Divalent Cations and the Activation Kinetics of Potassium Channels in Squid Giant Axons

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ABSTRACT The effects of external Zn\(^{2+}\) and other divalent cations on K channels in squid giant axons were studied. At low concentration (2 mM) Zn\(^{2+}\) slows opening kinetics without affecting closing kinetics. Higher concentrations (5–40 mM) progressively slow opening and speed channel closing to a lesser degree. In terms of "shifts," opening kinetics are strongly shifted to the right on the voltage axis, and OFF kinetics much less so. The shift of the conductance-voltage relation along the axis is intermediate. Zinc’s kinetic effects show little sign of saturation at the highest concentration attainable. Zn does not alter the shape of the instantaneous current-voltage relation of open channels. Some other divalent cations have effects similar to Zn\(^{2+}\), Hg\(^{2+}\) being the most potent and Ca\(^{2+}\) the least. After treatment with Hg\(^{2+}\), which is irreversible, Zn\(^{2+}\) still slows opening kinetics, which suggests that each channel has at least two sites for divalent cation action. The results are not compatible with a simple theory of fixed, uniform surface charges. They suggest that external cations interact directly with a negatively charged element of the gating apparatus that moves inward from the membrane's outer surface during activation. Examination of normal kinetics shows that there is a slow step somewhere in the chain leading to channel opening, but the slowest step must not be the last one.

INTRODUCTION

Potassium-selective channels in nerve membrane, like Na channels, are sensitive to certain divalent cations in the external medium. In many cases both channels are said to be affected by these cations in qualitatively similar ways (squid, Frankenhaeuser and Hodgkin, 1957; lobster, Blaustein and Goldman, 1968; frog, Hille, 1968; Xenopus, Vogel, 1974; Arhem, 1980). The reported effects of divalent cations have usually been interpreted in terms of the fixed surface charge theory. In the accompanying paper (Gilly and Armstrong, 1982), we showed that the action of Zn (II) on Na channel activation does not fit the predictions of fixed surface charge theory. When added to the external medium, Zn\(^{2+}\) slows

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opening (on) kinetics of Na channels as judged from both ionic and gating currents, but does not much alter closing (off) kinetics. The fixed surface charge theory predicts equivalent effects on both opening and closing kinetics. We proposed instead a more specific interaction of Zn\(^{2+}\) with Na channel gating charge: electrostatic attraction of an aqueous Zn ion and mobile, negative charges of the Na channel's gating apparatus. The charges are exposed on the membrane's outer surface when the channel is in the closed state. Depolarization normally causes inward migration of these negative charges, generating an outward gating current transient, and leading to Na channel activation. When Zn\(^{2+}\) is present, it electrostatically attracts the gating charges, thereby stabilizing the closed conformation of the channel. Interaction of Zn\(^{2+}\) and gating charge is weak enough to be overcome by sufficient depolarization, and once the gating charge migrates inward some distance, the external Zn ion is no longer electrically visible to it. Thus, the functional Zn\(^{2+}\) "receptor" disappears when the channel is activated, and the off kinetics with which the channel returns to the resting state are largely unaltered.

We report here that Zn\(^{2+}\) affects K channels in qualitatively the same way as Na channels, slowing on kinetics much more than it affects off kinetics or the conductance-voltage relation. K channels are far more sensitive to Zn\(^{2+}\) than are Na channels, but the interactions of Zn\(^{2+}\) and gating charges appear to be similar in both cases. As with Na channels, there is more than one Zn\(^{2+}\) receptor per channel, and more than one step in activation is affected by Zn\(^{2+}\). The discussion in the previous paper of models fitting these facts is continued here. Several models are possible, but a common feature in all of them is the presence of negative gating charge at the membrane's outer surface when a Na or K channel is in the resting state.

**METHODS**

Except for solutions, the methods and apparatus were identical to those described in the preceding paper (Gilly and Armstrong, 1982). The sole internal solution used contained 225 mM K glutamate, 50 mM KF, 420 mM sucrose, and 10 mM Tris 7.0. The pH was 7.0-7.2 and osmolality was 1,060 mosmol/kg. Three external solutions differing in their K concentrations were used: 50, 100, or 150 mM KCl, with, respectively, 430, 380, or 330 mM Tris 7.0, 50 mM CaCl\(_2\), and 2 \(\times\) 10\(^{-7}\) M tetrodotoxin. Divalent transition metals were substituted for Tris. Osmolality of external solutions was 950 mosmol/kg in all cases, and pH was titrated to 7.0-7.1 with Tris-free base.

Temperature for all experiments was 10°C, and the holding potential was -70 mV. Data sampling was at 20 μs per point. Compensation for 3 ohm·cm\(^2\) of series resistance was used. Linear ionic and capacity currents were subtracted from all records using the P/4 method with control pulses starting from a level of -130 mV.

**RESULTS**

Zn\(^{2+}\) Affects K Channel Opening (on) Kinetics More Than Closing (off)

At a concentration of 2 mM, ZnCl\(_2\) affects K channels in a manner almost identical to the effect of 30 mM Zn\(^{2+}\) on Na channels (Gilly and
Armstrong, 1982). Fig. 1A shows the action of 2 mM Zn\(^{+2}\) in the presence of 50 mM Ca\(^{+2}\). The upper pair of traces are control \(I_K\) for a large depolarization recorded before application of 2 mM Zn\(^{+2}\) and after washing it away. They are nearly indistinguishable. The 2-mM Zn\(^{+2}\) trace turns on more slowly and is slightly smaller, but turns off at the normal rate. In Fig. 1B the Zn\(^{+2}\) trace has been scaled to match the steady value of the averaged control \(I_K\). Slowing of \(I_K\) on is obvious, but there is no change in off kinetics.

**Figure 1.** Effect of 2 mM ZnCl\(_2\) in the presence of 50 mM CaCl\(_2\) on \(I_K\) on (+80 mV) and off (−70 mV). A. The largest trace is control before Zn\(^{+2}\) application, the smallest is in 2 mM Zn\(^{+2}\), and the middle trace is after Zn\(^{+2}\) washout. \(I_K\) on in Zn\(^{+2}\) is slower and smaller than normal. B. Zn\(^{+2}\) trace is scaled (1.087X) to match final amplitude of averaged control traces from A. \(I_K\) on in Zn\(^{+2}\) is slowed; \(I_K\) off is not altered. Axon MA200A (50 K).
Higher concentrations of Zn produce larger effects of K channel opening and can also affect the rate of channel closing. Several $I_K$ on traces are plotted in Fig. 2A. All were recorded at +80 mV in the presence of 0–40 mM Zn$^{+2}$ as indicated, and have been scaled to the same final amplitude. Slowing of opening kinetics becomes more profound with increasing Zn$^{+2}$ concentrations up to 40 mM, a level near the limit of solubility at pH 7. The steady state reduction of $I_K$ caused by Zn$^{+2}$ also grows with increasing concentration from 5% at 5 mM to 20% at 40 mM. Reversibility of this amplitude reduction was usually fairly complete, but seldom perfect. The effect on opening kinetics was more readily reversible.

Fig. 2B shows the off current ($I_K$ off) as K channels closed at −70 mV. 5 mM Zn$^{+2}$ leads to a small but significant increase in the rate of $I_K$ decay, and the effects of 10–40 Zn$^{+2}$ are larger. This contrasts with our results for 30 mM Zn$^{+2}$ on Na channels where the rate of Na channel closing is not increased (Gilly and Armstrong, 1982). As discussed later, this effect on K channel closing is important to consider in developing a model for zinc's action by modifying the one presented in the preceding paper for Na channels.

**Zinc and ON Kinetics of the $g_K$**

Zinc's action on activation kinetics cannot be explained by a voltage shift. In Fig. 3A current recorded at +80 mV is compared kinetically to control currents (scaled) recorded at +40, 50, and 60 mV. The early part of the Zn$^{+2}$ trace is most similar to the control at 60 mV, and the later part most similar to the one at 40 mV. The same difference between early and late phases is apparent at a higher Zn$^{+2}$ concentration in Fig. 3C, where $I_K$ in Zn$^{+2}$ at 100 mV is compared to scaled control currents at 20, 30, and 40 mV. The early part of the Zn trace is similar to the 30-mV control trace, the final part to the 20-mV control.

