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Modulation of Gating of Cloned Rat and Human K^+ Channels by Micromolar Zn^{2+}

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SUMMARY

The actions of zinc ions on three species of K^+ channels were studied using mouse fibroblasts stably transfected with a plasmid containing both the appropriate K^+ channel gene and a steroid-inducible promotor. The channels studied were rKv1.1 and hKv1.5, delayed rectifiers cloned from rat and human tissue, respectively, and hKv1.4, an inactivating human K^+ channel. Zn^{2+} shifted the activation curves for all three K^+ currents in the depolarizing direction and also shifted the steady state inactivation curve for hKv1.4 in the depolarizing direction. The effect of Zn^{2+} was concentration dependent between 2 and 1000 μM. As a consequence of the modulation of gating, the activation kinetics of the K^+ currents were slowed by Zn^{2+}, an effect likely to delay repolarization of the neuronal action potential. The action of Zn^{2+} on these diverse K^+ channels suggests the existence of a common Zn^{2+} binding domain, the occupation of which influences the voltage sensor. The resulting modulation of gating of hKv1.4 by Zn^{2+} may well be of physiological significance, in view of the localization of this channel in mossy fiber nerve terminals in the hippocampus, where Zn^{2+} is found in abundance.

K^+ channels play an important role in regulating the excitability of nerve and muscle cells (1). Multiple classes of K^+ channels are known to exist (2), and many K^+ channels have now been cloned from *Drosophila* (3, 4), mouse (5), rat (6, 7), and human (8, 9).

Divalent cations have been shown to alter the gating behavior of ion channels at millimolar concentrations. Voltage-dependent Na^+ channels in squid axon (10–12), voltage-dependent Ca^{2+} channels (13), and some voltage-dependent K^+ channels apparently share this property (14). Recently, we have demonstrated that the gating of transient outward currents in hippocampal neurons is modulated by Zn^{2+} at low micromolar concentrations (15). These observations may have important consequences for regulation of central nervous system excitability, because Zn^{2+} is endogenous to the hippocampus and other brain regions (16).

However, it is difficult to study K^+ channel modulation in neurons, because multiple channel species are present, even within one cell. For example, hippocampal neurons express delayed rectifier current, A current, and Ca^{2+}-dependent K^+ current (17). In addition, it is known that, even within a single neuron, each of these different classes of K^+ current may arise from the presence of multiple members of a single K^+ channel 'family' (18); for example, the transient outward current found in neurons may consist of several *Shaker*-like components. Study of ion channels at the molecular level is greatly facilitated by the expression of single gene products in host cells that have no detectable native ion channels. In the present study we have obtained functional expression of three cloned human and rat K^+ channels in mouse L cells and report here the effects of Zn^{2+} on the gating of these individual K^+ channel species.

Materials and Methods

Plasmid construction, transfection, and dexamethasone induction. Plasmid construction and transfection were carried out essentially as described previously (19). Fragments containing the coding region of each K^+ channel gene [rKv1.1, nucleotides −45 to 1548 (7); hKv1.4, nucleotides 290–2800 (8); hKv1.5, nucleotides 161–2059 (8)] were subcloned in the *Eco*RV site of pMSVNeo (20). This vector contains a dexamethasone-inducible murine mammary tumor virus promotor controlling transcription of the inserted cDNA and a gene conferring neomycin resistance driven by the simian virus 40 early promotor. The cDNA-containing expression vector was transfected into mouse Ltk^- cells (21). After 24 hr, selection with 0.5 mg/ml G418 was initiated for 2 weeks or until discrete foci formed. Individual foci were isolated, maintained in G418, and screened for the relevant RNA by Northern analysis. One of the resultant cell lines was selected for each channel species. Transfected cell lines were cultured in Dulbecco's modified Eagle medium supplemented with 10% horse serum and 0.2 mg/ml G418, under a 5% CO_2 atmosphere. The cultures were passaged every 3–4 days, using a brief trypsin treatment. Before experimental use, subconfluent cultures were incubated with 2–20 μM dexamethasone for 24 hr. The cells were then removed from the dish with a rubber policeman, a procedure that leaves the vast majority of the cells intact

ABBREVIATIONS: BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

and avoids the use of enzymatic treatment. The cell suspension was then allowed to reattach for several hours before recordings were initiated. Sham-transfected cells, untransfected cells, and transfected cells that did not receive dexamethasone treatment have all been tested (19), and no voltage-activated outward currents were recorded.

