Neuron, Vol. 32, 649–656, November 20, 2001, Copyright ©2001 by Cell Press

# Tight Steric Closure at the Intracellular Activation Gate of a Voltage-Gated $K^+$ Channel

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## Summary

**In voltage-gated $K^+$ channels (Kv), an intracellular gate regulates access from the cytoplasm to the pore by organic channel blockers and by chemical modifiers. But is ion flow itself controlled instead by constriction of the narrow selectivity filter near the extracellular surface? We find that the intracellular gate of Kv channels is capable of regulating access even by the small cations $Cd^{2+}$ and $Ag^+$. It can also exclude small neutral or negatively charged molecules, indicating that the gate operates by steric exclusion rather than electrostatically. Just intracellular to the gated region, channel closure does not restrict access even to very large reagents. Either these Kv channels have a broader inner entrance than seen in the KcsA crystal, even in the closed state, or the region is highly flexible (but nevertheless remains very securely closed nearby).**

## Introduction

Voltage-dependent $K^+$ channels (Kv channels) respond to membrane depolarization with a rapid opening or "activation gating." This process involves a voltage sensor responsible for sensing the voltage across the membrane and a gate responsible for the actual opening and closing of the pore. Armstrong (1966, 1971, 1975) first proposed that the activation gate was located at the intracellular entrance to the pore. He discovered that tetraethylammonium and its long chain derivatives block the squid axon delayed rectifier $K^+$ channel only after it has been opened, as if the access of these quaternary ammonium (QA) blockers to their binding site is regulated by the gate that opens and closes the pore. Once these blockers are bound to their site in the pore, they can interact with the activation gate in either of two ways, both consistent with the existence of a gate at the intracellular mouth: the blocker can prevent the gate from closing (by a foot in the door effect) (Yeh and Armstrong, 1978; Choi et al., 1993), or the blocker can become trapped behind a closed activation gate (Armstrong, 1971; Armstrong and Hille, 1972; Holmgren et al., 1997).

More recently, the site of this intracellular gate has been determined for a cloned, expressed Kv channel (the *Drosophila* Shaker Kv channel) by studying gating-dependent changes in chemical modification rates (Liu et al., 1997). Cysteines introduced in the intracellular half of the pore-lining transmembrane segment S6 were used as targets for chemical modification by MTS (methanethiosulfonate) reagents applied to the intracellular side. For cysteines located in the part of S6 closest to the intracellular side, chemical modification occurs both when channels are open and when they are closed, but for sites closer to the middle of the transmembrane region, reaction can occur only in the open state. Access of the chemical reagents to these sites of very strong state dependence is apparently prevented by the intracellular gate when it is closed.

There is a remarkable correspondence between this functional study in Shaker and the three-dimensional structure determined for a non-voltage-dependent bacterial $K^+$ channel, KcsA (Doyle et al., 1998). The bacterial channel is formed of four subunits surrounding a central pore. Each subunit has only two transmembrane segments (versus six in Kv channels) and a P region (which forms the selectivity filter). The M2 segments (corresponding to S6) line the inner vestibule of the channel and are nearly straight helical rods that are spread apart at the top (extracellular side) to accommodate the selectivity filter, but they cross as a bundle near the bottom of the channel. The Shaker residues whose accessibility is strongly state dependent lie above the "bundle crossing" of the KcsA M2, while those that are rapidly modified in either the open or closed states lie below. The boundary between these two behaviors is located at the bundle crossing in the KcsA structure.

This and other evidence, both for Shaker and KcsA, support the conclusion that the intracellular gate for both quaternary ammonium channel blockers and cysteine modifying MTS reagents is located at the bundle crossing (Holmgren et al., 1998; Yellen, 1998). An engineered metal bridge between sites above and below the bundle crossing can lock the channel in the open state (Holmgren et al., 1998). Quaternary ammonium blockers bind (and are trapped) in the internal cavity lying between the bundle crossing and the selectivity filter (Yellen et al., 1991; Holmgren et al., 1997; Zhou et al., 2001). And, spin-label studies on KcsA suggest significant motion of the lower S6 (Perozo et al., 1998), consistent with gating motions in this region.

Several important questions remain concerning this intracellular gate. First, is this gate, which clearly governs access of channel blockers and cysteine modifying chemicals, also the key site for gating of $K^+$ movements? An alternative site for activation gating of ion movements is the narrow selectivity filter located closer to the extracellular side of the membrane. There are several reasons to consider seriously the role of the selectivity filter in gating. Permeant ions produce changes in deactivation gating (Chandler and Meves, 1965; Swenson and Armstrong, 1981; Neyton and Pelleschi, 1991; Demo and Yellen, 1992; Townsend et al., 1997), and because this effect is so sensitive to the species of permeant ion, it seems very likely to occur in the selectivity filter itself. Conversely, channel gating can produce conductance substates (Chapman et al., 1997) with varying selectivity (Zheng and Sigworth, 1997, 1998). Most significantly, in cyclic nucleotide-gated (CNG) channels, the bundle crossing does not effectively restrict access to S6 cys-

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Table 1. Physical Properties of Permeant and Probe Ions

| | Pauling Radius (Å) | Diffusion Coefficient ($cm^2/s$) |
|---|---|---|
| $K^+$ | 1.33 | $1.96 \times 10^{-5}$ |
| $Na^+$ | 0.95 | $1.33 \times 10^{-5}$ |
| $Cd^{2+}$ | 0.97 | $0.72 \times 10^{-5}$ |
| $Ag^+$ | 1.26 | $1.65 \times 10^{-5}$ |

Values from Marcus, 1997.

teines, leading (by elimination) to the conclusion that these channels are indeed gated at the selectivity filter (Flynn and Zagotta, 2001).

