

Voltage-Independent Gating Transitions in Squid Axon Potassium Channels

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ABSTRACT We have investigated the actions of internal and external Zn^{2+} on squid axon K channel ionic and gating currents. As has been noted previously, application of Zn^{2+} to either membrane surface substantially slowed the activation of these channels with little or no change in deactivation. Internal Zn^{2+} (near 200–300 nM) slowed channel activation by up to sixfold over the range of membrane voltages from -30 to $+50$ mV. External Zn^{2+} (10 mM) produced an approximate twofold slowing of activation from -40 to $+40$ mV. We found that the changes in ionic current activation kinetics were accompanied by less than a twofold slowing of channel-gating currents in a narrow range of potentials near -30 mV. There was, at most, only a few percent reduction of charge movement associated with Zn^{2+} application. We conclude that these ions interact with channel components involved in weakly voltage-dependent conformational changes. Although there are some differences in detail, the general similarity of the actions of both internal and external Zn^{2+} on channel function suggests that the modified channel-gating step involves amino acids accessible to both the internal and external membrane surface.

INTRODUCTION

Membrane voltage controls the transitions among the conformational states of many ion channel proteins. Consequently, the ionic current through these voltage-gated channels is time- and voltage-dependent. This voltage sensitivity arises from electric charges or dipoles on the proteins, and the movement of these components can be detected as channel-gating currents (see reviews by Armstrong, 1981; Bezanilla, 1985).

Because macroscopic ionic and gating currents reflect the same underlying protein conformational changes, there must be a general similarity between these two types of measurements. However, because ionic currents only appear after the channels attain an open (conducting) conformation, transitions among closed states are not directly observed. In contrast, any voltage-dependent transition will contribute to gating current, but fast, highly voltage-sensitive steps will be more heavily weighted. Consequently, analysis of ionic and gating currents obtained under similar conditions provides substantially more information on the ion channel-gating process than is available from either one alone.

Many types of divalent cations induce substantial modifications of ion channel-gating (e.g., see Chapter 17 of Hille, 1992). External application of Zn^{2+} ions greatly slows the opening of squid axon potassium channels but slightly increases closing rates (Gilly and Armstrong, 1982). This is true even for low concentrations of Zn^{2+} in a high background of Ca^{2+} and Mg^{2+} (Spires and Begenisich, 1992), indicating that these actions are likely directly on the channel

protein and not a modification of membrane surface charges. Cloned K channels, including *Shaker* B channels, are similarly modified (Spires and Begenisich, 1994). Internal divalent cations, including Zn^{2+} , produce even larger yet rather specific modification of squid axon K channel ionic currents (Begenisich and Lynch, 1974).

There have been relatively few studies of the actions of divalent cations on K channel-gating currents, but the available data suggest that, in contrast to the large effects on ionic currents, internal Zn^{2+} has only a modest effect on squid axon K channel-gating currents (Spires and Begenisich, 1989). In the study reported here, we compared the actions of internal and external Zn^{2+} on squid axon K channel ionic and gating currents. As previously described (Begenisich and Lynch, 1974; Gilly and Armstrong, 1982), application of Zn^{2+} to either membrane surface substantially slowed the activation of these channels with little or no change in deactivation. We found that the large changes in ionic current activation kinetics were accompanied by only a slight slowing of channel-gating currents in a narrow range of potentials near -30 mV. There was, at most, only a few percent reduction of total charge movement associated with Zn^{2+} application. We conclude that these ions interact with channel components involved in weakly voltage-dependent conformational changes. Although there are some differences in detail, the general similarity of the actions of both internal and external Zn^{2+} on channel function suggests that the modified channel-gating step may involve amino acids accessible to both the internal and external membrane surface.

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MATERIALS AND METHODS

Biological preparation

The data in this report were obtained with giant axons from the squid *Loligo pealei* at the Marine Biological Laboratory (Woods Hole, MA).

Voltage-clamp and internal perfusion

The axons used in this study were internally perfused and voltage-clamped using techniques that have previously been described in detail (Begenisich and Lynch, 1974; Busath and Begenisich, 1982). A description of our techniques for measuring K channel-gating currents can be found in Spires and Begenisich (1989). All voltages have been corrected for the junction potential between the internal 0.56 M KCl electrode and the internal solutions. External potentials were measured with an agar-filled 3 M KCl electrode. Unless otherwise noted, measurements of ionic currents were done at a temperature of 15°C. Gating currents were measured at 20°C. Series resistance compensation was used.

Membrane currents were measured with a 12 bit analog/digital converter controlled by a laboratory computer. The voltage-clamp pulses were generated by a 12 bit digital/analog converter controlled by the computer system. Linear capacitive and leakage currents were subtracted from most records using a $\pm P/4$ procedure (Bezanilla and Armstrong, 1977). For ionic current measurements, the subtraction potential for the P/4 pulses was the holding potential and a potential of about -100 mV was used for gating current measurements. To improve the signal-to-noise ratio of gating current measurements, 16 or 32 records were averaged.

Solutions

The external solution used for measurements of ionic currents was an artificial sea water (50 K ASW) containing (in mM) 390 NaCl, 50 KCl, 10 CaCl₂, 50 MgCl₂, 10 HEPES buffer, pH near 7.4. The elevated potassium was used to reduce the effects of K⁺ accumulation in the periaxonal space associated with these axons (Frankenhaeuser and Hodgkin, 1956). Na⁺ channel currents were blocked by addition of 1 mM tetrodotoxin (TTX). The external solutions had osmolarities of about 975 mOsm/kg. The standard internal solution (K SIS) contained (in mM) 50 KF, 270 K glutamate, 15 K₂HPO₄, 390 glycine, pH 7.4 and 970 mOsm/kg.

The solutions used for measurement of K channel-gating currents were designed to minimize contamination by ionic and Na channel-gating currents (Gilly and Armstrong, 1980; White and Bezanilla, 1985). Our external solution (Tris-NO₃ ASW) for K channel-gating current measurements consisted of (in mM): 415 TrisNO₃, 50 Ca(NO₃)₂, 10 CsNO₃, 1 mM TTX, pH 7 (with 25 Tris base), 60 sucrose, 960–980 mOsm/kg. The internal solution (Cs SIS) was (in mM): 270 Cs-glutamate, 50 CsF, 360 glycine, 0.2 dibucaine, 10 HEPES, pH 7.4, 970 mOsm/kg. The local anesthetic dibucaine reduces Na channel-gating currents (Gilly and Armstrong, 1980; White and Bezanilla, 1985). The use of internal and external Cs⁺ slows the loss of K channel function in K⁺-free solutions (Chandler and Meves, 1970; Almers and Armstrong, 1980; see also Pardo et al. (1992) for a similar effect on cloned K channels) and so may preserve the component of gating current lost in K-free media (Gilly and Armstrong, 1980). However, because of the small permeability of these K channels to Cs⁺ (Chandler and Meves, 1965), some gating current records may be contaminated by ionic current, especially at potentials more positive than about $+10$ mV. To inhibit this current, we included 1 mM K channel blocker, 4-aminopyridine (4-AP), in the internal solution in most experiments. Although McCormack et al. (1994) have reported small effects of this compound on *Shaker* K channel-gating currents, we were unable to detect any effects on squid axon K channel-gating (Begenisich and Spires, 1989) (see also Fig. 8 B).