Solutions. The whole-cell patch-clamp technique was used to record membrane currents. Patch pipettes were filled with a solution of (in mM) 140 KCl, 5 K_2ATP, 2 $MgCl_2$, 5 BAPTA, H8, and 5 HEPES/KOH, pH 7.2. The osmolarity of the pipette solution was measured using an osmometer and was adjusted to 310 mosmol. Seal resistances were ≥5 GΩ and pipette resistances were ≤4 MΩ. The extracellular medium contained (in mM) 140 NaCl, 3 KCl, 6 D-glucose, 1.5 $CaCl_2$, and 1 $MgCl_2$, buffered to pH 7.4 with 10 mM HEPES/NaOH. The neurons were continuously perfused at 2 ml/min at 25°. Zn^{2+} was dissolved directly in the recording medium and applied by superfusion or from an array of 200-μm-diameter "flow-pipes."

Data acquisition and analysis. Currents were low-pass filtered at 5–10 kHz (−3 dB, Bessel filter 902; Frequency Devices Inc.), sampled and digitized by using a TL-1–125 interface (Axon), and stored for off-line analysis. Voltage commands, acquisition, and analysis of stored data were achieved using AXOBASIC (Axon). The reversal potential, E_R, for each of the currents studied was measured using tail current analysis, and the peak chord conductance values, g, at each potential were calculated from the peak currents as follows: $g = I/(V_{test} - E_R)$. Activation curves were then fitted using the following equation: $g/g_{max} = [1 + \exp(V_{1/2} - V_{test})/k]^{-1}$, in which g_{max} is the maximum conductance, $V_{1/2}$ is the voltage at which the current is half-activated, and k is a factor describing the steepness of the activation curve. Similarly, steady state inactivation curves were fitted using the following equation: $I/I_{max} = [1 + \exp(V_{hold} - V_{1/2})/k]^{-1}$, in which I_{max} is the maximum current, $V_{1/2}$ is the voltage at which the current is half-inactivated, V_{hold} is the conditioning potential, and k is a slope factor.

Results

Delayed rectifier currents. In cells expressing the delayed rectifier current hKv1.5, a depressant effect of Zn^{2+}, which was fully reversible on washing out of Zn^{2+}, was observed by using a single-step command from −80 mV to 0 mV (Fig. 1A). To quantify the effects of Zn^{2+}, activation curves were constructed for hKv1.5 in the presence and absence of various concentrations of Zn^{2+}; the holding potential was −80 mV throughout. Fig. 1B shows an example of activation curves obtained under control conditions and in the presence of 200 μM Zn^{2+}. The control curve was best fitted by a Boltzmann-type equation (see Materials and Methods) with $V_{1/2} = -2.8$ mV and $k = 10.8$ mV. In the presence of 200 μM Zn^{2+}, the corresponding values were $V_{1/2} = 20.0$ mV and $k = 15.4$ mV. Zn^{2+} at 200 μM thus caused a rightward shift (by 22.8 mV) of the activation curve for hKv1.5 and somewhat depressed the slope of the activation curve. In experiments with cells expressing the rat delayed rectifier channel rKv1.1, an analogous effect of Zn^{2+} was observed (Fig. 2, A and B). The shift in the activation curve was approximately parallel in this case, with a small decrease in the slope of the curve in the presence of Zn^{2+}. From a number of experiments of this type it was possible to construct a concentration-response curve for the modulation of rKv1.1 by Zn^{2+} (Fig. 2C). This curve was fitted according to a single-binding site model, yielding best-fit estimates of 48 μM for the apparent dissociation constant, K_d, and 38 mV for the maximal rightward shift of the activation curve; the Hill slope was estimated to be 1.03.

