

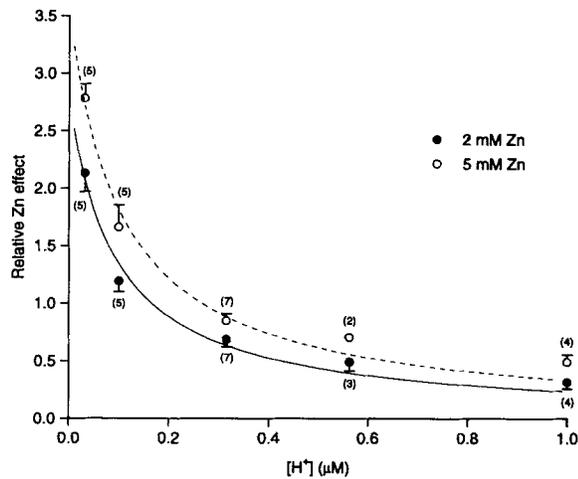


FIGURE 4. Competitive inhibition between Zn and H ions. The relative effect of 2 and 5 mM Zn at 22 mV is plotted as a function of the H ion concentration. The data were obtained at pH values of 7.5, 7.0, 6.5, 6.25, and 6.0. Numbers in parentheses indicate the number of experiments. Standard error limits shown for data with three or more measurements. Lines are from a model of competitive inhibition between Zn and H ions (see text) for 2 mM (*solid line*) and 5 mM Zn (*dashed line*). Inhibitory pK values of 7.2 and 7.1 were obtained from the model fits for 2 and 5 mM Zn, respectively.

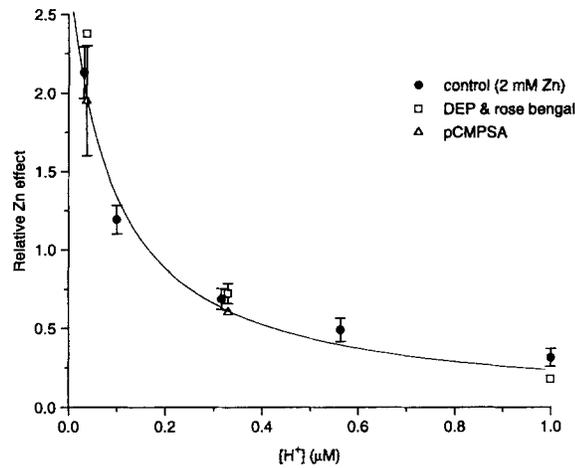


FIGURE 5. Zn effect after treatment with histidyl and sulfhydryl reagents. The 2 mM Zn data of Fig. 4 are replotted (*filled circles*) along with the competitive inhibition model fit (*solid line*). The effect of 2 mM Zn was computed from Eq. 3 using time constants obtained after treatment with the histidyl reagents DEP and rose bengal (*open squares*) and after treatment with *p*-CMPSA (*open triangles*). Treatment data with error bars are from three measurements; those without error bars

are single observations. For some of the treatment data the points have been slightly displaced along the abscissa for clarity.

Also, addition of the low pH buffer (MES) to the higher pH solutions had no effect on Zn action. These results are consistent with the reported negligible binding of divalent cations to these buffers (Ferguson, Braunschweiger, Braunschweiger, Smith, McCormick, Wasmann, Jarvis, Bell, and Good, 1966). These experiments were constrained to pH values 7.5 and lower because the complex formed between Zn and OH ions becomes significant at higher pHs.

We found that a simple model (see Methods) derived from standard enzyme kinetics can account for the inhibition of the Zn effect by H ions seen in Fig. 4. In this

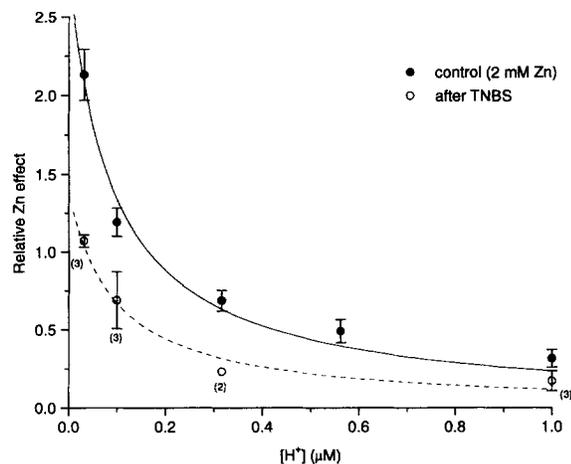


FIGURE 6. Reduction of Zn effect after treatment with the amino group reagent, TNBS. The 2 mM Zn data of Fig. 4 are replotted (*filled circles*) along with the competitive inhibition model fit (*solid line*). Data obtained after treatment with TNBS are plotted as open circles. Numbers in parentheses are the number of experiments with TNBS treatment. Standard error bars plotted for data with three measurements. The dashed line represents the model fit scaled by 0.5. The

