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Role of Surface Electrostatics in the Operation of a High-Conductance Ca^{2+}-Activated K^+ Channel†

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ABSTRACT: This paper demonstrates that local electric fields originating from negatively charged groups on a K^+-specific ion channel modify its behavior. Single high-conductance, Ca^{2+}-activated K^+ channels were studied in neutral phospholipid bilayers. The channel protein surface charges were manipulated experimentally by carboxyl group esterification using trimethyloxonium (TMO) or by electrolyte screening. Three channel properties—ion conduction, ion blockade, and voltage-dependent gating—are affected by surface electrostatics. Negative charges increase the affinity of cationic pore blockers by establishing a local negative potential at the pore entrance; these charges modify channel gating by establishing a potential gradient across the ion channel; finally, both effects influence ion permeation through the pore.

Electrostatic forces originating from charged groups on protein surfaces influence a variety of macromolecular functions. The distribution of charges on the surface of superoxide dismutase is important for electrostatic guidance of substrate into the active site (Getzoff et al., 1983; Sharp et al., 1987), and a similar electrostatic steering mechanism may act in the encounter of cytochrome *c* with cytochrome *c* peroxidase (Northrup et al., 1988). Likewise, charged groups are known to affect the binding of protons in subtilisin (Russell & Fersht, 1987) and of Ca ions in subtilisin (Pantoliano et al., 1988) and intestinal calbindin (Linse et al., 1988). In these well-studied cases, for which high-resolution structures are available, it is clear that the charged groups influence ion binding at distant sites through electrostatic forces operating over distances up to 2 nm. The effects are as expected intuitively: a negative charge near to, but not right at, a binding site for a positively charged ligand enhances the binding of that ligand.

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These electrostatic effects, well-known in soluble proteins, must also operate in ion channels, a class of integral membrane proteins which allow the pore-mediated diffusion of ions across biological membranes (Hille, 1984). Little is known about ion channel structures, but in several cases, it is clear that strategically located charged groups exert long-range electrostatic forces to control ion access to the channel conduction pore or to provide a background potential near the protein surface which influences ion permeation and channel gating (Jordan, 1987; Dani, 1986). For example, Imoto and colleagues (Imoto et al., 1988) showed that a "ring" of negatively charged residues lining the entryway into the nicotinic acetylcholine receptor channel enhances the conduction of Na^+ through this cation-selective pore and raises the affinity of a cationic pore-blocking inhibitor, Mg^{2+}.

In this study, we examine the role of surface charges on the functions of an ion channel protein, the high-conductance Ca^{2+}-activated K^+ channel from mammalian skeletal muscle plasma membrane. We know neither the structure nor the amino acid sequence of this channel, but by studying the behavior of single channels in a simple model membrane system, we provide strong evidence that the channel carries ionized carboxyl groups near its pore entrance and that these groups influence ion permeation, voltage-dependent gating, and ionic blockade in a manner consistent with a simple electrostatic mechanism.

Materials and Methods

Chemicals and biochemical preparations utilized were as in the preceding paper (MacKinnon & Miller, 1989), as were all planar bilayer and data collection techniques. Many of the experiments presented in this paper involve varying the ionic concentration and composition of the bilayer chamber solutions. The liquid junction potentials which occur at the salt bridge solution interface affect the data only slightly. Nevertheless, we used the Henderson equation to correct the membrane voltage (MacInnes, 1961).

Results

All experiments reported here are aimed at the following question: How do surface charges on the Ca^{2+}-activated K^+ channel influence its function? The preceding paper (MacKinnon & Miller, 1989) suggested that this channel carries anionic carboxylate groups close to the pore's entryway, or "mouth", and that these groups set up a negative potential which acts to enhance the permeation of K ions and to promote the binding of charybdotoxin, a highly basic peptide inhibitor. To examine this question further, we study the properties of single Ca^{2+}-activated K^+ channels reconstituted into *electrically neutral* planar phospholipid membranes. There are three types of channel functions under study here: ion permeation through the open pore, blocking of the conducting pore by nonconducting ions, and channel "gating", defined as the conformational changes involved in the opening and closing of the channel. For each of these channel functions, we assess the role of surface electrostatics by two experimental maneuvers: (1) chemical modification of the reconstituted channel by the carboxyl esterifying agent trimethyloxonium (TMO) and (2) variation of bulk solution ionic strength on the particular function under study. Taken together, these two lines of attack can be used to demonstrate that long-range electrostatic forces are involved in all three of these channel behaviors.

Negative Charges Raise the Single-Channel Conductance. This study begins with the observation shown in Figure 1: that

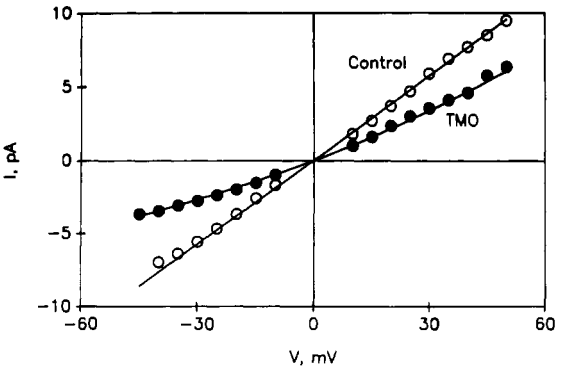

FIGURE 1: External TMO modification reduces the single-channel current. The single-channel current was measured as a function of membrane voltage before (open circles) and after modification of the outside using TMO (closed circles). The solution on both sides of the membrane contained 14 mM KCl, 10 mM MOPS, and 6 mM KOH, pH 7.4. Internal [Ca^{2+}] was 20 μM except for data points obtained at voltages more negative than 0 mV after channel modification. In this case, it was necessary to raise [Ca^{2+}] stepwise to 200 μM in order to achieve channel opening.

