Modulation of Potassium Channel Gating by External Divalent Cations

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ABSTRACT We have examined the actions of Zn^{2+} ions on *Shaker* K channels. We found that low (100 μM) concentrations of Zn^{2+} produced a substantial (~threefold) slowing of the kinetics of macroscopic activation and inactivation. Channel deactivation was much less affected. These results were obtained in the presence of 5 mM Mg^{2+} and 4 mM Ca^{2+} in the external solution and so are unlikely to be due to modification of membrane surface charges. Furthermore, the action of 100 μM Zn^{2+} on activation was equivalent to a 70-mV reduction of a negative surface potential whereas the effects on deactivation would require a 15-mV increase in surface potential. External H^+ ions reduced the Zn-induced slowing of macroscopic activation with an apparent pK of 7.3. Treatment of *Shaker* K channels with the amino group reagent, trinitrobenzene sulfonic acid (TNBS), substantially reduced the effects of Zn^{2+}. All these results are qualitatively similar to the actions of Zn^{2+} on squid K channels, indicating that the binding site may be a common motif in potassium channels. Studies of single *Shaker* channel properties showed that Zn^{2+} ions had little or no effect on the open channel current level or on the open channel lifetime. Rather, Zn^{2+} substantially delayed the time to first channel opening. Thus, K channels appear to contain a site to which divalent cations bind and in so doing act to slow one or more of the rate constants controlling transitions among closed conformational states of the channel.

INTRODUCTION

It has long been known that divalent cations influence the operation of ion channels including voltage-gated K channels. Following the suggestion of A. F. Huxley (Frankenhaeuser and Hodgkin, 1957), the actions of divalent cations on channel gating have been described by various forms of surface charge theory (Hille, 1992). This simple formalism has been so successful in quantitatively describing many of the actions of divalent cations that it seems very likely that there are some fixed charges that can influence the gating of K channels.

But there are also many observations that are not easily reconciled with anything but very complicated versions of surface charge theory and so suggest a more direct role of these ions in channel gating (Armstrong and Lopez-Barneo, 1987; Begenisich,

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1988; Grissmer and Cahalan, 1989; Armstrong and Miller, 1990; Hille, 1992). The effects of increases in external Ca^{2+} or small concentrations of added Zn^{2+} on K channel gating kinetics cannot be described by a shift of voltage-dependent parameters as expected from surface charge models (Gilly and Armstrong, 1982; Armstrong and Matteson, 1986). Rather, it appears that for delayed rectifier K channels in squid neurons, Zn^{2+} substantially slows channel opening (activation) by a constant amount at all potentials while simultaneously slightly speeding channel closing (Spires and Begenisich, 1992*a*). In these cells, external H^+ ions compete with Zn^{2+} with an apparent inhibitory pK_a near 7.2 and an amino group modifying reagent significantly reduces the magnitude of the Zn^{2+} effects (Spires and Begenisich, 1992*a*).

If divalent cations play an important, direct role in K channel function, it might be expected that the actions of Zn^{2+} described above for squid channels would be a common finding. Therefore, we have investigated the actions of Zn^{2+} on *Shaker* K channels expressed in an insect cell line. We found that the effects of this divalent cation on *Shaker* channels share all the properties seen in squid neurons: differential actions of Zn^{2+} on channel activation and deactivation, competition between Zn^{2+} and external H^+ ions, and reduction of the Zn^{2+} effects after amino group modification.

To investigate the mechanism of action of Zn^{2+} on channel function, we examined the actions of this cation on some single-channel properties. We found that Zn^{2+} had little or no effect on single-channel current or mean channel open times but substantially increased the time to first channel opening.

The results of this study suggest that the divalent cation binding site (as probed with Zn^{2+} ions) is a common property of voltage-gated K channels. It appears that divalent cations bind to an external site on these channels and stabilize one or more of the closed channel conformations, thus, delaying channel opening. The ϵ-amino group of a lysine group may be part of or quite near the divalent cation binding site.

A report of some of these results has appeared in abstract form (Begenisich and Spires, 1991*a*,*b*).

MATERIALS AND METHODS

Expression System

Drosophila Shaker (H4) K channels were expressed with the baculovirus (*Autographa californica*) insect (*Spodoptera frugiperda*) cell line Sf9 system (Klaiber, Williams, Roberts, Papazian, Jan, and Miller, 1990). We used standard methods for growing and maintaining cells and for propagating the recombinant virus (Summers and Smith, 1987). Cells to be used for electrophysiological recording were grown on glass coverslips and were used ~24 (for single-channel measurements) or 48 h after viral infection.

Macroscopic K channel currents from Sf9 cells were obtained with the whole cell configuration of the patch clamp technique. Owing to the large size of the currents expressed in the Sf9 cells, we used a circuit of our own design that allowed compensation for 92–95% of the measured series resistance (Spires and Begenisich, 1992*a*). When filled with internal solution, the patch electrodes (fabricated with Corning model 8161 glass, Garner Glass, Claremont, CA) had resistance values of 1.5–2 MΩ.

Single-channel currents were measured from cell-attached patches. For these measurements, we used conventional patch clamp electronics with a 10 GΩ headstage. The pipettes were

fabricated with Corning model 7052 glass and had resistance values of 2–10 MΩ when filled with the solution described below. Most of the capacity current was subtracted as described by Sigworth (1983). Residual capacity current was subtracted from single-channel records with a template obtained under identical voltage clamp conditions. This template was made either from the average of many records with no channel openings or by fitting an exponential function to a single record with no openings.

Membrane currents were acquired with a 12-bit analogue/digital converter controlled by a laboratory personal computer. The voltage clamp pulses were generated by a 12-bit digital/analogue converter controlled by the computer system. The macroscopic current records were usually blanked for ~35 μs, eliminating most or all of the capacitative transient (Spires and Begenisich, 1989). For some macroscopic current measurements, residual linear capacity and leakage currents were subtracted using a −P/4 procedure (Bezanilla and Armstrong, 1977). Currents were filtered at 10 or 20 kHz (macroscopic currents) or at 2 or 5 kHz (unitary currents) with a four-pole Bessel filter. All experiments were performed at room temperature (22–24°C).

Solutions

For measurements of macroscopic currents, the external solution consisted of (in millimolar): 122 NaCl, 10 KCl, 5 $MgCl_2$, 4 $CaCl_2$, 5 glucose, and 10 MOPS, pH 7.2 (with NaOH). The high concentration of divalent cations was used to minimize possible effects of membrane surface charges (see Results). The internal (pipette) solution contained (in millimolar): 60 KF, 50 KCl, 1 $MgCl_2$, 10 EGTA, and 10 MOPS; the pH was adjusted to 7.2 with KOH which produced a final K^+ concentration of ~135 mM.