Second, to the extent that the S6 bundle crossing does act as a gate, how does it work? Is it steric, acting as a physical barrier, or electrostatic, selectively permitting or denying access to particularly charged molecules?

Finally, what motion does the gate make in order to open and close the pore? From our previous investigation of the sites of blocker binding and the structure of open Shaker channels, we proposed a model for the open channels in which the S6 helices are bent near the intracellular surface of the membrane (del Camino et al., 2000), unlike the relatively linear inner helices seen in the KcsA crystal. Because the determined KcsA structure most likely corresponds to the closed channel, we wondered if Kv channels might close by switching from a "S6 bent" structure to a KcsA-like structure.

To address these questions, we studied state-dependent accessibility of two cysteines near the bundle crossing in Shaker channels, using a series of reagents with different sizes and charges. Our results suggest that the intracellular gate in Kv channels, in addition to being the gate for small organic molecules, is also a gate for $K^+$ ions. We also show that this is a steric gate, and that most likely, even when it closes, it does not adopt a structure with a narrow "inner pore" like that seen in the KcsA crystal structure.

## Results and Discussion

### The Intracellular Activation Gate Prevents Access of $Cd^{2+}$ to 474C

First we asked whether the intracellular gate is responsible for regulating the movement of $K^+$ across the channel. Our approach to this question was to determine whether small cations that can react with cysteines show state-dependent access to a cysteine introduced at position 474. This position is particularly appropriate for this study for several reasons. First, it has been shown previously that among all cysteines introduced in the S6, 474C is the residue that shows the largest difference in the MTSET (2-trimethylaminoethyl methanethiosulfonate) modification rates between the open and closed states (Liu et al., 1997). Also, 474C residues from the four subunits form a high affinity binding site for $Cd^{2+}$, so that when $Cd^{2+}$ is bound, it blocks conduction of the channels. This blockade is essentially irreversible, although $Cd^{2+}$ can be released from the channel by intracellular application of the dithiol reagent DMPS (dimercaptopropanesulfonate). The voltage dependence of $Cd^{2+}$ release by DMPS closely matches the voltage dependence of conductance in this mutant, which indicates that the energetics of gating when $Cd^{2+}$ is bound are similar to the energetics of gating without $Cd^{2+}$ bound to the channels. All of these results suggest that 474 residues are located lining the pore behind the intracellular gate, and that they do not move much during gating (Liu et al., 1997; Webster and Yellen, 2001). Thus, the rate of metal inhibition appears to be governed, not by conformationally-induced burial of the cysteine in the body of the protein, but rather by gated access to this cysteine that lines the lumen of the pore.

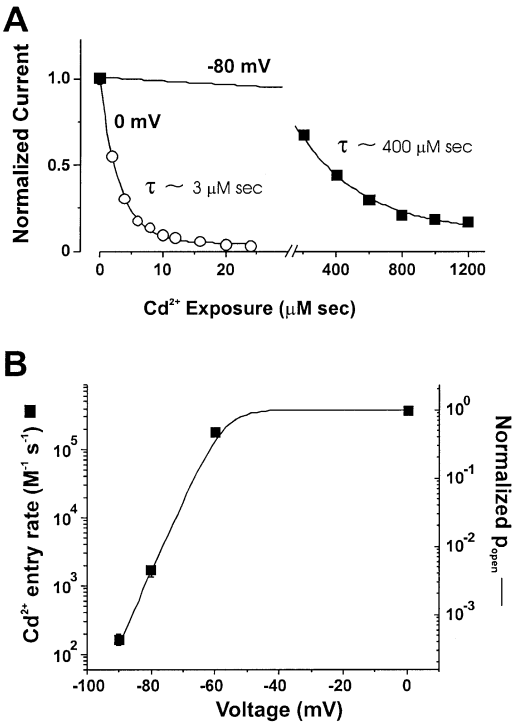


Figure 1. Gating State Dependence for $Cd^{2+}$ Modification of 474C

(A) Time course of the normalized current decrease produced by repeated $Cd^{2+}$ applications to 474C channels at 0 or −80 mV. The abscissa shows the cumulative exposure to $Cd^{2+}$ (modification time × $[Cd^{2+}]$). The lines are best fits to a single exponential function. (B) Voltage dependence for the $Cd^{2+}$ modification rate of 474C channels. The closed squares represent the mean modification rates measured from three to five experiments at each voltage. The solid line is an averaged Boltzmann fit to the voltage-activation curves obtained from seven experiments (midpoint of −57.5 ± 1.79 mV; slope factor of 4.1 ± 0.19 $mV^{-1}$).

