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Effects of Permeant Cations on K^+ Channel Gating in Nerve Axons Revisited

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Abstract. An increase in extracellular potassium ion concentration, K_o, significantly slows the potassium channel deactivation rate in squid giant axons, as previously shown. Surprisingly, the effect does not occur in all preparations which, coupled with the voltage independence of this result in preparations in which it does occur, suggests that it is mediated at a site outside of the electric field of the channel, and that this site is accessible to potassium ions in some preparations, but not in others. In other words, the effect does not appear to be related to occupancy of the channel by potassium ions. This conclusion is supported by a four-barrier, three-binding site model of single file diffusion through the channel in which one site, at most, is unoccupied by a potassium ion (single-vacancy model). The model is consistent with current-voltage relations with various levels of K_o, and, by definition, with multiple occupancy by K^+. The model predicts that occupancy of any given site is essentially independent of K_o (or K_i). The effects of extracellular Rb^+ and Cs^+ on gating are strongly voltage dependent, and they were observed in all preparations investigated. Consequently, the mechanism underlying these results would appear to be different from that which underlies the effect of K^+ on gating. In particular, the effect of Rb^+ on gating is reduced by strong hyperpolarization, which in the context of the occupancy hypothesis, is consistent with the voltage dependence of the current-voltage relation in the presence of Rb^+. The primary, novel, finding in this study is that the effects of Cs^+ are counterintuitive in this regard. Specifically, the slowing of channel deactivation rate by Cs^+ is also reduced by hyperpolarization, similar to the Rb^+ results, whereas blockade is enhanced, which is seemingly inconsistent with the concept that occupancy of the channel by Cs^+ underlies the effect of this ion on gating. This result is further elucidated by barrier modeling of the current-voltage relation in the presence of Cs^+.

Key words: K^+ channel — Ion effects on gating — Nerve axon

Introduction

The effects of permeant ions on ion channel gating have been well established, especially for potassium channels (Arhem, 1980; Swenson & Armstrong, 1981; Cahalan et al., 1985; Clay, 1986; Matteson & Swenson, 1986; Spruce, Standen & Stanfield, 1989; Sala & Matteson, 1991; Shapiro & DeCoursey, 1991; Demo & Yellen, 1992; Safronov & Vogel, 1995; Mienville & Clay, 1996). In particular, the alteration of K^+ channel gating in nerve axons by K^+, Rb^+, and Cs^+ can be summarized by the observation that these ions slow channel deactivation when added to the extracellular medium. Indeed, these effects appear to be limited to channel closing which has led to the hypothesis that gating is related to occupancy of the channel by permeant ions, perhaps by a ''foot-in-the-door'' mechanism (Swenson & Armstrong, 1981; Matteson & Swenson, 1986). However, the most that can be said for the available evidence is that channel gating may be correlated with occupancy of the channel by Rb^+, K^+, or Cs^+. The results in this report call into question this correlation and suggest that the mechanism underlying these results may require reevaluation.

Materials and Methods

Experiments were performed on giant axons from the common North Atlantic squid (*Loligo pealei*) at the Marine Biological Laboratory in Woods Hole, MA with axial wire voltage clamp and intracellular per-

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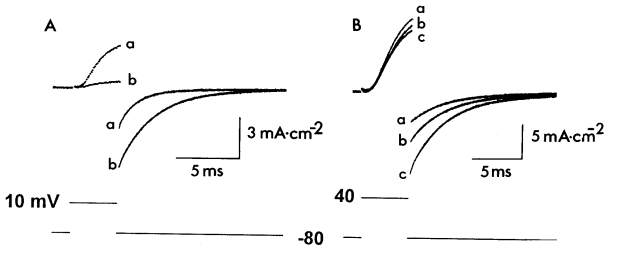

Fig. 1. Variability of the effect of K_o on gating. (*A*) Records were obtained with a 4 msec duration pulse to +10 mV followed by a return to holding potential (−80 mV) in either 50 K_o (*a*), or 300 K_o (*b*). The increase in K_o produced a marked slowing of tail current, as shown more clearly in Fig. 2*A*. (*B*) Records obtained from another preparation than in *A* with a 5 msec step to +40 mV followed by return to holding potential (−80 mV) in 50 K_o (*a*), 2 min after switching to an extracellular solution containing 300 K_o (*b*), and 5 min after switching to 300 K_o (*c*), which is approximately the wash-in time for the experimental chamber. The tail current time course was not significantly affected in this experiment, as shown more clearly in Fig. 2*B*.