We added ZnCl₂ to either the internal or external solution used in a particular experiment. Most of the data presented here were obtained with 10 mM external Zn²⁺. Because many of the ingredients of our internal solutions (including F⁻ and glutamate; Sillén, 1964) complex Zn²⁺ ions, we attempted to determine the free Zn²⁺ concentrations in these solutions using the Zn-sensitive dye "Zincon" (1-(2-hydroxy-5-sulphophenyl)-3-phenyl-(2-carboxyphenyl)-formazan) (Rush and Yoe, 1954) with the methods of Kalfakakou and Simons (1990). We found a dissociation constant for the Zn-Zincon interaction in our calibrating solutions of 6.4 mM close to the value of 5.9 mM of Kalfakakou and Simons (1990) and consistent with the different values of ionic strength. Even though the total ZnCl₂ added to our internal solutions was 3 mM, the measurements with Zincon indicated that the concentration of free Zn²⁺ was quite low, near 200–300 nM. A more

accurate estimate was not possible because these levels are considerably below the Zn-Zincon dissociation constant.

Although the solutions and temperatures for ionic and gating current experiments were, in general, different, we tested the actions of both internal and external Zn²⁺ on ionic currents (at 20°C) using gating current solutions (but with K⁺ replacing Cs⁺ and without 4-AP). The results of one such experiment are illustrated in Fig. 7. Except for the slightly faster kinetics at this elevated temperature, the effects were the same as in the usual solutions at 15°C.

Data analysis

The quantitative analysis of our data included the fitting of exponential time functions to ionic and gating current records and determinations of gating charge movements. As shown in White and Bezanilla (1985), the final approach to steady-state K channel ionic 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 about 20% or less. The time constant obtained in this way is called the deactivation time constant.

The major part of K channel-gating current ON and OFF responses can also be described by a single exponential time function (White and Bezanilla, 1985; Spires and Begenisich, 1989), and we fit such a function to the data. The first 20–100 ms of data were omitted from the fits to reduce contamination from the much faster, residual Na channel-gating current. The gating current ON and OFF time constants were obtained from these fits. The charge movement, Q , was computed from the product of the amplitude and time constant of the fits to the OFF response (White and Bezanilla, 1985; Spires and Begenisich, 1989). This method avoids problems associated with the rising phase of K channel-gating currents (White and Bezanilla, 1985) and, because Na channel-gating currents immobilize during the pulse (Armstrong and Bezanilla, 1977), will result in less contamination from this source.

The voltage dependence of the gating charge movement was fit by a two-state Boltzmann function of the form

$$Q = \frac{1}{1 + \exp[-q(V_m - V_{1/2})F/RT]}, \quad (1)$$

where q represents the equivalent charge difference between the two states, measured here in units of electronic charge (e); $V_{1/2}$ is the voltage of the midpoint of this function; F , R , and T have their usual thermodynamic meaning. All fits of theoretical functions used the "simplex" algorithm (Caceci and Cacheris, 1984).

RESULTS

Internal Zn²⁺ slows the late phase of K channel ionic current activation

Internal Zn²⁺ has a substantial, reversible effect on squid axon delayed rectifier K channels (Begenisich and Lynch, 1974). An example of this behavior is illustrated in Fig. 1. Fig. 1 A shows superimposed K channel ionic currents elicited by 4 ms pulses to several potentials between -43 and $+37$ mV. The usual voltage- and time-dependence of these currents are apparent, including the sigmoid activation time course. Also apparent are the exponentially declining "tail" currents initiated upon repolarization to the holding potential (-63 mV in this case). Fig. 1 B illustrates the action of internal Zn²⁺ on these ionic currents. For identical voltage pulses, the currents in the presence of internal Zn²⁺ are quite reduced and appear to have a much slower time course. The

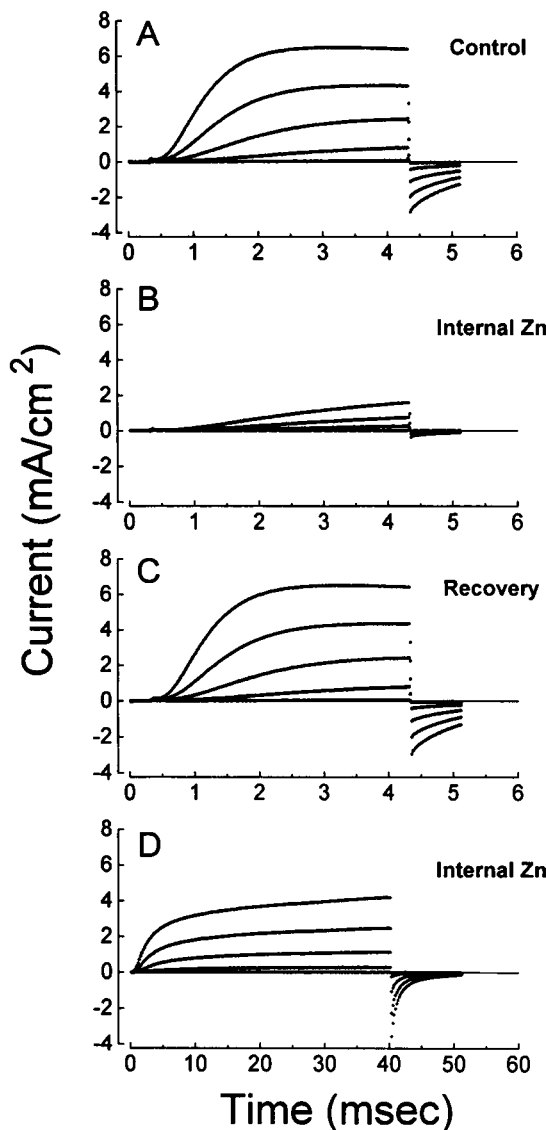


FIGURE 1 Internal Zn^{2+} and squid K channel ionic currents. Superimposed squid axon K channel currents from voltage steps to -43 , -23 , -3 , $+17$, and $+37$ mV from a holding potential of -63 mV. (A) Control currents recorded before Zn^{2+} application. (B) Currents in the presence of internal Zn^{2+} : same current and time scales as in A. (C) Currents recorded after washing out internal Zn^{2+} . (D) Currents recorded in the presence of internal Zn^{2+} but with longer duration pulses and displayed with a 10-fold slower time scale. The free Zn^{2+} concentration was near 200–300 nM (see Materials and Methods).

records in Fig. 1 C show that these large actions of internal Zn^{2+} were totally reversible.

The reduced current seen in the presence of internal Zn^{2+} in Fig. 1 B was mostly due to a substantial slowing of channel kinetics. As illustrated in Fig. 1 D, if the duration of the voltage pulse is lengthened 10-fold, the currents in the presence of Zn^{2+} begin to approach those in control conditions. Seen on this time scale, the activation kinetics appear to have lost much of the usual sigmoid character. In other words, kinetic events early in time appear to have been less effected by Zn^{2+} than events later in time. The records in Fig. 1 D also

suggests that tail currents were less altered than were the late parts of channel activation.