We first used $Cd^{2+}$ as a probe to test gated access of small cations to the pore because this ion is slightly smaller than $K^+$ (see Table 1), and it can interact with 474C residues blocking the channels. In an earlier characterization of 474C modification by $Cd^{2+}$, it was found that $Cd^{2+}$ access to this residue is much faster in the open state than in the closed state (Liu et al., 1997). We further studied $Cd^{2+}$ state-dependent accessibility to 474C, measuring the modification rate of this residue at several voltages and comparing the voltage dependence of modification with the voltage dependence of gating in these channels. This would permit us to see whether $Cd^{2+}$ access to 474C correlates with the open

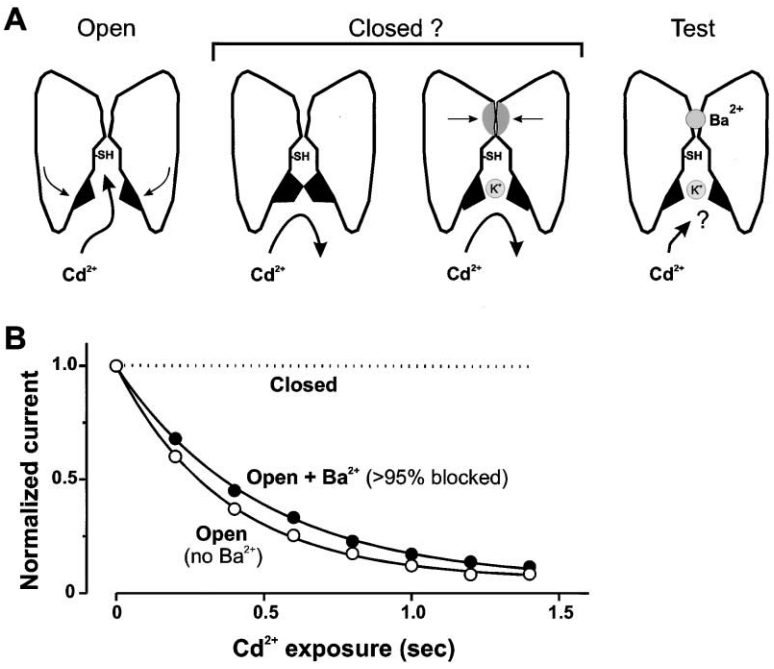


Figure 2. $Cd^{2+}$ Access to 474C in the Open State Is Not Prevented by Blocking the Selectivity Filter with $Ba^{2+}$

(A) The cartoon shows two possible explanations for $Cd^{2+}$ gated access to 474C. In the closed state, access could be prevented either by the direct action of an intracellular gate or by an indirect effect of closure at the selectivity filter. The right hand model shows the experimental test to distinguish between the two possibilities, using $Ba^{2+}$ to mimic selectivity filter closure.

(B) Time course of 474C channel modification by 10 $\mu$M $Cd^{2+}$ application to open channels (open circles; $\tau = 0.36$ s) or to open channels blocked by $Ba^{2+}$ at 50 $\mu$M (closed circles; $\tau = 0.48$ s). The solid lines are best fits to a single exponential function. For comparison, the dotted line is a fit to a single exponential function corresponding to the time course of modification in the closed state.

probability ($p_{open}$) of 474C channels, as estimated from an averaged conductance voltage (g-V) relation measured for this mutant. Figure 1A shows the current decrease produced by repeated applications of $Cd^{2+}$ at 0 mV or at −80 mV to inside-out patches containing 474C channels. The inverse of the time constant for the single exponential fit was divided by the reagent concentration to obtain a second-order rate constant, which is more than 100 times slower at −80 mV than at 0 mV. The average of similar experiments done at different voltages is shown in Figure 1B, together with an averaged Boltzmann fit to several g-V relations obtained for 474C channels. This figure shows that the reduction in $Cd^{2+}$ modification rate at negative voltages agrees reasonably well with the g-V curve, extrapolated down to a very small fractional open probability. At −90 mV, the reaction rate is ~2500 times slower than at 0 mV. This large reduction in $Cd^{2+}$ on-rate is apparently a consequence of channel gating rather than any intrinsic voltage dependence for the divalent cation binding in the pore; there is no significant difference between the rates measured at 0 mV and at +60 mV (data not shown), as would be expected if substantial intrinsic voltage dependence were present. Thus, compared with open channels, $Cd^{2+}$ reacts with closed channels at least 2500 times slower, and the actual effect of channel closure may be even larger, because the rate measured at −90 mV may include rapid reaction by the tiny percentage of channels that are open. These results show that, as for small organic molecules like MTSET, the intracellular activation gate also regulates the access of $Cd^{2+}$ to 474C.

### Intracellular Gated Access Cannot Be an Indirect Effect of Closure at an Extracellular Gate

Because it has been suggested that the selectivity filter might actually be the site of activation gating (Van-Dongen and Chapman, 2001), we worried that our results showing intracellular gated access to 474C might be explained simply as an indirect effect of closure at the selectivity filter. In other words, for $Cd^{2+}$ to enter the pore from the inside, it might be necessary to push a resident $K^+$ ion outward though the selectivity filter. Closure of an outer gate might prevent this "pressure relief" and thus prevent $Cd^{2+}$ access, even with no actual gating at the bundle crossing (Figure 2A).

