Tuning the Voltage Dependence of Tetraethylammonium Block with Permeant Ions in an Inward-Rectifier K^+ Channel

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abstract To understand the role of permeating ions in determining blocking ion–induced rectification, we examined block of the ROMK1 inward-rectifier K^+ channel by intracellular tetraethylammonium in the presence of various alkali metal ions in both the extra- and intracellular solutions. We found that the channel exhibits different degrees of rectification when different alkali metal ions (all at 100 mM) are present in the extra- and intracellular solution. A quantitative analysis shows that an external ion site in the ROMK1 pore binds various alkali metal ions (Na^+, K^+, Rb^+, and Cs^+) with different affinities, which can in turn be altered by the binding of different permeating ions at an internal site through a nonelectrostatic mechanism. Consequently, the external site is saturated to a different level under the various ionic conditions. Since rectification is determined by the movement of all energetically coupled ions in the transmembrane electrical field along the pore, different degrees of rectification are observed in various combinations of extra- and intracellular permeant ions. Furthermore, the external and internal ion-binding sites in the ROMK1 pore appear to have different ion selectivity: the external site selects strongly against the smaller Na^+, but only modestly among the three larger ions, whereas the internal site interacts quite differently with the larger K^+ and Rb^+ ions.

key words: inward-rectifier K^+ channel • rectification • ionic blocker • ion selectivity • tetraethylammonium

introduction

Inward-rectifier K^+ channels act as K^+-selective diodes in the cell membrane (Katz, 1949; Hodgkin and Horowicz, 1959; Horowicz at al., 1968; Noble and Tsien, 1968; Adrian, 1969; Hagiwara and Takahashi, 1974; Hille and Schwarz, 1978; Hille, 1992). In a normal intracellular environment, when the extracellular K^+ concentration is raised to the same level as the intracellular concentration, inward K^+ current is much larger than the outward current. This property is commonly called inward rectification. Although the detailed mechanisms underlying rectification are still controversial (e.g., Aleksandrov et al., 1996; Lee et al., 1999), rectification in most inward-rectifier K^+ channels is induced by intracellular cations.

It was first discovered that some inward-rectifier K^+ channels are blocked by intracellular Mg^{2+} and that the extent of block depends on membrane voltage (Matsuda et al., 1987; Vandenberg, 1987; Horie et al., 1987). More recently, inward-rectifier K^+ channels were also found to be blocked by intracellular polyamines in a voltage-dependent manner (Lopatin et al., 1994; Ficker et al., 1994; Fakler et al., 1995; Taglialatela et al., 1995; Yang et al., 1995). Since both Mg^{2+} and polyamines are cationic, the simplest explanation for the voltage dependence of channel blockade would be the Woodhull hypothesis (Woodhull, 1973), which proposes that the blocking ion site is located within the transmembrane electrical field. Therefore, to block the ion conduction pore, a blocking ion must travel across a portion of the electrical field, which results in the voltage dependence. Consequently, the degree of voltage dependence of the block, or rectification, is determined by the blocker's valence and the fraction of the electrical field that it needs to traverse to reach the blocking site.

However, as early as a half century ago, it was observed that inward rectification is also sensitive to the concentration of extracellular K^+ (Katz, 1949). An increase in the concentration of extracellular K^+ anomalously increases the outward current rather than decreases it as one would predict from a simple change in the K^+ equilibrium potential. Furthermore, altering the concentration of extracellular K^+ causes a "parallel" shift of the current–voltage (I–V)[1] curve along the voltage axis. Thus, inward rectification does not simply depend on membrane voltage. Instead, on an empirical basis it appears to depend on the difference between the membrane voltage (V_m) and the equilibrium potential for K^+ (E_K); i.e., the driving force for K^+ ($V_m - E_K$) (Katz, 1949; Hodgkin and Horowicz, 1959; Horowicz at al., 1968; Noble and Tsien, 1968; Adrian, 1969;

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[1]*Abbreviations used in this paper:* I–V, current–voltage; NMG^+, *N*-methyl-d-glucamine; TEA, tetraethylammonium.

Hagiwara and Takahashi, 1974; Hille and Schwarz, 1978; Hille, 1992). Subsequently, a seemingly related phenomenon was observed: the extent of channel blockade by intracellular tetraethylammonium (TEA) in a voltage-activated K^+ channel can be reduced by raising the concentration of extracellular K^+; this is commonly referred to as "trans knock-off of TEA by K^+" (Armstrong and Binstock, 1965). Since then, the binding of most cationic K^+ channel blockers has been found to be sensitive to K^+ concentration on the trans side of the membrane (Hille, 1992). The knock-off effect has been suspected to be somehow related to rectification (e.g., Hille and Schwarz, 1978; Hille, 1992; Lopatin and Nichols, 1996).

Recently, not only the extent but also the voltage dependence of blockade of the ROMK1 inward-rectifier K^+ channel by both TEA and Mg^{2+} were shown to depend on the concentration of extracellular K^+ (Oliver et al., 1998; Spassova and Lu, 1998). The discovery that the voltage dependence varies with the concentration of extracellular K^+ led to a further investigation of why the degree of rectification, or the voltage dependence of channel blockade, depends on both the membrane potential and the concentration of extracellular K^+ (Spassova and Lu, 1998). In that study, the binding of extracellular K^+ to the pore was found to be voltage dependent, compatible with the K^+-binding site being located halfway through the transmembrane electrical field. Since the binding of extracellular K^+ into the pore lowers the affinity of intracellular blocking ions such as TEA and Mg^{2+}, the K^+ ion bound at the outer part of the pore and the blocking ion at the inner part are energetically coupled. Therefore, the voltage dependence of channel blockade must be determined by the movement of all energetically coupled ions in the electrical field. A variation in the concentration of extracellular K^+ will alter the saturation level of the external K^+ site ($K_d \approx 10$ mM), and thus the degree of rectification. In other words, for a given intracellular ionic condition, the degree of rectification reflects the level of ion saturation at the external site.

Thus far, it is unclear how much of the voltage dependence is due to the movement of permeant ions versus the movement of blocking ions in the electrical field. However, it is clear that a significant fraction of the voltage dependence results from the movement of permeant ions (Spassova and Lu, 1998). Thus, the degree of rectification induced by a given intracellular blocking ion can be tuned by adjusting the concentration of extracellular permeant ion K^+. To gain more insight into the role of permeant ions in determining the degree of rectification, we examined block of the ROMK1 channel by intracellular TEA when the extra- as well as the intracellular solutions contain various alkali metal ions.

methods

Molecular Biology and Oocyte Preparation

ROMK1 cDNA was cloned into the p-SPORT1 plasmid (GIBCO BRL) (Ho et al., 1993). RNA was synthesized using T7 polymerase (Promega Corp.) from Not1-linearized ROMK1 cDNA. Oocytes harvested from *Xenopus laevis* (Xenopus One) were incubated in a solution containing (mM) 82.5 NaCl, 2.5 KCl, 1.0 $MgCl_2$, 5.0 HEPES, pH 7.6, and 2–4 mg/ml collagenase. The oocyte preparation was agitated using a platform shaker (80 rpm) for 60–90 min. It was then rinsed thoroughly and stored in a solution containing (mM): 96 NaCl, 2.5 KCl, 1.8 $CaCl_2$, 1.0 $MgCl_2$, 5 HEPES, pH 7.6, and 50 μg gentamicin. Defolliculated oocytes were selected and injected with RNA at least 2 and 16 h after collagenase treatment, respectively. All oocytes were stored in an incubator at 18°C.