A quantitative analysis of the actions of internal Zn^{2+} on four axons is presented in Fig. 2. As described in Materials and Methods, single exponential time functions were fit to the late part (approximately 50–90%) of the current activation. These values, obtained at potentials more positive than -40 mV, are plotted as open symbols for control conditions and as filled symbols in the presence of internal Zn^{2+} . Although a single exponential function is an excellent representation of this phase of channel activation in control conditions (White and Bezanilla, 1985; Spires and Begenisich, 1989), the fit was not as good in the presence of internal Zn^{2+} at the most positive potentials—there appeared to be an additional, very slow kinetic component (e.g., Fig. 1 D). As a result, the degree of Zn^{2+} -induced kinetic slowing, assayed by the single exponential fitting process, may be somewhat underestimated. Nevertheless, it is clear that internal Zn^{2+} produced a substantial (fivefold or more) slowing of the activation of squid K channel ionic currents.

In sharp contrast to the large slowing of ionic current activation, Zn^{2+} had little effect on deactivation kinetics. Deactivation time constant values are included in Fig. 2 at potentials more negative than -40 mV. Control data are shown as open symbols and time constants obtained in the presence of Zn^{2+} as filled symbols. No significant effect of internal Zn^{2+} on ionic current deactivation is apparent.

The sigmoid character of the activation of squid axon macroscopic K channel ionic currents is enhanced if the acti-

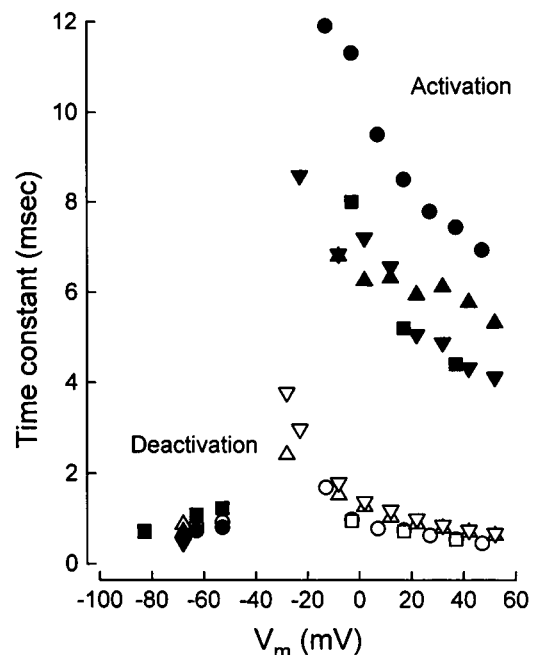


FIGURE 2 Internal Zn^{2+} and squid axon ionic current time constants. Activation and deactivation (for V_m more negative than -40 mV) obtained from exponential fits to data. Open symbols in the absence and filled symbols in the presence of internal Zn^{2+} . Data from four axons identified by different symbols. The concentration of free Zn^{2+} concentration was near 200–300 nM (see Materials and Methods).

vating depolarization is preceded by a hyperpolarization (Cole and Moore, 1960). In addition, this behavior has significant implications for kinetic models of delayed rectifier K channels (Cole and Moore, 1960; Hill and Chen, 1971a, b; Palti et al., 1976; Begenisich, 1979; Zagotta et al., 1994). Fig. 3 A illustrates the delay of channel currents induced by application of a 30 mV hyperpolarization—the currents with the prepulse appear to be simply shifted in time. Ionic current kinetics in the presence of internal Zn^{2+} are also sensitive to prepulse hyperpolarization (Begenisich and Lynch, 1974) but in quite a different manner, as illustrated in Fig. 3 B.

The records in Fig. 3 B were obtained in the presence of internal Zn^{2+} with the same pulse protocol as those in Fig. 3 A. The slowing of channel activation by internal Zn^{2+} is reflected in the slower time scale of Fig. 3 B. In contrast to the hyperpolarization-induced delay in activation seen in Fig. 3 A, the same protocol in the presence of internal Zn^{2+} causes an increased rate of activation. That is, hyperpolarization tended to reverse the Zn^{2+} -induced slowing of K channel ionic current kinetics. The overlap of the early part of the

records in Fig. 3 B is consistent with the suggestion above that Zn^{2+} affects mostly late steps in channel activation.

Internal Zn^{2+} has little effect on K channel-gating current

Examples of squid axon K channel-gating currents in the absence and presence of internal Zn^{2+} are presented in Fig. 4. The left panel shows currents recorded during 5 ms depolarizations to -47 , -32 , and -17 mV before application of internal Zn^{2+} . An early fast phase of current followed by a slower component is apparent in the record obtained at -47 mV. As described in White and Bezanilla (1985) and Spires and Begenisich (1989), much, if not all, of the fast component is residual Na channel-gating current. The data in the center panel of this figure were obtained in the presence of internal Zn^{2+} and the currents in the right panel after Zn^{2+} removal. In contrast to the very large effects on K channel ionic currents, internal Zn^{2+} appears to have had, at most, rather little affect on K channel-gating currents.

The results of a quantitative analysis of the K channel-gating currents are illustrated in Fig. 5. As described in Materials and Methods, a single exponential time function was fit to the data, avoiding the fast, residual Na channel component. The amount of gating charge moved at each potential was determined from the fits to the OFF currents. In six axons the maximum charge moved after washing out the internal Zn^{2+} was $88 \pm 2.8\%$ of the original value. The maximum charge moved in the presence of internal Zn^{2+} was $94 \pm 2.3\%$ of the average of the control and recovery values. Thus, Zn^{2+} reduced the maximum charge movement by, at most, only a few percent.

Fig. 5 A illustrates the voltage dependence of the relative amount of charge moved in the absence (*open symbols*) and presence (*filled symbols*) of internal Zn^{2+} . The data in the absence of Zn^{2+} were obtained as the average of the control and recovery data normalized by the average maximum charge moved. The solid line is a fit of Eq. 1 to the data obtained in the absence of Zn^{2+} . The fitted parameters q and $V_{1/2}$ were $2.3 e$ and -35 mV, respectively, which compare well with previously determined values of $2.2 e$ and -38 mV (Spires and Begenisich, 1989). The data in the presence of Zn^{2+} were normalized by the maximum charge movement with Zn^{2+} . As for maximum charge moved (see preceding paragraph), Zn^{2+} appears to have had no significant effect on the voltage dependence of charge movement.