To test for this possibility, we mimicked the putative closure of the selectivity filter by occluding it with a $Ba^{2+}$ ion (Neyton and Miller, 1988; Jiang and MacKinnon, 2000). We compared the $Cd^{2+}$ modification rate of 474C for channels that were completely open with the rate for channels that were conformationally open but blocked with $Ba^{2+}$. To make this measurement, we opened channels with voltage, blocked them with $Ba^{2+}$, applied $Cd^{2+}$ for a defined period of time, and then removed both ions and measured the persistent reduction in current; this process was repeated to find the time course of $Cd^{2+}$ inhibition for $Ba^{2+}$ blocked channels. We found that $Ba^{2+}$ blockade produced almost no difference in the rate of $Cd^{2+}$ inhibition of open 474C channels (Figure 2B). Therefore, stoppage of ion flow at the selectivity filter alone cannot explain $Cd^{2+}$ gated access to 474C. Instead, this gated access reflects the action of an intracellular gate.

### Even the Monovalent $Ag^+$ Ion Shows Gated Access to 474C from the Intracellular Solution

The intracellular gate constitutes a barrier for the access of $Cd^{2+}$ to the pore. But is $Cd^{2+}$ a good model for $K^+$? As mentioned before, the ionic radius of $Cd^{2+}$ is smaller than that of $K^+$, but it is a divalent cation and therefore it is more strongly attached to its hydration shell, as evidenced by its much smaller diffusion coefficient (Table 1). Another small cation that can be used as a cys-

teine-reactive probe is $Ag^+$. As pointed out by Lü and Miller (1995), $Ag^+$ makes an ideal reagent to study the narrow parts of the pore because: 1) it is very similar in size to $K^+$ ions; 2) like $K^+$, $Ag^+$ allows rapid exchange of inner-shell water molecules in aqueous solutions; and 3) $Ag^+$ reacts strongly with the thiol group of a cysteine. These properties make $Ag^+$ a very good probe to test whether the intracellular gate is likely to restrict $K^+$ flow through the channel.

There are several experimental difficulties in working with $Ag^+$. Because AgCl is insoluble, it is necessary to work with non-chloride-containing nitrate solutions. Nitrate solutions alone make patches unstable, and this problem is worse when $Ag^+$ is added. Also, we found initially that the results obtained with $Ag^+$ application were very variable; unbuffered $Ag^+$ solutions appear to wash in very slowly (seconds), as though some part of the experimental system has a binding capacity for $Ag^+$. To avoid this problem, we used EDTA as a buffer to give the desired concentration of free $Ag^+$ (this entailed using no divalent cations in the intracellular solution, which further compounds the problems with patch stability).

When applied to 474C channels, $Ag^+$ produces a complete and irreversible inhibition of the current; under the same conditions it only causes a small reduction (~10%–20%) in the current of wild-type channels (data not shown). We then studied whether $Ag^+$ access to 474C is state dependent. Figure 3A shows an example of 474C channel modification by $Ag^+$ in the open state. Upon opening the channels, 0.2 μM free $Ag^+$ produced a very rapid current inhibition, from which we directly estimated the reaction rate. To obtain the reaction rate in the closed state, 474C channels were repeatedly exposed to 2 μM free $Ag^+$ (Figure 3B), and the current decrease produced after each $Ag^+$ application was plotted to determine the modification rate constant.

Figure 3C shows the mean apparent second-order reaction rate constants for the open and closed channels measured for $Ag^+$ and, for comparison, $Cd^{2+}$. These mean rate constants are plotted on a logarithmic axis, so that the length of the bar connecting the symbols for open and closed rates shows the fold-change in cysteine reactivity between the two states. In the open state, $Ag^+$ reacts with 474C at a much faster rate than $Cd^{2+}$, with a second-order rate constant (~$10^8$) that is close to the diffusion limit. $Ag^+$ access to this position is also strongly state dependent, with the modification rate in the closed state about 700 times slower. There is no significant difference in the $Ag^+$ on-rate measured at 0 mV or at 60 mV, so, as for $Cd^{2+}$, channel gating (and not an intrinsic voltage dependence for $Ag^+$ binding in the pore) seems to be responsible for the voltage-dependent modification of 474 cysteines by $Ag^+$.

The apparent open/closed ratio (based on the modification rates at 0 and −90 mV) is at least 3 times larger for $Cd^{2+}$ than for $Ag^+$. There are two possible explanations for this difference. On the one hand, the solutions used for both reagents are quite different; $Cd^{2+}$ experiments were done in regular $Cl^-$ solutions containing 0.5 mM $Mg^{2+}$, while the $Ag^+$ experiments required $NO_3^-$ solutions with high EDTA and zero divalents. These changes might produce a higher $p_{open}$ at −90 mV in the conditions used for $Ag^+$, and therefore a smaller apparent open/closed ratio. Alternatively, the closed intracellular gate might exclude $Cd^{2+}$ better than $Ag^+$, allowing a small leak of $Ag^+$ ions even in the closed state.