Patch Recording

ROMK1 currents were recorded in the inside-out configuration from *Xenopus* oocytes (injected with ROMK1 cRNA) with an Axopatch 200B amplifier (Axon Instruments, Inc.). The recorded signal was filtered at 1 kHz and sampled at 5 kHz using an analogue-to-digital converter (DigiData 1200; Axon Instruments, Inc.) interfaced with a personal computer. pClamp6 software (Axon Instruments, Inc.) was used to control the amplifier and acquire the data. Macroscopic current–voltage curves were recorded as membrane voltage was linearly ramped (50 mV/s). Background leak current correction was carried out as previously described (Lu and MacKinnon, 1994a; Spassova and Lu, 1998).

Recording Solutions

Pipette solutions contained specified concentrations of alkali metal ions with (mM): 0.3 $CaCl_2$, 1.0 $MgCl_2$, and 10 HEPES, pH 7.6. Na^+ and *N*-methyl-d-glucamine (NMG^+) had nearly identical effects on all examined properties (see results). Thus, when K^+, Rb^+, or Cs^+ concentrations were reduced, Na^+ was used to maintain the total concentration of alkali metal ions at 100 mM. The bath solutions contained the specified concentrations of TEA with (mM): 90 KCl, 5 K_2EDTA, and 10 HEPES, pH 7.6. In some experiments, K^+ in the bath solution was replaced by Rb^+.

results

Current-Voltage Relation of the ROMK1 Channel under Either Symmetric K^+ or Biionic Conditions

In the presence of 100 mM K^+ on both sides of the membrane and the absence of intracellular blocking ions, the I–V curve of the ROMK1 channel was remarkably linear (Fig. 1 A). However, when extracellular K^+ was replaced by an equal concentration of Rb^+, the channel conducted larger outward than inward current with a reversal potential of -5.3 ± 0.3 mV (mean ± SEM, $n = 5$) (Fig. 1 B). As shown in Fig. 1, C–E, no inward currents can be seen when extracellular K^+ was replaced by either Cs^+, Na^+, or NMG^+. The reversal potentials were less than -60 mV in extracellular Cs^+ and less than -120 mV in extracellular Na^+ or NMG^+. From these, we estimated the permeability ratios (P_K/P_X) using the Goldman-Hodgkin-Katz equation as $P_K/P_{Rb} = 1.2$, $P_K/P_{Cs} >$

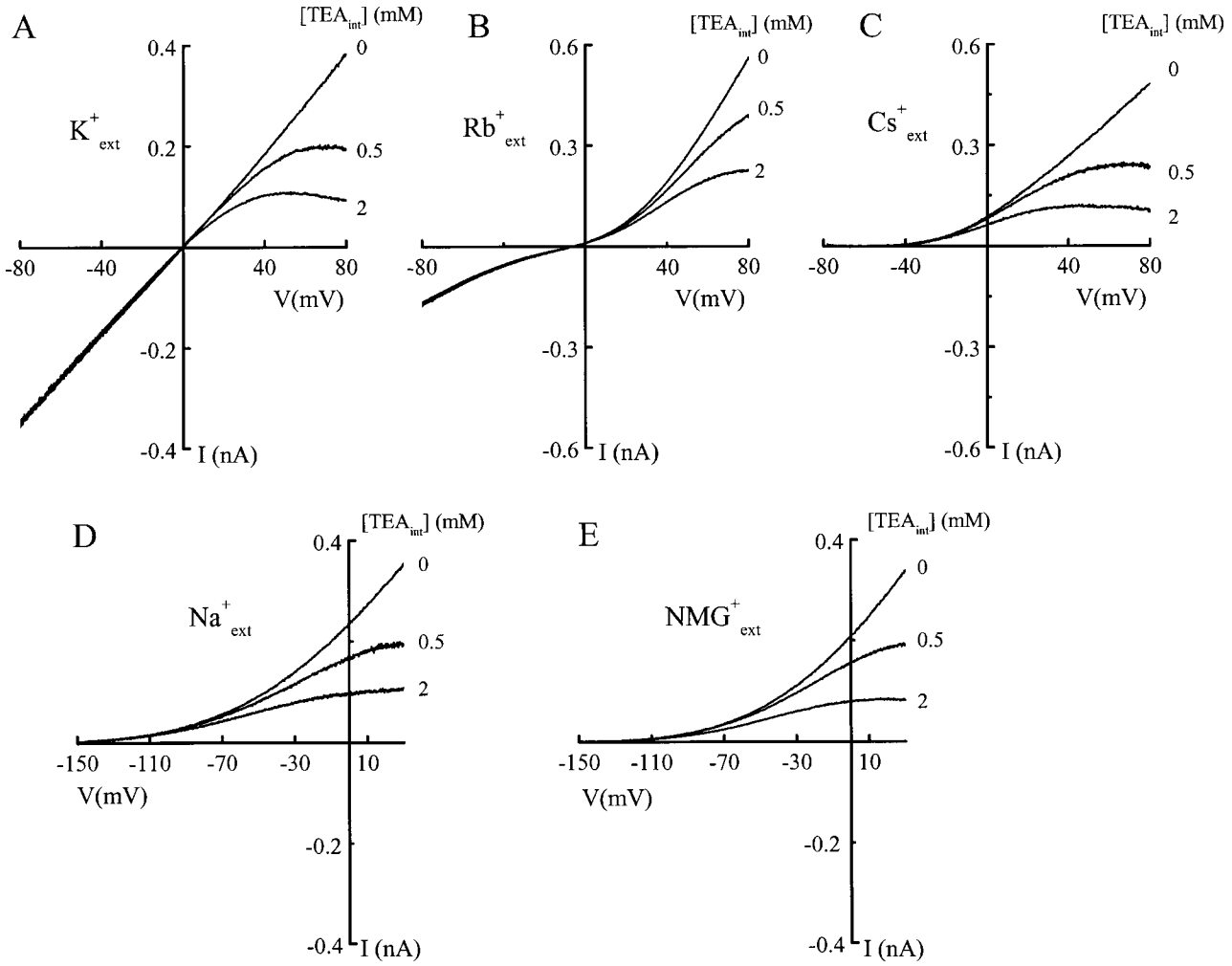

Figure 1. Voltage-dependent block of the ROMK1 channel by intracellular TEA in various extracellular alkali ions. Macroscopic I–V curves of the ROMK1 channel were recorded in the absence or presence of various concentrations of intracellular TEA. The pipette solutions contained 100 mM of either K^+, Rb^+, Cs^+, Na^+, or NMG^+. The bath solution contained 100 mM K^+ in all cases.