Transient outward current. In cells expressing the transient outward current hKv1.4, Zn^{2+} depressed outward currents evoked by single-step commands in a concentration-dependent manner (Fig. 3A) and shifted the activation curve for the current in the depolarizing direction. Fig. 3B shows an example of activation curves obtained under control conditions and in the presence of 20 μM Zn^{2+}. The control curve was fitted best by a Boltzmann equation with $V_{1/2} = -14.1$ mV and $k = 13.8$ mV. In the presence of 20 μM Zn^{2+}, the corresponding values were $V_{1/2} = 0.5$ mV and $k = 14.8$ mV. Zn^{2+} at 20 μM thus caused a parallel rightward shift (by 14.6 mV) of the activation curve for hKv1.4. In addition, Zn^{2+} also modulated the inactivation behavior of hKv1.4 (Fig. 3C). Fig. 3C shows inactivation curves obtained under control conditions and in the presence of 20 and 200 μM Zn^{2+}. The control curve was best fitted by a Boltzmann equation with $V_{1/2} = -39.7$ mV and $k = 3.7$ mV. In

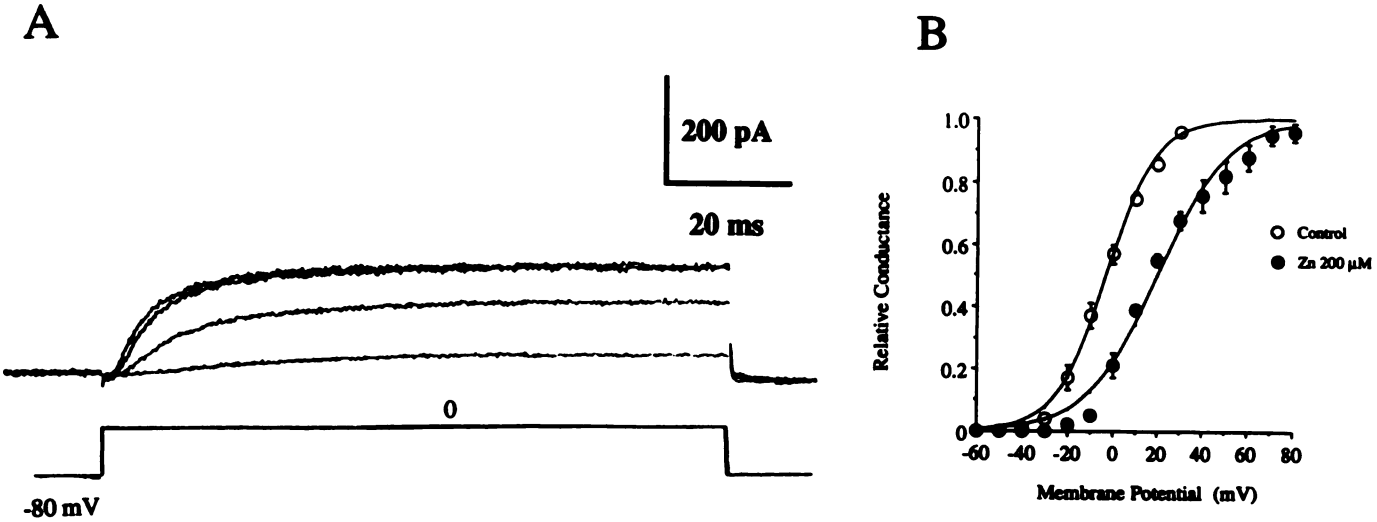

Fig. 1. Concentration-dependent effect of Zn^{2+} on hKv1.5. A, Effect of three different Zn^{2+} concentrations on the hKv1.5 current elicited by a 200-msec depolarizing command to 0 mV from −80 mV. *Traces* were recorded in the presence of 0, 2, 20, and 200 μM Zn^{2+}. B, Effect of 200 μM Zn^{2+} on the activation curve for hKv1.5. Conductance calculations and activation curve fitting were performed as described in Materials and Methods. The control data were best fitted by an equation with slope factor $k = 10.8$ mV and half-activation voltage $V_{1/2} = -2.8$ mV. In the presence of 200 μM Zn^{2+}, the corresponding values were $k = 15.4$ mV and $V_{1/2} = 20.0$ mV. Each *point* represents the mean ± standard error of four to six experiments.