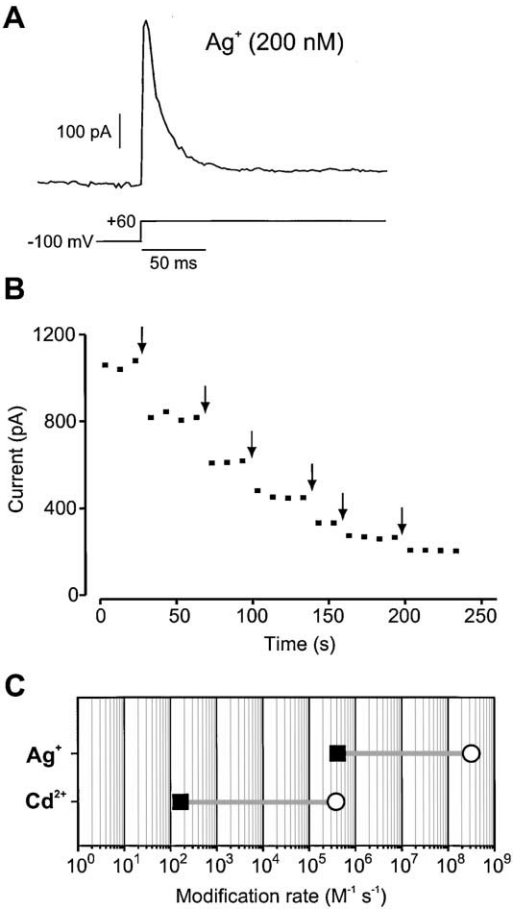


Figure 3. $Ag^+$ Modification of C474 Is Also Strongly Gated

(A) $Ag^+$ modification of 474C channels in the open state. $Ag^+$ (200 nM free) was applied to an inside-out patch for the 200 ms prior to opening and during the time the channels were held open. Upon opening, the channels were rapidly modified by $Ag^+$. The current decay was fitted to a single exponential, and the time constant obtained was used to compute a second-order rate constant of $3.3 \times 10^8\ M^{-1}\ s^{-1}$.

(B) $Ag^+$ modification of 474C channels in the closed state. The channels in the inside-out patch were held closed at −90 mV and the dots represent the steady-state current in response to 10 ms long pulses to 0 mV. The arrows indicate the times at which 2 μM free $Ag^+$ was applied for 500 ms in the closed state at −90 mV, and then washed out before the following test pulse.

(C) Second-order rate constants for 474C modification by $Ag^+$ and $Cd^{2+}$ measured at 0 mV (open circles) or at −90 mV (closed squares). The length of the bar connecting the two symbols represents the fold-change in rate between the indicated voltages. Each point is the mean of 3 or more determinations; error bars are smaller than the symbols.

To decide between these two possibilities, we calculated the extrapolated $p_{open}$ at −90 mV from an average single Boltzmann fit to several g-V relations obtained for 474C channels in $NO_3^-$ solutions. This $p_{open}$ (~0.2%) is indeed higher than the one measured in $Cl^-$ solutions, and the modification rate of 474C channels by $Ag^+$ at −90 mV is thus roughly that expected from this small

fraction of open channels reacting at the high open channel modification rate. However, if this results from a voltage shift in $p_{open}$, we would expect to be able to reduce the $Ag^+$ modification rate further by using a more negative voltage of −120 mV. Although working with $Ag^+$ at very negative potentials made it difficult to measure an accurate rate (because patches were so unstable), it appears that the rate of $Ag^+$ modification at −120 mV was comparable to that at −90 mV (in contrast to the result with $Cd^{2+}$) (data not shown). So although altered $p_{open}$ in $NO_3^-$ solutions might in part account for the smaller apparent open/closed ratio for $Ag^+$, it remains possible that $Ag^+$ ions can enter closed channels at an extremely low rate.

Even if all the modification seen for $Ag^+$ at −90 mV were due to $Ag^+$ leaking into closed channels, the intracellular gate could still effectively gate the flow of $K^+$ ions. If $K^+$ ions behave like $Ag^+$ ions, the conductance of the closed channel at −90 mV would be ~0.0014 times the conductance in the open state. This difference is probably large enough to consider a channel closed. Certainly for single channel measurements (as Sigworth [1994] has pointed out), reducing the ion flow to less than 1% of the open channel would make a "closed" state indistinguishable from any other closed state; even macroscopic measurements do not establish that closed channels are more than ~700 times less conductive than open channels. Therefore, all of these results suggest that the intracellular gate, in addition to being a gate for small organic molecules, is also a significant barrier for the movement of $K^+$ across the channel.

### The Intracellular Gate Is Not a "Field-Effect" Gate

How does the intracellular gate exclude ions, QA blockers, and MTSET? Because all of these molecules are positively charged, we wondered whether the intracellular gate might work as a "field-effect" gate (Hille, 1992) by altering a local electrical potential within the pore that attracts or repels the ions. If this is the way the gate operates, an easy way to test it is to measure the effect of gating on the entry rate for species with different charges. It has been shown previously that MTSET (positively charged) modification of 474C is very strongly state dependent (Liu et al., 1997). We therefore studied whether the MTS reagents MTSES (2-sulfonatoethyl methanethiosulfonate, negatively charged) and MTSACE (2-aminocarbonylethyl methanethiosulfonate, uncharged but polar) react with the cysteine at position 474 in a state-dependent manner. Both reagents have very little effect on the outward current, but clearly affect the tail current of 474C; the inward tail current becomes larger and slower upon modification. We do not know the reason for this particular consequence of chemical modification, but we can use it to monitor the rate of reaction. Using state-dependent applications (as for $Cd^{2+}$ and $Ag^+$), we determined the mean modification rate in the open state and an upper limit for the modification rate in the closed state. Figure 4 shows on a logarithmic axis these mean rates for MTSES and MTSACE, together with the previously determined rates for MTSET. The results clearly show that modification of 474C by all of these reagents is much slower in the closed state than in the open state. The intracellular gate can thus regulate the access to the pore by reagents having positive, negative or no charge, indicating that this is a steric and not a field-effect gate.