10, and P_K/P_{Na} and $P_K/P_{NMG} > 100$. Based on this empirical measure of ion selectivity, the channel has the same ion-selective sequence as other K^+ channels, $K^+ \approx Rb^+ > Cs^+ >> Na^+, NMG^+$.

The Degree of Rectification Induced by Intracellular TEA in the Presence of Various Extracellular Cations

Fig. 1 A shows the I–V curves recorded in symmetric 100 mM K^+ without or with intracellular TEA at the concentrations indicated. As shown previously, intracellular TEA causes the ROMK1 channel to conduct in an inwardly rectifying manner (Oliver et al., 1998; Spassova and Lu, 1998). This is because TEA blocks the channel in a voltage-dependent manner, manifested by the downward deflection of the I–V curves. Interestingly, when we replaced extracellular K^+ by other monovalent cations, we found that the I–V curves of the channel exhibit various degrees of curvature depending on the substituting ion species. As shown in Fig. 1 B, the curvature of the I–V curves appears to be, if anything, only slightly reduced when extracellular K^+ was replaced by Rb^+. However, the curvature of the I–V curves was significantly reduced when K^+ was replaced by Cs^+, and even further reduced when K^+ was replaced by Na^+ and NMG^+ (Fig. 1, C–E). Thus, although inward rectification is induced by the binding of intracellular cations to the pore, the degree of rectification is at least in part determined by extracellular ions.

The Affinity of TEA and the Voltage Dependence of Channel Blockade by TEA

In Fig. 2, the fractions of unblocked current in the presence of 100 mM extracellular K^+, Rb^+, Cs^+, or Na^+ were plotted against TEA concentration for several representative membrane voltages. Although the increment in membrane voltage between the adjacent curves is

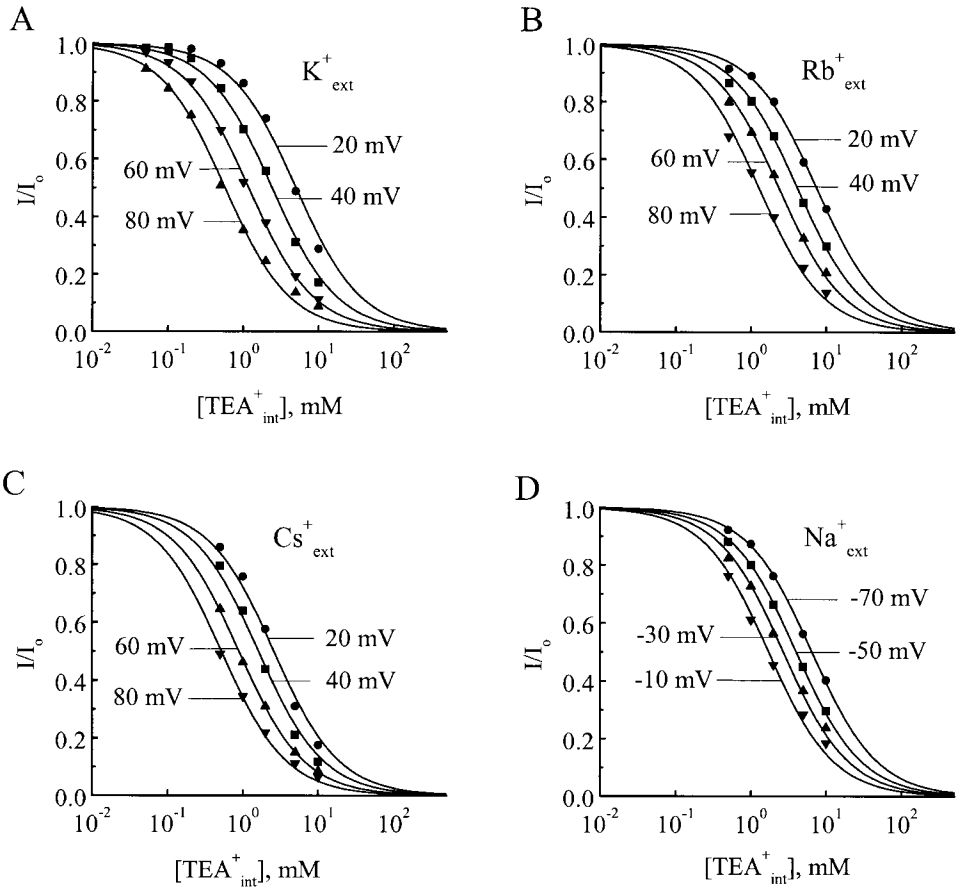

Figure 2. Tetraethylammonium inhibition curves. The fractions of unblocked currents (I/I_o) were plotted against the concentration of intracellular TEA ($[TEA_{int}]$) for several representative membrane voltages. Curves superimposed on the data correspond to least-squares fits using $I/I_0 = {}^{TEA}K_{obs}/({}^{TEA}K_{obs} + [TEA_{int}])$, where ${}^{TEA}K_{obs}$ is the observed TEA equilibrium dissociation constant. The alkali metal ions (100 mM) contained in the pipette solution were as indicated.

the same (20 mV) in all four plots, the distance between adjacent curves is not the same in the four cases. It decreases in the order of K^+, Rb^+, Cs^+, and Na^+, illustrating how voltage dependence of channel blockade by TEA varies with various extracellular alkali metal ions. The curves superimposed on the data are least-squares fits of an equation that assumes a 1:1 stoichiometry between the channel and TEA. From the fits, we determined the observed equilibrium dissociation constants for TEA (${}^{TEA}K_{obs}$) in the presence of each of the four alkali metal ions.

In Fig. 3, we plotted ${}^{TEA}K_{obs}$ determined in the presence of 100 mM of each of the four species of extracellular ions as a function of membrane voltage. The lines superimposed on the data represent least-squares fits of the Woodhull equation (Woodhull, 1973). From the Woodhull analysis we determined ${}^{TEA}K_{obs}(0\ mV)$ (the observed TEA equilibrium dissociation constant at 0 mV) and ${}^{TEA}(z\delta)_{obs}$ (an empirical measure of the voltage dependence).