This differential Zn$^{+2}$ effect on early and late phases of $I_K$ on is difficult to quantify because the time course of $I_K$ is determined both by the rate of K channel opening and also by the rate of K accumulation in the limited extracellular space between axon and Schwann cell (Frankenhaeuser and Hodgkin, 1956; Adelman et al., 1973). The external solution in Figs. 3C and D contained 150 mM K to minimize the effects of K accumulation, and the greater effect of Zn on the final phase of $I_K$ on was still observed. Analysis of control and Zn-treated $g_K$ time courses using the method of tail currents (see below) also consistently showed this effect.

**OFF Kinetics Are Affected Less Than ON**

As noted in Fig. 1B, 2 mM Zn$^{+2}$ slows $I_K$ on without detectably altering off kinetics. This conclusion is more thoroughly established in Fig. 3B. $I_K$ off in 2 mM Zn$^{+2}$ at −70 mV is compared with control traces at −60, −70, and −80 mV. The Zn$^{+2}$ trace at −70 mV (dotted) is indistinguishable from the control −70 mV record. Thus, a low concentration of Zn$^{+2}$ slows on kinetics without affecting off.
FIGURE 2. Effect of Zn\(^{2+}\) on the rates of K channel opening at +80 mV (A) and closing at −70 mV (B) over a large concentration range. Zn\(^{2+}\) concentration is indicated for each trace. Sequence of measurement was: control (0 Zn), 5 Zn, 0, 10, 0, 20, 0, 40, 0. Illustrated control traces are the averages of five traces, one from each set of control measurements. Axon SE060A,B (100 K). A. Zn\(^{2+}\) slows \(I_K\) on and the effect increases steadily from 5 to 40 mM. Vertical scale bar applies to averaged control (0 Zn) trace only. Zn\(^{2+}\) traces have been scaled as follows: 5 Zn\(^{2+}\) 0.934X; 10 Zn\(^{2+}\) 1.057X; 20 Zn\(^{2+}\) 1.255X; 40 Zn\(^{2+}\) 1.535X. B. Zn\(^{2+}\) speeds \(I_K\) off kinetics, and the effect also increases with concentration. Scaling of Zn traces as follows: 5 Zn\(^{2+}\) 0.973X; 10 Zn\(^{2+}\) 1.216X; 20 Zn\(^{2+}\) 1.651X; 40 Zn\(^{2+}\) 2.131X.
Figure 3. Equivalent voltage shifts of activation kinetics produced by 2 mM (A and B) and 20 mM (C and D) Zn$^{2+}$. In each part of the figure four traces are superimposed: one trace in Zn$^{2+}$ (dotted) at the indicated voltage and three control traces (scaled) at the voltages indicated with arrows. The equivalent voltage shift is approximated by the number of millivolts separating the control and Zn$^{2+}$ trace that best superimpose. Vertical scale bar applies to Zn$^{2+}$ traces only and represents 1.87 mA/cm$^2$ in A and 2.0 mA/cm$^2$ in B–D. A. $I_K$ on effect of 2 mM Zn$^{2+}$. The Zn$^{2+}$ (+80 mV) trace matches the control (+60 mV) during the early rise of $I_K$, then crosses the control (+50 mV) trace and follows the control (+40 mV) record during the final slow approach to its steady value. Thus, the Zn$^{2+}$ effect is not a simple voltage shift of activation kinetics. Scaling of control traces: +40 mV, 1.628X; +50 mV, 1.383X; +60 mV, 1.196X. Same axon as Fig. 1. B. $I_K$ off effect of 2 mM Zn. $I_K$ off was measured after a 13.5-ms pulse to +80 mV. Only a very slight, if any, increase in the rate of $I_K$ off is apparent. Scaling of control traces: −60 mV, 1.12X; −70 mV, 0.937X; −80 mV, 0.79X. Same axon as A. C. $I_K$ on effect of 20 mM Zn$^{2+}$. The same phenomenon as described in A is evident with this high Zn$^{2+}$ concentration; the final slow phase of $I_K$ on is slowed more by Zn$^{2+}$ than is the rapid, early phase. Scaling of control traces: +20 mV, 2.054X; +30 mV, 1.415X; +40 mV, 1.092X. Axon AU280A (150K). D. $I_K$ off effect (measured as in B) of 20 mM Zn$^{2+}$. $I_K$ off decay is speeded by Zn$^{2+}$, and the effect is roughly equivalent to a hyperpolarization of ~25 mV. Scaling of control traces: −80 mV, 0.687X; −90 mV, 0.552X; −100 mV, 0.610X. Same axon as C.

A similar analysis shows that 20 mM Zn$^{2+}$ speeds the rate of K channel closing about as much as would a 20- or 25-mV hyperpolarization (Fig. 3D). This is much less than the 70- or 80-mV “shift” in on kinetics caused by 20 mM Zn$^{2+}$.
Zn Shifts the $g_K$-$V$ Relation Less Than It Does on Kinetics

The fixed surface charge theory of divalent cation action predicts an equal voltage shift of on kinetics, off kinetics, and of the equilibrium $g_K$-$V$ relation. These three quantities are compared in Fig. 4 for a low Zn concentration. The measures used for on and off kinetics were half-time ($t_{1/2}$ ON) and time constant ($\tau_{OFF}$), respectively. $g_K$ was determined as $\Delta I/\Delta V$ as shown in the inset.

In 2 mM Zn$^{+2}$, on half-time was shifted from +14 to +34 mV (indicated by arrows), and off time constant was shifted by 2 mV (Fig. 4A). The shift of the $g_K$-$V$ relation was intermediate, about +6 mV (Fig. 4B). This pattern is the same as for 30 mM Zn$^{+2}$ acting on Na channels (Gilly and Armstrong, 1982).

Results from a 20-mM Zn$^{+2}$ experiment are plotted in Fig. 5. Half-time to peak $I_K$ is shifted by +52 mV in 20 mM Zn$^{+2}$ at the negative end of the voltage range covered, and +74 mV for the most positive point in Zn (+130 mV). Off time constant is shifted from +18 to +21 mV, and again the shift of the $g_K$-$V$ curve is intermediate (+36 mV).

A complete summary of the effect of Zn$^{+2}$ on gating parameters over a large concentration range is presented in Table I. In all cases on kinetics are most shifted, off kinetics are least affected, and the shift of the $g_K$-$V$ curve is intermediate. This is in qualitative agreement with the result on Na channels.

Zn Does Not Cause a Voltage-dependent Block of $K$ Channels

In the preceding paper (Gilly and Armstrong, 1982) we discussed the possibility that Zn$^{+2}$ acted on Na channels by a voltage-dependent plugging mechanism similar to that proposed by Woodhull (1973) for protons and Ca ions. We found no evidence of such an effect of Zn$^{+2}$ on Na channels. We also tested the possibility that Zn$^{+2}$ directly blocks K channels by measuring its effect on the instantaneous current voltage curve. The channels were activated by a first pulse to +100 mV for 10.5 ms, and voltage was then stepped to a new value, which is given on the abscissa in Fig. 6. Current was measured 60 $\mu$s after the second step, and its value is given by the ordinate of each point for a fiber in 100 K $\pm$ 20 Zn (275 K internal medium). In both solutions the points are adequately fit by a straight line whose slope reflects the K conductance. Conductance is reduced by Zn$^{+2}$ to 83% of its control value, and the reduction does not depend on membrane voltage. The mechanism by which Zn$^{+2}$ reduces $g_K$ is unknown, but the lack of voltage dependence indicates that Zn$^{+2}$ does not penetrate into the channel far enough to be influenced by the membrane field.

Effects of Other Divalent Cations

With the exceptions of Cd$^{+2}$ and Hg$^{+2}$, all other divalent cations that we have tested were much less effective than Zn$^{+2}$ in slowing on kinetics. $I_K$ on records in normal and low Ca$^{+2}$ and with the addition of the divalent form of several transition metals are shown in Fig. 7. Changing Ca$^{+2}$ from 12 to 50 mM had a slight slowing effect, as did addition of 10 mM Mn$^{+2}$, Ni$^{+2}$, or Cu$^{+2}$ to an external solution containing 50 mM Ca$^{+2}$. For comparison the effects of 0.5 mM Zn$^{+2}$ (added to 50 mM Ca$^{+2}$) are illustrated. Zn$^{+2}$ is ~20-fold more
Comparison of voltage shifts on (A) kinetic (\(t_{1/2 \text{ ON}}\) and \(\tau_{\text{OFF}}\)) and (B) steady state (\(g_K\)) gating parameters produced by 2 mM Zn\(^{2+}\). Parameters were measured as indicated in inset; \(g_K = \Delta I/\Delta V\) Same axon as in Fig. 1. Data before (\(\bullet\)) and during (\(\bigcirc\)) application of Zn\(^{2+}\) and after washout (\(\triangle\)) are shown. Voltage shifts produced by Zn\(^{2+}\) are indicated by arrows. A. Plot of \(I_K\) OFF time constant (\(\tau_{\text{OFF}}\), left-hand ordinate) and half-time to steady \(I_K\) ON value (\(t_{1/2 \text{ ON}}\), right-hand ordinate) vs. voltage. B. Normalized \(g_K-V\) plots. Zn\(^{2+}\) points have been scaled 1.064X.
effective than the other cations. A complete summary of our results with these weakly effective metal ions is presented in Table II. Curiously, Cu$^{2+}$ at low concentrations (2 and 5 mM) speeds activation kinetics slightly, but at higher concentrations (10 mM) causes a slight slowing.