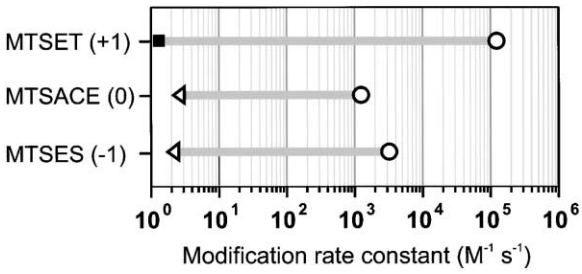


Figure 4. Gated Access to 474C Does Not Depend on Reagent Charge

Second-order rate constants for 474C modification by MTSET (positively charged), MTSACE (neutral), and MTSES (negatively charged) were measured at 0 mV (open circles). For MTSET, the closed rate (at −90 mV) is shown as an closed square. The error bars are smaller than the symbols. For MTSACE and MTSES, we measured only small changes in the current, even with several applications at −110 or −120 mV; the triangles represent an upper limit for the possible rate constant under these conditions.

Because a neutral polar molecule like MTSACE (about 5 × 5.5 Å in the smallest profile) is excluded by the gate, the gate also does not act simply as a "dielectric barrier" that would prohibit charged compounds from passing (because of the loss of their favorable polarization interaction with water). Nevertheless, hydration energies are of course likely to be important. To exclude small ions like $K^+$ or $Ag^+$, it is sufficient for the gate to narrow just enough to exclude the hydrated ions, because these ions have such a strong energetic interaction with water that the rate of entry for the smaller dehydrated ion would be vanishingly small.

### Channel Closure Does Not Restrict Access to 478C, Even for Very Large Reagents

We recently proposed a model for the architecture of the pore of open Kv channels (del Camino et al., 2000) that differs from the crystal structure of KcsA by supposing that the S6 segments are bent near the intracellular surface of the membrane, unlike the relatively linear inner helices seen in the KcsA crystal. The bend is placed at the level of two prolines (positions 473 and 475 in Shaker) that are absolutely conserved in the Kv1–4 family of voltage-gated $K^+$ channels, but not found in KcsA. One possible mechanism for closure of the intracellular gate in Shaker would be a switch from an open state with a bent S6 structure to a closed state with straight S6 helices, as seen in the KcsA structure (Figure 5A). If gating occurs in this way, the accessibility of cysteines introduced at positions located right below the bundle crossing should be very sensitive to the narrowing of the pore when the channels close. A good residue to study this effect is 478C, because the evidence suggests that this is the first residue below the bundle crossing that faces the pore; as for 470C and 474C, $Cd^{2+}$ binds with high affinity to cysteines at this position, and the binding of intracellular open channel blockers effectively protects this position from chemical modification (Liu et al., 1997; del Camino et al., 2000).

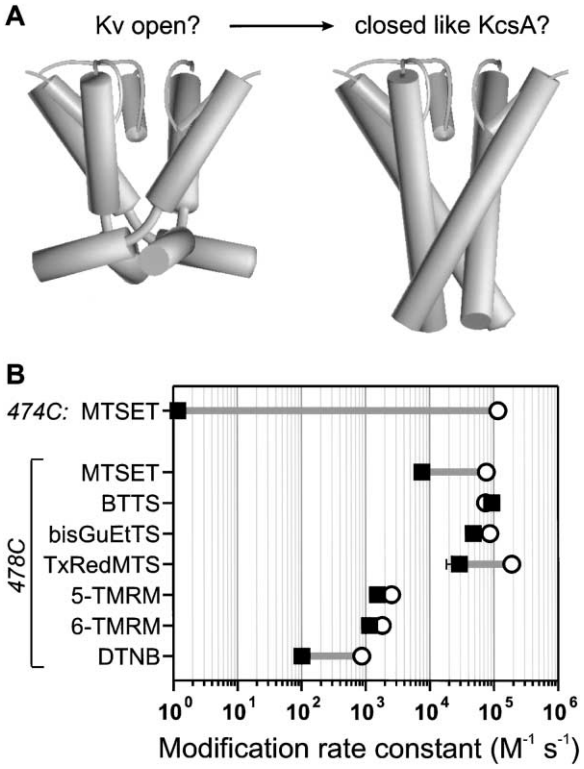


Figure 5. Does Channel Closure Involve a Constriction of the Inner Approach to the Pore?

(A) The hypothesis is that the "bent-S6" open state proposed for Kv channels by del Camino et al. (2000) may, upon closing, adopt a more linear arrangement of the inner helices, as found in the crystal structure of KcsA (Doyle et al., 1998). If so, larger reagents should have a progressively harder time reaching position 478 in Shaker, which would be located just below the bundle crossing.

(B) Measured rate constants for modification of Shaker 478C channels by a series of cysteine modifying reagents. Rates were determined in the open (O) and closed (■) states, at 0 mV and −90 mV, respectively. For comparison, the rate constants for MTSET reaction with the strongly gated position 474 are also shown.

The previous work on the state-dependent accessibility of S6 cysteines found that gating produces only a modest effect on the rate of MTSET reaction with 478C; the reaction rate is only ten times slower in the closed state than in the open state (Liu et al., 1997). We thought that perhaps the protein does constrict below the bundle crossing during closure, but that MTSET is sufficiently small that it can still reach 478C at a fairly high rate even in the closed state. If so, larger reagents should show much greater state dependence.