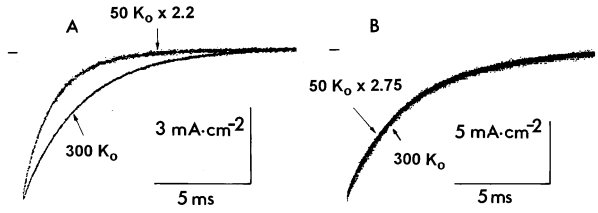

Fig. 2 (*A*) Same tail currents as in Fig. 1*A*, with the 50 K_o result scaled upward by a factor of 2.2 The 300 K_o result from Fig. 1*A*, also shown here, has a time constant approximately twice as great as the 50 K_o result. (*B*) Same tail currents as in records *a* and *c* from Fig. 1*B*. Record *a* has been scaled upward by a factor of 2.75. These results are essentially superimposable, as shown here.

fusion techniques described elsewhere (Clay & Shlesinger, 1983). The temperature in these experiments ranged between 6 and 9°C. In any single experiment, it was maintained constant to within 0.1°C by a negative feedback circuit connected to a Peltier device located within the experimental chamber. The intracellular perfusate for all experiments save for the 100 K_i results in Fig. 5 contained (in mM): 400 sucrose, 25 K_2HPO_4, 50 KF, and 200 K-glutamate (pH = 7.2). This solution is labeled 300 K_i in the text. The 100 K_i solution contained 25 KF, 75 K-glutamate, and 550 sucrose. The extracellular solution for all results contained (in mM): 10 $CaCl_2$, 50 $MgCl_2$, 1 μM tetrodotoxin (TTX, Sigma), and 10 Tris-HCl (pH = 7.2). The additional constituents are as follows. In Figs. 1 and 2, the 50 and 300 K_o solutions contained either 390 NaCl and 50 KCl (50 K_o), or 140 NaCl and 300 KCl (300 K_o), respectively. The 10 K_o solution for the results in Fig. 5 contained 10 KCl and 430 NaCl. The solutions for the results in Fig. 6 contained either 440 NaCl (control) or 340 NaCl and 100 CsCl. Similar solutions were used for Fig. 7*B* with 100 CsCl replaced by 100 RbCl. The control solution for Fig. 8 was 100 K_o, which contained 100 KCl, and 340 NaCl. The test solutions contained 100 KCl, 240 NaCl, and either 100 CsCl or 100 RbCl.

Results

Variability of the Effect of Extracellular K^+ (K_o) on Gating

An example of a significant slowing of the ''tail'' current time course following an increase in K_o is illustrated in Fig. 1*A*. In this experiment, the membrane potential was stepped to +10 mV for 4 msec followed by a return to the holding level (−80 mV) in either 50 (records labeled *a*), or 300 mM K_o (*b*). The tail currents are shown on expanded scales in Fig. 2*A* with the 50 mM record scaled upward by a factor of 2.2. The tail current time constant was increased by a factor of ~2 for these conditions following the increase in K_o (Clay, 1986), which is similar to the original report of this effect by Swenson and Armstrong (1981). In contrast, Figs. 1*B* and 2*B* illustrate a striking lack of this effect, which is one of the main observations in this study. Specifically, the results in Fig. 1*B*, which were obtained from a different preparation than Fig. 1*A*, illustrate membrane currents obtained during a voltage step to +40 mV and then following a return to −80 mV in 50 K_o (record *a*), two minutes after switching the extracellular solution to 300 K_o (*b*), and, finally, 5 min after the switch to 300 K_o (*c*). The results show a lack of effect of K_o on channel closing rate as shown explicitly in Fig. 2*B*. (The difference in results between Figs. 1 and 2*A* and 1 and 2*B* was not related to prepulse potential. Similar results were obtained from various different preparations regardless of the depolarizing pulse amplitude). In a large series of experiments (n ~50) the increase in tail current time constant by a factor of two following a change in K_o from 50 to 300 mM was observed roughly half of the time, whereas a lack of effect was observed in the rest of the experiments. No correlation of this result with any obvious factor in experimental protocol was apparent.