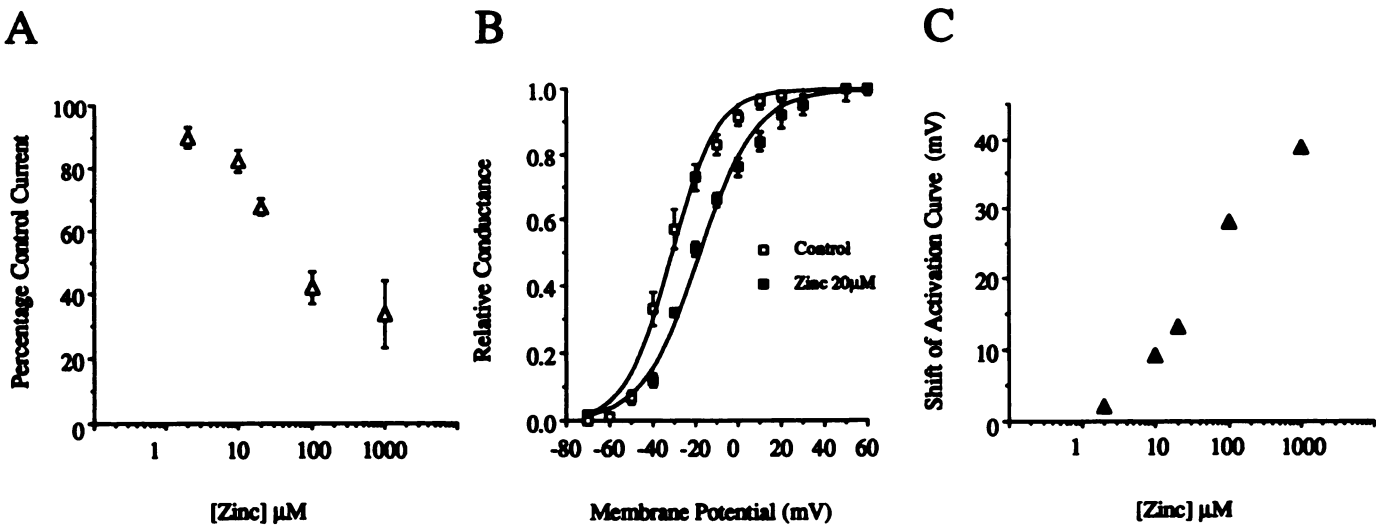

Fig. 2. Concentration-dependent effect of Zn^{2+} on rKv1.1. A, Concentration-effect curve for Zn^{2+} inhibition of the steady state rKv1.1 current elicited by a step to −20 mV from −80 mV. Each *point* represents the mean ± standard error of four experiments. B, Effect of 20 μM Zn^{2+} on the activation curve for rKv1.1. Conductance calculations and activation curve fitting were performed as described in Materials and Methods. The control data were best fitted by an equation with slope factor k = 10.5 mV and $V_{1/2}$ = −30.8 mV. In the presence of 20 μM Zn^{2+}, the corresponding values were k = 13.1 mV and $V_{1/2}$ = −17.7 mV. Each *point* represents the mean ± standard error of four or five experiments. C, Dose-effect curve for Zn^{2+} in shifting the activation curve for rKv1.1. The Zn^{2+} concentrations used were 2, 10, 20, 100, and 1000 μM.