Cd$^{2+}$ and Hg$^{2+}$, like Zn$^{2+}$, are in group II B of the periodic table and have a strong Zn-like effect as Fig. 8 illustrates. 10 mM Cd$^{2+}$ and 0.1 mM Hg$^{2+}$ are comparable in their effects to 5 mM Zn$^{2+}$. The effect of 1 mM Hg$^{2+}$ is roughly
equivalent to 40 mM Zn$^{+2}$, the highest concentration used. Cadmium, like Zn$^{+2}$, is reversible, but Hg$^{+2}$ is not.

**Mercury Has an Irreversible Zinclike Effect**

Hg$^{+2}$ is of particular interest because of its extreme potency and lack of reversibility. The effect of 0.1 mM Hg$^{+2}$ develops progressively over the first few hundred seconds of application, and then continues to grow at a slower rate during a prolonged exposure. Short exposures were therefore used in this study (see also Table III). The developing Hg$^{+2}$ effect was halted upon washing out the Hg$^{+2}$ solution but could not be reversed even after long periods of washing (hours). Addition of 2 mM EDTA to the external medium did not result in reversal. The progressive Hg$^{+2}$ effect and its irreversibility, at least with a short exposure, is not caused by gross damage of the axon, as evidenced by the lack of a greatly increased leakage conductance. This contrasts with the effects of certain organic mercurials, e.g., methylmercury,

\[
\begin{array}{c|c|c|c}
\text{Zn concentration (mM)} & \text{Shift of } g_K-V \text{ at } 0.5 g_{\text{Max}} & \text{Shift of } t_{1/2 \text{ ON}}-V \text{ at } +100 \text{ mV} & \text{Shift of } \tau_{\text{OFF}}-V \text{ at } -70 \text{ mV} \\
\hline
40 & 0 & +84 (1) & +20 (1) \\
30 & +32 \pm 3.46 (3) & +92.7 \pm 5.49 (3) & +28 (2) \\
20 & +31.8 \pm 1.31 (6) & +76.1 \pm 2.04 (10) & +20.6 \pm 2.02 (10) \\
10 & +16 (1) & +61 (2) & +10.5 (2) \\
7.5 & +15 (1) & +54 (1) & 0 (1) \\
5 & +12.9 \pm 1.16 (4) & +44.4 \pm 2.99 (5) & +8.6 \pm 1.5 (5) \\
2 & +6.8 (2) & +35.5 (2) & +1 (2) \\
0.5 & +2 (2) & +12.5 (2) & -0.5 (2) \\
\end{array}
\]

Concentration dependence of the effect of Zn (in the presence of 50 mM Ca) on parameters of K channel gating. Equivalent shifts in millivolts were determined from plots of $g_K$, $t_{1/2 \text{ ON}}$, and $\tau_{\text{OFF}}$ vs. $V$ as discussed in the text. Shifts caused by Zn are expressed relative to averaged control measurements from before Zn application and after washout. Mean values ± SEM are indicated; number of fibers is given in parentheses.

which blocks K channels and increase leakage (Shrivastav et al., 1976) without greatly altering $g_K$ kinetics (T. Narahashi, personal communication).

The Hg$^{+2}$ effect is very similar to that of Zn$^{+2}$. This is best shown by plots similar to those in Fig. 4 (for Zn), which show that on kinetics are shifted most, off kinetics are affected least, and the shift of the $g_K$-$V$ curve is intermediate. Fig. 9 illustrates data for 0.1 mM Hg$^{+2}$ in this manner, and the similarities between Figs. 4 and 9 are obvious. As is the case with a high concentration of Zn$^{+2}$, 0.1 mM Hg$^{+2}$ also reduced maximum conductance by 20–30% in this experiment.

Despite the overall similarity between the Zn$^{+2}$ (Fig. 4) and Hg$^{+2}$ (Fig. 9) plots, there are slight differences between them that may be significant: (a) near 0 mV the shifts of on kinetics and the $g_K$-$V$ relation are more nearly equal in Hg$^{+2}$ than with Zn$^{+2}$; (b) the shifts of the $t_{1/2 \text{ ON}}$ and $\tau_{\text{OFF}}$ curves seem to depend more on voltage than is the case with Zn$^{+2}$. In Hg$^{+2}$ the
increase in the shift of both curves as voltage goes positive is somewhat more pronounced. Irreversibility of Hg$^{+2}$ makes definitive demonstration of such differences difficult.

Shifts of gating parameters ascribed to 0.1 Hg$^{+2}$ for a number of axons are given in Table III, along with the corresponding Cd$^{+2}$ data. Both ions exert a large effect on K channel opening kinetics and a far smaller effect on the rate of closing. When Tables I and III are compared, 0.1 mM Hg$^{+2}$ appears to act very much like 10–20 mM Zn$^{+2}$, whereas Cd$^{+2}$ is roughly half as potent as Zn$^{+2}$. All three of the IIB transition metals are far more active than any of the divalent cations in Table II.

*K Channels Have More Than One Receptor for IIB Metal Ions*

Although brief Hg$^{+2}$ application leaves K channels in a permanently modified, zincline state (i.e., resembling the presence of a high concentration of Zn$^{+2}$), application of Zn$^{+2}$ after Hg$^{+2}$ treatment still results in a strong Zn$^{+2}$ effect. That is, the effects of the two ions are additive.

Fig. 10A shows three $I_K$ ON traces at +90 mV: in 50 Ca$^{+2}$ before Hg$^{+2}$
Figure 7. Effects of several divalent transition metal ions on $I_K$ on at +80 mV. The faster-rising trace of each superimposed pair is the average of control records taken before application of the particular divalent cation and after washout. 0.5 mM Zn trace indicates the relative potency of these weakly effective ions. Ca was tested by comparing 12 vs. 50 mM Ca$^{2+}$; other metals were tested in the presence of 50 mM Ca$^{2+}$. Time base for all records is identical. Vertical $I_K$ scale bar represents for the control traces (in mA/cm$^2$): 3.83 (Zn$^{2+}$), 3.92 (Ca$^{2+}$), 4.21 (Mn$^{2+}$), 4.18 (Cu$^{2+}$), 3.85 (Ni$^{2+}$). In the presence of the divalent cations the bar represents (mA/cm$^2$): 3.72 (Zn), 4.2 (Ca), 4.43 (Mn), 4.37 (Cu), 3.71 (Ni). Zn$^{2+}$ and Ca$^{2+}$ data are from axon MA300A (50 K); Mn$^{2+}$, Cu$^{2+}$, and Ni$^{2+}$ are from axon AU300A (50 K).
TABLE II

| Ion species | Fiber | Shift of $g_e-V$ at 0.5 $g_{MAX}$ mV | Shift of $i_{1/2-ON-V}$ at +100 mV mV | Shift of $\tau_{OFF-V}$ at -70 mV mV |
|-------------|-------|-----------------------------------|--------------------------------------|-------------------------------------|
| Ca (12.5±37.5) | MA300A | +3 | +8 | +4 |
| Mn (5) | MA280A | +4 | +10 | +2 |
| (5) | MA300A | 0 | +4 | -1 |
| (10) | MA280A | +2 | +8 | +3 |
| (25) | MA280A | +8 | +20 | +5 |
| Ni (2) | MA220A | +2.5 | +6.5 | -1 |
| (10) | AU300A | +6 | +12 | +1.5 |
| Cu (2.5) | MA200A | -4 | 0 | -4 |
| (5) | MA300A | -3 | -2 | -6 |
| (10) | AU300A | 0 | +5 | +0.5 |
| Mn (5) | MA280A | +4 | +10 | +2 |
| (5) | MA300A | 0 | +4 | -1 |
| (10) | MA280A | +2 | +8 | +3 |
| (25) | MA280A | +8 | +20 | +5 |
| Ni (2) | MA220A | +2.5 | +6.5 | -1 |
| (10) | AU300A | +6 | +12 | +1.5 |
| Cu (2.5) | MA200A | -4 | 0 | -4 |
| (5) | MA300A | -3 | -2 | -6 |
| (10) | AU300A | 0 | +5 | +0.5 |

Effect of divalent cations other than Zn on parameters of K channel gating. Equivalent shifts were determined as discussed in the legend to Table I. All measurements were made in the presence of 50 mM Ca, except for the test of Ca itself, which compared 12.5 mM with 50 mM Ca.