We used trinitrobenzene sulfonic acid (TNBS, P-3402, Sigma Chemical Co., St. Louis, MO) to modify *Shaker* K channels with techniques similar to those used for amino group modification of squid axon and neuron delayed rectifier K channels (Spires and Begenisich, 1992*a*,*b*). Because TNBS reacts with the neutral form of amino groups, treatment was done in a solution similar to the normal external solution but with elevated pH (pH 9.0 buffered with 10 mM CHES).

The bath solution used for the on cell, single-channel measurements consisted of (in millimolar): 125 K-aspartate, 1 $MgCl_2$, 40 glucose, and 10 MOPS, pH 7.2. Because the K^+ concentration of this solution is quite near the measured internal K^+ concentration of Sf9 cells (He, Wu, Knauf, Tabak, and Melvin, 1993), the membrane voltage should have been near zero and so would not bias the patch potential. The pipette solution for the on cell patches was the same as the external solution used for the macroscopic current measurements except that it did not contain K^+.

Data Analysis

The quantitative analysis of our data included the fitting of exponential time functions to macroscopic current records. As illustrated in the inset of Fig. 2, the later part of the macroscopic current produced by a depolarizing voltage pulse was fit well by a nonmonotonic, two exponential time function: $A_1 \cdot \exp(-t/\tau_1) + A_2 \cdot \exp(-t/\tau_2) + A_3$. To produce a nonmonotonic function, the first amplitude term (A_1) was forced to be negative; the second term was positive.

Fits of this biexponential and all other theoretical functions to the data used the "simplex" algorithm (Caceci and Cacheris, 1984). While this algorithm assures convergence, like any nonlinear fitting procedure (especially with several parameters), there is some variability in the values of the fitted parameters. This variability shows up as "noise" in the time constant values as can be seen, for example, in Fig. 2 *B*. Part of our analysis includes computing the ratio of time constants obtained in the presence and absence of Zn^{2+} (e.g., Fig. 3) and the "noise" in the time constant values is amplified in the ratio.

Ionic tail currents were 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.

Analysis of single-channel current data included current amplitude, open time, and first latency histograms. Single-channel current levels were obtained by fitting a Gaussian function to the amplitude histograms.

The number of channels in a patch was determined by observing the current amplitude with large voltage clamp pulses (to +50 mV). The patches used in this study contained one or two channels. Open times were determined from idealized records with a threshold of 50% of mean channel current amplitude (Colquhoun and Sigworth, 1983). When the activity of two channels overlapped, the open durations were randomly assigned (Aldrich, Corey, and Stevens, 1983). Open time histograms were fit with an exponential function to estimate the mean open time.

Time to first opening (first latency) histograms were also determined from idealized records. As with open time determinations, the presence of more than a single channel in a patch complicates the interpretation of first latency data since it is impossible to tell which channel was the first to open. We corrected the first latency distributions of the two channel patches as has previously been described (Patlak and Horn, 1982; Aldrich et al., 1983).

Shaker Channels without Inactivation

To examine some of the actions of Zn^{2+} on *Shaker* channel gating in the absence of inactivation, we expressed the deletion mutant ShB Δ6-46 (Hoshi, Zagotta, and Aldrich, 1990) in *Xenopus* oocytes. We used standard methods for preparation and injection of cRNA (e.g., see Hoshi et al., 1990). The currents of expressed channels were recorded with a two microelectrode voltage clamp. The oocytes were bathed in a high divalent cation solution (analogous to that used for the Sf9 cells [see above]) containing (in millimolar): 75 Na, 10 K, 4 Ca, 5 Mg, 10 MOPS (pH 7.2). To minimize endogenous oocyte outward currents, the major anion was methanesulfonate.

RESULTS

Zn^{2+} Slows Macroscopic Current Kinetics

Fig. 1 illustrates the basic actions of low concentrations of Zn^{2+} on macroscopic *Shaker* K channel currents. Fig. 1 *A* shows superimposed current records in response to 40-ms pulses to membrane voltages of −20, 0, 20, and 40 mV. Fig. 1 *B* shows that 100 μM Zn^{2+} slowed both the activation and inactivation phases of the macroscopic currents. These actions of Zn^{2+} were readily reversible as seen in the recovery data (Fig. 1 *C*). The P/4 procedure (see Materials and Methods) was not used for the records of Fig. 1 so the data shown represent total cell current. While not investigated in detail, inspection of the records in Fig. 1 suggests that Zn^{2+} had little or no effect on "leakage currents."

A quantitative measure of the kinetics of the macroscopic currents was obtained as described in Materials and Methods by fitting a biexponential function to the current records. An example of the result of this procedure is illustrated in the inset of Fig. 2. Shown in the inset are current records in response to step depolarizations to +30 mV, before and during exposure to 100 μM Zn^{2+}. The arrows in each record denote the beginning and ending time points for the biexponential fitting procedure. Superimposed on the data (*dots*) are the best-fit exponential functions (*lines*) which appear to be reasonable representations of the data.

The slower time constant derived from the biexponential fitting procedure is a measure of macroscopic inactivation kinetics and these values obtained at several membrane voltages are plotted in Fig. 2 *A*. The open symbols represent time constants in the absence of 100 μM Zn^{2+} and the filled circles show that this concentration of Zn^{2+} greatly slowed macroscopic inactivation except at the most depolarized potentials. As illustrated in Fig. 2 *A*, the macroscopic inactivation time constant of *Shaker* K channels approached a limiting voltage-independent level. This behavior is a reflection of the underlying single-channel activity in which depolarized voltages drive channels into an open state from which they inactivate in a voltage independent manner (Hoshi et al., 1990; Zagotta and Aldrich, 1990). The complexity of this time constant makes it a poor choice for further analysis except to note that the converging of the Zn^{2+} and control values at large positive potentials suggests that Zn^{2+} had little or no effect on the underlying single-channel inactivation process.