The averages of ${}^{TEA}K_{obs}(0\ mV)$ and ${}^{TEA}(z\delta)_{obs}$ determined in the presence of 100 mM extracellular K^+, Rb^+, Cs^+, and Na^+ are presented in Fig 4. ${}^{TEA}K_{obs}(0\ mV)$ determined in K^+ was slightly smaller than that in Rb^+, but larger than those in Cs^+ and Na^+. Judged from ${}^{TEA}(z\delta)_{obs}$, the voltage dependence of channel blockade by TEA is similar in both K^+ and Rb^+, although it may be slightly smaller in Rb^+. However, the voltage dependence is significantly attenuated in Cs^+ and even more in Na^+. The value of ${}^{TEA}(z\delta)_{obs}$ in Na^+ is only about half what it is in K^+.

Dependence of TEA Affinity on the Concentration of Extracellular Alkali Metal Ions

We next examined how channel blockade by TEA varies with the concentration of each ion species. When the concentrations of K^+, Rb^+, or Cs^+ were reduced, we used Na^+ as a substituting ion instead of the more commonly used NMG^+, because in the present study extracellular Na^+ and NMG^+ behaved similarly and membrane patches tolerated Na^+ better. Both the I–V curves of the channel and its blockade by TEA were very similar in the presence of extracellular Na^+ or NMG^+ (Fig. 1). Furthermore, TEA binds to the channel with nearly identical affinities in the presence of extracellular Na^+ or NMG^+ (Fig. 5 D). Assuming NMG^+ does not bind at the external ion-binding site, we estimated the equilibrium dissociation constant of the site for Na^+ is in the molar range (see discussion).

Generally, the values of both ${}^{TEA}K_{obs}(0\ mV)$ and ${}^{TEA}(z\delta)_{obs}$ increase with the concentration of extracellu-

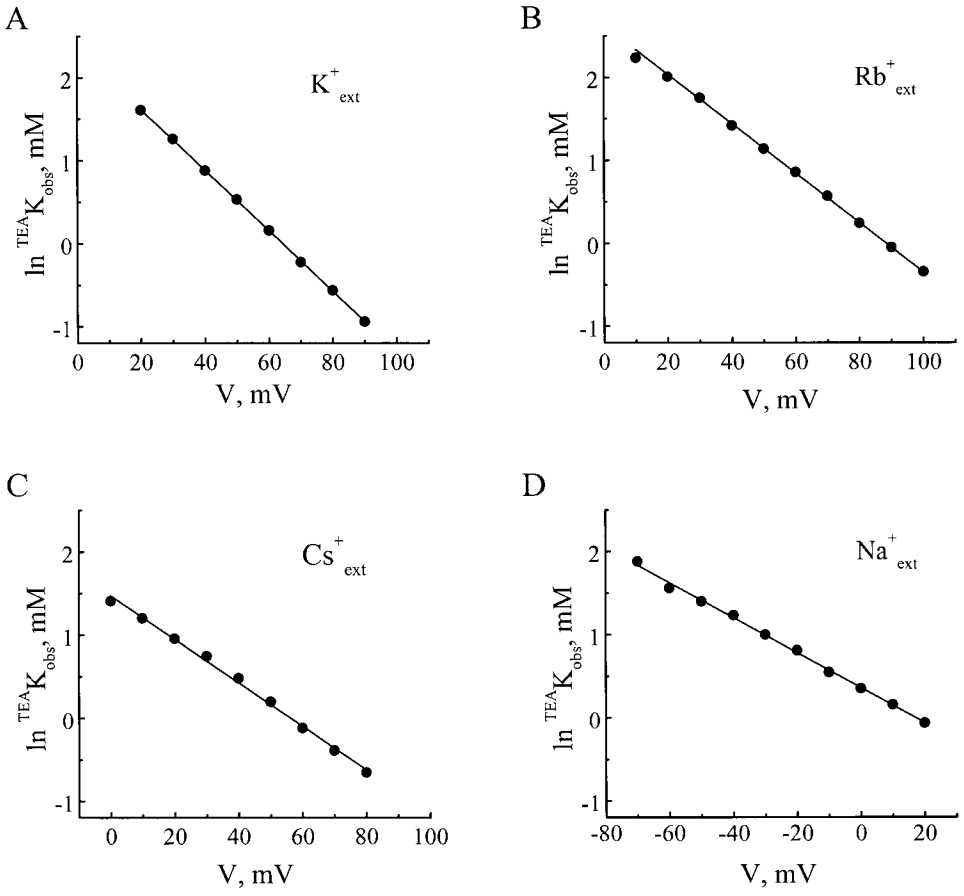

Figure 3. Dependence of ${}^{TEA}K_{obs}$ on membrane voltage. The natural logarithms of ${}^{TEA}K_{obs}$ values obtained in Fig. 2 were plotted against membrane voltage. The lines superimposed on the data are least-squares fits of the Woodhull equation (Woodhull, 1973), $\ln {}^{TEA}K_{obs} = \ln {}^{TEA}K_{obs}(0\ mV) - {}^{TEA}(z\delta)_{obs}FV_m/RT$. The alkali metal ions (100 mM) contained in the pipette solution were as indicated.

lar alkali metal ions. In Fig. 5, we plotted ${}^{TEA}K_{obs}(0\ mV)$ as a function of the concentration of all four alkali metal ions. In the presence of K^+, Rb^+, and Cs^+, ${}^{TEA}K_{obs}(0\ mV)$ increases linearly with ion concentration. The plots for K^+ and Rb^+ are very similar, but the slope of the plot for Cs^+ is much smaller. In the case of Na^+, the ${}^{TEA}K_{obs}(0\ mV)$ value is nearly the same in either 0 mM (i.e., 100 mM NMG^+) or 100 mM extracellular Na^+. Furthermore, the value of ${}^{TEA}(z\delta)_{obs}$ also increases with the concentration of alkali metal ions. For presentation purposes, the ${}^{TEA}(z\delta)_{obs}$ data are presented in Fig. 7 (below).

Channel Blockade by TEA in the Presence of Intracellular Rb^+

To gain insight into how intracellular permeating ions affect channel blockade by intracellular TEA, we exam-