**Figure 8.** Comparative efficacy of group IIb transition metal ions (Zn$^{2+}$, Cd$^{2+}$, Hg$^{2+}$) in slowing $i_K$ on at +80 mV. Larger amplitude traces are controls (average of before and after exposure to Zn$^{2+}$ or Cd$^{2+}$; before exposure to Hg$^{2+}$). Time base for all records is identical. Cd$^{2+}$ is roughly half as effective as Zn$^{2+}$; Hg$^{2+}$ is much more effective than either Zn$^{2+}$ or Cd$^{2+}$. Vertical scale bar represents for the controls (in mA/cm$^2$): 3.48 (5 Zn), 5.15 (10 Cd), 4.06 (0.1 Hg), 2.32 (1 Hg); and in the presence of test cations: 3.22 (Zn), 4.50 (Cd), 2.96 (0.1 Hg), 1.56 (1 Hg). Axons MA280A (5 Zn, 50 K), AU300A (10 Cd, 50 K), MA230A (0.1 Hg, 50 K), MA220B (1 Hg, 50 K).
FIGURE 9. Comparison of effects of 0.1 mM Hg$^{+2}$ (500-s exposure) on the voltage dependence of K channel opening kinetics (half-time, $t_{1/2 \text{ ON}}$), closing kinetics (time constant, $\tau_{\text{OFF}}$), and the $g_K-V$ relation. The Hg effects are very similar to those of Zn$^{+2}$ (Fig. 4): on kinetics are affected most, off kinetics least, and the $g_K-V$ relation to an intermediate extent. Axon MA230A (50 K).

(control), in the presence of 0.1 mM Hg$^{+2}$ after 250 s of exposure, and finally in 20 mM Zn$^{+2}$ after Hg$^{+2}$ treatment and washout. Clearly Zn$^{+2}$ has a strong effect and could not possibly have been acting only on channels with completely normal kinetics that escaped Hg treatment.
Thus, Zn$^{+2}$ must act on Hg-modified channels, and its effect is quantitatively the same as that before Hg$^{+2}$. In either case, $\sim$70–80 mV of additional depolarization is required to overcome the action of Zn$^{+2}$, as demonstrated in Fig. 10B. The solid trace was recorded at +90 mV in 0.1 Hg$^{+2}$. The dotted trace of similar amplitude is the control current at +10 mV obtained before Hg$^{+2}$ application. Thus, Hg$^{+2}$ caused an 80-mV shift in $I_K$ on kinetics. The smaller dotted trace was recorded in 20 mM Zn$^{+2}$, also at +10 mV, but before Hg$^{+2}$ treatment. Comparison of parts A and B indicates that the effect of 20 mM Zn$^{+2}$ before and after Hg$^{+2}$ treatment seems identical.

Fig. 10C illustrates this effect for another axon in terms of $t_{1/2}$ ON–V plots. The curves were obtained in the control solution (filled circles), in 0.1 Hg$^{+2}$ (open circles), and in 20 Zn$^{+2}$ after Hg treatment and washout (open triangles).

### Table III

| Ion species | Fiber  | Shift of $g_K$–V at 0.5 $G_{max}$ | Shift of $t_{1/2}$ ON–V at +100 mV | Shift of $\tau_{OFF}$ at -70 mV |
|-------------|--------|----------------------------------|---------------------------------|-------------------------------|
| Cd (2)      | MA220A | $+3$                             | $+14$                           | $+1$                          |
| (5)         | MA300A | $+5$                             | $+20$                           | $+6$                          |
| (10)        | AU300A | $+14$                            | $+36$                           | $+14$                         |
| Hg (0.02, 200 s)* | JL031A | $+5$                             | $+10$                           | $+5$                          |
| (0.02, 250 s) | JL031A | $+8$                             | $+16$                           | $+5$                          |
| (0.1, 500 s) | MA230A | $+30$                            | $+58$                           | $+10$                         |
| (0.1, 150 s) | JN030B | $+26$                            | $+75$                           | $+21.5$                       |
| (0.1, 70 s)  | JL010A | $+46$                            | $+75$                           | $+$                           |
| (0.1, 250 s) | AU260B | $-$                              | $+80$                           | $-$                           |
| (1.0, 160 s) | MA220B | $+42$                            | $+88$                           | $+20$                         |

Effect of group IIb metal ions other than Zn on parameters of K channel gating. Equivalent shifts for Cd were determined as described in legend to Table II. Effect of Hg is irreversible and the shifts are expressed relative to controls taken before Hg application only.

* Exposure times to Hg are indicated in parentheses with concentration. Irreversible effect of 0.02 mM Hg was determined twice in the same axon after separate exposures and washout. Other Hg results were obtained in the presence of Hg (i.e., before washout) after exposures of the indicated times; time required for a complete set of measurements was typically 2–3 min.

At +130 mV Hg$^{+2}$ caused a 90-mV shift of $t_{1/2}$ ON relative to the control value. After Hg$^{+2}$ treatment, 20 mM Zn$^{+2}$ caused an additional 85-mV shift (relative to the Hg$^{+2}$ curve), a value similar to that normally caused by 20 Zn$^{+2}$ (i.e., without previous Hg$^{+2}$ treatment; see Table I). Thus, Zn$^{+2}$ exerts its usual effect on the rate of K channel opening even though all, or a large fraction, of the channels are permanently altered by Hg$^{+2}$. This implies that K channels have more than one site at which they are sensitive to the action of divalent cations.

### Normal Kinetics of Potassium Channels

Hodgkin and Huxley (1952) described $g_K$ kinetics with their $n^4$ formulation, commenting that $n^4$ would be more accurate but for their purposes was not worth the trouble. In attempting to model the effect of Zn$^{+2}$ we found it
necessary to use a different formulation for \( g_K \) kinetics, as described briefly in this section. The main feature of the new formulation is the presence of several fast steps and a much slower one in the chain leading to activation. This is similar to the Na channel model presented in an earlier paper (Armstrong and Gilly, 1979).

Accurate measure of \( g_K \) kinetics is complicated by accumulation of \( K^+ \) in the confined space near the membrane, with a resultant change in driving force (Frankenhaeuser and Hodgkin, 1956; Adelman et al., 1973). To minimize this problem we measured the time course of both K conductance and current in a fiber immersed in 150 mM K to minimize the effects of accumulation. Conductance as a function of time was measured as \( \Delta I/\Delta V \) upon termination of pulses of varying duration (see inset to Fig. 4). Both sets of measurements are plotted in Figs. 11A–C for an experiment in which there was only a small difference between conductance (filled circles) and current (dotted trace) time course. Conductance has been arbitrarily scaled to match the current time course during the early phase of \( I_K \) on. The two measurements thus coincide for the first 4 ms, but the current trace then falls slightly below the conductance as \( K^+ \) accumulation reduces the driving force.

The continuous traces in each part of the figure show the “best fits” (determined by trial and error and judged visually) of three specific cases of a six-state model for \( g_K \) activation with the following general form:

\[
\begin{align*}
X_6 & \xrightarrow{\alpha_5} X_5 & \xrightarrow{\alpha_4} X_4 & \xrightarrow{\alpha_3} X_3 & \xrightarrow{\alpha_2} X_2 & \xrightarrow{\alpha_1} X_1^*.
\end{align*}
\]

\( X_6 \) through \( X_2 \) are closed states; \( X_1^* \) is the only conducting state. Variations of this model that were tested differed only in the values for the forward rate constants for the various steps. Six states were necessary to reproduce the delay in activation after a step depolarization. The reverse rate constants for all steps were assumed to be zero, a reasonable simplification since the depolarization was to +40 mV and most of the K channels are open in the steady state.