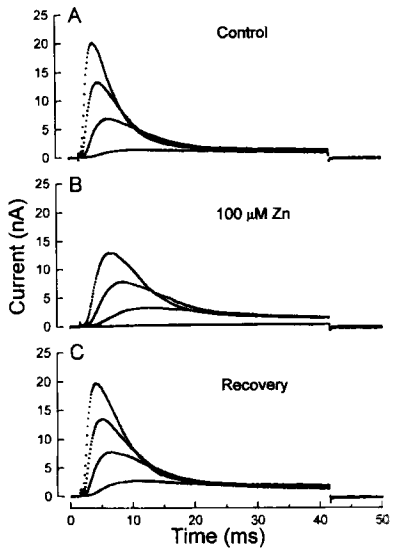

FIGURE 1. Actions of Zn^{2+} on macroscopic ionic currents. Superimposed currents in response to 40-ms depolarizations to −20, 0, 20, and 40 mV before (*A*), during (*B*), and after (*C*) exposure to 100 μM Zn^{2+}. These data were obtained without using the P/4 procedure (see Materials and Methods).

The voltage dependence of the faster time constant obtained from the biexponential fitting process is illustrated in Fig. 2 *B*. These values are labeled "activation" and include data in the absence (○) and presence (●) of 100 μM Zn^{2+} and show that Zn^{2+} slowed this parameter at all voltages examined. The control values (○) are quite similar to time constants associated with the late phase of macroscopic currents from the *Shaker* inactivation deletion mutant, ShB Δ6-46 (e.g., Fig. 7 of Zagotta, Hoshi, Dittman, and Aldrich, 1994) indicating that the presence of inactivation does not significantly interfere with estimates of channel activation.

One of the characteristics of the kinetic effects of Zn^{2+} on delayed rectifier K channels in squid neurons and axons is that, in contrast to the large slowing of channel activation, Zn^{2+} slightly speeds channel closing determined from "tail" currents (Gilly and Armstrong, 1982; Spires and Begenisich, 1992*a*). With the conditions used here, *Shaker* K channel tail currents were well described by a single

exponential time function (data not shown). The time constants obtained from such fits at potentials from −140 to −70 mV in the absence (□) and presence (■) of 100 μM Zn^{2+} are shown in Fig. 2 *B*. While Zn^{2+} did not speed channel closing as in squid K channels, there was certainly a much smaller effect on *Shaker* K channel deactivation than activation.

The presence of inactivation should contribute little to the "tail" currents measured at potentials more negative than −60 mV. Furthermore, the control deactivation time constants values of Fig. 2 *B* (□) are similar to data from the inactivation deletion

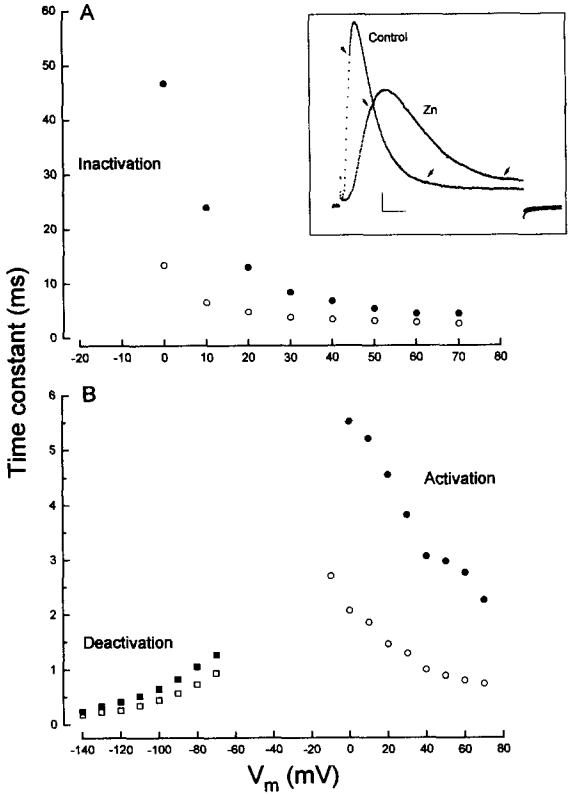

FIGURE 2. Quantitative assessment of the actions of Zn^{2+} on time constants. (*Inset*) Macroscopic currents (*dots*) in the presence and absence of Zn^{2+} superimposed with the fitted biexponential functions (*solid lines*). The arrows mark the range over which the fit was made. Calibration: 2 nA, 5 ms. (*A*) Macroscopic inactivation time constant in the absence (○) and presence (●) of 100 μM Zn^{2+}. (*B*) Activation (*circles*) and deactivation (*squares*) time constants in the absence (*open symbols*) and presence (*filled symbols*) of 100 μM Zn^{2+}. A 15 ms pulse to +40 was used to open channels for measurement of deactivation (*tail*) currents.

mutant, ShB Δ6-46 (e.g., Fig. 12 of Zagotta et al., 1994). Thus, the presence of inactivation does not appear to have compromised the estimation of channel deactivation.

In many studies on voltage-gated ion channels, the actions of divalent cations were described in terms of surface charge theory (e.g., see Frankenhaeuser and Hodgkin, 1957; Gilbert and Ehrenstein, 1969). In the context of such models, added divalent cations interact with negative external surface charges to reduce the transmembrane potential and so produce a "shift" of the observed voltage dependence of ion channel properties. The data in Fig. 2 show that such an interpretation is not appropriate for the results presented here. As described in Materials and Methods, these experi-

ments were done with a total of 9 mM of Ca^{2+} and Mg^{2+} ions. This large background of divalent cations would, to a large extent, reduce the external surface potentials and so prevent the low concentrations of Zn^{2+} used here from producing any further surface charge effects. The ~70 mV apparent Zn^{2+}-induced shift of the activation time constant seen in Fig. 2 is much too large to be consistent with any reasonable surface charge explanation. Furthermore, the apparent shift of the deactivation time constant is much smaller (near 15 mV) and in the opposite direction. That is, the effects of Zn^{2+} on activation would be described as a 70-mV reduction of a negative external surface potential and deactivation by a 15-mV more negative surface potential. Thus, it seems unlikely that, under the conditions used here, Zn^{2+} acts to alter channel kinetics by interacting with membrane surface charges.

To more clearly examine the voltage dependence of Zn^{2+} action on the time constants, we computed the ratios of the time constants in Zn^{2+} to control values. The results of these computations are plotted in Fig. 3. In this figure, a value of 1 represents no effect of Zn^{2+} and values greater than 1 indicate Zn-induced kinetic slowing.