The outward current elicited by the step to +10 mV in Fig. 1*A* was significantly reduced followed the change in the extracellular solution from 50 to 300 K_o, whereas relatively little reduction in outward current occurred under these conditions for the results in Fig. 1*B*, in which the prepulse potential was +40 mV. These results together with the marked increase in inward current in both preparations at −80 mV with 300 K_o are all consistent with the outward rectification of the squid I_K channel, as has been shown previously (Clay & Shlesinger, 1983; Clay, 1991). This effect is illustrated by the current-voltage (I-V) relations in Fig. 3 for 50 and 300 K_o together with a description of these results by the model given below.

Single Vacancy Model of Ion Diffusion: Relation to the Effects of K^+ on Gating

As has been previously shown (Clay, 1991), the voltage and potassium ion dependence of fully activated I_K in

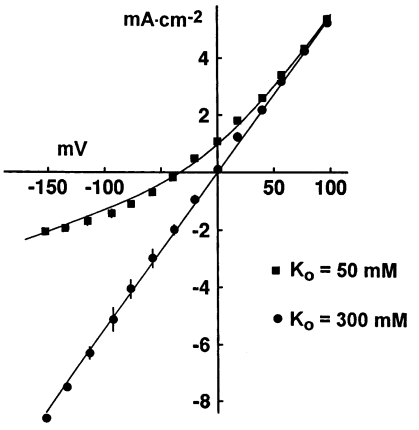

Fig. 3. Current-voltage relations in 50 and 300 K_o. Experimental results taken from Clay (1991). The theoretical description of these results corresponds to Eq. (1) in the text with $[K_o] = 75$ and 300 mM for the 50 and 300 mM results, respectively. The former ($[K_o] = 75$ mM) is greater than the bulk concentration (50 mM) to account for ion accumulation during the prepulse which was used to activate the I_K conductance in these experiments.

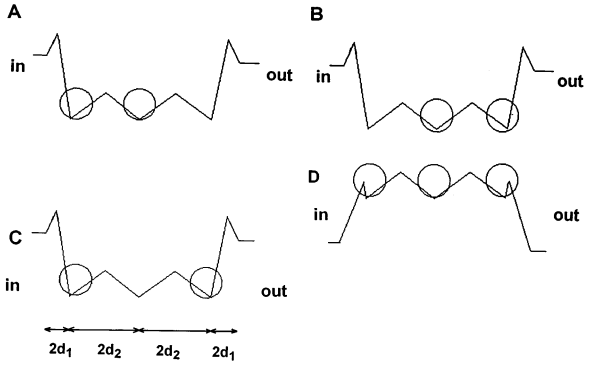

Fig. 4. Free energy diagrams of the single vacancy model used to describe the current-voltage relations in Fig. 3 and Eq. (1) in the text. The model has three binding sites with four energy barriers. Panels *A-C* illustrate the three possible ways in which the channel can be occupied by two ions. Panel *D* illustrates the model when all sites are occupied. The barriers have been modified to indicate that an ion rapidly exits the channel in this case from either the innermost or outermost site. The electrical distances used in Fig. 3 correspond to $d_1 = 0.08$ and $d_2 = 0.17$.