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**Figure 10.** (opposite) Comparison of the effect of 20 mM Zn\(^{2+}\) on \( I_K \) on before and after permanent modification of K channels by Hg\(^{2+}\). A. Control \( I_K \) on at +90 mV compared with that in 0.1 mM Hg\(^{2+}\) after a 250-s exposure. Hg\(^{2+}\) exerts an irreversible slowing of \( I_K \) on. Smallest trace was recorded in 20 mM Zn\(^{2+}\) after Hg\(^{2+}\) washout. Zn\(^{2+}\) still shows a large effect on Hg\(^{2+}\)-treated K channels. Axon AU260A,B (50 K). B. Control \( I_K \) on at +10 mV from same experiment as in A is compared with \( I_K \) on at +90 mV in 0.1 Hg. Smallest trace shows \( I_K \) at +10 mV in 20 mM Zn\(^{2+}\) before Hg\(^{2+}\) treatment. The 20 Zn\(^{2+}\) effect after (A) and before (B) Hg\(^{2+}\) treatment is very similar despite the difference in control reference voltage (+90 mV in A and +10 mV in B). This suggests Zn\(^{2+}\) and Hg\(^{2+}\) do not bind to a single receptor. C. \( t_{1/2} \) on-\( V \) relation for a fiber before (●) treatment with 0.1 mM Hg\(^{2+}\) (70 s exposure), after (○) washout of Hg\(^{2+}\), and finally in 20 mM Zn (Δ) after Hg\(^{2+}\). Hg\(^{2+}\) shifts \( t_{1/2} \) on for a large pulse by about +90 mV. 20 mM Zn\(^{2+}\) after Hg\(^{2+}\) treatment results in an additional +85 mV, its normal effect before Hg\(^{2+}\) treatment. Axon JL010A (before Hg in 50 K, after Hg and 20 Zn in 150 K).
FIGURE 11. Modeling of normal K channel opening kinetics. In each part of the figure the lightly dotted trace is experimentally observed $I_{K}$ ON at $+40$ mV. Large, solid circles (●) represent the values of $g_{K}$ measured as $\Delta I_{K}/110$ mV upon return to $-70$ mV after pulses of varying duration (see inset to Fig. 4). $g_{K}$ was arbitrarily scaled to give a good match to $I_{K}$ ON during the early part of the trace; the two measurements then deviate as K accumulation reduces the driving force. Solid traces represent calculations using variations of the six-state kinetic model described in the text. Axon AU280A (150 K). A. Model with all forward rate constants set equal to 2.5/ms. Backwards rate constants equal to zero for all calculations. B. Rate constants, going from first step to last, set equal to 5, 4, 3, 2, 1/ms. This is equivalent to a Hodgkin-Huxley $n^{6}$ formulation. C. Rate constants for first four steps are fast and equal to 4.5/ms. Rate constant for the slow last step equals 0.6/ms. Fit to $g_{K}$ is good over the entire time course.

The continuous trace in Fig. 11A is the best fit with all the rate constants set equal to 2.5/ms. The fit to the initial part of the trace is fair, but the calculated trace then rises much more rapidly than the data. In Fig. 11B the continuous line is predicted from a Hodgkin-Huxley-like formulation in which
the rate constants, going from the first to last step, are 5, 4, 3, 2, and 1/ms, thus making this case equivalent to $n^6$ rather than $n^4$ (Fitzhugh, 1965; Armstrong, 1969). The theoretical curve again fits fairly well at the outset of $I_K$, but then rises too quickly. The last model tested (Fig. 11C) is similar to the one proposed for Na channels (Armstrong and Gilly, 1979), and assumes that the first four steps are rapid (and, arbitrarily, all alike) and the last step much slower. The continuous curve gives the best fit, which is very good over the entire time course. The rate constant for the first four steps was 4.5/ms, and 0.6/ms for the last step.

Further examination showed that a slow step anywhere in the chain yields a good fit to the data, and the predicted kinetics of $g_K$ are unaffected by the position of the slow step in the chain. Fig. 12 shows three $I_K$ ON traces calculated with rate constants from first step to last of: $(I_K \text{ I}) 4.5, 4.5, 4.5, 4.5, 0.6; (I_K \text{ II}) 0.6, 4.5, 4.5, 4.5, 4.5; (I_K \text{ III}) 4.5, 4.5, 0.6, 4.5, 4.5$. Thus the position of the slow step is the only difference among the three. The three curves are all plotted in Fig. 12 and, surprisingly, are completely indistinguishable. Thus, on the basis of $I_K$ alone, it is impossible to say where in the chain the slow step occurs.

If transfer of a gating charge of valence 1 across the membrane accompanies each of the individual transitions in the kinetic scheme above, a gating current trace can be calculated for comparison to the conductance time course (see also Armstrong and Gilly, 1979, and preceding paper). The easily distinguishable gating current predictions for the three sets of rate constants above are also illustrated in Fig. 12 ($I_g \text{ I}, \text{ II, III}$). Curve I looks most similar to the gating current proposed previously to be associated with K channels (Gilly and Armstrong, 1980), and we conclude that the slow step in K channel activation must be at or near the end of the chain, as it is for the Na channel.
Another experiment, however, shows that the K channel's slow step cannot be the last in the chain. One can get a fairly direct measure of the kinetics of the last step with the three-pulse method diagrammed in Fig. 13 (see also Oxford, 1981). A first pulse to +60 mV for 13.5 ms opens all, or nearly all, of the channels (puts them in state \( X_f^p \)) and a brief (0.5 ms) repolarization to −70 mV then returns a small fraction to state \( X_2 \) (and possibly also to other states between \( X_2 \) and \( X_6 \)). A third step back to +60 mV then mainly reflects the transition of that fraction of closed channels (about 20% in Fig. 13) from \( X_2 \) back to \( X_f^p \), in theory a one-step reaction with exponential kinetics. \( I_K \) at +60 mV is plotted in Fig. 13 as the dotted traces (\( I_K \) during the 0.5 ms at −70 mV is not illustrated). \( K \) current during the third step while channels are re-opening does have approximately exponential kinetics, and the time constant (obtained by a least-squares exponential fit) is 0.325 ms. The time course of channel re-opening is also obviously faster than the final, slow approach of \( I_K \) on to its maximum value during the first pulse. Thus, the last step, as seen in isolation during the third pulse, cannot be rate limiting for \( g_K \) activation at +60 mV. Conti and Neher (1980) have also proposed that the last step in K channel activation is not the slowest from an independent line of evidence.

A complete fitting of \( I_K \) during the first and the third pulse is also plotted.
(superimposed solid traces) in Fig. 13. The specific six-state scheme used had the rate constants (first step to last) 7.5, 7.5, 7.5, 0.85, and 2.8/ms. At the beginning of the first step, all channels were assumed to be in state $X_s$, and at the beginning of the third, 80% were in $X_2^*$ and 20% were taken to be in $X_b$. It would be more accurate to assume a distribution among states $X_2$ through $X_b$, but in the absence of information on what that distribution is we have used the assumptions just cited, which probably do not seriously affect the calculations. The fits are very good, but do not take into account the accumulation artifact because conductance time course was not measured in the three-pulse experiment.

Our conclusions regarding normal kinetics can be summarized as follows. (a) Fitting the time course of conductance requires a slow step somewhere in the chain leading to activation. (b) Gating current experiments (Gilly and Armstrong, 1980) show that the slow step is late in the chain. (c) Kinetics of the last step, as measured directly from the three-pulse experiment, are moderate in speed and definitely not the slowest. It should be noted that the three-pulse experiment of Fig. 13 on Na channels in pronase-treated axons (Gilly et al., 1981; Oxford 1981) shows that the last step is the slowest in the sequence, consistent with the Na channel model we presented previously (Gilly and Armstrong, 1981; Armstrong and Gilly, 1979).

Opening Kinetics of Zn$^{2+}$ K Channels

As with Na channels, the effect of Zn$^{2+}$ on K activation kinetics can be formally described as a selective reduction in forward rate constants in the six-state activation sequence. The magnitude of the reduction in rate constants by Zn$^{2+}$ was estimated by repeating the three-pulse experiment of Fig. 13 on the same axon in the presence of 20 mM Zn$^{2+}$. This procedure shows directly the effect of Zn on the rate constant for the $X_2$ to $X_2^*$ transition. Fig. 14A gives the experimental results. Both control $I_K$ and $I_K$ in 20 mM Zn$^{2+}$ are plotted during the first and third pulses. Slowing of $I_K$ on by Zn$^{2+}$ during the first pulse is obvious, and re-opening of K channels during the third pulse is also slowed. Exponentials fitted to $I_K$ during the third pulse are superimposed on the experimental records, and the respective time constants are 0.362 (control) and 0.798 ms (Zn), corresponding to a reduction of the rate constant by a factor of 0.45.