the single-channel conductance, measured from the slope of the open-channel current–voltage relation (I–V curve), is reduced by treatment of the channel with TMO. Here, a single channel was observed with 20 mM KCl on both sides of the membrane; treatment with TMO from the *external* solution reduced the channel current by about 40% across the entire voltage range. Both inward and outward currents were reduced by modification with the esterifying agent. This symmetrical lowering of current by an asymmetric carboxyl modification might seem counterintuitive, but as we will show below, this is the effect expected from a removal of negative surface charge near to the pore mouth. The result suggests qualitatively that negatively charged groups on the outer face of the channel may attract K^+ ions into the pore and thus enhance the channel conductance. It is this idea that we will test further.

It is known that the variation in this channel's conductance with K^+ concentration is consistent with a role for local negatively charged groups (Moczydlowski et al., 1985). In particular, as [KCl] is lowered symmetrically (along with ionic strength), the channel conductance does not approach zero, but rather a finite value, as though the local concentration of K^+ is being "buffered" by surface electrostatic effects (Bell & Miller, 1984). This behavior is shown in Figure 2, which also demonstrates that the effectiveness of TMO in lowering channel conductance is strongly dependent on ionic strength. At low ionic strength (5 mM), where electrostatic forces are large and far-reaching, TMO treatment results in a 50% lowering of conductance, while at high ionic strength (450 mM), the effect of TMO is barely discernible (<5% inhibition). The lack of effect of TMO at high ionic strength, where the channel conductance approaches a saturation value, additionally argues that TMO does not affect the "deep" regions of the conduction pathway which make intimate contact with K^+ ions, but rather operates only on more periferally located surface carboxylate groups.

External Negative Charges Increase the Outward Current by Polarizing the Pore. It is easy to visualize how externally positioned negative surface charge can promote inward K^+ current; a negative surface potential will raise the local K^+ concentration near the pore, and thus, the current through it. However, we have seen (Figure 1) that removal of ionized carboxylate groups on the channel's external surface reduces both inward and outward current. How can external negative surface charges influence *outward* K^+ currents? In this sec-

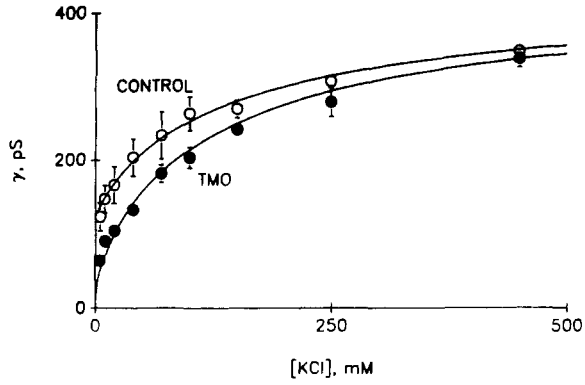

FIGURE 2: Current–voltage relationship, as measured in Figure 1, was determined at different concentrations of symmetric KCl. The slope at zero voltage, γ, is plotted as a function of the KCl concentration for control (open circles) and externally TMO-modified channels (closed circles). Each point represents an average of 2–10 measurements in separate channels. The control curve corresponds to a symmetric two-barrier, single-ion binding site permeation model that includes fixed surface charges at a density of 9×10^{-4} charges/A^2 on both sides of the channel. The TMO curve corresponds to the same model after reduction of the external charge density to 10^{-4} charges/A^2.

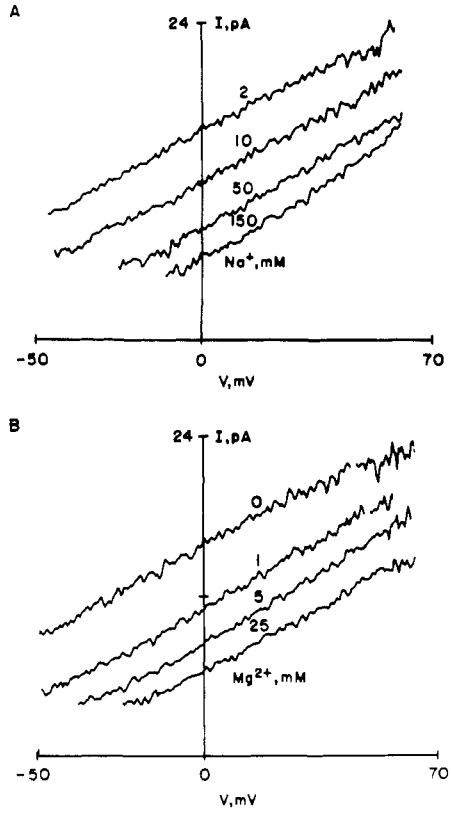

FIGURE 3: Outward K^+ current is reduced by screening external negative charges. The current–voltage relationship was determined by measuring the single-channel current during applied voltage ramps. The internal solution contained 150 mM KCl, and the external solution was initially 4 mM MOPS–NaOH, pH 7.4. NaCl (A) and $MgCl_2$ (B) were added to the outside at the indicated concentrations, and the current measurement was repeated.

tion, we focus upon the effects of external surface electrostatics on outward currents.