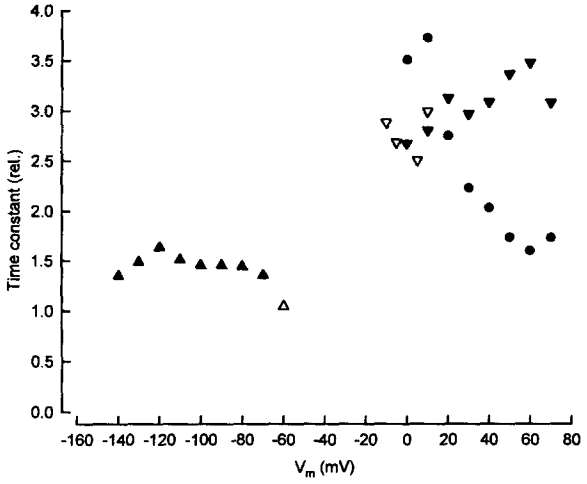

FIGURE 3. Effects of Zn^{2+} on macroscopic time constants. The ordinate is the ratio of the time constants in 100 μM Zn^{2+} over control values for the activation (▼) inactivation (●), and deactivation (▲) time constant data of Fig. 2. Also shown are the effects of 500 μM Zn^{2+} on the deactivation time constant (△) and activation time constants (▽) for the *Shaker* mutant (ShB 6-46) that lacks inactivation.

As seen in Fig. 3 (●), Zn^{2+} appeared to slow the macroscopic inactivation time constant in a voltage-dependent manner: a ~3.5-fold slowing near 0 mV and only a ~50% slowing at the more depolarized potentials. However, as discussed above, this finding may be because this parameter includes both voltage-dependent and voltage-independent microscopic rate constants (Hoshi et al., 1990; Zagotta and Aldrich, 1990).

In this experiment, Zn^{2+} slowed the activation time constant (▼) by about a factor of 3 to 3.5 with little systematic voltage dependence. This lack of voltage dependence is seen more clearly in Fig. 5 in which average data from several experiments are presented. The deactivation time constant data in Fig. 3 (▲) shows that Zn^{2+} slowed this parameter by ~50% in a voltage-independent manner.

As discussed above, the *Shaker* channel inactivation process is not likely to have significantly distorted the conclusions about the actions of Zn^{2+} on channel activation and deactivation. However, to more directly address this issue, we obtained data from the *Shaker* deletion mutant, ShB Δ6-46, that lacks (fast) inactivation (Hoshi et al., 1990). In the absence of inactivation, a single exponential function is sufficient to describe the latter part of *Shaker* channel activation (Zagotta et al., 1994). We fit such a function to ShB Δ6-46 currents in the presence and absence of Zn^{2+} and the ratio of the time constants are included in Fig. 3 (▽). These data show that Zn^{2+} produced about the same approximate threefold slowing of activation as in channels with inactivation intact (▼). This figure also illustrates the action of Zn^{2+} on the deactivation time constant (▲) of ShB Δ6-46 and the results support the conclusion that this divalent cation exerts a much larger effect on *Shaker* channel activation than deactivation.

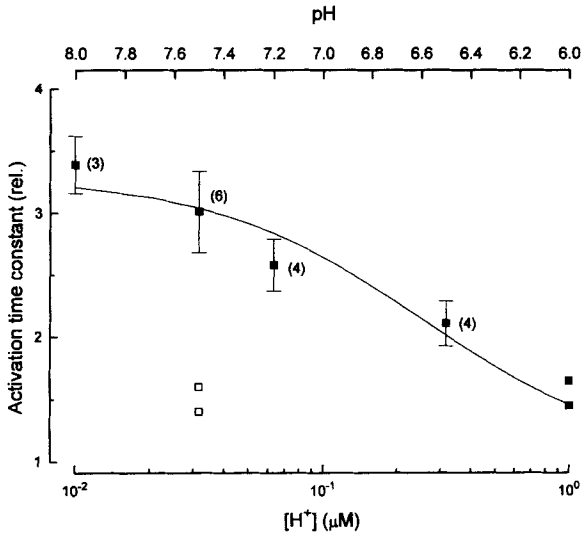

FIGURE 4. Titration of Zn^{2+} effect with external H^+ ions. The ordinate is the ratio of the activation time constant in 100 μM Zn^{2+} over control values, averaged over the voltage range from 20 to 50 mV. Average values with SEM limits are shown along with the number of experiments. Data points without error bars represent single measurements. The data from two cells at pH 7.5 indicated by (□) appeared to be quite different than the data from the other six cells at this pH and so were not included in the mean value at this pH. The solid line is the result of fitting a standard competitive inhibition equation $y = y_{max}/\{1 + K_m/[Zn^{2+}](1 + [H^+]/K_I)\} + 1$ to the data (except for the values indicated by □). In this equation, K_m represents the binding constant of Zn^{2+} with the site and K_I is the inhibitory binding affinity for H^+ ions. The K_m value obtained from the fit was 26 μM and the inhibitory pK value was 7.3.

Zn^{2+}-induced Kinetic Slowing and External pH

As described in Introduction, the effect of Zn^{2+} on squid K channel currents is a function of external solution pH (Spires and Begenisich, 1992*a*). The Zn^{2+}-induced slowing of the activation time constant of *Shaker* K channels was also pH dependent as can be seen in Fig. 4. The ordinate in this figure is the ratio of the activation time constant in 100 μM Zn^{2+} to control values and, as in Fig. 3, a value of 1 represents no effect. To reduce some of the "noise" in this ratio (see Fig. 3 and Materials and Methods), the data in Fig. 4 have been averaged over the voltage range from 20–50

mV. This averaging procedure is justified by the lack of voltage dependence of the effect of Zn^{2+} on the activation time constant over this voltage range (see Fig. 5).

It appears from the data of Fig. 4 that increased H^+ ion concentration inhibited the action of Zn^{2+} on *Shaker* channel activation kinetics. In an external solution of pH 8, 100 μM Zn^{2+} slowed the activation kinetics by a factor of 3.4 and by a significantly ($p < 0.01$) smaller value of 2.1 at pH 6.5. The solid line in this figure is the result of fitting a competitive inhibition function to the data represented by the filled squares (Fig. 4, legend). The inhibitory pK value obtained from this fit was 7.3, very close to the value of 7.1–7.2 for squid K channels (Spires and Begenisich, 1992*a*).