As a first step toward modeling the Zn$^{2+}$ effect on $I_K$, a 0.45-fold reduction in rate constant was assumed to apply to all five steps of the model used in Fig. 13. Rate constants in Zn$^{2+}$, from first to last step, were 6.8, 6.8, 6.8, 0.77, and 2.54/ms. The calculation is plotted in Fig. 14B (dashed trace) along with the experimental record from Fig. 14A on an expanded time scale. The fit is good for the final half of the trace during the slow approach of $I_K$ to its final value, but the calculated delay is too long and the early rise is too slow. The solid curve in Fig. 14B that fits the data points extremely well for the entire 14 ms was generated by increasing the rate constant for the first three steps from 6.8/ms to 8.8/ms, amounting to a reduction of the control value by a factor of 0.59. Zinc thus seems to act more powerfully on the slower two steps than on the fast, early ones. This is in agreement with Fig. 3.
Figure 14. Action of 20 mM Zn$^{2+}$ on kinetics of the last step in the activation sequence (A) and modeling the Zn effect on $I_K\text{ON}$ (B). Same axon as Fig. 11. A. Records of $I_K$ in the absence (control) and presence of 20 mM Zn$^{2+}$ at +60 mV and during re-opening at +60 mV after a 0.5-ms return to −70 mV. Exponential fits to re-opening $I_K$ traces are superimposed: control time constant = 0.362 ms, zincked time constant = 0.798 ms. Zn$^{2+}$ slows both opening and re-opening of K channels. B. Dotted trace is $I_K\text{ON}$ at +60 mV in 20 mM Zn$^{2+}$ from A plotted on an expanded time scale. Same vertical scale as in A. Dashed trace is calculated $I_K\text{ON}$ obtained by reducing all $\alpha$ values from Fig. 13 by a factor of 0.45 (i.e., change in re-opening time course caused by Zn$^{2+}$). Solid curve through the data points was generated by reducing the last two $\alpha$ values by 0.45 but the first three by only 0.59. Vertical scaling factor for Zn$^{2+}$, calculations have been reduced by a factor of 0.83 relative to control values used in the fitting in Fig. 13.
The increase in K channel closing rate described earlier for high Zn\(^{2+}\) concentration (e.g., Figs. 2 and 3D) clearly cannot be accounted for by a selective slowing of the forward rate constants only and requires an increase in the reverse rate constants at negative voltages. We describe a physical model and qualitative interpretation for the increase in closing rate in the Discussion, but we have chosen here not to illustrate modeling of \(I_K\) OFF. In the absence of a gating current trace, which usefully constrains the fitting of closing rate, detailed modeling of the Zn\(^{2+}\) effect on \(I_K\) OFF does not seem profitable.

**DISCUSSION**

The main findings of this paper are the following.

(a) The action of externally applied Zn\(^{2+}\) and other divalent cations on potassium conductance can be distinguished from the effect of a membrane voltage change. The same point was made in the last paper for Zn\(^{2+}\) acting on the Na conductance. As explained there, the effects of these cations cannot result from an alteration of a diffuse surface charge. Instead, a more specific interaction with the charges of the gating apparatus is implied. Similar conclusions have been reached for the action of protons (Shrager, 1974) and epinephrine (Tsien, 1974) on other excitable membranes.

(b) Two or more of the several steps in channel activation are affected by Zn\(^{2+}\). At least two steps must be slowed to fit both ionic current and gating current records for Na channels. With K channels, Zn\(^{2+}\) retards the last step, as well as the slowest step, and perhaps others also (Fig. 14A).

(c) More than one metal binding site exists for each K channel. Zinc still affects \(g_K\) activation after permanent modification of K channels by Hg\(^{2+}\) (Fig. 10), which shows the presence of at least two receptors.

(d) Group IIB metal ions (Zn\(^{2+}\), Cd\(^{2+}\), and Hg\(^{2+}\)) are extremely effective at interfering with gating charge movement, although all the divalent cations we tested, including Ca\(^{2+}\), show at least a small zinclike effect. Fig. 15 summarizes the data in Tables I–III graphically. The ordinate is the shift in millivolts of activation kinetics (\(t_{1/2}\) on) in the presence of various divalent cations at the concentrations given on the abscissa. Hg\(^{2+}\) lies in the lower right of the graph, and the least effective ion, Ca\(^{2+}\), is at the upper left. The sequence of potency is Ca\(^{2+}\) < Cu\(^{2+}\) < Mn\(^{2+}\) < Ni\(^{2+}\) < Cd\(^{2+}\) < Zn\(^{2+}\) < Hg\(^{2+}\). On a molar basis, Hg\(^{2+}\) is probably 200 times more potent than Zn\(^{2+}\), and roughly 20,000 times more effective than Ca\(^{2+}\).

This sequence for squid axon is very similar to that for Na (Hille et al., 1975) and perhaps K (Hille, 1968) channels in frog node. It is rather different from that in *Xenopus* node, where Cu\(^{2+}\) and Ni\(^{2+}\) are more effective than Zn\(^{2+}\) at slowing K and Na channel opening, and where Cd\(^{2+}\), and even Hg\(^{2+}\), apparently have small effects on K channel kinetics (Arhem, 1980).

**A Model for Divalent Cations Acting on K Channels**

One possible model for divalent cation action is diagrammed in Fig. 16. It is an extension of the model presented for Na channels in the preceding paper...
FIGURE 15. Comparison of the effectiveness of different cations in slowing on kinetics. The most effective cation, Hg$^{2+}$, is at lower left; the least effective, Ca$^{2+}$, is at upper right. See text for additional details.

(Gilly and Armstrong, 1982). Like that model, it takes into account the likelihood that gating charge has stable positions only at the membrane surfaces rather than in the hydrophobic interior, and that the charge polarizes the adjacent aqueous medium. We present this more complicated model because we feel it is physically more plausible than the simple Na channel model. The ideas to be subsequently developed could equally well apply to Na channel gating, but nothing in our data can rigorously justify a choice between these two possibilities.
Parts A and B of Fig. 16 show the closed (OFF) and open (ON) positions of a single subunit of the gating apparatus of one channel. The complete machinery would be composed of several such units, each of which must be in the ON position in order for the channel to conduct. The six-state model of K channel activation presented above, for example, might require five such subunits, one for each transition. The mechanical attachment of the voltage-sensing subunits to the gate itself, or of the three-dimensional arrangement of subunits into a channel complex, is not expressed in the drawing (see also Armstrong, 1981).
The mechanism of charge movement postulated in Figs. 16A–B is an alternative to the one in the preceding paper in which a discrete charge moved all the way through the membrane. Instead, there is here a concerted movement of all the charges on a predominantly negatively charged protein chain comprising part of the subunit. This chain moves relative to another that is predominantly positively charged, which for descriptive purposes is assumed to be fixed. Each of the five negative charges (at locations 1–5 in A) moves one-fifth of the way through the membrane in the transition from off to on, equivalent to the transfer of one electronic charge across the membrane. The selection of five charges in the chain is arbitrary; the important feature is that each of several charges moves only a fraction of the way through the membrane's electric field. Such a mechanism, in which a small movement of a charged macromolecule provides voltage sensitivity, seems in consonance with current knowledge of proteins that form channels in biological membranes (e.g., Unwin and Zampighi, 1980).

In the two stable positions shown, four of the five negative charges are closely paired with the positive charges, making possible their residence in the membrane’s interior, a region of low dielectric constant that is hostile to unpaired charge. Electrostatic interactions of this sort appear to be very important in determining the three-dimensional structure of globular proteins (Wada and Nakamura, 1981; Hol et al., 1981).

A fifth negative charge is unpaired and is located either at the external (off position) or internal (on position) surface of the membrane, where it polarizes the appropriate medium and draws a counterion. An internal counterion is illustrated for the on position in Fig. 16B. Externally, divalent cations are attracted to the immediate vicinity of the exposed charge in the off position and can bind to a proximate site S (see Fig. 16A). The nature and specificity of this site is discussed later. At negative voltages, when the channels are closed (off), formation of divalent cation-S complexes is thus promoted by the locally enriched concentration of divalents around the outermost, exposed charge on the negative chain. When the subunit moves to the on position, this attraction greatly diminishes, because the outermost negative charge, now in position 2, no longer polarizes the medium to a significant extent. The probability of finding a divalent cation at S thus drops, and in on configuration (Fig. 16B) this site is diagrammed without an occupying cation. This idea of a “disappearing” receptor accompanying activation is the one we presented in the preceding paper on Na channels. The possibility of a divalent cation remaining bound to S at all times will be considered below.