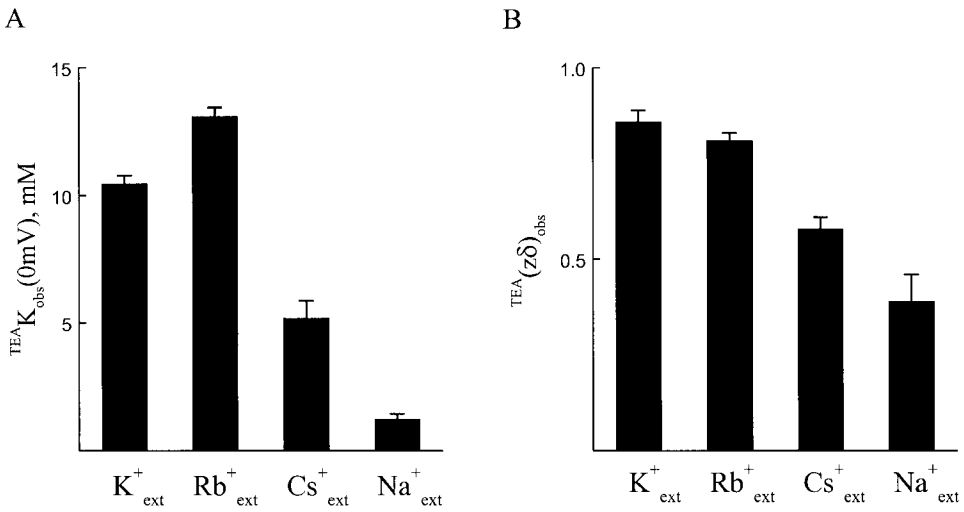

Figure 4. The values of ${}^{TEA}K_{obs}(0\ mV)$ and ${}^{TEA}(z\delta)_{obs}$ determined in various extracellular alkali metal ions. ${}^{TEA}K_{obs}(0\ mV)$ and ${}^{TEA}(z\delta)_{obs}$ values (mean ± SEM, $n = 5$) obtained in the presence of 100 mM of each of the four alkali metal ions are presented in A and B, respectively. The data were obtained as shown in Fig. 3.

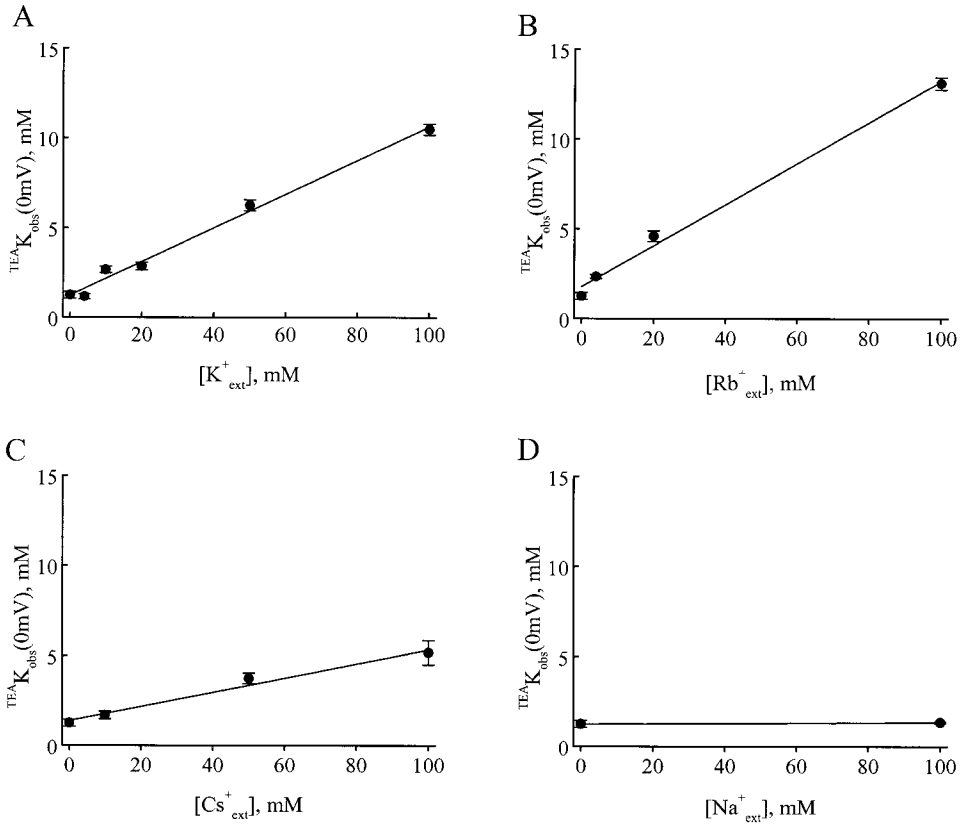

Figure 5. Dependence of $^{TEA}K_{obs}$(0 mV) on the concentration of extracellular alkali metal ions. $^{TEA}K_{obs}$(0 mV) values (mean ± SEM, n = 5) are plotted against the concentration of extracellular K^+, Rb^+, Cs^+, or Na^+. The values of $^{TEA}K_{obs}$(0 mV) were obtained as shown in Fig. 3. (A–C) When the concentration of K^+, Rb^+, or Cs^+ was reduced, Na^+ was used as a substitute. (D) The Na^+-free solution contained 100 mM NMG^+. The lines superimposed on the data represent fits of Eq. 5 (see discussion).

ined how TEA-blocking behaviors would change if we replaced intracellular K^+ by Rb^+. Fig. 6 A shows the I–V curves obtained in the presence of 100 mM intracellular Rb^+ and 100 mM extracellular K^+. The inward current carried by K^+ is larger than the outward current carried by Rb^+, and the currents reverse at approximately +10 mV. Addition of TEA to the intracellular solution significantly reduced the outward current. However, the curvature of the outward current induced by intracellular TEA is significantly smaller than that observed when 100 mM K^+ is present in both the intracellular and extracellular solutions (compare Fig. 1 A with 6 A). The I–V curves in Fig. 6 B were recorded in the presence of 100 mM Rb^+ on both sides of the membrane. Unlike the remarkably linear I–V curve in symmetric K^+, the I–V curve in symmetric Rb^+ is nonlinear. The outward current is slightly smaller than the inward current, which reveals the asymmetric property of the channel. Addition of intracellular TEA also significantly reduced the outward current. Again, the TEA-induced curvature of the I–V curves obtained in the presence of intracellular Rb^+ is much less than that obtained in intracellular K^+ (compare Fig. 1 B with 6 B).

Fig. 6, C and D, shows the effects of reducing the concentration of extracellular permeant ions on channel blockade by intracellular TEA. All I–V curves in Fig. 6, C and D, were recorded in the presence of 100 mM intracellular Rb^+. The extracellular permeant ions were 20 mM K^+ and Rb^+ for C and D, respectively. The concentrations of intracellular TEA were as indicated. In the presence of 20 mM extracellular K^+ or Rb^+, both the extent and the voltage dependence of channel blockade by TEA were very similar to those obtained in 100 mM corresponding extracellular ions (Fig. 6, A vs. C, and B vs. D).

Fig. 7, A and B, plots $^{TEA}K_{obs}$(0 mV) determined in the presence of 100 mM intracellular K^+ (open symbols) or 100 mM intracellular Rb^+ (closed symbols) as a function of the concentration of extracellular K^+ and Rb^+, respectively. Replacing intracellular K^+ by Rb^+ dramatically reduced the dependence of $^{TEA}K_{obs}$(0 mV) on the concentration of either extracellular ion.