Figure 3 displays single-channel I–V curves under highly asymmetric ionic conditions. In these experiments, 150 mM K^+ was always present in the internal solution, and *no* permeant ion was ever present in the external solution. Thus, under these conditions, ionic current was always carried by the outward, unidirectional flow of K^+. We ask how outward

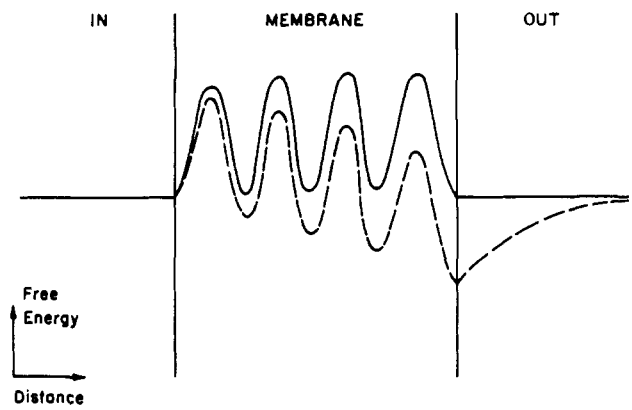

FIGURE 4: Asymmetric surface charges electrically polarize the ion conduction pathway. The energy peaks and valleys within the membrane correspond to a hypothetical chemical free energy profile encountered by an ion diffusing through an ion channel. External surface charge superimposes an electrical energy which "tilts" the energy profile as shown.

K^+ current changes as we manipulate the external surface potential by changing the external ionic strength, using impermeant ions. As illustrated in Figure 3A, as the strictly impermeant salt NaCl is added externally, outward K^+ current is reduced at all voltages. Likewise, external Mg^{2+} (also strictly impermeant) lowers outward K^+ currents in a similar fashion, but the divalent cation is effective at lower concentrations than the monovalent Na^+ ion. (Figure 3B) The effect of these ions is to shift the I–V curves horizontally to the right along the voltage axis.

The reduction of outward K^+ currents by impermeant ions is easily explainable in terms of electrostatic screening, as is depicted in Figure 4. If the external face of the channel carries negative surface charge, a local negative surface potential will produce a voltage gradient through the conduction pore, thus "tilting" the free energy profile experienced by K^+ ions diffusing through the channel. When the external electrolyte concentration is *low*, the magnitude of this negative potential will be large, and the outward flux of K^+ will be kinetically favored. As external impermeant electrolyte is added, the local negative charges are screened, and the outward K^+ flux is accordingly diminished. Since the transmembrane voltage applied by the external electronics is simply additive with the local potential due to surface charges, as long as the rate-determining step for ion permeation occurs inside the pore, we expect that a change in local potential should simply shift the I–V curve along the voltage axis, as is experimentally observed (Figure 3).

This explanation based on surface electrostatics is not unique. It might be argued that the inhibitory effects of external Na^+ and Mg^{2+} on outward K^+ currents are simply due to the physical blocking of the channel by these impermeant cations: that these ions may occupy the pore and prevent K^+ conduction. This alternative explanation is ruled out by the experiments shown in Figure 5. Here, outward K^+ currents at a fixed voltage are measured, as in the I–V curves above, at varying concentrations of externally added impermeant cations. Inhibition of K^+ currents by Mg^{2+} (Figure 5A) is seen to be biphasic, with a partial inhibition exerted in the concentration range 0–10 mM, and then a much more sluggish reduction in current as concentration is raised up toward 200 mM. This type of inhibition curve is inconsistent with a simple blocking mechanism but is quantitatively predicted by surface electrostatics. As Mg^{2+} concentration is raised in the low concentration range, initially large local potentials are effectively screened, and the K^+ current is

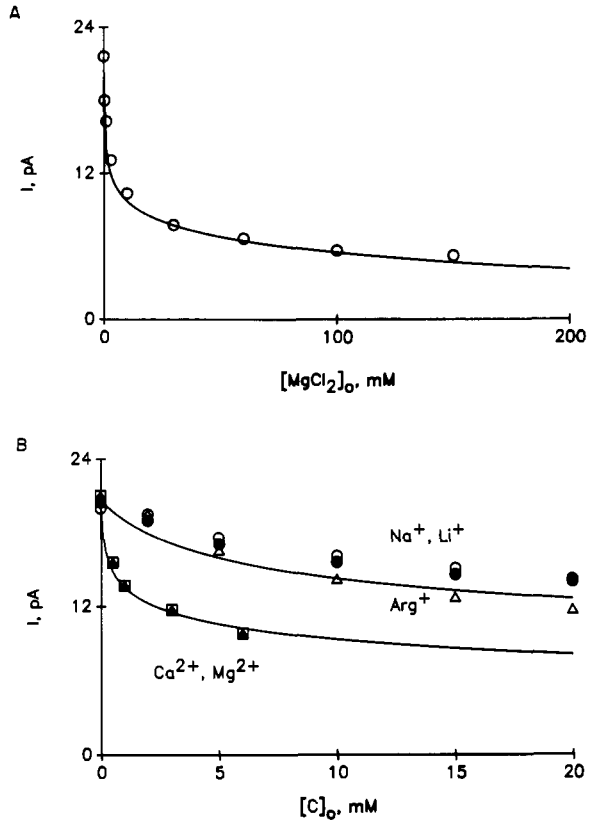

FIGURE 5: Reduction of outward current by external impermeant ions is inconsistent with a simple blocking mechanism. The experimental conditions were exactly as described in Figure 3, but here the current corresponding to a constant membrane voltage of 30 mV (A) or 20 mV (B) is plotted as a function of the external impermeant cation concentration. The curves correspond to eq 1 and 2. In (A), $I([X] = 0) = 22$ pA, $\gamma = 190$ pS, $K_D = 300$ mM, and $\sigma = 7 \times 10^{-4}$ charges/A^2. In (B), $I([X] = 0) = 21$ pA, $\gamma = 180$ pS, $\sigma = 7 \times 10^{-4}$ charges/A^2, $K_D = 2500$ mM (monovalents), and $K_D = 250$ mM (divalents).