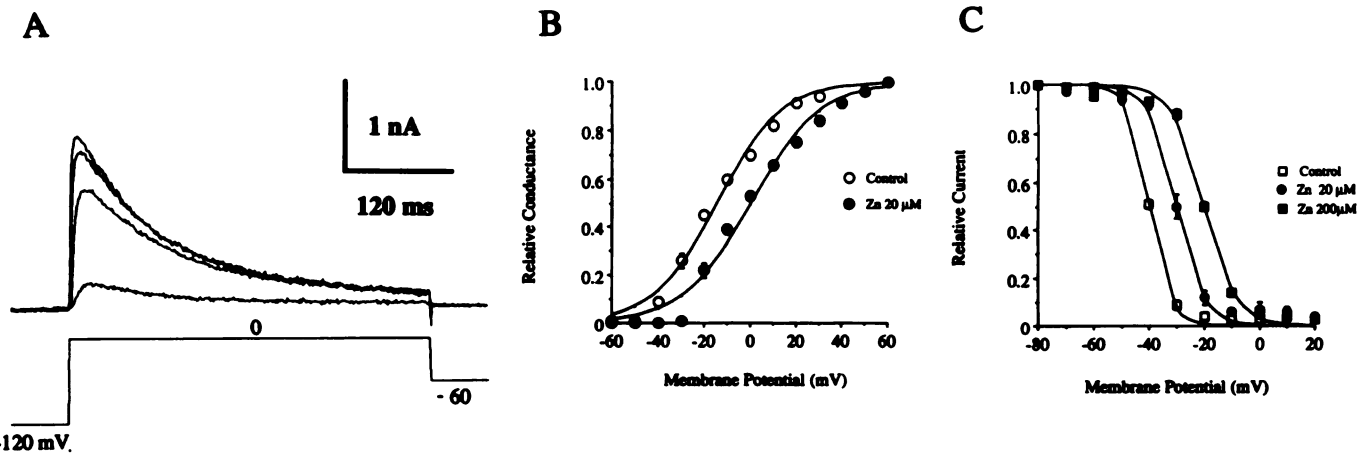

Fig. 3. Zn^{2+} shifts inactivation and activation curves for hKv1.4. A, Effect of three different Zn^{2+} concentrations on the hKv1.4 current elicited by a 200-msec depolarizing command to 0 mV from −120 mV. *Traces* shown (from the *top*) were recorded in 0, 2, 20, and 200 μM Zn^{2+}. B, Effect of Zn^{2+} on the activation curve for hKv1.4. The control data were best fitted by an equation with slope factor k = 13.8 mV and $V_{1/2}$ = −14.1 mV. In the presence of 20 μM Zn^{2+}, the corresponding values were k = 14.8 mV and $V_{1/2}$ = 0.5 mV. Each *point* represents the mean ± standard error of four to seven experiments. C, Effect of 20 and 200 μM Zn^{2+} on the inactivation curve for hKv1.4. Conductance calculations and curve fitting were performed as described in Materials and Methods. The control data were best fitted by an equation with slope factor k = 3.7 mV and $V_{1/2}$ = −39.7 mV. The dwell time at the conditioning potential was 500 msec. In the presence of 20 μM Zn^{2+}, the corresponding values were k = 5.0 mV and $V_{1/2}$ = −29.8 mV. In the presence of 200 μM Zn^{2+}, the corresponding values were k = 5.8 mV and $V_{1/2}$ = −19.9 mV. Each *point* represents the mean ± standard error of four to seven experiments.

the presence of 20 μM Zn^{2+}, the corresponding values were $V_{1/2}$ = −29.8 mV and k = 5.0 mV. In the presence of Zn^{2+}, the corresponding values were $V_{1/2}$ = −19.9 mV and 200 μM k = 5.8 mV.

Modulation by Zn^{2+} of hKv1.5 activation kinetics. At any given membrane potential, Zn^{2+} slowed activation of outward currents activated by depolarizing commands. An example of this effect is seen in Fig. 1, in which the delayed rectifier current hKv1.5 activated progressively more slowly in the presence of increasing concentrations of Zn^{2+}. The effect of membrane potential on the activation kinetics of hKv1.5 and the modulatory effect of Zn^{2+} on the activation kinetics are displayed graphically in Fig. 4A. The activation kinetics for hKv1.5 were slow at modest levels of depolarization and were speeded by stronger depolarization, approaching an asymptote at about +40 mV. In the presence of Zn^{2+}, the activation kinetics of hKv1.5 slowed still more at modest depolarizations but tended to approach the same asymptotic value at stronger depolarizations. The degree of slowing at a given membrane potential was dependent upon the Zn^{2+} concentration. The influence of Zn^{2+} on activation kinetics is more clearly illustrated in Fig. 4B, in which the same data are displayed as reciprocals of the time to half-activation. In this plot, it is quite clear that at low concentrations of Zn^{2+} there was a pure parallel rightward shift of the curve relating speed of channel opening to membrane potential. This would tend to support the idea