Figure 6 shows the relative size of the MTS reagents used, compared to MTSET and the pore structure of KcsA. When applied to 478C channels, all of these reagents produced (like MTSET) almost complete current inhibition. For each one of them, we determined the apparent second-order reaction rate constant in the open and closed states, and the mean values obtained are plotted on a logarithmic axis in Figure 5B. Because MTSET modification of 474C channels is very strongly state dependent, the values for the closed and open state rates at this position are also shown for comparison. As indicated before, MTSET modification of 478C is only about 10 times slower in the closed state than in the open state, which is a small difference, especially when compared with the strong gated access to 474C.

None of the larger reagents reacted with 478C in a more state-dependent manner than MTSET itself. This result suggests that in the region below the bundle crossing, the S6 helices do not constrict significantly with channel closure, and thus that Shaker channels do not gate by flipping between a S6 bent structure and a structure like that in the KcsA crystal.

How significant is this absence of size-selective state dependence at this location? Obviously the result is inconsistent with a rigid narrow pore with a structure like that of KcsA. Some of the reagents we used (Texas Red MTS and 5- or 6-TMRM [tetramethylrhodamine maleimide]) have a bulky group at one end, but are linked by a narrow tether to a relatively small reactive group (methanethiosulfonate or maleimide). These would not fit easily into the KcsA pore, but neither would the bulky group need to penetrate very far up into the pore. On the other hand, three of the larger reagents (BTTS [S-benzyl-p-toluenethiosulfonate], bis-(guEt)TS [bis-{guanidinoethyl} thiosulfonate], and DTNB [5,5′-dithio-bis-{2-nitrobenzoic acid}]) have a reactive group in the middle of the molecule. These can potentially react in two ways. They could snake up linearly into the pore and react with 478C, but this would force the leading end of the compound up to the level of 474C, which we know cannot react even with $Ag^+$ when the channel is closed. This seems particularly unlikely for bis-(guEt)TS and DTNB, which are charged at both ends and thus likely to experience at least the same unfavorable energy as a $Ag^+$ ion. The alternative is for these molecules to lie sideways, with the two sulfurs at the top center and the bulky groups bent down. This would be impossible without major deformation of the inner pore structure in KcsA.

All of the reagents we used react quite rapidly with the 478C channel, indicating that the reactive conformation of the channel is probably not a rare one. The methanethiosulfonates all react at $\sim 10^5\ M^{-1}\ s^{-1}$, comparable to the rate of reaction with free cysteine (Stauffer and Karlin, 1994). The maleimides and DTNB react ~100-fold slower than this, probably because of the slower intrinsic rate of chemical reaction.

The results with large reagents thus compel us to conclude either that the closed Shaker channel has an average structure that is substantially more open than the narrow inner pore seen in the KcsA crystal structure, or that the structure in this region is very flexible, permitting even large reagents to reach 478C in the closed state. There is, however, a stringent limit on how flexible the lower S6 can be; it must reliably (>99.8% of the time) prevent very small probes like $Cd^{2+}$ or $Ag^+$ from reaching a cysteine at position 474. Thus, in the closed state this flexibility (if present) must be limited to the part of S6 below the bundle crossing, and the secure closure of this intracellular gate occurs at a point located below the level of 474 but not below 478. This intracellular S6 gate limits blocker access to the cavity, and it limits ion access to the cavity and thence through the selectivity filter. Our results do not bear directly on whether activation gating affects ion movement in the selectivity filter, but we conclude that the action of the