GFL cell current kinetics were slowed by TNBS treatment as are axon channel kinetics (Spires and Begenisich, 1992; Nealey, T., S. Spires, R. A. Eatock, and T. Begenisich, manuscript in preparation). Solutions of pH 9 in the absence of TNBS produced no irreversible effects.

competitive inhibition model, H and Zn ions compete in a mutually exclusive manner for a binding site on the K channel protein. We assumed that the relative Zn effect, as defined by Eq. 1, is proportional to the fraction of sites occupied by Zn. The lines in Fig. 4 are fits of Eq. 1 to the 2 mM (solid line) and 5 mM (dashed line) data. The fits are reasonably good descriptions of the data at both concentrations of Zn and yield approximately equal pK_i ($-\log K_i$) values of 7.2 and 7.1 for 2 and 5 mM Zn, respectively. The K_m values for Zn binding to the channel site were 4 and 11 mM for the fits to the 2 and 5 mM Zn data, respectively.

The pK values obtained were not specific to the competitive inhibition model. A fit of a noncompetitive inhibition model (see Methods) yielded slightly lower pK values of 7 for both the 2 and 5 mM Zn data. This model produced estimates of the binding constant, K_m , of 75–90 μM . Such low values are not consistent with the data of Gilly and Armstrong (1982a) that showed that the effects of Zn did not saturate until near 20 mM.

The pK_i values obtained from the model fits are close to pK values for histidyl residues in proteins. Treatment of squid axon K channels with histidyl-modifying reagents slows channel activation kinetics with little or no effect on deactivation (Spires and Begenisich, 1990)—results similar to those produced by Zn. Consequently, we investigated the ability of the histidyl-modifying reagents DEP and rose bengal (see Methods) to alter the actions of Zn on K channel activation kinetics. While these compounds slowed the intrinsic kinetics of GFL K channels as they do axon channel kinetics (Spires and Begenisich, 1990), the results in Fig. 5 (open squares) show that treatment with these reagents had no effect on Zn activity compared with untreated cells (filled circles). This figure also shows that treatment with the sulfhydryl reagent *p*-CMPSA (open triangles) was without effect on Zn activity.

In contrast to the negative results with histidyl and sulfhydryl reagents, the data in Fig. 6 show that treatment with an amino group reagent, TNBS, reduces the ability of Zn to alter K channel kinetics. The filled circles are the 2 mM Zn data from untreated cells already seen in Figs. 4 and 5. The data identified by open circles are from cells treated with 5 mM TNBS for 10 min at pH 9. While we did not extensively examine the issue of the reversibility of TNBS treatment, the reduced sensitivity to Zn persisted for > 30 min.

The solid line in this figure is the fit of the competitive inhibition model for 2 mM Zn from Fig. 4. The dashed line is this same curve simply scaled by 0.5, as if treatment rendered half the channels insensitive to Zn.

We do not know if a more extensive TNBS treatment would completely eliminate the Zn effect. Our choice of treatment conditions was a compromise between conditions that produced significant effects and more extensive treatment that seemed to make the cells fragile and increased electrical leak currents. A milder treatment (2.5 mM TNBS at pH 8.2 for 10–20 min) was significantly less effective at inhibiting Zn action. The relative effect of 2 mM Zn at pH 7.5 after this milder treatment was 1.51 ± 0.14 (SEM, $n = 3$), midway between the untreated, control value of 2.1 ± 0.16 (7) and the 1.07 ± 0.04 (3) value after the more extensive treatment.

DISCUSSION

Our observations of the actions of external Zn ions on the gating kinetics of squid neuron, delayed rectifier K channels are consistent with those of Gilly and Armstrong (1982*a*) on squid axon K channels. We agree with those authors that the effects cannot result from divalent cation interactions with diffuse surface charges. Indeed, with the experimental conditions used here, little or no such interaction of Zn with surface charges is expected for several reasons: (*a*) analysis of Ca-induced shifts of K channel conductance suggests that the voltage sensors of these channels are in a region of relatively low surface charge density, at least compared with Na channels (Begenisich, 1975); (*b*) the high ionic strength and large concentration (60 mM) of Ca and Mg ions in our external solutions would tend to "screen" much of the surface charge density; (*c*) the low concentration (2 mM) of Zn ions used would not be expected to produce much change in surface potential unless there was high specific binding of these ions to the surface charges. It may be that the K channel gating apparatus is influenced by fixed surface charges under other experimental conditions.