Fig. 5 B presents an analysis of the kinetics of K channel-gating currents. Shown are the fitted time constants to ON currents (potentials more positive than -60 mV) in the absence (*open symbols*) and presence of internal Zn^{2+} (*filled symbols*). Time constants at potentials more negative than -60 mV were obtained from OFF currents. Except near -30 mV, internal Zn^{2+} had little or no effect on gating current kinetics. There does appear to have been an approximate 50% slowing of the activation time constant obtained at -32 mV. The mean time constant obtained at -32 mV after Zn^{2+} removal is also shown (Δ , displaced a few mV for clarity)

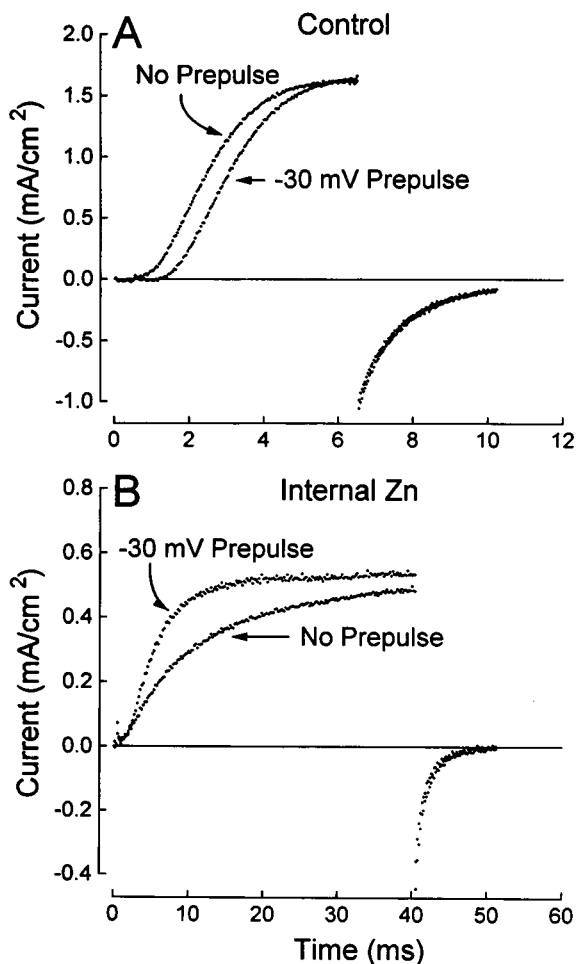
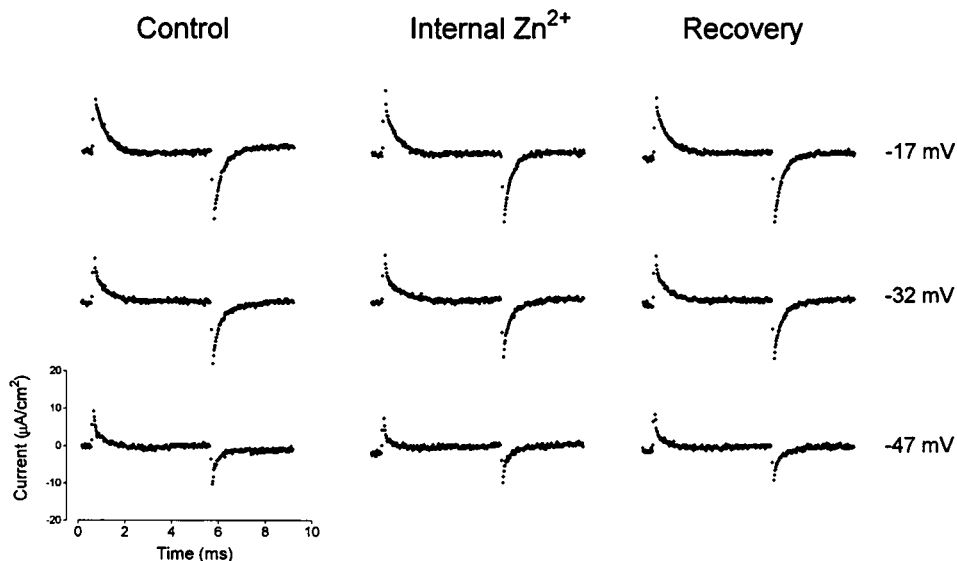


FIGURE 3 Hyperpolarization reversal of Zn^{2+} -induced slowing. Currents at -13 mV with or without a 50 ms prepulse to -93 mV from a holding potential of -63 mV. (A) In the absence of Zn^{2+} ; (B) same axon as in A but with internal Zn^{2+} .

FIGURE 4 Internal Zn^{2+} and K channel-gating currents. Currents recorded during pulses to -47 , -32 , and -17 mV (bottom to top, as indicated) from a holding potential of -67 mV. (left) Control currents before Zn^{2+} application; (middle) currents in the presence of internal Zn^{2+} ; (right) currents after washing out Zn^{2+} .



and is essentially identical with the control value. The value in Zn^{2+} is statistically different (at the 0.01 level) from the control data. Thus, the only effects of internal Zn^{2+} appears to have been a small slowing of a gating current component recorded near -30 mV.

The Cole-Moore effect (Cole and Moore, 1960; see Fig. 3 A) has a correlate in squid axon K channel-gating currents. Bezanilla et al. (1982) showed that a hyperpolarizing prepulse produces a delay in the activation of gating current elicited by the succeeding test pulse. Under some conditions, the effect is to reveal a clear rising phase in the gating current record (White and Bezanilla, 1985). Stühmer et al. (1991) have pointed out that such rising phases could be artifactually produced by the protocol for linear capacity current subtraction. However, the rising phase in squid axon (and *Shaker*) K channel-gating currents occurs with several different subtraction protocols (White and Bezanilla, 1985; Zagotta et al. 1994) and is therefore likely to be genuine property of these channels.

Internal Zn^{2+} appears to induce or exaggerate the rising phase of squid axon K channel-gating currents (Spires and Begenisich, 1989). This point was examined in more detail by the experiment illustrated in Fig. 6. Parts A and B of this figure contain gating currents recorded in the absence of internal Zn^{2+} at -2 mV (A) and -12 mV (B). These control data show little evidence of a rising phase. The solid lines in this figure represent the results of fitting a biexponential time function to the data, and the equivalent rising phase time constants of 90 and 62 ms are certainly too fast to be well resolved. However, the inset in part B shows that a hyperpolarizing prepulse slowed a channel-gating kinetic step sufficiently to allow the observation of a rising phase.

The records in Fig. 6, C and D show that internal Zn^{2+} appeared to induce or enhance the gating current rising phase. This effect is quite noticeable at -2 mV (part C) but is also apparent at -12 mV (part D). The inset in Fig. 6 D indicates that the presence of Zn^{2+} has enhanced the ability

of the prepulse to delay the current record. This latter effect is the opposite of what was observed with prepulses and ionic current (see Fig. 3 B). The Na channel contamination of these records, especially the fact that prepulses may remove Na channel-gating current immobilization (Armstrong and Bezanilla, 1977), precludes a quantitative assessment of this action of internal Zn^{2+} on K channel-gating currents.

External Zn^{2+} and K channel-gating

Similar to internal application, external Zn^{2+} slows the late phase of squid axon K channel ionic current activation (Gilly and Armstrong, 1982). Analogous actions of Zn^{2+} occur on many types of K channels (e.g., Stanfield, 1975) including *Shaker* (Boland et al., 1994; Spires and Begenisich, 1994) and other flavors of cloned channels (e.g., Kv2.1 (De Biasi et al., 1993) and Kv1.5 (our unpublished observations)). Unlike the slowing of activation, deactivation kinetics are actually faster in the presence of external Zn^{2+} (Gilly and Armstrong, 1982; Spires and Begenisich, 1992). These effects are not mediated through membrane surface charges (Gilly and Armstrong, 1982; Spires and Begenisich, 1992).

Because the external solution used for recording K channel-gating currents contained a large concentration of Tris NO_3 , we examined (Fig. 7 A) the actions of external Zn^{2+} with an external solution very similar to that used to record gating currents. The usual effects of external Zn^{2+} are apparent, including a slowing of the late phase of current activation and a depolarizing shift of the fraction of open channels (Gilly and Armstrong, 1982; Spires and Begenisich, 1992). Fig. 7 B shows that under these conditions external Zn^{2+} produced a two- to threefold slowing of the ionic current activation time constant and the expected (Gilly and Armstrong, 1982; Spires and Begenisich, 1992) slight (25% in this experiment at -64 mV) speeding of deactivation kinetics.