Curve I in Fig. 16D is an energy diagram of the subunit at the voltage where, in the absence of a divalent cation in site S, on and off positions are equally probable. The diagram assumes the presence of an internal counterion in on configuration: otherwise the on energy level would be higher than shown. Each gating subunit has only two stable positions (energy minima), and in both there is maximum charge pairing. The minima are separated by an energy barrier, the peak of which represents the transitional state, with maximal separation of negative and positive charges as the scallops are sliding.
The distance between the two energy minima along the reaction coordinate represents the fraction of membrane thickness through which each negative charge moves in the $\text{OFF} \leftrightarrow \text{ON}$ transition (one-fifth in the model of Figs. 16A and B).

Addition of divalent cations, in our case $\text{Zn}^{+2}$, to the external medium will stabilize the closed OFF state. When a Zn ion is bound to S, the electrostatic field around it affects the potential energy of each of the negative charges on the gating subunit in inverse proportion to their distance from $\text{Zn}^{+2}$. A diagram of the $\text{Zn}^{+2}$-induced energy bias on the negative charges at the different locations 1–5 in the membrane is given in Fig. 16C. As a result, the outermost gating charge, which can be in position 1 (Fig. 16A) or 2 (Fig. 16B) is strongly biased toward position 1. The innermost negative charge is only slightly affected; i.e., $\text{Zn}^{+2}$ causes a negligibly small change in the relative energies of its possible positions, 4 and 5.

Zinc thus changes the composite energy diagram from curve I to curve II in Fig. 16D. The OFF energy level is significantly depressed, the peak of the barrier is slightly depressed, and the ON level is scarcely affected. Three basic points emerge from this idea. (a) When $\text{Zn}^{+2}$ is present at S, the OFF state is stabilized by a heightened barrier for the $\text{OFF} \leftrightarrow \text{ON}$ transition. This results in a rate constant that is smaller than normal (i.e., in the absence of $\text{Zn}^{+2}$). Once the ON state is reached, two possibilities exist for describing the $\text{ON} \leftrightarrow \text{OFF}$ transition. (b) If as discussed above, $\text{Zn}^{+2}$ diffused away from the S-site, the energy diagram would revert to curve I, and the ON to OFF barrier height and transition rate constant would be absolutely normal. (c) If $\text{Zn}^{+2}$ remained bound to S with the gating subunit in ON position, curve II would also apply to the ON to OFF transition, resulting in a slightly depressed barrier height and correspondingly faster rate constant. This ON to OFF acceleration would in no case be as large, energetically speaking, as the OFF to ON retardation.

Applying these ideas to a population of zincified K channels leads to the following considerations supported by our data. Point a underlies the strong slowing of K channel opening rate, which is roughly equivalent to a large depolarizing shift of ON kinetics. Point b appears to be satisfied only at low $\text{Zn}^{+2}$ concentrations ($\leq 2$ mM), where closing (OFF) kinetics are unaltered. The model thus predicts that ON but not OFF kinetics can be affected because $\text{Zn}^{+2}$ might disengage from site S in the ON configuration. This was the case we presented for Na channels as well (Gilly and Armstrong, 1982). K channels are obviously more sensitive to $\text{Zn}^{+2}$, however, and at higher $\text{Zn}^{+2}$ concentrations a fraction of the K channels' S-sites may contain Zn even when ON. Point c requires a slightly accelerated rate of channel closing under these conditions, an effect that we definitely observe with $\text{Zn}^{+2}$ at concentrations above 2 mM or with $\text{Hg}^{+2}$. Because the effects of $\text{Hg}^{+2}$ are irreversible, this cation presumably remains bound at all times, and the explanation for the speeding of $I_K \text{OFF}$ by $\text{Hg}^{+2}$ is therefore straightforward. There are two equally plausible explanations for the reversible effects of $\text{Zn}^{+2}$ on closing kinetics. The first is that $\text{Zn}^{+2}$ dissociates too slowly from S to reach equilibrium during a pulse tens of milliseconds long. The other possibility is that $\text{Zn}^{+2}$ at high
concentration remains bound at all times to an appreciable number of sites despite lower affinity in the on configuration.

Application of the Model to Low Internal Ionic Strength
The model in Fig. 16 can also explain what is known about the effect of low internal ionic strength on channel gating. In the off to on transition a negative gating charge disappears from the outer surface and, in effect, appears at the inner surface, where it draws a counterion from the internal medium (Fig. 16B).

Removing the counterion by lowering the internal ionic strength would raise the energy of the on configuration, thereby destabilizing it. An effect interpretable in this way has been reported for Na channels (Baker et al., 1964; Moore et al., 1964). Action potential threshold can be near 0 mV when the internal medium contains 10 mM KCl as the only ions. Chandler et al. (1965) invoked a layer of fixed negative charge on the internal membrane surface to account for the effects of low internal ionic strength. We present here an alternative explanation, but at present there is no evidence on which to base a choice between the two hypotheses. Effects of low internal ionic strength on K channels have not been reported in detail, but preliminary results are consistent with the model presented here (López-Barneo and Armstrong, 1982).

Positive Gating Charges Moving Outward Cannot Explain the Zinc Effect
Both the effects of external divalent cations and of low internal ionic strength are easily explained if one assumes that a major element of gating current is produced by negative charge moving inward from the external surface as the channels activate. Neither set of observations can be explained if only positive charge moves outward during activation. Fig. 17 shows an energy diagram for a model similar to the one in Fig. 16, but reversed in the sense that a positive charge disappears from the internal surface during activation. Curve I in Fig. 17 is the energy diagram of such a gating subunit at the voltage where on and off configurations are equally probable in the absence of unusual divalent cations in the external medium and at normal internal ionic strength. External addition of Hg\(^{+2}\) would change the energy profile to curve III (Hg\(^{+2}\) is used for this argument because it is irreversible and would remain attached to the site at all times). One would predict a small effect on on kinetics and a large effect on off kinetics, the opposite of our experimental observations. This model also cannot account for the effect of lowering internal ionic strength.

In conclusion, the effects of Zn\(^{+2}\) and other divalent cations on Na and K permeabilities can be explained by a model in which at least part of the gating apparatus is a negative charge that migrates inward from the membrane surface during activation. The same model can account for the effects on Na permeability of lowering the internal ionic strength. The opposite model, in which a positive charge present at rest on the inner surface migrates outward during activation, can explain neither set of observations. Negative gating charges for axon K channels have also been proposed to account for the action
of the dipolar compound phloretin, which impedes the movement of lipophilic anions in artificial bilayer membranes (Strichartz et al., 1980).

**Chemical Nature of the IIB Metal Binding Sites**

The high specificity of IIB metal ions argues strongly for binding sites on both K and Na channels that have very low affinity for other divalent metal ions in the first transition series. Specificity cannot be accounted for simply by the cations' charge density, which has no correlation with potency. Mercury is the largest of the ions we tested (i.e., lowest charge density), Cu$^{2+}$ is the smallest, and Ca$^{2+}$ and Cd$^{2+}$ are almost exactly the same size.

**Figure 17.** Energy diagram of a hypothetical gating subunit that operates by transferring a positive charge from inside to outside during activation. As explained in the text, a strongly bound external cation like Hg$^{2+}$ would affect OFF kinetics more than ON, contrary to the experimental results.

It is more likely that the electronic structure of the IIB metal ions, a filled outer d-shell and an empty s-shell, is the crucial factor in determining specificity. These ions are very “soft” or polarizable and can bind covalently to uncharged molecules, including amino acid residues of proteins. Calcium, at the other extreme of potency, has a noble gas electronic configuration and generally binds well only to charged ligands.