strongly reduced. Above 20 mM Mg^{2+}, most of the local screening has already occurred, and the K^+ current levels off. The expected outward current, $I([X])$, as a function of externally added cation concentration, [X], may be easily shown to follow the relation:

$$I([X]) = \{I([X = 0]) + \gamma[\psi([X = 0]) - \psi([X])]\} \times \left\{1 + \frac{[X]}{K_D} \exp\left[\frac{-zF\psi([X])}{RT}\right]\right\}^{-1} \quad (1)$$

Here, we admit the possibility that in addition to the major electrostatic effect, the impermeant cation may also weakly block the channel, with dissociation constant K_D. The local electrostatic potential at the outside of the channel's conduction pathway is $\psi([X])$, and γ is the channel conductance. The local potential is related to the bulk ionic composition in a geometry-dependent and generally unknown way, but as a first approximation, we use the Gouy–Chapman model with an "effective" surface charge, σ, seen at the pore entrance:

$$\sigma = 2\epsilon_r\epsilon_o RT\sum c_i\left[\exp\left(\frac{-z_iF\psi}{RT}\right) - 1\right]^{1/2} \quad (2)$$

where c_i is the concentration of ions of valence z_i in the bulk aqueous phase, ϵ_r is the dielectric constant of water, and ϵ_o is the permittivity of free space (Grahame, 1947).

Using eq 1 and 2, we can then predict the dependence of K^+ current on external electrolyte composition. This is shown

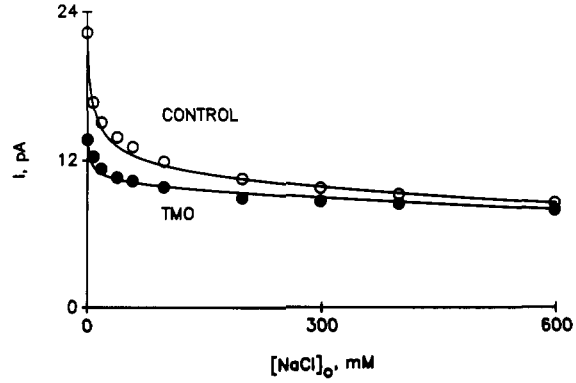

FIGURE 6: TMO modification weakens the dependence of outward current on external screening ions. The effect of external addition of NaCl on outward K^+ currents was measured as in Figure 5, for a control and a TMO-modified channel. Curves are drawn according to eq 1 and 2 with $\gamma = 180$ pS, $K_D = 2500$ mM; for control, $I([X] = 0) = 22.3$ pA, $\sigma = 6 \times 10^{-4}$ charges/A^2; for modified, $I[(X] = 0) = 13.7$ pA, $\sigma = 1.5 \times 10^{-4}$ charges/A^2.

for Mg^{2+} in Figure 5A (solid curve), with an effective surface charge density of 7×10^{-4} charges/A^2 and a blocking constant of 300 mM. This blocking constant is so weak that virtually all the reduction in K^+ current up to 20 mM Mg^{2+} is due to local electrostatic effects.

A very strong test of local electrostatics is that charge screening should be dependent on the *valence* of the screening ion but not on the particular species. Thus, all impermeant monovalent cations should, at lower concentrations, produce identical reduction in K^+ current. Furthermore, the relative effectiveness of monovalent and divalent species should be predicted a priori. Figure 5B shows that both of these predictions of the electrostatic mechanism are verified here. Reduction of K^+ currents by the divalents Ca^{2+} and Mg^{2+} is quantitatively identical with each other, as are the effects of the impermeant monovalents Li^+, Na^+, and arginine. Moreover, the effective surface charge density derived to fit the divalent inhibition curve adequately *predicts* the monovalent inhibition curve. We consider that these results strongly indicate that a pure electrostatic mechanism provides an adequate description of the effects of external impermeants on outward K^+ currents and that direct blocking of the pore by these impermeants is not significant, at least below 50 mM concentration for divalents and 300 mM for monovalents.

A further prediction of the ideas under consideration here (eq 1 and 2) is that the effect of external impermeant ions on outward K^+ currents should depend on the magnitude of the external surface charge density. We therefore compared the effect of external [NaCl] in a channel before and after TMO modification. In Figure 6, we plot the outward K^+ current at a fixed voltage against external NaCl concentration, in a control and a TMO-modified channel. K^+ currents are far less affected by NaCl after TMO treatment; quantitatively, the channel is acting as though the effective surface charge has been reduced 4-fold by TMO treatment.