squid axons can be well described by the single vacancy model of ion translocation of Kohler and Heckman (1979), and Schumaker and MacKinnon (1990). The version of the model used here has three binding sites and is always filled by two or three ions. In the latter case, an ion rapidly exits the channel (Fig. 4*D*), so that the conductance *vs.* potassium ion concentration relation does not saturate over the range of potassium concentrations used in these experiments. Indeed, this relation is approximately a straight line for equimolar K^+ in the 0–500 mM range (Clay, 1991). The current-voltage relation can be shown to be given by:

$$I_K = \frac{q\,N\,(a\,exp(-(2d_1 + d_2)\,qV/kT)([K_i]\,exp(qV/kT) - [K_o])}{2\,cosh(d_1\,qV/kT)\,(exp(3\,d_2\,qV/kT) + 2\,cosh(d_2\,qV/kT))} \quad (1)$$

where $[K_o]$ and $[K_i]$ are the extra- and intracellular potassium ion concentrations, respectively, N is channel density, q, k, and T all have their usual meanings with kT/q ~24 mV at 8°, d_1 and d_2 are the electrical distances defined in Fig. 4*C*, and $a = 2.5 \times 10^7$ sec^{-1} M^{-1} (Clay, 1991). A good fit of Eq. (1) to the *I-V* relations in Fig. 3 was obtained with $d_1 = 0.08$ and $d_2 = 0.17$. In particular, the model predicts a nearly straight line relation for $[K_o] = [K_i]$ over the −200 to +200 mV potential range, which is consistent with experiment (*results not shown*). The model is also consistent, by definition, with multiple occupancy of the channel by *K* ions (Hodgkins & Keynes, 1955; Begenisich & DeWeer, 1980). An additional advantage of this analysis in the context of this study is that the probability of occupancy of various sites in the channel can be explicitly calculated. For example, the probability that the outermost site of the channel is occupied by a *K* ion is given by:

$$2\,exp(2\,d_2\,V')\,cosh(d_2\,V') + a/b \times \times \frac{\{terms\ which\ depend\ upon\ [K_o]\ and\ [K_i]\}}{cosh(d_1\,V')\,(exp(3\,d_2\,V') + 2\,cosh(d_2\,V'))} \quad (2)$$

where $V' = qV/kT$. However, $a[K] << b$, where $[K]$ is either $[K_i]$ or $[K_o]$ and $[K] < 500$ mM and b is the rate constant for an ion leaving the channel when all three sites are occupied (Clay, 1991). Consequently, Eq. (2) reduces to:

$$2\,exp(2\,d_2\,V')\,cosh(d_2\,V')/(cosh(d_1V')\,(exp(3\,d_2\,V') + 2\,cosh(d_2\,V')). \quad (3)$$

Similar results can be obtained for the occupancy of any site, or any combination of sites. In other words, occupancy is approximately independent of either $[K_o]$ or $[K_i]$. The marked increase in inward current at −140 mV, for example, with 300 K_o relative to 50 K_o is attributable in the model to an increased rate of movement of ions, or equivalently, the ''vacancy'' through the channel. However, average occupancy is independent of potassium concentration on either side of the membrane. Consequently, based on this model, the slowing of channel closing rate in preparations in which the effect was observed (Fig. 1*A*) would be predicted to be related to factors other than occupancy, especially since the change in the *I-V* relation following a change in K_o was always observed, whereas the effect of K_o on tail current time constant was observed only in some preparations (Discussion).

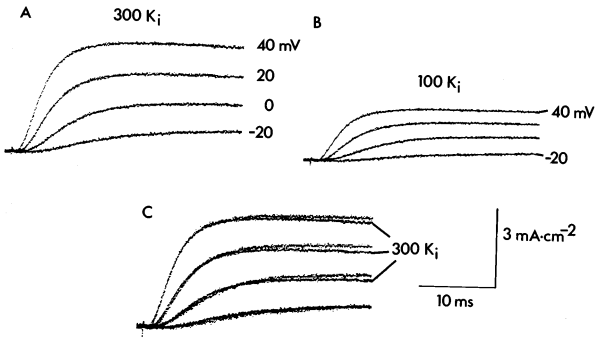

Fig. 5. Lack of effect of a change in K_i on I_K kinetics. (*A*) I_K records obtained for the steps indicated with standard intracellular perfusate, which contains 300 K_i (Materials and Methods). (*B*) Records from the same preparation following a change of intracellular perfusate to 100 K_i (Materials and Methods). (*C*) Results from *A* and *B* superimposed with the 100 K_i records scaled upward by a factor of 2.2.