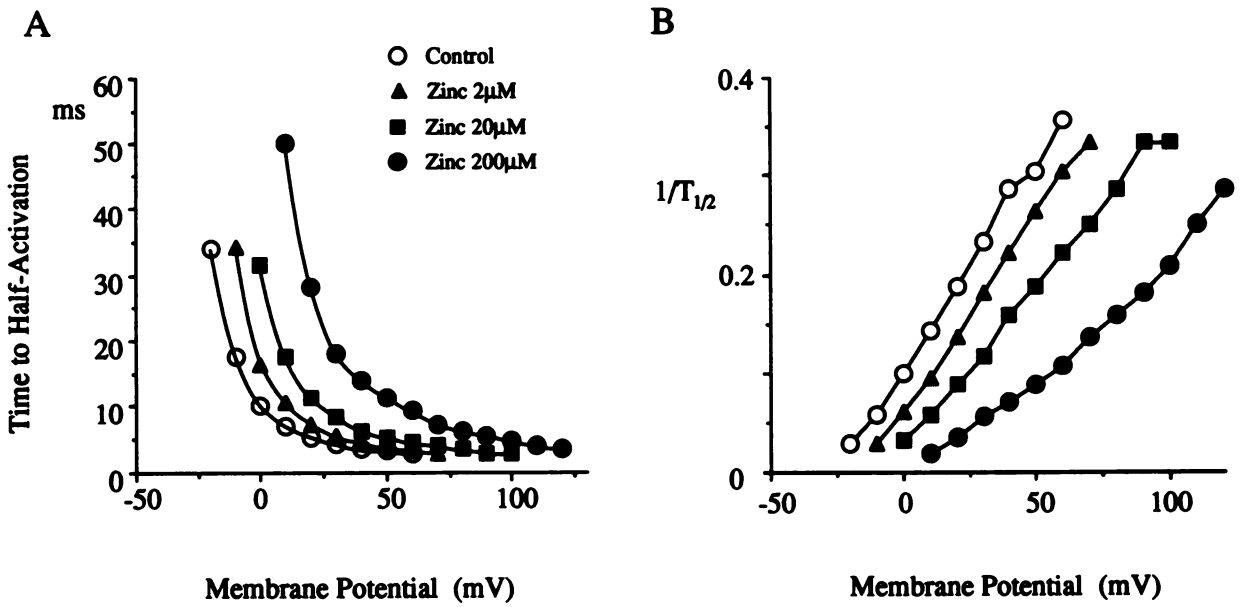

Fig. 4. Zn^{2+} slows activation kinetics of hKv1.5. A, Effect of three different Zn^{2+} concentrations on the time to half-activation for the hKv1.5 current elicited by depolarizing commands from −80 mV; data are taken from a single experiment. *Open symbols*, control kinetics; *filled symbols*, data obtained with 2, 20, and 200 μM Zn^{2+}. B, The same data plotted as reciprocals of the half-activation times, illustrating the rightward shift of the curve toward more positive membrane potentials in the presence of Zn^{2+}.

that the primary action of Zn^{2+} is to modify channel gating. At the highest concentration of Zn^{2+} shown in Fig. 4B, there was a decrease in the slope of the plot, perhaps indicating an additional effect of Zn^{2+} on the kinetics of channel opening that is independent of the shift in the voltage dependence of channel gating.