The fitting procedure also produced a measure of the binding affinity, K_m, of Zn^{2+} to the site controlling channel slowing. Since only a single concentration (100 μM) of

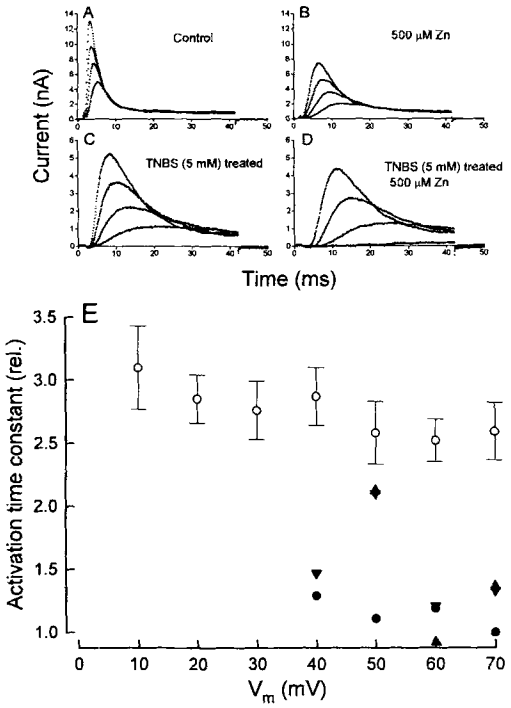

FIGURE 5. Effects of Zn^{2+} after extensive treatment with TNBS. (*A*) *Shaker* currents in the absence and presence (*B*) of 500 μM Zn^{2+}. Currents from another cell after a 7-min treatment with 5 mM TNBS in the absence (*C*) and the presence (*D*) of Zn^{2+}. The currents in all panels were recorded during depolarizations to 30, 50, 70, and 90 mV. (*E*) The ordinate is the activation time constant in 500 μM Zn^{2+} over the control values. The data shown are from six control (untreated) cells (○) and three cells (*filled symbols*) treated with TNBS. Each treated cell is represented by a different filled symbol.

Zn^{2+} was used for this procedure, the value of 26 μM obtained must be considered a rough estimate. However, the effects of 500 μM Zn^{2+} (○, Fig. 5 *E*) were essentially the same as those of 100 μM (Fig. 4), consistent with a K_m value much less than 100 μM.

Zn^{2+} Effect after TNBS Treatment

Another property of the Zn^{2+} binding site on squid K channels is the involvement of amino groups as judged by the reduction of the Zn^{2+} effect after cells are treated with the amino group modifying reagent, trinitrobenzene sulfonic acid (TNBS) (Spires and Begenisich, 1992*a*). Fig. 5 illustrates the results of experiments designed to

examine this issue in *Shaker* K channels. Fig. 5, *A* and *B*, shows *Shaker* K channel currents from an untreated cell in the absence and presence of 500 μM Zn^{2+}, respectively. As with the lower Zn^{2+} concentrations of Figs. 1–3, a substantial Zn-induced slowing of the channel activation kinetics is apparent.

Extensive TNBS treatment (5 mM for 7 min) substantially slowed *Shaker* K channel kinetics (Fig. 5 *C*) and caused channel activation to occur at more depolarized potentials (not shown). These are the same modifications that TNBS produces in squid axon and neuron K channels (Spires and Begenisich, 1992*a*,*b*). An example of the action of 500 μM Zn^{2+} on currents from *Shaker* channels modified by TNBS is shown in Fig. 5 *D*. A quantitative analysis of the effects of Zn^{2+} on the channel activation kinetics is illustrated in Fig. 5 *E*.

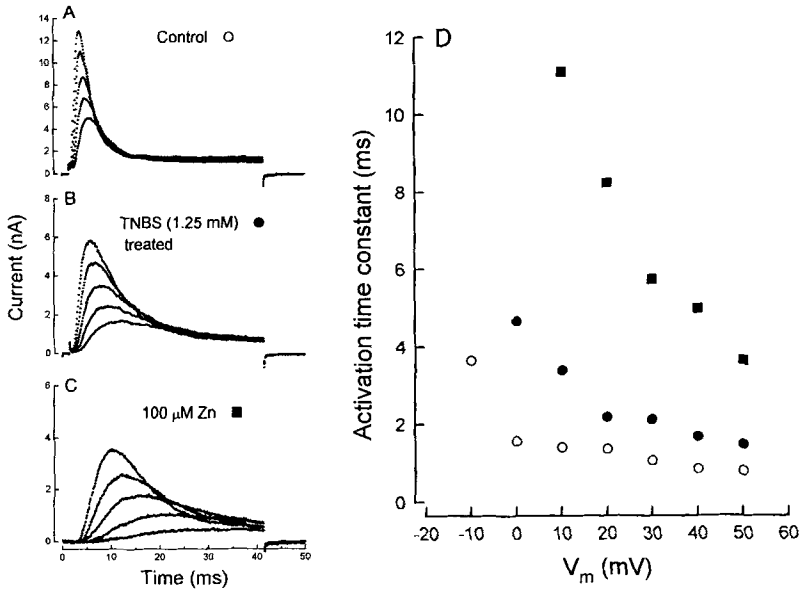

FIGURE 6. Effects of Zn^{2+} after mild treatment with TNBS. Currents elicited by depolarizations to 10, 20, 30, 40, and 50 mV before (*A*) and after (*B*) treatment with 1.25 mM TNBS for 8 min. (*C*) Currents from the same cell after TNBS treatment but in the presence of 100 μM Zn^{2+}. (*D*) Activation time constant before (○) and after (●) TNBS treatment and after treatment in the presence of 100 μM Zn^{2+} (■). The TNBS treatment slowed the activation time constant at 0, 10, 20, 30, 40, and 50 mV by a factor of 3.0, 2.4, 1.6, 2.0, 2.0, and 1.9, respectively.

In Fig. 5 *E*, the ratios of the activation time constants in 500 μM Zn^{2+} over control values are shown at several membrane potentials. Data from six untreated cells (○) are included with data from three cells treated with TNBS (*filled symbols*). As we found with squid K channels, the inhibition of Zn-induced slowing of *Shaker* K channels required much more extensive TNBS treatment than was required to produce the TNBS-induced kinetic slowing (see below and Fig. 6). As a consequence, many Sf9 cells did not survive the treatment procedure but the activation kinetics of currents

from the three that did were substantially less affected by 500 μM Zn^{2+} than were the untreated cells (*filled symbols*).

As illustrated in Fig. 5, TNBS treatment substantially slowed the activation kinetics and may have made the channels resistant to further slowing by any means, including Zn^{2+} ions. This was not the case as is illustrated by the experiment of Fig. 6. Currents recorded at several membrane potentials are illustrated in Fig. 6 *A*. After a moderate TNBS treatment (1.25 mM for 8 min), the kinetics were slowed (Fig. 6 *B*) similar to results of Fig. 5. However, unlike extensive treatment, the lower TNBS concentration did not alter the Zn-induced slowing (Fig. 6 *C*).