Lack of Effect of K_i on Gating

The results in Fig. 1*B* are indicative of a lack of effect of K_o on activation kinetics, as has been previously documented (Clay, 1984; Armstrong & Matteson, 1986). This result also holds true for changes in the intracellular potassium ion concentration (K_i), as shown in Fig. 5. The results in Fig. 5*A* illustrate a family of I_K records with 300 mM K_i for the potentials indicated in the figure. The results in Fig. 5*B* were obtained from the same preparation following a change of the intracellular perfusate to 100 mM K_i. The latter records are shown superimposed upon the 300 K_i results in Fig. 5*A* scaled upward by a factor of 2.3. The lack of effect of K_i on kinetics shown here was observed in all preparations in which K_i was changed ($n = 6$). Moreover, tail current kinetics were unaltered by changes in K_i, including preparations in which the effect of K_o on gating was observed.

Effects of Cs^+ and Rb^+ on Gating

The addition of Cs^+ to the extracellular solution significantly blocks inward current (Bezanilla & Armstrong, 1972; Adelman & French, 1978). Consequently, the tail current time constant cannot be directly determined for these conditions. To circumvent this problem, the three-pulse protocol of Swenson and Armstrong (1981) was used as illustrated in Fig. 6. A 5 msec pulse to +80 mV activated I_K followed by a return of the membrane potential of variable duration to a given level (usually a hyperpolarized potential) for which the deactivation time course was to be determined. The potential was then stepped again to +80 mV for 5 msec, followed by a return to holding level (−80 mV). The protocol is illustrated for −80 and −120 mV in Fig. 6, conditions for which the conductance is zero in steady state. The amplitude of current, I_o, immediately following the second step to +80 mV (3rd step of the protocol) is a relative measure of conductance which has not yet had time to deactivate during the time the membrane potential was held at either −80 or −120 mV, during the second step of the protocol. Consequently, I_o as a function of the duration of the step to −80 or −120 mV, or whatever the potential of that step may be, provides a measure of the channel deactivation time course at that potential. The small filled circles in Fig. 6*A* and *C* represent the time constant for deactivation in control and with 100 mM Cs^+ respectively, for −80 mV. These results were 8 and 33 msec, respectively. That is, 100 mM Cs^+ increased the channel deactivation time constant by a factor of 4.1 at −80 mV. The corresponding results for −120 mV were 2.3 and 6.1 msec, respectively, i.e., an increase of the time constant by a factor of 2.7, significantly less than the increase at −80 mV. This reduction of the effect of Cs^+ on kinetics with hyperpolarization is a primary result of this study. Similar results are shown for two other preparations with 100 mM Cs^+ in Fig. 7*A* and for 100 mM Rb^+ in Fig. 7*B*. The latter results demonstrate that the effect on the tail current time constant with Rb^+ was also diminished with hyperpolarization, as was the case with Cs^+.

In contrast to the results given above for K_o, the effects of Cs^+ ($n = 8$) and Rb^+ ($n = 6$) on gating were observed in all experiments in which these effects were investigated.

Effects of Cs^+ and Rb^+ on Ion Permeation (Correlation with Effects on Gating)

The effects of extracellular Cs^+ and Rb^+ on the I_K current-voltage relation are well known. These results are illustrated in Fig. 8 for 100 K_o in control, and either 100 Cs^+ or 100 Rb^+ in the test solution, Fig. 8*A* and *B*, respectively. Fig. 8*A* illustrates the familiar N-shaped *I-V* produced by Cs^+ (Bezanilla & Armstrong, 1972; Adelman & French, 1978). That is, the current increased slightly in the inward direction with modest hyperpolarizations with Cs^+ in the extracellular solution and was then reduced with increased hyperpolarization, so that it was essentially nil at −120 mV. A secondary increase of inward current was not observed, even with potentials as negative as −200 mV (*results not shown*). By contrast, Rb^+ appears to block I_K in the vicinity of E_K and the block is relieved by hyperpolarization (Clay & Shlesinger, 1983), which is consistent with the reduction in the effect of Rb^+ on gating with hyperpolarization (Fig. 7*B*). The effects of Cs^+ differ significantly in this regard. In particular, the alteration of gating produced by Cs^+ was reduced by hyperpolarization, whereas the degree of block was enhanced, which would appear to be incon-