To further illustrate how different intracellular ions alter the voltage dependence of channel blockade, in Fig. 7, C and D, we plotted the $^{TEA}(z\delta)_{obs}$ values determined under various intra- and extracellular conditions. Fig. 7 C plots $^{TEA}(z\delta)_{obs}$ values against the concentration of extracellular K^+, while Fig. 7 D plots the values against the concentration of extracellular Rb^+. In both plots, the data represented by open and closed symbols were obtained in intracellular K^+ and Rb^+, respectively. The values of $^{TEA}(z\delta)_{obs}$ increased with increasing concentrations of extracellular K^+ or Rb^+, compatible with a scenario where the observed changes in the voltage dependence result from titrating an ion-binding site in the external part of the pore. When K^+ in the intracellu-

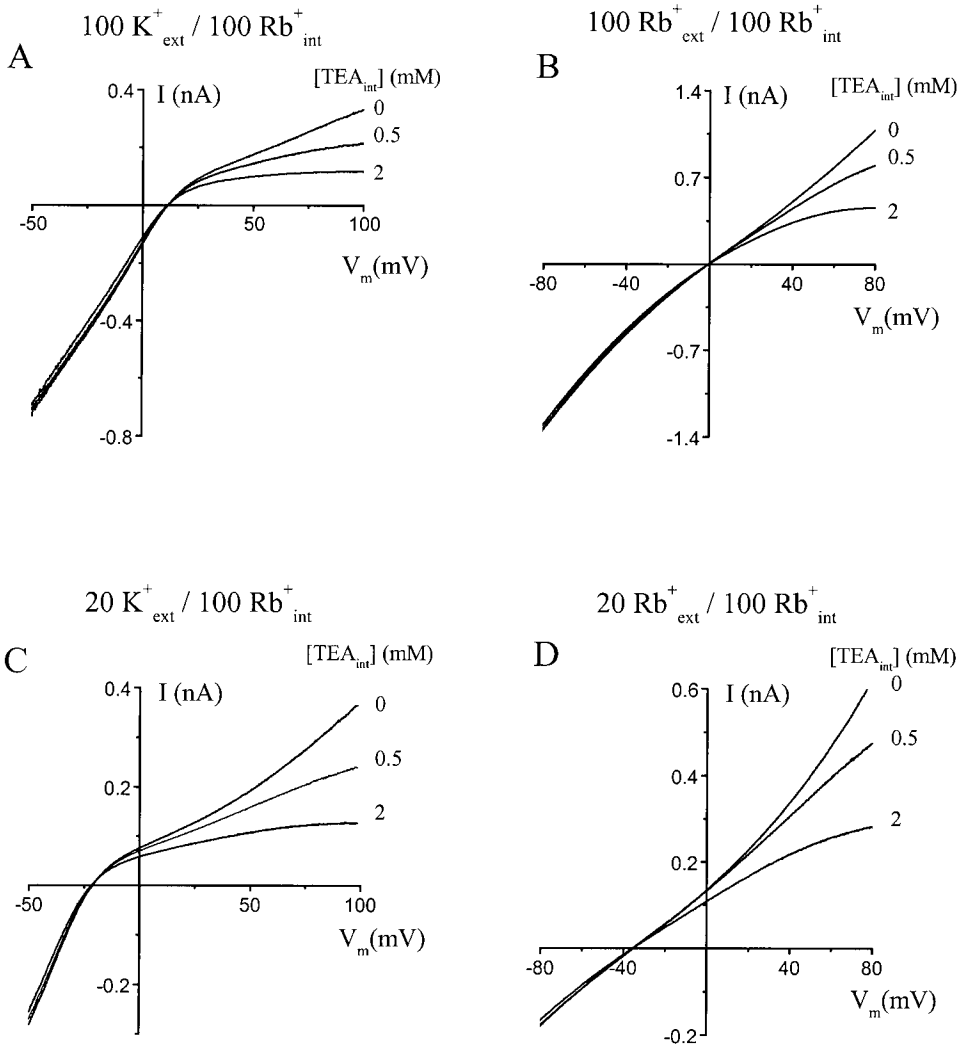

Figure 6. Blockade of the ROMK1 channel by intracellular TEA in the presence of intracellular Rb^+. Macroscopic I–V curves of the ROMK1 channel were recorded in the absence or presence of various concentrations of intracellular TEA. The pipette solutions contained 100 mM K^+ (A), 100 mM Rb^+ (B), 20 mM K^+ (C), and 20 mM Rb^+ (D). The bath solution contained 100 mM of Rb^+ in all four cases.

lar solution was replaced by Rb^+, the value of ${}^{TEA}(z\delta)_{obs}$ was significantly reduced at all tested concentrations of extracellular K^+ or Rb^+. However, regardless of whether the intracellular ion was K^+ or Rb^+, the ${}^{TEA}(z\delta)_{obs}$ values converged to the same minimum at vanishing extracellular K^+ or Rb^+ concentrations. These observations show that replacing intracellular K^+ by Rb^+ dramatically reduces the ability of extracellular ions to alter the voltage dependence of channel blockade.

discussion

In the present study, we found that both the extent and the voltage dependence of channel blockade by TEA depend on the type of alkali metal ions, including K^+, Rb^+, Cs^+, and Na^+, in the extracellular solution (Figs. 1 and 4). Since the voltage dependence is determined by the movement of all energetically coupled ions in the transmembrane electrical field (Spassova and Lu, 1998), these results can be understood if the external ion site has different affinities for these ions. To test this idea, we determined the affinities of the site for the four alkali metal ions by examining how ${}^{TEA}K_{obs}(0\ mV)$ varies with the concentration of each ion.

As shown in Fig. 5, ${}^{TEA}K_{obs}(0\ mV)$ increases linearly with not only the concentration of K^+, as observed previously (Spassova and Lu, 1998), but also the concentrations of Rb^+ and Cs^+. Altering Na^+ concentration has very little effect on ${}^{TEA}K_{obs}(0\ mV)$. The linear dependence of ${}^{TEA}K_{obs}(0\ mV)$ on the concentration of extracellular ions argues that extracellular ions in effect compete with intracellular TEA for binding to the pore. However, the apparent competition undoubtedly does not result from the binding of extracellular permeant ions and intracellular TEA to the same site. Rather, the competition is most likely an indirect process. For example, the binding of K^+ at the external site may perturb the binding site for intracellular TEA, which in turn affects TEA binding, and vice versa. This scenario is in line with the finding that mutations at