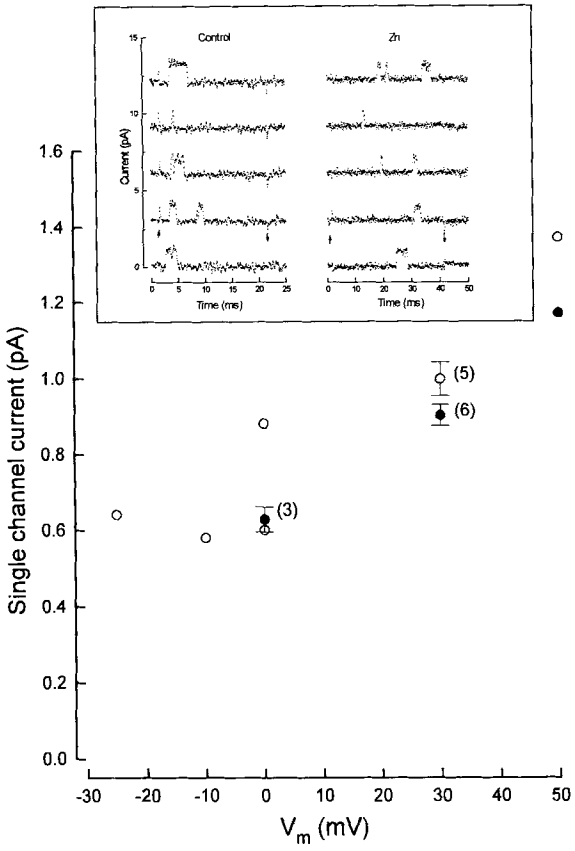

FIGURE 7. Single-channel current level and Zn^{2+}. (*Inset*) Selected current records in response to a voltage step (*arrows*) to +30 mV for an on cell patch in the absence (*Control*) of Zn^{2+} and records (Zn) from another on cell patch with 500 μM Zn^{2+}. Note the time scale difference in the two sets of records. (*Main figure*) Single-channel current amplitude at several voltages obtained as described in Materials and Methods. Values are shown in the absence (○) and in the presence (●) of 500 μM Zn^{2+}. SEM limits and number of experiments are included for $N \geq 3$.

The activation time constants from the data of Fig. 6, *A–C*, are shown in *D* of this figure. The values obtained in the absence of Zn^{2+} (○) are very similar to those of Fig. 2. The mild TNBS treatment (●) produced a two- to threefold slowing of the activation time constant over the voltage range from 0 to 50 mV (see legend for details). In the three experiments with high TNBS concentration, the amount of slowing was also in the range of two- to threefold with a mean value of 2.9. In spite of similar large slowing of channel activation kinetics, the milder treatment with 1.25

mM TNBS did not alter the ability of Zn^{2+} to slow the channels by an additional factor of 3 (Fig. 6 *D*). The finding that *Shaker* K channel kinetics were modified by rather milder TNBS treatment than that needed to reduce Zn-induced slowing is consistent with similar observations on squid K channels (Spires and Begenisich, 1992*a*,*b*).

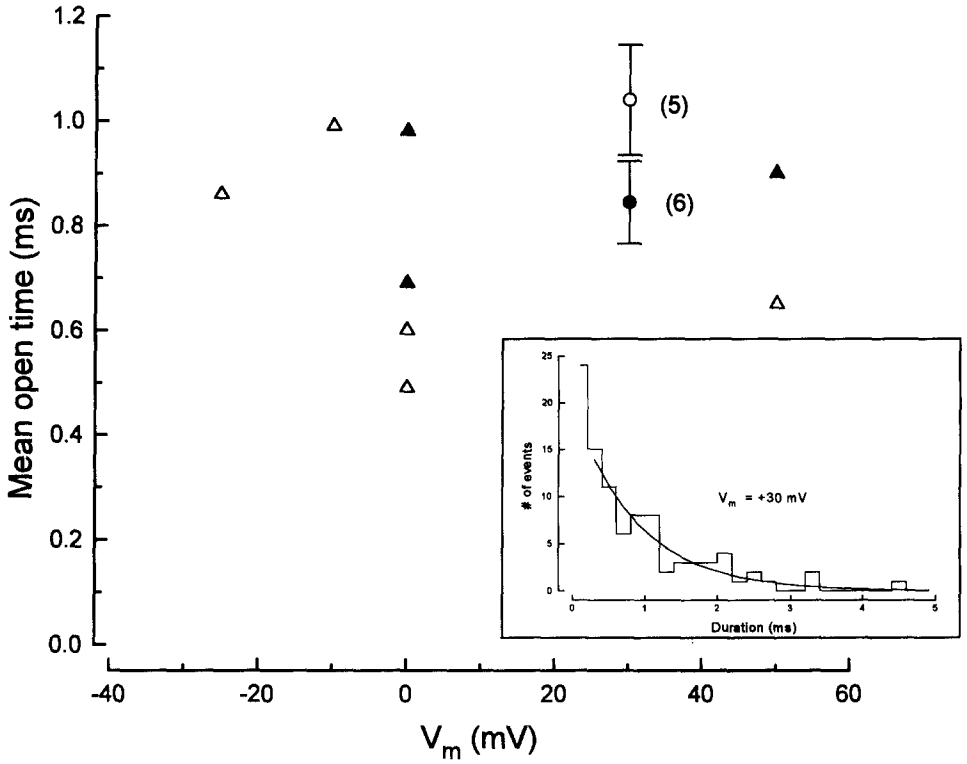

FIGURE 8. Channel open time and Zn^{2+}. (*Inset*) Open duration histogram at +30 mV for a single-channel patch in the absence of Zn^{2+}. The smooth curve is an exponential function fit to the histogram data (excluding the first bin) and has a time constant of 0.89 ms. This time constant represents the mean channel open duration. This and other values at various potentials are shown for control (*open symbols*) patches and for patches (*filled symbols*) exposed to 500 μM Zn^{2+}. SEM limits and number of experiments are included for $N \geq 3$.

Zn^{2+} and Single Shaker Channels

To investigate the mechanism by which Zn^{2+} alters K channel behavior, we measured some of the properties of single *Shaker* channels. Examples of some single-channel data are illustrated in the inset of Fig. 7. The left panel contains records obtained in the absence of Zn^{2+} during a 20-ms pulse to a potential of +30 mV. The right panel of the inset are currents in response to a 40-ms pulse to the same potential from another patch treated with 500 μM Zn^{2+}. The onset and termination of the voltage

pulses are marked by up and down arrows, respectively. Some uncompensated capacity current can be seen at some of the voltage transitions.