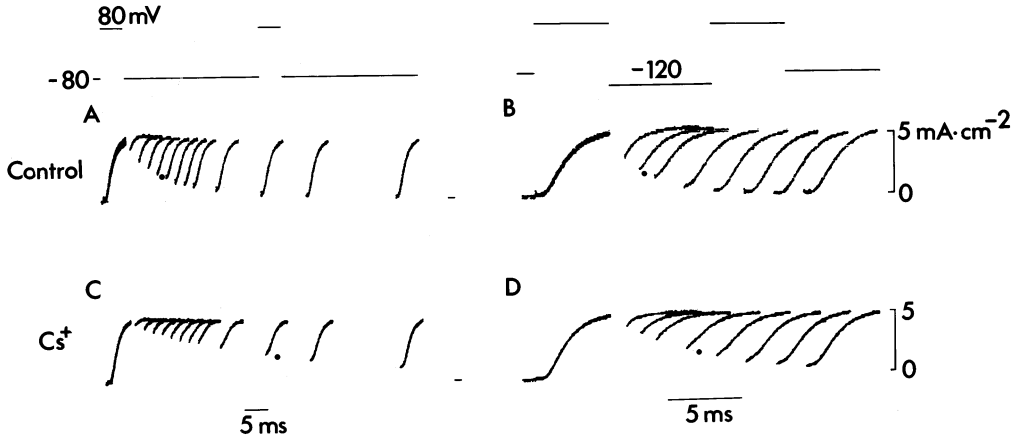

Fig. 6. Effects of 100 Cs^+ on closing kinetics at −80 and −120 mV using the three-step protocol described in the text. The protocol is illustrated explicitly above *A* and *C* for −80 mV and a duration of the second step equal to 30 msec, and above *B* and *D* for −120 mV and a duration of the second step equal to 7 msec. The currents, I_o, obtained from the beginning of the response to the third step in the protocol (the 2nd step to +80 mV) were plotted semilogarithmically and fitted with a straight line to give the time constant of deactivation for each condition. These results are represented by the small filled circle in each panel.

sistent with the occupancy hypothesis. However, this simple qualitative view does not take into account multiple binding sites within the channel, as noted above in connection with results with K_o. A similar analysis was carried out for Cs^+, as demonstrated in Fig. 9. In this case a two binding site model was used (top panel, Fig. 9) and the single vacancy concept was employed. That is, at least one of the two sites was assumed to be always occupied either by K^+ or by Cs^+. Once inside the channel, Cs^+ was assumed to translocate in the same way as K^+, except that a cesium ion was not allowed to enter the axon. The current-voltage relations in control and with Cs_o^+ were determined by straightforward algebraic manipulation. The results are shown in the bottom panel of Fig. 9 (same experimental results as in Fig. 8*A*). One interesting outcome of this analysis was the observation that a cesium ion is ~50% as effective at entering the channel compared with K^+, whereas the probability of Cs_o^+ crossing through the channel is zero, by definition. The probability that either site in the channel is occupied by a cesium ion can be calculated directly. These results are shown in the top panel of Fig. 9. The probability that the inner site is occupied by Cs^+ was significantly enhanced with hyperpolarization, which is not surprising, because this site is where blockade occurs in the model. This result is clearly counter to the effect of Cs_o^+ on gating as described above. The outer site, that is the site adjacent to the external solution, has only a slight probability of occupancy by Cs^+ at any potential. This result is diminished with hyperpolarization. Consequently, the effect of Cs_o^+ on gating could conceivably be correlated with occupancy at this site although this interpretation is problematic given the relatively small probability of occupancy at ~−50 mV where the effect on gating is the greatest. Moreover, the probability of occupancy of this site at −50 mV is not greatly different from that at 0 mV,