Ionic Blockers Sense the Surface Potential. Just as conducting ions are influenced by fixed protein surface charges, ionic pore blockers should also sense the presence of these charges. We therefore use ionic blockers as probes of the electrostatic potential near the outer mouth of the channel. Tetraethylammonium (TEA) blocks the high-conductance Ca^{2+}-activated K^+ channel by entering the conduction pathway from the outside and preventing ionic conduction (Vergara et al., 1984; Villarroel et al., 1989). Because the kinetics of TEA block are fast compared to the response time of our measuring system, TEA block is manifested as a reduction in the open-

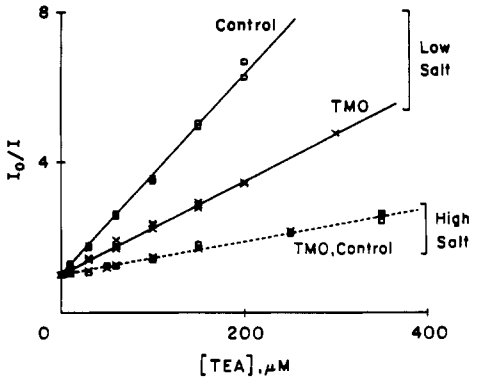

FIGURE 7: Modification diminishes the ionic strength dependence of external TEA block. The single-channel current in control (squares) and external TMO-modified channels (crosses) was measured as a function of the external TEA concentration. The internal solution was 150 mM KCl and the external 4 mM MOPS/2.5 mM KOH, pH 7.4, with (high salt) or without (low salt) 148 mM NaCl. Membrane voltage was 30 mV. I_0/I is the reciprocal of the fraction of unblocked current.

channel current (Yellen, 1984). As the concentration of TEA is raised, the current falls in a simple manner:

$$I_0/I = 1 + [\text{TEA}]/K_D \quad (3)$$

where I is the single-channel current in the presence of TEA, I_0 is the current in the absence of TEA, and K_D is the apparent dissociation constant of TEA for its blocking site. Because TEA is a monovalent cation:

$$K_D = K_D(0) \exp\left(\frac{F\psi}{RT}\right) \quad (4)$$

where $K_D(0)$ is the zero-potential dissociation constant and ψ is the electrostatic potential at the TEA blocking site. If there are ionized carboxyl groups on the outside of the channel, close to where TEA binds, then K_D should be increased by external TMO modification. Figure 7 shows that this is exactly what is observed. At low ionic strength, TMO modification reduces the affinity of TEA for its blocking site. This effect supports a pure electrostatic mechanism since at high ionic strength, when fixed surface charges are screened, TEA blocks control and modified channels with equal affinity. This result argues strongly that TMO modification has not simply altered the structural integrity of the TEA blocking site.

Charybdotoxin (CTX) is another inhibitor of the high-conductance Ca^{2+}-activated K^+ channel that is well-suited for probing the electrostatic potential near the outer mouth of the channel. It is a 37 amino acid peptide which is known to inhibit K^+ conduction by physically occluding the pore's external entryway (MacKinnon & Miller, 1988; Miller, 1988). Since CTX has a valence of approximately +5 at neutral pH (Gimenez-Gallego et al., 1988), its interaction with the channel should be particularly sensitive to local electrostatic fields near its binding site. Indeed, in the preceding paper (MacKinnon and Miller, 1989) we showed that TMO modification of the channel's external face reduces the CTX affinity, as would be expected if surface carboxyl groups provide such local fields. We now subject this idea to further quantitative tests. According to a simple electrostatic mechanism, the association rate, α, of CTX should be influenced by the local electrostatic potential at its binding site, ψ:

$$\alpha = \alpha(0) \exp\left(\frac{ZF\psi}{RT}\right) \quad (5)$$

where $\alpha(0)$ is the association rate that would be observed in the absence of a local potential and z is the effective valence of CTX. If the surface potential varies with ionic composition as in eq 2, then it is easy to show that the CTX on-rate should obey the relation:

$$\alpha = \alpha(0)\left\{\frac{136\sigma}{\sqrt{c}} + \left[\left(\frac{136\sigma}{\sqrt{c}}\right)^2 + 1\right]^{1/2}\right\}^{+2Z} \quad (6)$$

where σ (charges per angstrom squared) is the surface charge density near the CTX binding site and c is the molar concentration of electrolyte in the solution (1:1 monovalent).