It appears from the records in the inset of Fig. 7 that Zn^{2+} had little effect on the single-channel current level. We determined the single-channel current levels at several voltages in the absence and presence of 500 μM Zn^{2+} and these are illustrated in the main part of Fig. 7. There appears to be at most a slight reduction in single-channel current produced by this treatment.

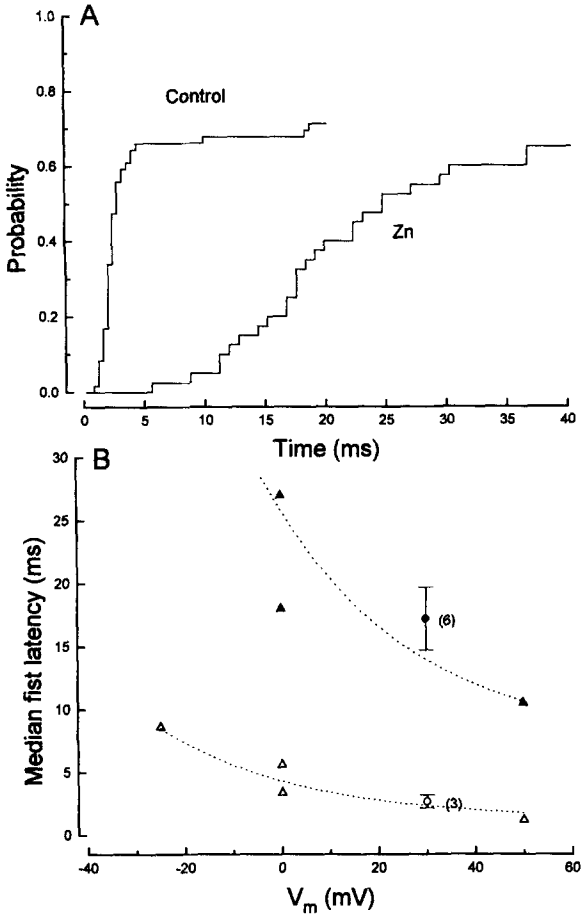

FIGURE 9. First latency and Zn^{2+}. (*A*) Cumulative first latency histograms from an on cell patch in the absence of Zn^{2+} and another patch exposed to 500 μM Zn^{2+}. V_m of +30 mV. (*B*) Median first latency values from patches in the absence (*open symbols*) and presence (*filled symbols*) of 500 μM Zn^{2+}. SEM limits and number of experiments are included for $N \geq 3$.

We also examined the actions of Zn^{2+} on the open times of *Shaker* channels. The inset in Fig. 8 contains a histogram of the open times measured at +30 mV from a single-channel patch. The data appear well fit by a single exponential function as has been noted previously for *Shaker* channels in native cells and as expressed in *Xenopus* oocytes (Zagotta and Aldrich, 1990; Zagotta, Hoshi, and Aldrich, 1989; Shao and Papazian, 1993). The time constant of this function is an estimate of the channel mean open time (MOT). MOT values in the absence (*open symbols*) and presence

(*filled symbols*) of 500 μM Zn^{2+} are shown for several membrane voltages in the main part of Fig. 8. As has been observed previously (Zagotta and Aldrich, 1990; Zagotta et al., 1989; Shao and Papazian, 1993), the MOT showed little voltage dependence. The data of Fig. 8 indicate that Zn^{2+} had little effect on the channel MOT.

It appears from the current records in the inset in Fig. 7 that Zn^{2+} might have caused *Shaker* channels to open later after the application of the voltage pulse. A quantitative measure of this parameter can be obtained by analyzing the latencies to first opening as described in Materials and Methods. Examples of cumulative first latency distributions in the presence and absence of 500 μM Zn^{2+} are presented in Fig. 9 *A*. It is clear that Zn^{2+} greatly delayed the time to channel opening.

The time at which the first latency distribution crosses half the final value (the median first latency) was obtained from data like that in Fig. 9 *A*. As previously observed for *Shaker* channels in native cells (Zagotta and Aldrich, 1990) and channels expressed in oocytes (Zagotta et al., 1989; Shao and Papazian, 1993), the median first latency values in the absence (*open symbols*) of Zn^{2+} were strongly voltage-dependent as can be seen in Fig. 9 *B*. The data obtained in the presence of 500 μM Zn^{2+} (*filled symbols*) show that Zn^{2+} substantially increased this strongly voltage-dependent parameter. The dotted line through the control data is a fit of a single exponential function of voltage and is a reasonable representation of the data. The dashed line drawn through the Zn^{2+} data is the same function multiplied by a factor of 6. This line is rather similar to the Zn^{2+} data and suggests that the actions of Zn^{2+} can be roughly described as a voltage-independent, sixfold increase of the median first latency.

DISCUSSION

Properties of Zn^{2+} Action on K Channels

The data in this study of the effects of Zn^{2+} on *Shaker* K channels can be summarized as follows: (*a*) Zn^{2+} substantially slowed the activation kinetics of macroscopic currents with a much smaller effect on deactivation kinetics; (*b*) the slowing of the activation time constant was essentially independent of membrane voltage; (*c*) these actions were unlikely to be mediated through interaction with nonspecific membrane surface charges; (*d*) increases in external H^+ ion concentration produced a reduction in the magnitude of the Zn^{2+} effect; (*e*) treatment of channels with TNBS substantially reduced the Zn^{2+} effect.

These results are similar to but not identical with those produced by Zn^{2+} on squid axon and squid neuron K channels. While the inhibitory pK of the Zn^{2+} effect in *Shaker* (7.3) was similar to the value found for squid channels (7.1–7.2), our estimates of the Zn^{2+} affinity for the two channels were quite different. The Zn-induced slowing of squid K channels occurs in the mM range of Zn^{2+} concentration (Spires and Begenisich, 1992*a*) and the effect saturates at concentrations near 20 to 40 mM (Gilly and Armstrong, 1982). In contrast, we estimate that the binding affinity of Zn^{2+} for *Shaker* channels is near 25 μM (see Fig. 4 and associated text) and the Zn-induced slowing appeared to saturate above 100 μM.