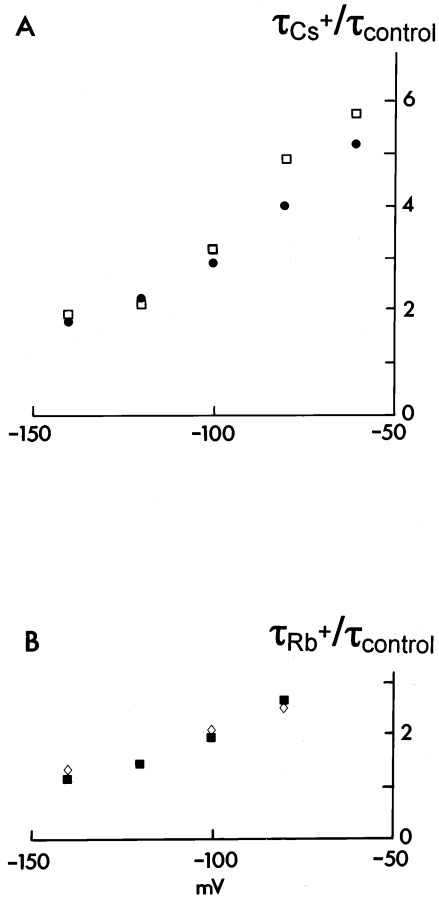

Fig. 7. Reduction of the effect of Cs^+ (*A*), and Rb^+ (*B*) on deactivation time constant with hyperpolarization. Each symbol represents the ratio of the time constant with either Cs^+ or Rb^+ and the corresponding control result for the potentials indicated, following the procedure illustrated in Fig. 6. Each different symbol type represents a different preparation.

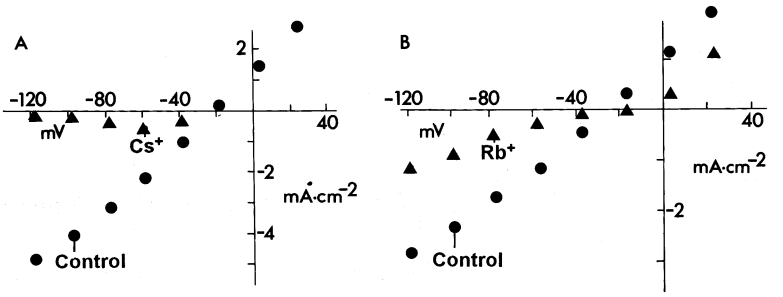

Fig. 8. Effects of Cs^+ (*A*) and Rb^+ (*B*) on I_K current-voltage relations. The control results in *A* and *B* (represented by the symbols (●)) were obtained with a prepulse to −20 mV followed by test steps to the potentials indicated. The test results (represented by ▲) were obtained with the same protocol with either 100 Cs^+ (*A*), or 100 Rb^+ (*B*) in the extracellular solution.