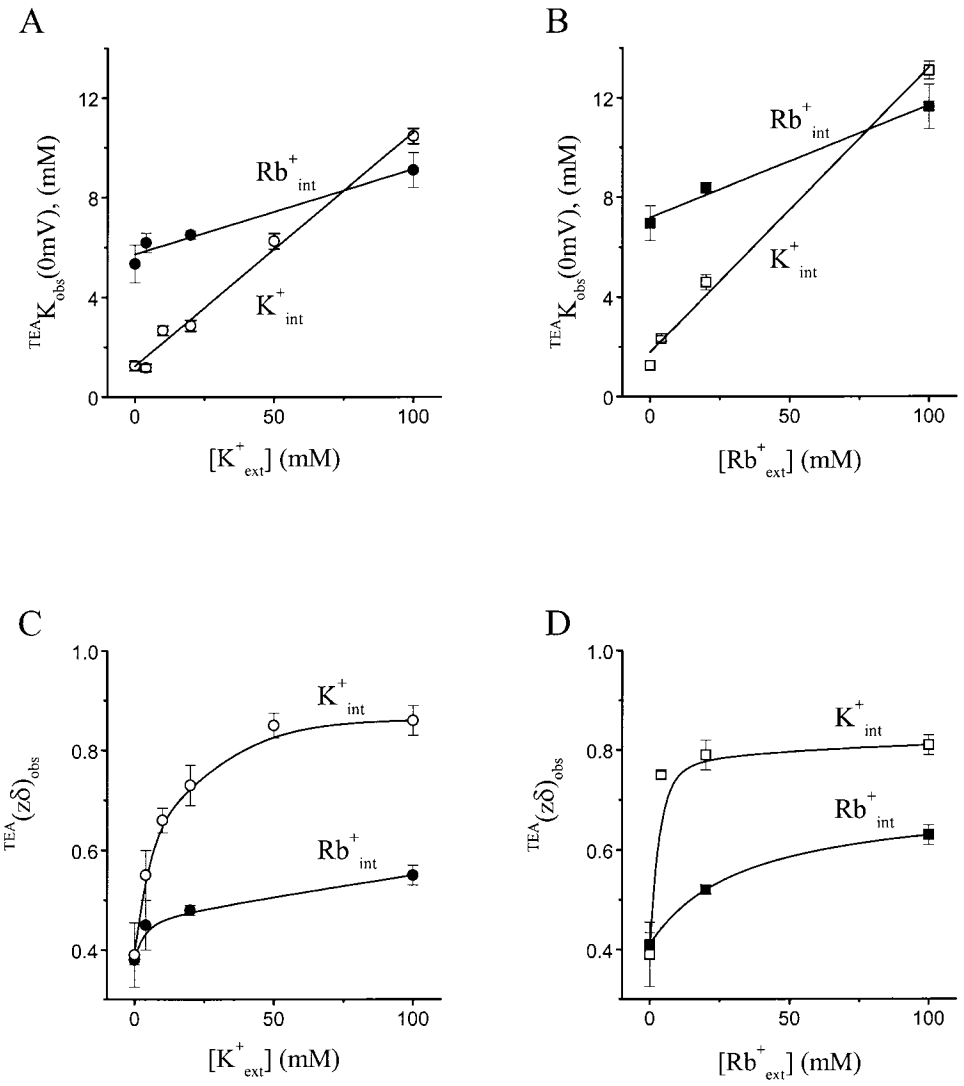

Figure 7. Effects of intracellular permeant ions on channel blockade by intracellular TEA. (A and B) $^{TEA}K_{obs}$(0 mV) (mean ± SEM, n = 5) are plotted against the concentration of extracellular K^+ in A and Rb^+ in B. Data in the open symbols were obtained in 100 mM intracellular K^+, while data in the closed symbols were obtained in 100 mM intracellular Rb^+. The lines superimposed on the data correspond to Eq. 5. (C and D) The values of $^{TEA}(z\delta)_{obs}$ (mean ± SEM, n = 5) are plotted against the concentration of extracellular K^+ in C and Rb^+ in D. Data in the open symbols were obtained in 100 mM intracellular K^+, while data the in closed symbols were obtained in 100 mM intracellular Rb^+. The curves superimposed on the data have no theoretic meaning.

some residues around the permeant ion-binding sites in the narrow region of the voltage-activated K^+ channels can significantly affect the binding of intracellular TEA to the more internal region of the pore (e.g., Yellen et al., 1991; Taglialatela et al., 1993). Furthermore, as discussed below, we show here that ion binding at an internal site in the ROMK1 pore can dramatically alter ion binding at the external site (Fig. 8).

The diagram in Fig. 8 is a simplified version of the kinetic models that we previously used to interpret the dependence of $^{TEA}K_{obs}$(0 mV) on the concentration of extracellular K^+ (Spassova and Lu, 1998). The dissociation constants (^{TEA}K and ^{X}K) for the two competitors (TEA_{int} and X_{ext}) are defined as:

$$^{TEA}K = \frac{[Ch][TEA_{int}]}{[ChTEA_{int}]} \quad (1)$$

and

$$^{X}K = \frac{[Ch][X_{ext}]}{[X_{ext}Ch]}. \quad (2)$$

The fraction of unblocked channel is

$$\theta = \frac{[Ch] + [X_{ext}Ch]}{[Ch] + [X_{ext}Ch] + [ChTEA_{int}]}, \quad (3)$$

where $[X_{ext}Ch]$, $[ChTEA_{int}]$, and [Ch] are concentrations of the channels bound with an additional extracellular ion X, intracellular TEA, or neither of them. Combining Eqs. 1–3 yields:

$$\theta = \frac{1}{1 + \frac{[TEA_{int}]}{^{TEA}K_{obs}}}, \quad (4)$$

where

$$^{TEA}K_{obs} = \frac{^{TEA}K}{^{X}K}[X_{ext}] + {}^{TEA}K. \quad (5)$$

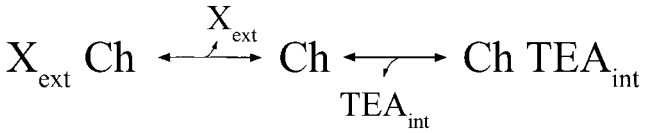

Figure 8. A kinetic state diagram. Extracellular ion X and intracellular TEA bind competitively to a channel (Ch).

Undoubtedly, the state diagram in Fig. 8 is an over simplification for the interaction between extracellular permeant ions and intracellular TEA. Nevertheless, it provides a simple and instructive way for considering the interaction between the extracellular permeant ions and intracellular TEA. In fact, Eq. 5 derived based on this simple state diagram is the same as one we previously derived based on a more complex model (Spassova and Lu, 1998).

According to Eq. 5, we determined the equilibrium dissociation constant for TEA at 0 mV and in zero extracellular K^+ [$^{TEA}K(0\ mV)$] from the y intercept of the plots in Fig. 5 as 1.4 ± 0.3 mM. We also determined the equilibrium dissociation constants of the channel for the four ions from the ratio of the y intercept and slope of the corresponding plots (mM): ^{K}K = 13.2 ± 2.4 (mean ± SEM), ^{Rb}K = 15.6 ± 3.1, ^{Cs}K = 34.0 ± 3.5, and ^{Na}K = 1.4 M. Since the slope of plot D is minimal, a ^{Na}K value of 1.4 M may not be very precise. Nevertheless, it does indicate that the affinity of the site for Na^+ is much lower. The results of the analysis indicate that the external ion-binding site strongly selects K^+ over smaller Na^+, but only minimally or modestly selects K^+ over larger Rb^+ or Cs^+.