On the basis of the largely empirical information available regarding transition metal ions interacting with small molecules and proteins (see Eichorn, 1973; Williams, 1976), we suggest that Zn$^{2+}$ binds to a histidine residue on the proteins comprising the ionic channels. Most enzymes binding Zn$^{2+}$ with high affinity do so via chelation by several histidines, and the actual binding generally occurs between Zn$^{2+}$ and the uncharged imidazole nitrogen (Freeman, 1973). Zinc is preferred over other first-row transition metals, and Cd$^{2+}$ and Hg$^{2+}$ can usually substitute for Zn$^{2+}$ at the binding site. Mercury exerts its toxic effect in some cases by irreversibly replacing Zn$^{2+}$.
There are thus several parallels in this outline to our data. Clearly, Zn$^{+2}$ binding to K channels cannot be as specific as binding by the Zn$^{+2}$ enzymes, as the Zn$^{+2}$ effect is not readily saturable. A single histidine molecule in solution binds Zn$^{+2}$ with a strength of about $-3.8$ kcal/mole (Freeman, 1973), but even such a relatively weak bond might confer considerable selectivity for Zn$^{+2}$ over Ca$^{+2}$, for example. Selectivity could be greatly increased through chelation.

Although histidine is a likely Zn$^{+2}$ binding site, the sulfhydryl group of cysteine is another possibility. This site is usually not as selective as histidine for IIB metals, but sulfhydryl groups very readily bind Hg$^{+2}$ (see Means and Feeney, 1971), as do K channels (and Na; unpublished data). Mercury does react with other amino acids, however, as indicated by the loss of activity of carboxypeptidase, a Zn$^{+2}$ enzyme containing no sulfhydryls (Lipscomb, 1967), after treatment with $p$-mercuribenzoate (Vallee et al., 1960). Our results indicate that different binding sites probably exist for Zn$^{+2}$ and Hg$^{+2}$, but imply that both must be close to the resting location of gating charge.

We obviously cannot identify with certainty the binding site for IIB metal ions on Na and K channels, but it is unlikely that the large difference in efficacy between the IIB metals and the others we have tested could be achieved by a charged binding site, such as the gating charges themselves or a nearby, permanently charged phospholipid head group. It is interesting that Shrager (1974) also suggested that histidine was the important proton binding site on the K channel in crayfish axon. The effects of external pH that he described closely resemble those of Zn$^{+2}$, and similar results have been reported for squid axon (Carbone et al., 1978). Whether protons bind to the same histidine residue that we postulate for Zn$^{+2}$, or whether other less reactive divalent cations (e.g., Ca$^{+2}$, Mn$^{+2}$, etc.) affect channel gating from the Zn binding site or from some other remains to be established.

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REFERENCES

ADELMAN, W. J., Y. PALTI, and J. P. SENFT. 1973. Potassium ion accumulation in a periaxonal space and its effects on the measurement of membrane potassium ion conductance. J. Membr. Biol. 13:387-410.

ARHEM, P. 1980. Effects of some heavy metal ions on the ionic currents of myelinated fibres from Xenopus laevis. J. Physiol. (Lond.). 306:219-231.

ARMSTRONG, C. M. 1969. Inactivation of the potassium conductance and related phenomena caused by quaternary ammonium ion injection in squid axons. J. Gen. Physiol. 54:553-575.

ARMSTRONG, C. M. 1981. Sodium channels and gating currents. Physiol. Rev. 61:644-683.

ARMSTRONG, C. M., and W. F. GILLY. 1979. Fast and slow steps in the activation of sodium channels. J. Gen. Physiol. 74:691-711.

BAKER, P. F., A. L. HODGKIN, and H. MEVES. 1964. The effect of diluting the internal solution on the electrical properties of a perfused giant axon. J. Physiol. (Lond.). 170:541-560.

BLAUSTEIN, M. P., and D. E. GOLDMAN. 1968. The action of certain polyvalent cations on the voltage-clamped lobster axon. J. Gen. Physiol. 51:279-291.
CARBONE, E., R. FIORVANTI, G. PRESTIPINO, and E. WANKE. 1978. Action of extracellular pH on Na and K membrane currents in the giant axon of Loligo vulgaris. J. Membr. Biol. 43:295–315.

CHANDLER, W. K., A. L. HODGKIN, and H. MEVES. 1965. The effect of changing the internal solution on sodium inactivation and related phenomena in giant axons. J. Physiol. (Lond.). 180:821–836.

CONTI, F., and E. NEHER. 1980. Single channel recordings of K currents in squid axons. Nature (Lond.). 285:140–143.

EICHHORN, G. L., editor. 1973. Inorganic Biochemistry. Elsevier-North Holland, New York.

FITZHUGH, R. 1965. A kinetic model of the conductance changes in nerve membrane. J. Cell. Comp. Physiol. 66(Suppl. 2):111.

FRANKENHAUSER, B., and A. L. HODGKIN. 1956. The after-effects of impulses in the giant nerve fibres of Loligo. J. Physiol. (Lond.). 131:341–376.

FRANKENHAUSER, B., and A. L. HODGKIN. 1957. The action of calcium on the electrical properties of squid axons. J. Physiol. (Lond.). 137:218–244.

FREEMAN, H. C. 1973. Metal complexes of amino acids and peptides. In Inorganic Biochemistry. G. L. Eichhorn, editor. Elsevier-North Holland, New York. 121–166.

GILLY, W. F., and C. M. ARMSTRONG. 1980. Gating current and potassium channels in the squid giant axon. Biophys. J. 29:485–492.

GILLY, W. F., and C. M. ARMSTRONG. 1982. Slowing of sodium channel opening kinetics in squid axon by extracellular zinc. J. Gen. Physiol. 79:935–964.

GILLY, W. F., R. P. SWENSON, and C. M. ARMSTRONG. 1981. Sodium channel activation in pronased squid axons: the slow last step. Proc. VII Int. Biophys. Congress. Mexico City. 330.

HILLE, B. 1968. Charges and potentials at the nerve surface. Divalent ions and pH. J. Gen. Physiol. 51:221–236.

HILLE, B., A. WOODHULL, and B. I. SHAPIRO. 1975. Negative surface charge near sodium channels of nerve: divalent ions, monovalent ions and pH. Phil. Trans. R. Soc. Lond. B Biol. Sci. 270:301–318.

HODGKIN, A. L., and A. F. HUXLEY. 1952. A quantitative description of membrane current and its application to conductance and excitation in nerve. J. Physiol. (Lond.). 117:500–544.

HOL, W. G., L. M. HALIE, and C. SANDER. 1981. Dipoles of the α-helix and β-sheet: their role in protein folding. Nature (Lond.). 294:532–536.

LIPSCOMB, W. N. 1967. Am. Chem. Soc. Abstr. 155:2-M. Cited in Means and Feeney, 1971, p. 204.

López-Barneo, J., and C. M. Armstrong. 1982. Internal slow ionic strength (LIS) and voltage dependence of Na and K channels. Biophys. J. 37:257a.

MEANS, G. E., and R. E. FEENEY. 1971. Chemical Modification of Proteins. Holden-Day, Inc., San Francisco. 254 pp.

MOORE, J. W., T. NARAHASHI, and W. ULBRICHT. 1964. Sodium conductance shift in an axon internally perfused with a sucrose and low-potassium solution. J. Physiol. (Lond.). 172:163–173.

OXFORD, G. S. 1981. Some kinetic and steady-state properties of the sodium channels after removal of inactivation. J. Gen. Physiol. 77:1–22.

SHRAGER, P. 1974. Ionic conductance changes in voltage-clamped crayfish axons at low pH. J. Gen. Physiol. 64:666–690.

SHRIVASTAV, B. B., M. S. BRODWICK, and T. NARAHASHI. 1976. Methylmercury: effects on electrical properties of squid axon membranes. Life Sci. 18:1077–1082.

STICHARTZ, G. R., G. S. OXFORD, and F. RAMON. 1980. Effect of the dipolar form of phloretin on potassium conductance in squid axons. Biophys. J. 31:229–246.
Tsien, R. W. 1974. Effects of epinephrine on the pacemaker potassium current of cardiac Purkinje fibers. J. Gen. Physiol. 64:293-319.

Unwin, P. N. T., and G. Zampighi. 1980. Structure of the junction between communicating cells. Nature (Lond.). 283:545-549.

Vallee, B. L., T. L. Coombs, and F. L. Hoch. 1960. The "active site" of bovine pancreatic carboxypeptidase A. J. Biol. Chem. 235:PC45.

Vogel, W. 1974. Calcium and lanthanum effects at the nodal membrane. Pflugers Arch. Eur. J. Physiol. 350:25-39.

Williams, D. R., editor. 1976. An Introduction to Bio-inorganic Chemistry. Charles C Thomas, Springfield, Ill.

Wada, A., and H. Nakamura. 1981. Nature of the charge distribution in proteins. Nature (Lond.). 293:757-758.

Woodhull, A. M. 1973. Ionic blockage of sodium channels. J. Gen. Physiol. 61:687-708.