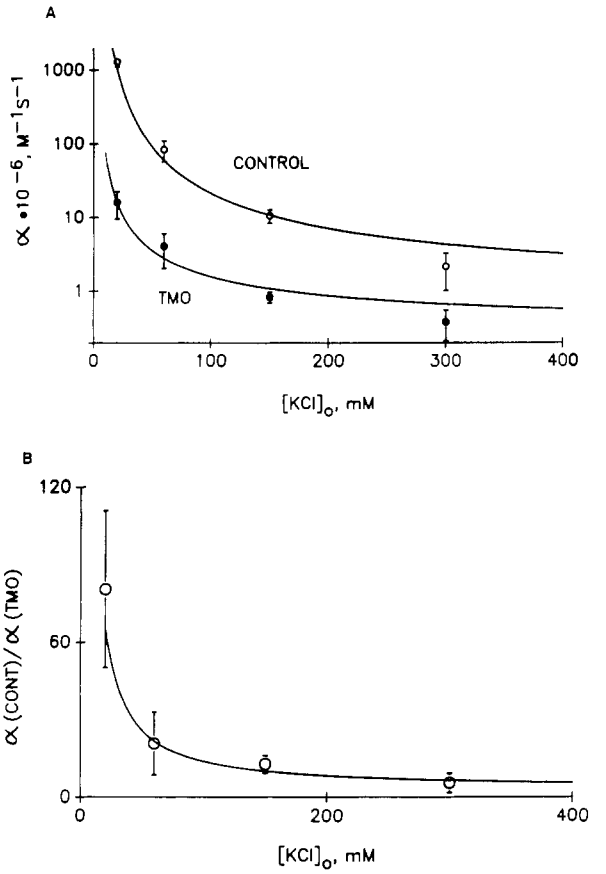

FIGURE 8: Effect of modification on the ionic strength dependence of the CTX association rate. (A) The rate constant, α, for CTX association with control (open circles) and modified channels (closed circles) was measured as a function of the external KCl concentration at 40 mV. Each point is the average of two or three determinations in separate channels. The internal solution was 150 mM KCl, and internal [$CaCl_2$] was adjusted to maintain the unblocked channel open probability above 0.8. (B) The ratios of α, determined in (A), are shown as a function of external [KCl]. The curves correspond to eq 6 with $z = 4$, $\sigma = 1.2 \times 10^{-3}$ charges/A^2 (control) and 6×10^{-4} (modified), $\alpha(0) = 4 \times 10^5$ M^{-1} s^{-1} (control) and 2×10^5 (modified).

For several reasons, eq 6 oversimplifies the situation (see Discussion), but even so, it makes strong qualitative predictions that we can test. First, in agreement with the known behavior of CTX (Anderson et al., 1988), eq 6 predicts that as the electrolyte concentration increases, the on-rate decreases. Equation 6 makes another prediction: as the effective surface charge density σ decreases, both α and the ionic strength dependence of α should decrease. This prediction offers a rigorous test of our proposal that TMO modification reduces the density of negative charges near the channel mouth. Figure 8A shows the relationship between the CTX on-rate and the external KCl concentration for control and TMO-modified channels. The prediction of eq 6 is clearly and strikingly confirmed. TMO modification not only reduces α but also greatly reduces its ionic strength dependence. This latter effect

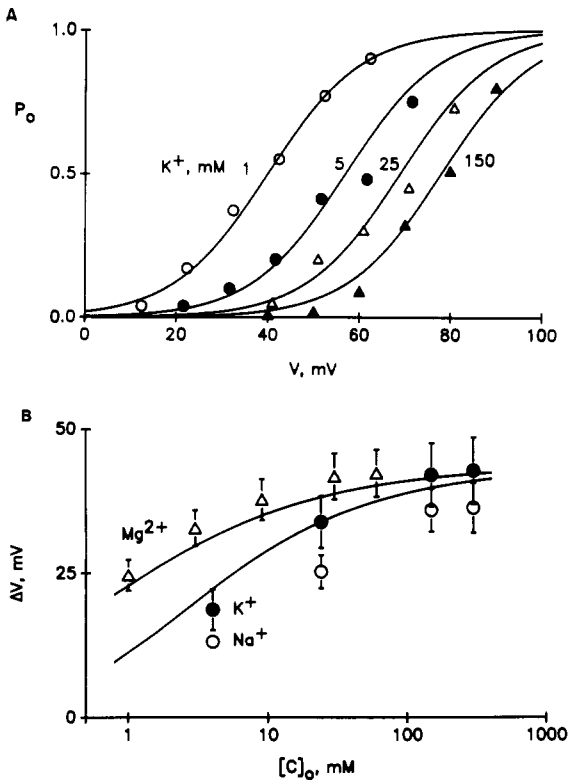

FIGURE 9: Effect of ionic screening on channel gating. (A) Single-channel open probability, P_o, was measured as a function of applied voltage, initially in the presence of external 2 mM MOPS/1.2 mM KOH, pH 7.4, and then after adding the indicated concentrations of KCl to the outside. The curves correspond to the equation $P_o = \{1 + \exp[zF(V - V_0)/RT]\}^{-1}$, where the "gating charge" $z = 2.4$, $V_0(1) = 40$, $V_0(5) = 57$, $V_0(25) = 69$, $V_0(150) = 78$ mV. (B) The voltage at $P_o = 0.5$, V_0, was determined as in (A) using external KCl (closed circles), NaCl (open circles), or $MgCl_2$ (triangles). The salt-induced shift in V_0, ΔV, is plotted as a function of external cation concentration. Each data point represents the average of two to six independent determinations. The curves are solutions to eq 2 with $\sigma = 2.3 \times 10^{-4}$ charges/A^2.

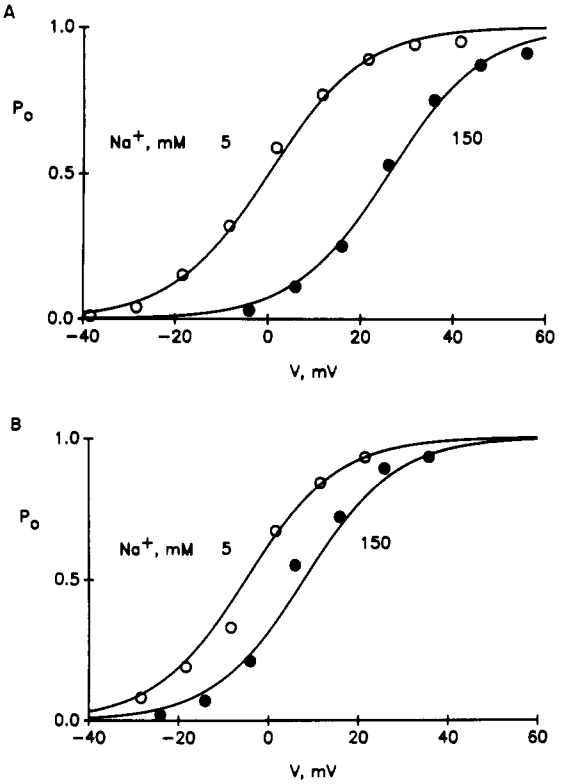

FIGURE 10: Channel gating is less sensitive to external screening ions after modification. The single-channel open probability was measured as a function of membrane voltage in a control (A) and TMO-modified (B) channel when the external solution was 10 mM MOPS/6 mM KOH, pH 7.4 (open circles), and then after the addition of 145 mM NaCl (closed circles). This experiment was repeated with four channels; the average open probability curve displacement was 25 ± 2 mV (control) and 8 ± 2 mV (modified). Internal [KCl] was 150 mM, and [$CaCl_2$] was held constant.