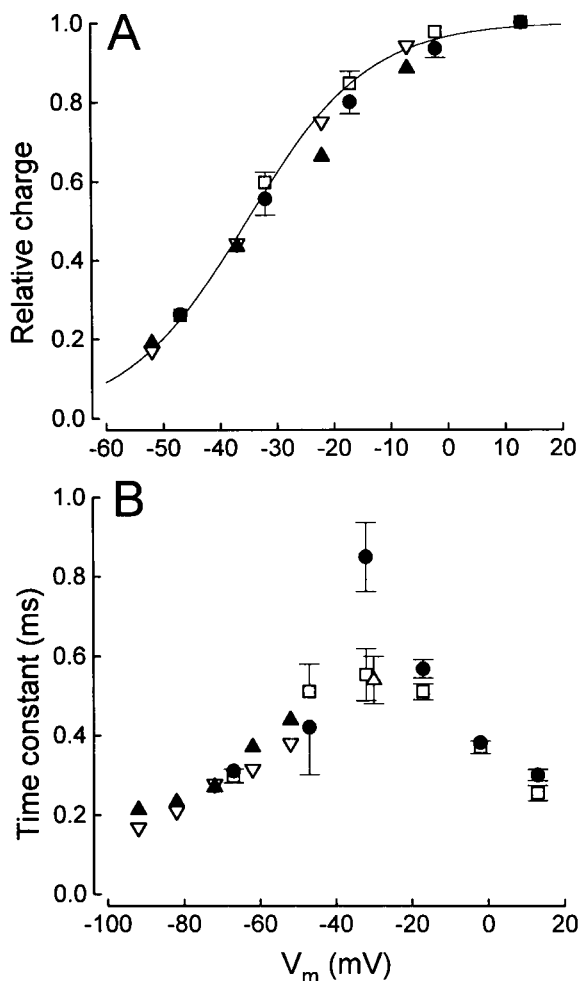


FIGURE 5 Internal Zn^{2+} and charge movement and gating current kinetics. Mean (\square , \bullet) and SEM values from four axons; SEM limits not shown if smaller than symbol. (∇ , \blacktriangle) Average data from two additional axons. (A) Voltage dependence of relative gating charge movement. Line: fit of Eq. 1 to the data in the absence of Zn^{2+} . Fitted values: $q = 2.3 e$; $V_{1/2} = -35$ mV. (B) Gating current ON (for potentials more positive than -60 mV) and OFF-time constants. The ON-time constant at -32 mV (D) recorded after Zn^{2+} washout has been displaced slightly along the voltage axis for clarity.

The data of Fig. 8, A and B illustrate the results of experiments on four axons designed to determine whether the actions of external Zn^{2+} on ionic currents are reflected in K channel-gating currents. Raw currents recorded at -47 and -32 mV in the absence and presence of 10 mM Zn^{2+} are shown in the inset of Fig. 8 A. No large effect of Zn^{2+} is apparent.

A quantitative analysis of the relative charge moved at various potentials is shown in Fig. 8 A in the absence (open squares) and presence of external Zn^{2+} (filled circles, filled triangles). The solid line in Fig. 8 A is a fit of Eq. 1 to the control values. The fitted parameters of q and $V_{1/2}$ were $2.4 e$ and -34 mV, quite close to those of Fig. 5 A. The maximum charge moved in the presence of external Zn^{2+} was $92 \pm 1.9\%$ of the average of the control and recovery values. These results indicate that external Zn^{2+} did not have an appreciable effect on the amount or voltage dependence of gating charge movement.

Gating current kinetic data are illustrated in Fig. 8 B. Time constants obtained in the absence (open symbols) and pres-

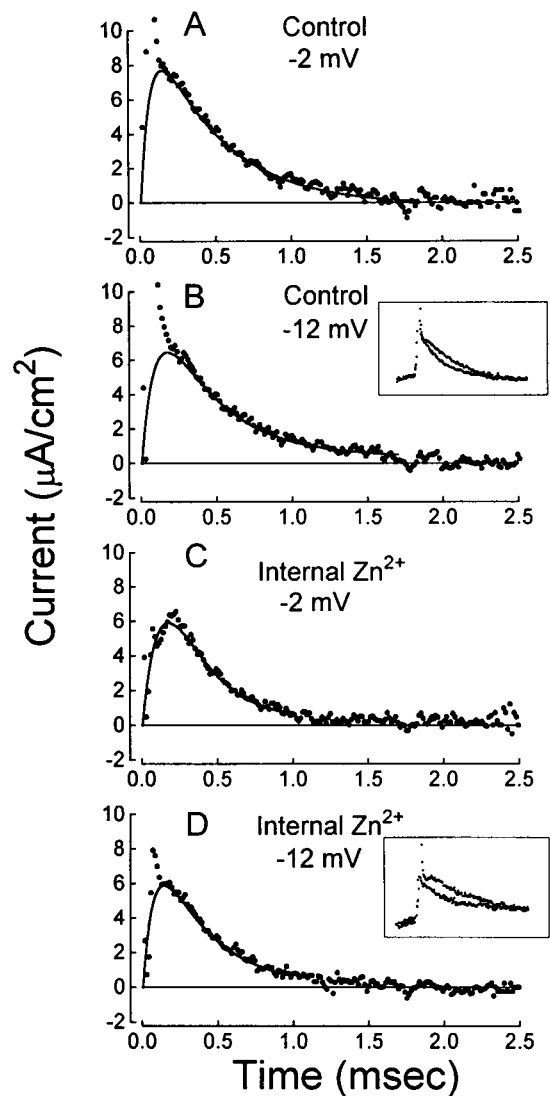


FIGURE 6 Internal Zn^{2+} and the gating current rising phase. (A, B) Gating currents recorded in the absence of internal Zn^{2+} at -2 and -12 mV. The solid lines are fits of a biexponential time function to the data with rising phase time constants of 62 and 90 ms at -2 and -12 mV, respectively. (inset) Gating current at -12 mV without and with a 25 ms prepulse to -112 mV. (C, D) Same as in A and B but obtained in the presence of internal Zn^{2+} .

ence of Zn^{2+} (filled symbols) are presented. Values more positive than -50 mV are gating current ON time constants, and those at negative voltages are from the OFF response. External Zn^{2+} certainly had little or no effect on the gating current kinetics except possibly near -30 mV where some slowing appeared to occur. The relatively few experiments (4) and the observed variability of the time constant at -30 mV in the presence of Zn^{2+} (SEM limits) require a cautious interpretation, but certainly the large slowing of ionic current activation over a large voltage range (Fig. 7 B) was not reflected in the gating current kinetics.