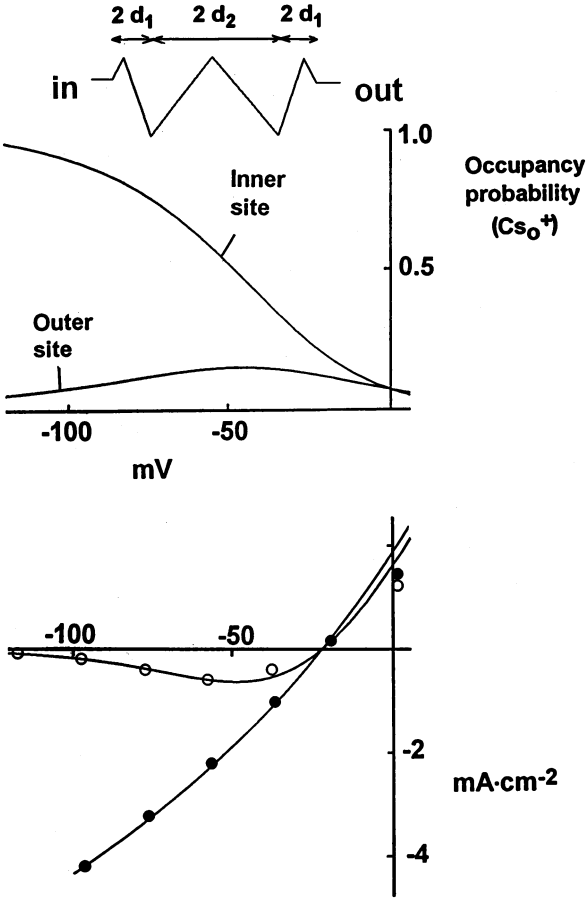

Fig. 9. Quantitative description of the Cs^+ results in Fig. 8*A*. The two-barrier model in the inset above the top panel was used in this analysis with $d_1 = 0.1$ and $d_2 = 0.3$. A description of the current-voltage relations in control (●) and with 100 Cs_o^+ (○) is given in the bottom panel. The probability of occupancy of either site by a cesium ion is given in the top panel.

and no effect of Cs_o^+ on gating was observed at this potential (*results not shown*).

Discussion

The significance of the original results by Arhem (1980), Swenson and Armstrong (1980), and Matteson and Armstrong (1986) is that they suggested that permeation and gating are coupled, in contrast to Hodgkin and Huxley (1952), who concluded that the two processes are independent of each other. The voltage dependence of these results provide significant additional information on this topic as shown by Clay (1986), Sala and Matteson (1991), and Demo and Yellen (1992). In particular, the effect of K_o on deactivation kinetics in squid axons is independent of membrane potential (Fig. 4, Clay, 1986). Moreover, the effect does not always occur, as shown in this report, even though ion permeation (the shape of the *I-V* relation) always changes following a change in K_o. These results argue against the occupancy hypothesis. Rather, they suggest that the effect on gating produced by a change in K_o occurs at a site located outside of the electric field of the channel, which is accessible to potassium ions in some, but not all, preparations for reasons which have yet to be elucidated. This mechanism can also describe the changes in the I_K activation curve reported by Matteson and Swenson (1986), i.e., a ''broadening'' of the voltage dependence of activation by elevated K_o or by Rb^+. This result can be accounted for by binding of a potassium ion to the channel gate at a position outside of the electric field of the channel, so that the deactivation rate constant is reduced with little or no effect on activation. In this scheme, gating and permeation are independent of each other. By contrast, the effects of Rb^+ and Cs^+ on gating are voltage dependent, and they were observed in all preparations investigated. The mechanism underlying these results is clearly different than it is for K^+, and it undoubtedly is more complicated than the simple occupancy hypothesis, as shown above for Cs^+.

The idea that the effects of K^+ on potassium channel gating differ from those of other ions appears to be consistent with results from other preparations. For example, Demo and Yellen (1992) found a lack of effect of K_o on gating in the Ca^{+2}-activated maxi-K^+ channel (BK) from rat skeletal muscle, in contrast to the effects of Cs^+ and Rb^+. Mienville and Clay (1996) reported a similar result for BK channels in the embryonic rat brain. They also found a lack of effect of changes in K_i on macroscopic current kinetics, although K_i does appear to influence ''flicker'' gating in this channel. Similarly, Shapiro

and DeCoursey (1991) found a lack of effect of potassium ions on gating of the L type K^+ channel in mouse lymphocytes. The effects of Rb^+ and Cs^+, which they reported, appeared to be mediated at a site located on the external mouth of the channel. A similar conclusion was reached by Safronov and Vogel (1995) concerning the effects of extracellular potassium ions on K^+ channels in demyelinated nerve axons from *Xenopus.* Indeed, evidence is accumulating that potassium ions affect gating of potassium channels *only* in nerve axons, and in these preparations the effects appear to be mediated at a site located outside of the permeation pathway. The mechanism underlying the effects of Cs^+ and Rb^+ is less clear. These results are voltage dependent in nerve axon K^+ channels and in K^+ channels from toadfish pancreatic islet cells (Sala & Matteson, 1991). Moreover, the effect of external Rb^+ on gating is reduced with hyperpolarization, which is consistent with the occupancy hypothesis, a noted by Sala and Matteson (1991). However, the results with Cs^+ reported above appear to run counter to this correlation. They suggest that the correlation between gating and permeation with Rb^+ may be fortuitous, although the clear voltage dependence of these results would seem to require that Cs^+ or Rb^+ interact with the channel in some way at a site within the electric field of the membrane.

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