is further emphasized in Figure 8B, where the ratios of the on-rates for normal and TMO-modified channels are displayed.

Electrostatic Effects on Channel Gating. The high-conductance Ca^{2+}-activated K^+ channel is a voltage-gated ion channel: membrane depolarization favors the open, conducting conformation of the protein. In this respect, the channel is a molecular voltmeter sensing the potential difference between the inside and the outside of the channel. Earlier we suggested that surface charges can influence ion permeation by electrically polarizing the channel (Figure 4); by a similar mechanism, we might expect surface charges to affect ion channel gating. We tested this idea by measuring the voltage dependence of the open probability while systematically varying the electrolyte concentration on only the outside of the channel, holding the internal solution constant. Typical results are illustrated in Figure 9. As external KCl concentration (and hence ionic strength) rises, the voltage activation curve shifts to more positive voltages (Figure 9A). This is precisely our expectation if there are negative charges on the outside of the channel which are screened by the electrolyte. This electrolyte-induced voltage shift is thus a *direct measure* of the change in the external surface potential as detected by the channel's "voltage sensor". In figure 9B, we plot the electrolyte-induced voltage shift for NaCl, KCl, and $MgCl_2$. The overall trend is entirely consistent with a screening mechanism; Na^+, K^+, and Mg^{2+} all cause voltage shifts, with the divalent Mg^{2+} ion being more effective than either monovalent cation. Furthermore, the maximal voltage shifts at high salt concentration, which represent complete screening of the surface charge, are similar for monovalent and divalent cations. The data agree well with solutions to eq 2 for both monovalent and divalent screening (solid curves).

In the preceding paper (MacKinnon & Miller, 1989), we showed that external TMO modification, at low external ionic strength, induces a shift in the voltage activation curve to more depolarized membrane voltages. We proposed that this is due to a reduction in the density of negative surface charge on the outside as a result of TMO modification. In agreement with this proposal, Figure 10 shows that the maximum shift induced by raising the external electrolyte concentration is smaller after the TMO modification. On the basis of four such experiments in control and TMO-modified channels, raising the external NaCl concentration from 5 to 150 mM induced a shift of 25 ± 2 (control) and 8 ± 2 mV (TMO modified).

Internal Surface Charges. We have described a variety of experiments which demonstrate the presence of a net negative charge density on the outside of the high-conductance Ca^{2+}-activated K^+ channel. Is the inside of the channel also negatively charged? This is a more difficult question to approach experimentally for the following reason: the channel is Ca^{2+} activated from the inside, and, therefore, changes in the internal electrolyte composition as well as internal TMO modification affect the channel's gating in a complicated manner, but we are still able to measure the open-channel conductance. The current–voltage curves in Figure 11 show that internal TMO modification reduces the single-channel conductance. Furthermore, these data were measured in the presence of 20 mM symmetric KCl; when the KCl concen-

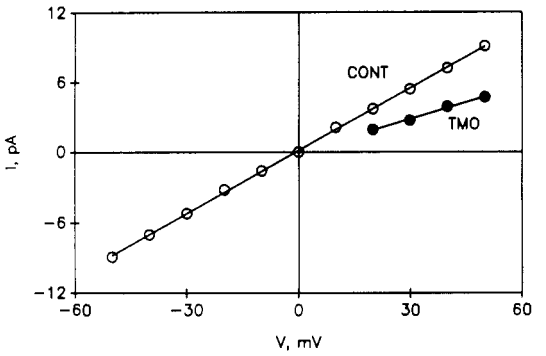

FIGURE 11: Internal TMO modification reduces the single-channel conductance. The single-channel current was measured as a function of membrane voltage before (open circles) and after (closed circles) the inside was modified by TMO. The internal and external solutions contained 20 mM KCl. Internal [$CaCl_2$] was 100 μM.

tration is raised, the relative reduction in conductance is less (data not shown). These results are the same as we saw for external TMO modification and suggest that there are fixed negative charges close to where the conduction pathway opens to the inside. We also know that the presumed carboxyl groups that are modified by internal TMO are different than those modified by external TMO because internal modification reduces the conductance but does not affect CTX block (data not shown).

Discussion

These experiments demonstrate that the high-conductance Ca^{2+}-activated K^+ channel carries negatively charged groups on its external side and that the local electrostatic field produced by these charges strongly affects the functions of this membrane protein. Since this is a simple reconstituted system consisting of single-channel molecules inserted into electrically neutral lipid membranes, it is clear that all the local electrostatic effects observed originate from charged groups on the channel protein itself, and not on membrane lipids. We have distinguished three types of channel functions which respond to changes in the local electrostatic field: ionic conduction through the open pore, ionic block of the pore, and voltage-dependent channel gating. Although the same overall electrostatic picture applies to each of these processes, it operates in slightly different ways in each case.