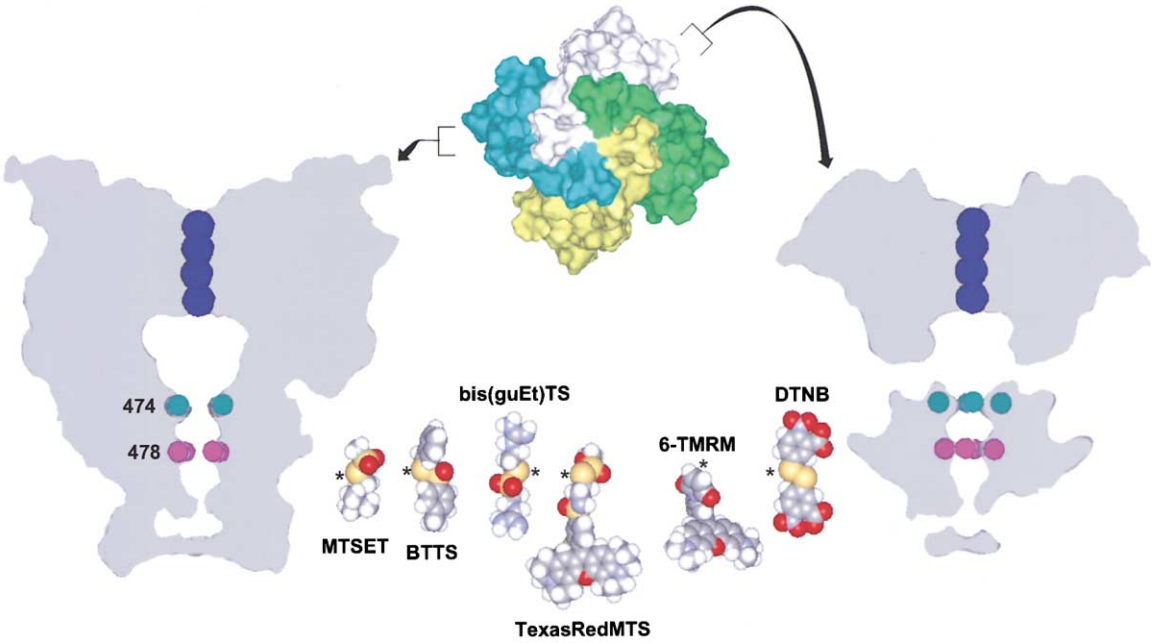


Figure 6. Cysteine Modification Reagents and the Pore of the KcsA Channel

Six of the modification reagents used in Figure 5 are shown in their most extended conformations, with an asterisk marking the reactive atom. On the same scale, two cross-sections (left and right) through the KcsA structure (PDB entry 1J95) (Zhou et al., 2001) are shown, taken from slices differing in orientation by 45° (as shown on the intracellular view of the structure, inset at top). The cross-sections are smoothed for a probe of 1.4 Å radius. The cross-sections indicate the four $K^+$ binding positions in the selectivity filter (blue balls at top) and the β carbons of residues 107 and 111, corresponding to Shaker positions 474 (green) and 478 (magenta), respectively. In the right-hand cross-section, a "hole" in the side of the central cavity is apparent, as are lateral passages at the very intracellular end of the structure. The modification reagents are vertically positioned at roughly the level needed for reaction with a downward facing thiol attached to the β-carbon of 478.

intracellular S6 gate is sufficient to explain ion gating in this class of Kv channels.

## Experimental Procedures

### Mutagenesis and Expression

Mutations V474C and V478C were introduced in the Shaker H4 $K^+$ channel (Kamb et al., 1988), carrying three additional modifications: a deletion between residues 6 and 46 that removes N-type inactivation (Hoshi et al., 1990), substitution of cysteines 301 and 308 by serines to diminish the wild-type effect of chemical modification (Holmgren et al., 1996), and mutation of threonine 449 to valine to reduce C-type inactivation (López-Barneo et al., 1993). Channels were transiently expressed in HEK 293 cells and used 1–3 days after transfection. Methods for mutagenesis, transfection, and identification of transfected cells have been described previously (Jurman et al., 1994).

### Physiological Recording and Chemical Modification

All experiments were performed from inside-out excised patches (Hamill et al., 1981). In the experiments studying $Cd^{2+}$ modification on 474C, the pipette solution contained 150 mM NaCl, 10 mM KCl, 3 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM HEPES at pH 7.4. The intracellular bathing solution contained 160 mM KCl, 0.5 mM $MgCl_2$, 10mM HEPES and typically 0.1 to 0.2 mM EGTA (no EGTA in the solutions containing $Cd^{2+}$) at pH 7.4.

For the experiments using organic cysteine-reactive reagents, similar solutions were used but containing 1 mM EGTA in the bath, and 100 mM NaCl and 60 mM KCl (experiments on 474C) or 150 mM NaCl and 10 mM KCl (experiments on 478C) in the pipette.

For the experiments with $Ag^+$, the solutions were, in mM: 110 $NaNO_3$, 47 $KNO_3$, 3 KCl, 3 $Ca(NO_3)_2$, 1 $Mg(NO_3)_2$, 10 HEPES at pH 7.4 (pipette); and 160 $KNO_3$, 10 HEPES, 10 EDTA at pH 7.4 (bath). A concentrated stock of $AgNO_3$ in water was prepared each day and stored protected from the light. For each experiment, this stock was used to freshly prepare bath solution containing a total of 52.5 μM or 501.5 μM $AgNO_3$. The EDTA present in the solution buffers most of the $AgNO_3$, giving a free $Ag^+$ concentration of 0.2 μM or 2 μM respectively.

The thiosulfonate reagents bis-(guEt)TS, MTSET, MTSES, MTSACE, and Texas Red-MTSEA were purchased from Toronto Research Chemicals Inc. (Downsview, Ontario). DTNB and BTTS were obtained from Aldrich (Milwaukee, WI), and 6-TMRM and 5-TMRM were purchased from Molecular Probes (Eugene, OR). The water soluble reagents (bis-(guEt)TS, MTSET, MTSES, MTSACE, and DTNB) were prepared as stocks in water each day, held on ice, and mixed into the recording solution immediately prior to use. The reagents with lower water solubility (BTTS, Texas Red-MTSEA, 6-TMRM, and 5-TMRM) were prepared as stocks in dimethylsulfoxide and stored at −20°C. An aliquot of the stock solution was thawed each day and used as in the case of water soluble reagents.

The methods for electrophysiological recordings and rapid perfusion switches have been described previously (Holmgren et al., 1996; Liu et al., 1997).

### Acknowledgments

We are grateful to Tatiana Abramson for expert technical assistance and to Sarah Webster for discussions and advice on the work. We also thank Ki Soon Shin and Brad Rothberg for discussions and Lise Heginbotham for her comments on the manuscript. Thanks also to Bertil Hille for prompting us with a question about charge selectivity. This work was supported by a National Institutes of Health research grant to G.Y. (NS 29693) and by a fellowship from the Spanish Ministry of Education to D.D.C.

Received August 17, 2001; revised September 17, 2001.

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