Discussion

We have demonstrated a potent effect of Zn^{2+} on the gating of the delayed rectifier currents rKv1.1 and hKv1.5 and the transient outward current hKv1.4, expressed in mouse L cells. Lower concentrations of Zn^{2+} cause approximately parallel shifts in the activation and inactivation curves for these currents, whereas at higher concentrations (≥200 μM) the slopes of these curves become more shallow. This suggests to us that the primary effect of Zn^{2+} is to modify channel gating at low concentrations, whereas at higher concentrations (≥200 μM) an additional channel-blocking effect may also become apparent. Divalent metal ion block of delayed rectifier channels (22, 23) and cardiac Na^+ channels (24) has been reported. In the case of the delayed rectifier channels, such interactions have occurred in the millimolar concentration range, as distinct from the micromolar potency of Zn^{2+} reported here in altering channel gating.

The concentration-response data for rKv1.1 do suggest that a single binding site for Zn^{2+} might be responsible for the changes in gating and that Zn^{2+} binds to this site with relatively high affinity, with K_d in the vicinity of 50 μM. Zn^{2+} also modulates gating of transient outward current in hippocampal neurons (15) and in cardiac myocytes (25) but does not alter gating of delayed rectifier current in cultured hippocampal neurons (15). This latter observation suggests that not all K^+ channels are modulated by Zn^{2+}, although all three channel species studied here show Zn^{2+} sensitivity. Sensitivity to Zn^{2+} may prove to be another useful tool for the correlation of cloned K^+ channel gene products with endogenous K^+ channel activity.

The identification of the binding site for Zn^{2+} would be greatly facilitated by the cloning of a Zn^{2+}-insensitive K^+ channel; unfortunately, such a cloned K^+ channel has yet to be identified, although delayed rectifier currents in cultured hippocampal neurons appear to be insensitive to Zn^{2+} (15). Examination of the amino acid sequence of a Zn^{2+}-insensitive K^+ channel and comparison with the sequences of the Zn^{2+}-sensitive channels would undoubtedly yield interesting information on the nature of the ion binding site. However, in the absence of such information we can only speculate on the nature of the binding site for Zn^{2+}. The "zinc finger" binding domain is not present in the K^+ channel polypeptides studied here. We can, however, speculate that the Zn^{2+} binding site is likely to be located close to the S4 segment, which is believed to function as a "voltage sensor" (26, 27). Binding of Zn^{2+} close to the four positively charged amino acid residues of S4 might be expected to modify gating by electrostatic interactions, as suggested previously for channel modification by phosphorylation (28). Clearly, further information will be required to pinpoint the Zn^{2+} binding site.

It has been reported that Zn^{2+} causes abnormal discharges ("giant depolarizing potentials") in the CA3 region of the hippocampus and that these may occur spontaneously and are prevented by selective chelators of Zn^{2+} (29). These giant depolarizing potentials are prevented by the γ-aminobutyric acid receptor antagonist bicuculline and thus appear to result from the release of excessive amounts of γ-aminobutyric acid, perhaps as a consequence of high frequency firing of interneurons (30). It is interesting to note that the distribution of Kv1.4 has recently been mapped using immunohistochemistry. The channel protein appears to be concentrated in axons and terminals (31). In the hippocampal formation, the Kv1.4 immunoreactivity was concentrated in mossy fiber terminals, where the highest concentrations of Zn^{2+} are also found (16). It has been suggested that Kv1.4 might play a role in regulating the excitability of nerve terminals and thereby controlling neuro-

transmitter release (31). The potent modulation by Zn^{2+} of gating of hKv1.4 reported here would, therefore, provide a physiologically relevant mechanism for the alteration by Zn^{2+} of transmission in the hippocampus; however it should be noted that Zn^{2+} also inhibits *N*-methyl-D-aspartate receptor-gated channels (32) and produces a voltage-dependent open channel block of cardiac Na^+ channels (24, 33). In conclusion, we have demonstrated a potent modulatory action of Zn^{2+} on the gating of cloned rat and human K^+ channels. Further investigation of K^+ channel sensitivity to Zn^{2+} might be expected to yield interesting structural information on the nature and location of the Zn^{2+} binding site on these channel molecules.

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