Ionic Conduction through the Open Pore. We have shown here that surface charges located near the mouth of a channel can act in *two ways* to enhance ionic permeation. First, as has been well understood in numerous channels (Apell et al., 1979; Bell & Miller, 1984; Moczydlowski et al., 1985), local negative surface charge raises the *local concentration* of current-carrying cations near the channel mouth. As has been discussed in detail (Bell & Miller, 1984; Miller & Garber, 1988), this effect can account for the fact that as K^+ (and ionic strength) is lowered symmetrically, the zero-voltage channel conductance does not approach zero, but rather a finite value, as occurs with channels in membranes containing known surface charge densities (Apell et al., 1979; Bell & Miller, 1984; Moczydlowski et al., 1985). The nonzero conductance intercept seen for this channel (Figure 2) is a signature strongly suggestive of local surface charge.

However, there is a second way, not previously documented, that surface charges can influence channel-mediated ion conduction: the "tilt" effect cartooned in Figure 4. The qualitative idea is that a local surface potential on only one side of the pore will lead to a voltage gradient *within* the pore, even when there is no voltage difference between the two solutions. We demonstrated this effect by setting up unnatural conditions in which conducting K^+ ions were present only on the inside of the channel, while local surface potentials were manipulated only on the outside (Figures 3, 5, and 6). We found that at a fixed *applied* voltage, outward current through the channel was reduced as the local external negative potential was decreased (by either screening or removing surface charge, via ionic strength variation and TMO modification, respectively). The effect of reducing the local electrostatic potential was to shift the open-channel I–V curve horizontally along the voltage axis, toward more positive voltages.

Superficially, this is the effect expected if we consider the voltage profile within the pore induced by asymmetric surface potentials to be equivalent to the profile produced by an externally applied potential, but in fact, there is no a priori reason to expect equivalent potential profiles for these two situations; in the case of asymmetric surface potentials, charge separation occurs within the double layers of each side of the membrane, while with an applied voltage, charges are separated across the membrane itself. Our results show empirically that the voltage profile along the channel's conduction pathway is similar for the two cases. Furthermore, recent calculations (P. Jordan, unpublished results) show that the electrical potential set up through a membrane-embedded aqueous pore should be very similar.

Open-Channel Block. If surface charges around the pore entryway locally concentrate conducting cations, then they should also increase locally the concentrations of cations which enter the pore and block it. We have confirmed this effect for two cationic pore blockers that act from the external solution: monovalent TEA^+ and pentavalent CTX. For both cases, we found that the affinity of block is increased by lowering the ionic strength, as expected for an electrostatic mechanism. Furthermore, we found that removal of surface charge by TMO treatment lowered the ionic strength dependence of the blocking affinity. This result makes a particularly compelling case for a nonspecific electrostatic influence on the affinities of these blocking cations.

Voltage-Dependent Channel Gating. The influence of surface electrostatics on the gating of voltage-dependent channels has been understood for many years (Frankenhaeuser & Hodgkin, 1957). As depicted in Figure 4 for ionic current through the open pore, asymmetric surface potentials will produce a transmembrane electric field in the absence of an applied voltage. This intramembrane field will be felt by the "voltage sensor" of a voltage-dependent channel. In these experiments, we have used both ionic strength variation and TMO modification to alter the local surface potential in the external solution and to observe the resulting shift in the voltage activation curve. We have observed the anticipated effects (Figures 9 and 10): increasing external ionic strength or reaction with external TMO shifts the voltage activation curve toward more positive voltages, as though these maneuvers alter the intramembrane electric field in a "hyperpolarizing" direction. These results further corroborate the existence of functionally important negative charges located on the external face of the channel.

Can We Estimate the Surface Charge Density? We have attempted to model our data using the charged interface theory of Gouy and Chapman. This theory has been used quite successfully to describe the electric potential near a charged lipid membrane, where the planar geometry conforms to the Guoy–Chapman solution of the Poisson–Boltzmann equation (McLaughlin, 1977). For the system under consideration here, a protein surface with discrete charges, the theory is concep-

tually inadequate. Furthermore, the surface charges on the channel are probably carboxyl groups, yet we have ignored the possibility of protonation or direct ion binding; in reality, the surface charge density may not remain constant as electrolyte concentration is varied. Another problem applying to CTX block is that this peptide is not a point charge, as eq 6 assumes. Therefore, any estimates of the absolute surface charge density are highly uncertain. Qualitatively, however, we arrive at self-consistent estimates of charge density from our various approaches. The conduction data point to an effective surface charge density near the ion entryway of 6×10^{-4} to 9×10^{-4} charges/A^2, in good agreement with 1.2×10^{-3} charges/A^2 as determined from the CTX block data. The charge density estimated from the shift in voltage-dependent gating, 2.3×10^{-4} charges/A^2, is much less than that estimated from ion conduction and block, but this discrepancy is reasonable; the conformational voltage-sensor region of the channel is not necessarily located near the ion conduction pore, and there is thus no reason to expect that the two processes will experience the same local fields.

The overall conclusion remains valid that the effects observed are due to local surface electrostatics near the external mouth of the channel. How relevant are these electrostatic effects in terms of the biological activity of the ion channel? Voltage-dependent gating is essentially unaffected by the presence of external protein surface charges since the charges are fully screened at physiological ion concentrations (Figure 9). However, our results demonstrate that the channel conductance under physiological conditions is enhanced (by approximately a factor of 2) by negative surface charges on both sides of the pore.

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