DISCUSSION

The main conclusion from this study is that it is possible to substantially alter squid axon K channel ionic current with

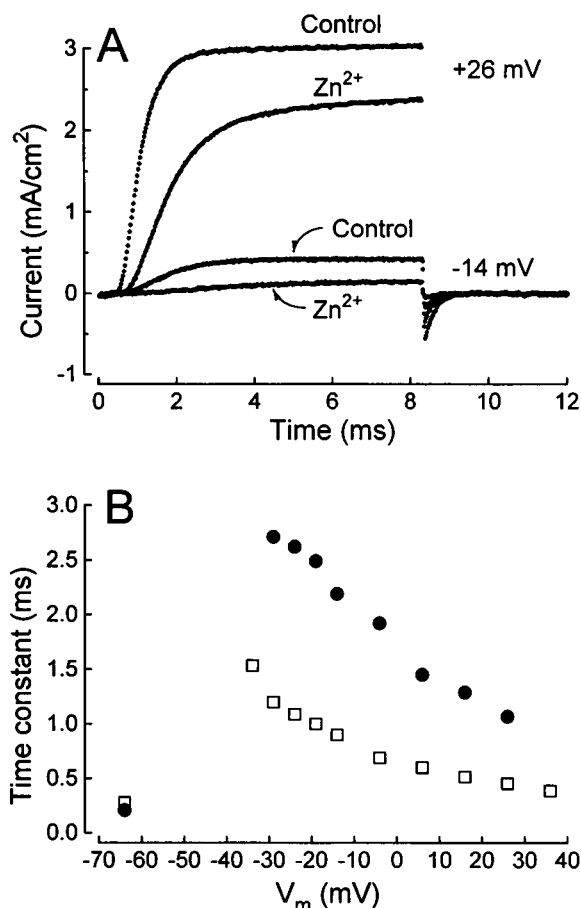


FIGURE 7 External Zn²⁺ and K channel ionic currents. (A) Raw currents recorded (20°C) at depolarizations to -14 and +26 mV before and during exposure to 10 mM external Zn²⁺ in Tris-NO₃ ASW without Cs. (B) Activation and deactivation (at -64 mV) time constants in the absence (○) and in the presence of external Zn²⁺ (●).

very little effect on channel-gating current. Application of internal or external Zn²⁺ slowed the late phase of ionic current activation over a broad range of membrane voltages by a factor of two to four with no concomitant slowing of gating current time constants (except for a small slowing in a narrow range of potentials near -30 mV). The effect of internal Zn²⁺ on fast events possibly associated with the gating current rising phase (see Fig. 6) may be significant, but these are much too fast to account for the slow, late phase of ionic current activation.

Both internal and external Zn²⁺ produced a modest (50% to twofold), reversible slowing of a component of gating current at voltages near -30 to -20 mV. This is the same voltage range in which the intrinsic major ionic current and gating current time constants are not equal (Spires and Begenisich, 1989). The actions of internal Zn²⁺ were more complex than those produced by external application: (1) internal Zn²⁺ appeared to slow the rising phase of gating current; (2) the slowing of ionic (but not gating) current produced by internal Zn²⁺ was reduced by hyperpolarizing prepulses. The former suggests that internal Zn²⁺ acts on at least two kinetic steps in channel-gating, one involved in the fast

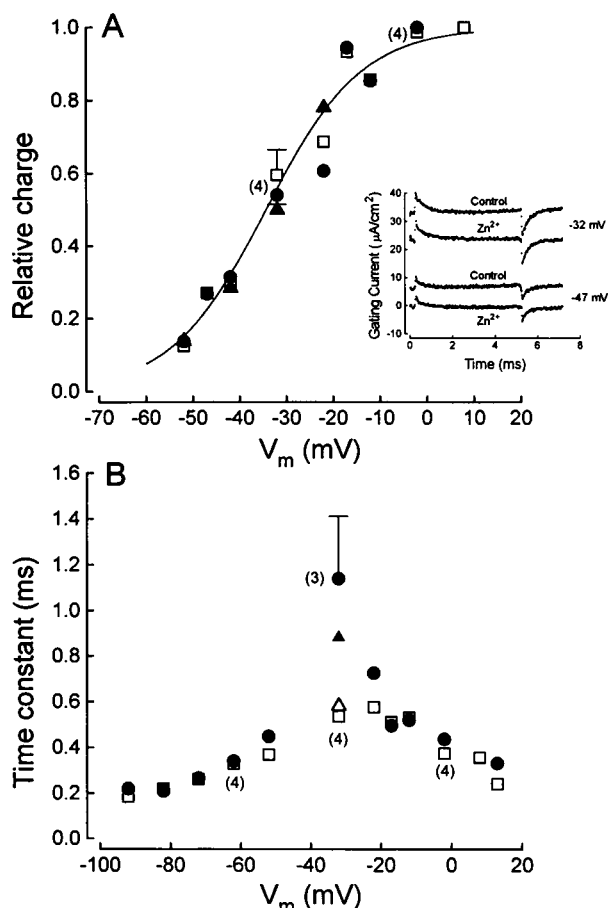


FIGURE 8 External Zn²⁺ and K channel-gating currents. (A) Voltage dependence of relative charge moved in the absence (□) and presence of external Zn²⁺ (●). Mean values from four axons as indicated; the remaining data are average values from two additional axons. SEM limits shown where larger than the symbols. Charge movement at -52, -42, -32, and -22 mV were obtained in the presence of Zn²⁺ from an axon not treated with 4-AP. These values were normalized (▲) by the extrapolated maximum value obtained from a fit of a Boltzmann function. (inset) Raw gating current records in the absence ("Control") and presence of internal Zn²⁺ recorded at -47 and -32 mV. (B) Gating current ON (for potentials more positive than -50 mV) and OFF-time constants in the absence (□) and presence (●) of external Zn²⁺. Where indicated, values are the average of data from four axons. SEM limits shown where larger than the symbols. Data at -32 mV in the absence (△) and presence (▲) of Zn²⁺ from an axon not treated with 4-AP are included.

events associated with the gating current rising phase and the other involved in the kinetic step reflecting the late phase of ionic current.

The ability of prepulse voltage to modulate the effects of internal Zn²⁺ on K channel-gating currents suggests that the site of action may be within the membrane electric field. Viewed in this way, hyperpolarization would decrease the number of sites occupied by Zn²⁺ and so reduce the kinetic effects. Alternatively, it could be that Zn²⁺ directly binds to some of the gating charges and changes their net valence from negative to positive. Thus, under these conditions, hyperpolarizing would have the same effect as depolarizing prepulses in the absence of Zn²⁺: a reduced delay

in channel opening (Cole and Moore, 1960; Palti et al., 1976; Begenisich, 1979). If so, these charges would necessarily be only a small component of the total charge movement because the data in Fig. 5 A show that any Zn^{2+} -induced change in gating charge valence must be quite small.

Implications for models of K channel-gating

Every voltage-dependent kinetic step in channel-gating must produce a component of gating current, and every time constant (eigenvalue) present in an ionic current record must also appear in the gating current recorded under comparable conditions. However, the amplitude of the gating current components is weighted by the speed and voltage dependence of the relevant kinetic step (e.g., see review by Almers (1978) and discussion in Hille (1992)). Consequently, it is possible that gating current records could be dominated by relatively few, strongly voltage-dependent steps, and so there may be components of ionic current that arise from slow and/or weakly voltage-dependent transitions that are not detectable in the equivalent gating current records. Indeed, Conti and Stühmer (1989) have provided evidence that most of the gating charge movement (at least for Na channels) occurs in two or three fast steps.