Since divalent cations appear to have a rather direct effect on K channel gating, important information on the structure/function relation of these channels includes data on the chemical nature of divalent cation effects. We found that H and Zn ions compete for binding to the K channels in squid neurons. There appears to be a single ionizable chemical group (over the pH range of 6.0–7.5) with a pK of ~ 7.2 (Fig. 4).

Our results with specific amino acid reagents suggest that amino groups may play a role in divalent cation binding. Our data with histidyl and sulfhydryl reagents do not allow us to conclude that there are no histidyl or sulfhydryl residues involved in the interactions of divalent cation with the channel. The lack of effect of these reagents may simply be because the site is not accessible to these reagents. However, these reagents are able to modify channel kinetics and are no larger than TNBS.

The ability of TNBS to modify the actions of Zn suggests the involvement of amino groups in divalent cation binding. TNBS has been shown to be an extremely specific and useful reagent for modifying amino groups. Although it also reacts with sulfhydryl residues, the *S*-trinitrophenyl derivative is unstable and the original sulfhydryl group is spontaneously restored (Means and Feeney, 1971; Lundblad and Noyes, 1984). The irreversibility (over a 30-min time course) of the TNBS modification we observed is consistent with amino group modification. This conclusion is further strengthened by the lack of effect of the sulfhydryl reagent *p*-CMPSA and of DEP, which can sometimes also modify sulfhydryl groups.

The simplest interpretation of our results is that the divalent cation binding site is a single amino group with an apparent pK_a near 7.2 and that divalent cations bind to the unprotonated (neutral) form. Since TNBS does not modify arginine residues, this group is either the ϵ -amino of a lysine or a terminal amine. Terminal amino group pK_a values are usually below 8 and values near 7.6 are common (Means and Feeney, 1971; Lundblad and Noyes, 1984)—not far from our pK₁ values near 7.2. However, since consensus folding models for K channels place the terminal amine of each subunit on the inner side of the membrane, we suggest that the amino group involved in divalent cation binding is the ϵ -amino of a lysine residue. The pK_a of the ϵ -amino group of lysine is usually >9 but much lower values may be expected if

positive residues (e.g., arginine) are nearby. The reported values cover an extremely wide range including a value as low as 5.9 (Schmidt and Westheimer, 1971).

The amino group involved in divalent cation binding is not likely to be in the channel pore because Gilly and Armstrong (1982a) showed that Zn had little effect on the instantaneous current-voltage relation of K channels in squid axons. While we have not directly examined this issue in GFL cell, the actions of Zn on the steady-state and "tail" currents (e.g., Fig. 1) suggest little or no voltage-dependent block of the pore.

The amino group involved in divalent cation binding is not likely to be the same amino group(s) that we have implicated in K channel gating (Spires and Begenisich, 1992). This conclusion is based on the very extensive TNBS treatment (5 mM at pH 9 for 10 min) needed to inhibit Zn activity. Maximum effects on gating kinetics of K channels of both squid axons (Spires and Begenisich, 1992) and GFL neurons (data not shown) are produced after 3–5-min treatments with only 2 mM TNBS at pH 9. Since Freedman and Radda (1968) have shown that the intrinsic reactivity of TNBS with amino groups increases with the group pK_a , our finding of a low reactivity of a group with a pK_a of 7.2 is not unexpected.

The actions of Zn observed here are not restricted to squid neuron or axon K channels. The activation kinetics of frog muscle delayed rectifier K channels are slowed 10-fold by 0.1 mM Zn (Stanfield, 1975). Squid axon Na channel kinetics are altered by Zn ions in much the same way as are K channel kinetics except that higher Zn concentrations are required (Gilly and Armstrong, 1982b). Non-voltage-gated channels are also sensitive to Zn. Skeletal muscle Cl flux is inhibited by Zn ions (Spalding, Taber, Swift, and Horowicz, 1990). Zn ions interact with excitatory and inhibitory amino acid-activated channels (Peters, Koh, and Choi, 1987; Westbrook and Mayer, 1987) and may play a physiological role in synaptic transmission in some parts of the central nervous system (Xie and Smart, 1991).

The identification of the particular amino acid(s) important for divalent cation binding is best done with site-directed mutagenesis techniques on a channel whose amino acid sequence has been determined. To this end, we have begun to examine the actions of Zn on *Shaker* A channels expressed in baculovirus-infected insect cells (Klaiber, Williams, Roberts, Papazian, Jan, and Miller, 1990). Our preliminary results (Spires and Begenisich, 1991) show that Zn slows activation (and inactivation) kinetics in this channel just as in the delayed rectifier K channel. Moreover, the Zn effect on *Shaker* channels is antagonized by low pH solutions and TNBS treatment slows the channel activation (and inactivation) kinetics. Finally, TNBS inhibits Zn action on *Shaker* channels. Thus, a divalent cation binding site with similar chemical properties may be common to many types of K channels.

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