The kinetic effects of extracellular Zn^{2+} may be a general phenomenon associated with K channels. In a manner qualitatively similar to its actions on squid axon, squid

neuron, and *Shaker* K channels, Zn^{2+} slows the activation of frog muscle delayed rectifier K channels (Stanfield, 1975) and KV1 channels expressed in *Xenopus* oocytes (unpublished observations). Furthermore, these effects are not restricted to Zn^{2+} ions. Several other divalent cations act in a manner similar to Zn^{2+} including physiologically important Ca^{2+} ions (Armstrong and Matteson, 1986).

Mechanism of Zn^{2+} Action on Shaker K Channels

The data of Fig. 7 shows that Zn^{2+} had little or no effect on the single-channel current. This indicates that the reduction of the peak macroscopic currents seen, for example, in Fig. 1 and Fig. 2 (*inset*) was likely due to a differential effect of Zn^{2+} on channel activation and inactivation. In particular, the macroscopic inactivation time constant data of Fig. 2 and the MOT data discussed below indicate that Zn^{2+} had little or no effect on the microscopic inactivation rate constants. If this conclusion is correct, then it would be expected that Zn^{2+} would have no effect on the steady state current of channels that lacked inactivation. This is indeed what has been observed for squid delayed rectifier K channels (Gilly and Armstrong, 1982; Spires and Begenisich, 1992*a*).

The length of time a channel spends in the open state is governed by the rate constants leading out of that state. One of these will be the forward rate constant leading to the inactivated state and the others would include any backward rate constants connecting to other nonconducting closed states (Hoshi et al., 1990; Zagotta and Aldrich, 1990; Zagotta et al., 1989). The lack of voltage dependence of the MOT seen in the control data (Fig. 8) suggests that these rate constants have little voltage sensitivity. This result has been previously noted for both native and expressed *Shaker* K channels (Hoshi et al., 1990; Zagotta and Aldrich, 1990; Zagotta et al., 1989; Shao and Papazian, 1993). The lack of any large effect of Zn^{2+} on the channel MOT shows that the important actions of Zn^{2+} are not on transitions out of the open state.

The time a channel spends before first opening represents sojourns among closed conformational states. The voltage dependence of the median first latency (see Fig. 9) reflects the voltage dependence of the rate constants connecting these states (Zagotta and Aldrich, 1990; Zagotta et al., 1989; Shao and Papazian, 1993). It is clear from Fig. 9 that Zn^{2+} acts to slow one or more of the rate constants controlling transitions among these closed states. This is a direct demonstration of the idea proposed by Gilly and Armstrong (1982) that Zn^{2+} stabilizes one of the preopening closed states.

It is unlikely that Zn^{2+} acts on the rate constants by modifying membrane surface potentials. The large background of Ca^{2+} and Mg^{2+} ions used in these experiments would neutralize much of any existing surface potential. The low (e.g., 100 μM) concentrations of Zn^{2+} used would be expected to produce little further neutralization, certainly too little to account for the large effects seen in Figs. 2 and 9. Furthermore, the actions of Zn^{2+} on the activation time constant (Fig. 2) and median first latency (Fig. 9) would require a very large (>70 mV) reduction in a negative surface potential but the effects on deactivation require an increase in surface potential.

Thus, it appears that the *Shaker* K channel contains a site to which Zn^{2+} binds and in so doing retards channel opening. The ability of TNBS to inhibit Zn^{2+} action (Fig.

5) suggests the involvement of an amino group in divalent cation binding. As discussed above, we estimate that the binding affinity of Zn^{2+} for the site on *Shaker* K channels to be near 25 μM; consequently, the very small effect of 500 μM Zn^{2+} after TNBS treatment indicates that this reagent produced a very substantial decrease in binding affinity.

TNBS is a reasonably specific agent for modifying the ϵ-amino group of lysine (but not arginine) residues and the terminal amino group of peptides (Means and Feeney, 1971). Although TNBS also reacts with sulfhydryl residues, the S-trinitrophenyl derivative is unstable and reverts to the original sufhydryl group (Means and Feeney, 1971; Lundblad and Noyes, 1984). While not investigated in detail, the lack of effects of Zn after TNBS treatment persisted for at least 20–30 min, suggesting an irreversible modification and so the involvement of an amino group. This conclusion is consistent with the lack of effect of histidyl and sulfhydryl modifying reagents on the Zn^{2+} effect on squid K channel (Spires and Begenisich, 1992*a*). It is also consistent with recent mutagenesis data on *Shaker* K channels that show that the Zn^{2+} effects persist even after all cysteines are removed (Boland, Jurman, and Yellen, 1994). Because the various folding models for *Shaker* K channels place the terminal amine on the inner membrane surface, the amino group involved in Zn^{2+} binding most likely resides with a lysine residue.

There are two possible roles for the lysine group in Zn^{2+} binding: (*a*) the amino group of lysine constitutes part of the binding site itself or (*b*) the amino group of lysine is near enough to the binding site to control access to it. Because the ϵ-amine of many lysine groups have pK_a values near 9 and so are positively charged at neutral pH, the first possibility may appear unlikely. However, there are many examples of lysine residues in proteins with apparent pK_a values near 7 (e.g., Schmidt and Westheimer, 1971) and even as low as 5.9 (Murdock, Grist, and Hirs, 1966). Such low values can occur if there are other positive charges near the target lysine. For example lysine-41 in ribonuclease has a pK_a near 7 perhaps due to the influence of arginine at position 39. The apparent pK_a for H^+ ion inhibition of the Zn^{2+} effect in squid K channels is ~7.2 and near 7.3 for *Shaker* channels and so it is possible that Zn^{2+} could interact with the neutral form of the lysine amino group.

The data in Figs. 5 and 6 show that the amino group involved in Zn^{2+} binding is distinct from other amino groups whose modifications with lower TNBS concentration produced a slowing of the macroscopic kinetics. *Shaker* K channels have this property in common with squid K channels (Spires and Begenisich, 1992*a*,*b*). Since Freeman and Radda (1968) have shown that the intrinsic reactivity of TNBS with amino groups increases with the group pK_a, the extensive treatment required to inhibit Zn^{2+} action is consistent with the low inhibitory pK of 7.3.

If not part of the site itself, the lysine residue would need to be near enough to the site so that the TNBS-induced trinitrobenzene substitution (Spires and Begenisich, 1992*b*) on the ϵ-amine blocks Zn^{2+} access. In this case the Zn^{2+} binding site would be formed by other amino acids. Regardless of the specific role for lysine, it seems likely that there is a lysine near the site of Zn^{2+} binding.

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