Thus, at any voltage, the gating current record contains several components but is dominated by only a few fast, voltage-dependent kinetic steps. At some voltages, the time constants of these components are similar or identical to the late phase of ionic current; but not at other potentials (-20 to -30 mV for squid K channels; Spires and Begenisich, 1989). Because slow, weakly voltage-dependent steps will contribute little to measured gating currents, a reasonable explanation for the effects of external and internal Zn^{2+} is that these ions slow such a step.

Several studies of intrinsic channel-gating have provided evidence for voltage-independent or weakly voltage-dependent steps in the opening of both native and cloned K channels (White and Bezanilla, 1985; Zagotta and Aldrich, 1990; Koren et al., 1990; Hoshi et al., 1994; Zagotta et al., 1994). Furthermore, treatment of squid K channels with histidine-specific reagents slows activation (but not deactivation) of ionic current with little or no effect on channel-gating currents, a result also consistent with the existence of a weakly voltage-dependent conformational transition. Because histidine (Spires and Begenisich, 1990) and amino groups (Spires and Begenisich, 1992) accessible from the external solution have been associated with voltage-insensitive or external Zn^{2+} -sensitive kinetic steps, these residues may be involved in this conformational change process.

It has been difficult to construct mathematical models of K channel-gating that completely accommodate the various observations on ionic and gating currents. For example, for squid axon K channels, the voltage dependence of charge movement occurs at potentials 30 mV (Spires and Begenisich, 1989) to 40 mV (White and Bezanilla, 1985)

more negative than the voltage dependence of channel opening. To account for this observation, White and Bezanilla (1985) found it necessary to invoke 15 sequentially coupled closed channel states with a single open state. Because external Zn^{2+} shifts the probability of channel opening (but not charge movement, Fig. 8 A) to even more positive voltages (by 16 mV for 10 mM; Gilly and Armstrong, 1983), presumably even more closed states must be considered in the context of sequentially coupled models.

The Cole-Moore shift also places severe constraints on possible channel models. Cole and Moore (1960) showed that at least 25 identical, independent channel subunits were required to account for this effect in squid axon K channels. Palti et al. (1976) found that no number of independent, identical subunits could adequately account for similar data in node of Ranvier K channels.

It is also possible to model the basic characteristics of axon K channel ionic and gating currents with two independent, nonidentical subunits (Spires and Begenisich, 1989). Models of this type easily accommodate differential changes in ionic and gating current kinetics (Spires and Begenisich, 1990, 1992). Native K channels may be heteromeric proteins (Rudy et al., 1988) but (based on results from cloned channels; MacKinnon, 1991) are most likely composed of more than two subunits. Adding more model subunits would improve the simulation of experimental data, especially the Cole-Moore shift (e.g., see Spires and Begenisich, 1989), but these more complex modeling efforts are hampered by the lack of molecular data on squid K channels.

Shaker K channels formed by in vitro expression systems are homotetramers (MacKinnon, 1991), and a recent study has considered macroscopic ionic, single-channel, and gating current data in formulating a gating model for this type of channel (Zagotta et al., 1994; Hoshi et al., 1994; Zagotta et al., 1994). However, these channels exhibit only a modest Cole-Moore shift (Zagotta et al., 1994), and there is only a 10–20 mV difference in the voltage dependence of charge movement and channel opening. (Bezanilla et al., 1991; Schoppa et al., 1992; Zagotta et al., 1994). Perhaps the added complexity of native (or at least squid) channel behavior results from a heteromultimeric structure. In any case, the properties of expressed *Shaker* channels places fewer constraints on and simplifies the mathematical modeling process.

It is interesting to note that a *Shaker* channel mutant named V2 displays a rather complex voltage-dependent, steady-state charge movement and a very large (more than 50 mV) difference between the voltage range for charge movement and that for channel opening (Schoppa et al., 1992). Although a partial model for these steady-state properties has been presented (Schoppa et al., 1992), a full kinetic treatment has not; indeed, the authors pointed out that the restricted model could not account for the temporal delay in channel opening. A more recent study (McCormack et al., 1994) described a model that could describe qualitatively the kinetics of the V2 mutant channel-gating currents. However, this

work did not attempt to produce a quantitative simulation of channel kinetics including Cole-Moore shifts.

Implications for K channel protein structure

External and internal Zn^{2+} both slow the late phase of squid axon K channel ionic current activation, and so the underlying protein conformational change may involve amino acids on both the external and internal face of the molecule. Because the action of both external and internal Zn^{2+} had little effect on channel-gating currents, this conformational change process has little intrinsic voltage dependence and so involves little net movement of charged or dipolar components of the channel.

The action of external Zn^{2+} on *Shaker* and squid axon K channel-gating is inhibited by an amino group reagent (Spires and Begenisich, 1992, 1994). The effects of external Zn^{2+} are not altered by sulfhydryl reagents (Spires and Begenisich, 1992) or by mutation of all cysteine residues (Boland et al., 1994). The actions of histidine-modifying reagents on squid axon K channels are very similar to the actions of external Zn^{2+} (Spires and Begenisich, 1990). Consequently, we suggest that externally accessible histidine and lysine amino acids on the squid K channel are involved in the conformational change that occurs in the late part of ionic current activation.

Perri et al. (1994) report a cDNA clone from squid that codes for a protein more than 50% identical with the Kv1 channel class. There are only four putative extracellular loops in this family of channels (e.g., Jan and Jan, 1992; Drewe et al., 1992), and only one of these (between membrane-spanning region S5 and the channel pore) contains a histidine in the squid K channel sequence. The squid sequence also contains a lysine residue in this loop. We therefore suggest that this region contains the binding site for Zn^{2+} and is involved in the protein conformational change underlying the late phase of ionic current activation. No data are currently available to suggest regions for binding of internal Zn^{2+} ions.

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REFERENCES

- Almers, W. 1978. Gating currents and charge movements in excitable membranes. *Rev. Physiol. Biochem. Pharmacol.* 82:96-190.
- Almers, W., and C. M. Armstrong. 1980. Survival of K^+ permeability and gating currents in squid axons perfused with K^+ -free media. *J. Gen. Physiol.* 75:61-78.
- Armstrong, C. M. 1981. Sodium channels and gating currents. *Physiol. Rev.* 61:644-682.
- Armstrong, C. M., and F. Bezanilla. 1977. Inactivation of the sodium channel. II. Gating current experiments. *J. Gen. Physiol.* 70:567-590.
- Begenisich, T. 1979. Conditioning hyperpolarization-induced delays in the potassium channels of myelinated nerve. *Biophys. J.* 27:257-266.
- Begenisich, T., and C. Lynch. 1974. Effects of internal divalent cations on voltage-clamped squid axons. *J. Gen. Physiol.* 63:675-689.
- Bezanilla, F. 1985. Gating of sodium and potassium channels. *J. Membr. Biol.* 88:97-111.
- Bezanilla, F., and C. M. Armstrong. 1977. Inactivation of the sodium channel. I. Sodium current experiments. *J. Gen. Physiol.* 70:549-566.
- Bezanilla, F., E. Perozo, D. M. Papazian, and E. Stefani. 1991. Molecular basis of gating charge immobilization in *Shaker* potassium channels. *Science.* 254:679-683.
- Bezanilla, F., M. M. White, and R. E. Taylor. 1982. Gating currents associated with potassium channel activation. *Nature.* 296:657-659.
- Boland, L. M., M. E. Jurman, and G. Yellen. 1994. Cysteines in the *Shaker* K^+ channel are not essential for channel activity or zinc modulation. *Biophys. J.* 66:694-699.
- Busath, D., and T. Begenisich. 1982. Unidirectional sodium and potassium fluxes through the sodium channel of squid giant axons. *Biophys. J.* 40:41-49.
- Caceci, M. S., and W. P. Cacheris. 1984. Fitting curves to data. *Byte.* 9:340-362.
- Chandler, W. K., and H. Meves. 1965. Voltage clamp experiments on internally perfused giant axons. *J. Physiol.* 180:788-836.
- Chandler, W. K., and H. Meves. 1970. Sodium and potassium currents in squid axons perfused with fluoride solutions. *J. Physiol.* 211:623-652.
- Cole, K. S., and J. W. Moore. 1960. Ionic current measurements in the squid giant axon membrane. *J. Gen. Physiol.* 44:123-167.
- Conti, F., and W. Stühmer. 1989. Quantal charge redistributions accompanying the structural transitions of sodium channels. *Eur. Biophys. J.* 17:53-59.
- De Biasi, M., J. A. Drewe, G. E. Kirsch, and A. M. Brown. 1993. Histidine substitution identifies a surface position and confers Cs^+ selectivity on a K^+ pore. *Biophys. J.* 65:1235-1242.
- Drewe, J. A., S. Verma, G. Frech, and R. H. Joho. 1992. Distinct spatial and temporal expression patterns of K^+ channel mRNAs from different subfamilies. *J. Neurosci.* 12:538-548.
- Frankenhaeuser, B., and A. L. Hodgkin. 1956. The after-effects of impulses in the giant nerve fibres of *Loligo*. *J. Physiol.* 131:341-376.
- Gilly, W. F., and C. M. Armstrong. 1980. Gating current and potassium channels in the giant axon of the squid. *Biophys. J.* 29:485-492.
- Gilly, W. F., and C. M. Armstrong. 1982. Divalent cations and the activation kinetics of potassium channels in squid giant axons. *J. Gen. Physiol.* 79:965-996.
- Hill, T. L., and Y. Chen. 1971a. On the theory of ion transport across the nerve membrane. III Potassium ion kinetics and cooperativity (with $x = 4, 6, 9$). *Proc. Natl. Acad. Sci. USA.* 68:2488-2492.
- Hill, T. L., and Y. Chen. 1971b. On the theory of ion transport across the nerve membrane. II. Potassium ion kinetics and cooperativity (with $x = 4$). *Proc. Natl. Acad. Sci. USA.* 68:1711-1715.
- Hille, B. 1992. *Ionic Channels of Excitable Membranes*, 2nd ed. Sinauer Associates, Sunderland, MA.
- Hoshi, T., W. N. Zagotta, and R. W. Aldrich. 1994. *Shaker* potassium channel gating I: transitions near the open state. *J. Gen. Physiol.* 103:249-278.
- Jan, L. Y., and Y. N. Jan. 1992. Structural elements involved in specific K^+ channel functions. *Annu. Rev. Physiol.* 54:537-555.
- Kalfakakou, V., and T. J. Simons. 1990. Anionic mechanisms of zinc uptake across the human red cell membrane. *J. Physiol.* 421:485-497.
- Koren, G., E. R. Liman, D. E. Logothetis, B. Nadal-Ginard, and P. Hess. 1990. Gating mechanism of a cloned potassium channel expressed in frog oocytes and mammalian cells. *Neuron.* 2:39-51.
- MacKinnon, R. 1991. Determination of the subunit stoichiometry of a voltage-activated potassium channel. *Nature.* 350:232-235.
- McCormack, K., W. J. Joiner, and S. Heinemann. 1994. A characterization of the activating structural rearrangements in voltage-dependent *Shaker* K^+ channels. *Neuron.* 12:301-315.
- Palti, Y., G. Ganot, and R. Stampfli. 1976. Effect of conditioning potential on potassium current kinetics in the frog node. *Biophys. J.* 16:261-273.
- Pardo, L. A., S. H. Heinemann, H. Terlau, U. Ludewig, C. Lorra, O. Pongs, and W. Stühmer. 1992. Extracellular K^+ specifically modulates a rat brain K^+ channel. *PNAS.* 89:2466-2470.

- Perri, M. A., J. J. C. Rosenthal, T. Liu, and W. F. Gilly. 1994. Cloning and distribution of KV-1 type potassium channel sequences in the squid stellate ganglia. *Biophys. J.* 66:104a. (Abstr.)
- Rush, R. M., and J. H. Yoe. 1954. Colorimetric determination of zinc and copper with 2-carboxy-2'-hydroxy-5'-sulfoformazylbenzene. *Anal. Chem.* 26:1345-1347.
- Rudy, B., J. H. Hoyer, H. A. Lester, and N. Davidson. 1988. At least two mRNA species contribute to the properties of rat brain A-type potassium channels expressed in *Xenopus* oocytes. *Neuron.* 1:649-658.
- Schoppa, N. E., K. McCormack, M. A. Tanouye, and F. J. Sigworth. 1992. The size of gating charge in wild-type and mutant *Shaker* potassium channels. *Science.* 255:1712-1715.
- Sillén, L. 1964. Stability Constants of Metal-Ion Complexes. London: Chemical Society, London.
- Spires, S., and T. Begenisich. 1989. Pharmacological and kinetic analysis of K channel gating currents. *J. Gen. Physiol.* 93:263-283.
- Spires, S., and T. Begenisich. 1990. Modification of potassium channel kinetics by histidine-specific reagents. *J. Gen. Physiol.* 96:757-775.
- Spires, S., and T. Begenisich. 1992. Chemical properties of the divalent cation binding site on potassium channels. *J. Gen. Physiol.* 100:181-193.
- Spires, S., and T. Begenisich. 1994. Modulation of potassium channel gating by external divalent cations. *J. Gen. Physiol.* 104:675-692.
- Stanfield, P. R. 1975. The effect of zinc ions on the gating of the delayed rectifier potassium conductance of frog sartorius muscle. *J. Physiol.* 251:711-735.
- Stuhmer, W., F. Conti, M. Stocker, O. Pongs, and S. H. Heinemann. 1991. Gating currents of inactivating and non-inactivating potassium channels expressed in *Xenopus* oocytes. *Pflügers Arch.* 418:423-429.
- White, M. M., and F. Bezanilla. 1985. Activation of squid axon K⁺ channels: ionic and gating current studies. *J. Gen. Physiol.* 85:539-554.
- Zagotta, W. N., and R. W. Aldrich. 1990. Voltage-dependent gating of *Shaker* A-type potassium channels in *Drosophila* muscle. *J. Gen. Physiol.* 95:29-60.
- Zagotta, W. N., T. Hoshi, and R. W. Aldrich. 1994. *Shaker* potassium channel gating. III. Evaluation of kinetic models for activation. *J. Gen. Physiol.* 103:321-362.
- Zagotta, W. N., T. Hoshi, J. Dittman, and R. W. Aldrich. 1994. *Shaker* potassium channel gating. II. Transitions in the activation pathway. *J. Gen. Physiol.* 103:279-319.