The ion affinity sequence of the site ($K^+ \approx Rb^+ > Cs^+ > Na^+$) correlates with the degree of voltage dependence of channel blockade by TEA in the presence of the corresponding alkali metal ions (Fig. 4 B). To account for the dependence of $^{TEA}K_{obs}$ on membrane voltage, we combined Eq. 5 and the Woodhull equation:

$$K_d(X\ mV) = K_d(0\ mV)e^{\frac{-(z\delta)FV_m}{RT}}, \quad (6)$$

which gives

$$^{TEA}K_{obs}(X\ mV) = \frac{[X_{ext}]^{TEA}K(0\ mV)e^{\frac{-^{TEA}(z\delta)FV_m}{RT}}}{^{X}K(0\ mV)e^{\frac{^{X}(z\delta)FV_m}{RT}}} + {}^{TEA}K(0\ mV)e^{\frac{-^{TEA}(z\delta)FV_m}{RT}}. \quad (7)$$

Rearranging Eq. 7, we obtain

$$^{TEA}K_{obs}(X\ mV) = \frac{[X_{ext}]^{TEA}K(0\ mV)e^{\frac{-[^{TEA}(z\delta)+{}^{X}(z\delta)]FV_m}{RT}}}{^{X}K(0\ mV)} + {}^{TEA}K(0\ mV)e^{\frac{-^{TEA}(z\delta)FV_m}{RT}}. \quad (8)$$

Quantity $^{X}(z\delta)$ in Eq. 8 is related only to the binding and unbinding of extracellular permeant ions to the external site. However, $^{TEA}(z\delta)$ is related to the movement of TEA and possibly other permeant ions (excluding the one bound to the external site) in the electrical field along the pore (for more details, see Spassova and Lu, 1998). According to Eq. 8, voltage dependence of channel blockade by intracellular TEA is determined by the movement of TEA as well as permeant ions in the transmembrane electrical field. Since the external site has different affinity for the various alkali metal ions, the site is occupied to a different extent in the presence of various ions at a given finite concentration. This explains why various degrees of voltage dependence are observed in the presence of 100 mM of the four extracellular alkali metal ions.

Although we exploited the apparent competition between extracellular alkali metal ions and intracellular TEA to determine K_d values for the alkali metal ions, these K_d values should characterize the interaction of alkali metal ions with the channel in the absence of TEA (see the diagram in Fig. 8). Even so, the absolute K_d values determined here do not necessarily reflect the intrinsic affinities of the site for the various alkali metal ions for the reason discussed below.

To learn how intracellular permeant ions modify blocking ion–induced rectification, we compared TEA block in 100 mM intracellular K^+ versus Rb^+. Regardless of whether K^+ or Rb^+ was in the extracellular solution, the effect on $^{TEA}K_{obs}(0\ mV)$ of changes in the concentration of extracellular permeating ions was much larger when the intracellular ion was K^+ than when it was Rb^+. For example, when the concentration of extracellular K^+ was decreased from 100 mM to near zero, $^{TEA}K_{obs}(0\ mV)$ decreased by eightfold in intracellular K^+, whereas it decreased by less than twofold in intracellular Rb^+ (Fig. 7 A). A similar phenomenon was observed when the concentration of extracellular Rb^+ was reduced (Fig. 7 B).

Analyzing the data acquired in intracellular Rb^+ using Eq. 5, we obtained ^{K}K = 155 ± 21 mM, ^{Rb}K = 159 ± 18 mM, and ^{TEA}K = 6.5 ± 0.4 mM (all at 0 mV). Thus, replacing intracellular K^+ with Rb^+ lowers channel af-

finity for extracellular K^+ and Rb^+ by ~10-fold (^{K}K = 13 vs. 155 mM, ^{Rb}K = 16 vs. 159 mM), and channel affinity for intracellular TEA by ~5-fold (^{TEA}K = 1.4 vs. 6.5 mM). The increase in ^{TEA}K accounts for the higher y intercepts of the plots corresponding to intracellular Rb^+ in Fig. 7, A and B, while the larger changes in both ^{K}K and ^{Rb}K than in ^{TEA}K account for the shallower slopes of the plots corresponding to intracellular Rb^+. These findings suggest that K^+ and Rb^+ interact quite differently with an internal ion-binding site, despite the fact that the external site has similar affinities for K^+ and Rb^+ (^{K}K = 13 mM vs. ^{Rb}K = 16 mM).

Since the binding of intracellular Rb^+ reduces the affinity of the external site for both K^+ and Rb^+, Eq. 8 predicts that in the presence of subsaturating concentrations of extracellular permeating ions, the voltage dependence of channel blockade should be less in intracellular Rb^+ than in K^+. Also, regardless of the types of permeating ions present in the intracellular solution, the voltage dependence should be the same when the concentration of extracellular permeating ions is zero, because in this case only the second term in Eq. 8 applies. These predictions are consistent with what was observed (Fig. 7, C and D). Therefore, the reduction in the voltage dependence due to replacing intracellular K^+ by Rb^+ can be explained by the resulting reduction in the affinity, and thus in the level of ion saturation, of the external ion-binding site.

It is unclear thus far why the affinity of the channel for blocking ions, such as TEA, and the voltage dependence of channel blockade are much less sensitive to the concentration of extracellular K^+ in the voltage-activated K^+ channels than in the inward-rectifier K^+ channels (e.g., Armstrong and Binstock, 1965; French and Shoukimas, 1981; Yellen et al., 1991; Choi, et al., 1993). Here, we found that in the presence of intracellular Rb^+, the affinity of the ROMK1 channel for extracellular permeating ions is significantly reduced. Consequently, in the presence of intracellular Rb^+, both the affinity of the channel for TEA and the voltage dependence of its block by TEA are much less sensitive to the concentration of extracellular permeating ions, a property reminiscent of voltage-activated K^+ channels. This finding makes one wonder whether the relatively low extracellular ion sensitivity of TEA block in the voltage-activated K^+ channels may in part result from a lower affinity of the channels for extracellular ions under comparable conditions. Interestingly, the single channel conductance of the ROMK1 inward-rectifier K^+ channel is half-maximal at 10 mM K^+ (Lu and MacKinnon, 1994b), whereas the half-maximal single-channel conductance of the *Shaker* voltage-activated K^+ channel is not reached until the concentration of K^+ is 300 mM (Heginbotham and MacKinnon, 1993).

We showed here that the external ion site in the ROMK1 channel interacts selectively with alkali metal ions with an affinity sequence $K^+ \approx Rb^+ > Cs^+ >> Na^+$. This sequence is similar to that previously determined in a Ca^{2+}-activated K^+ channel by examining the interaction between K^+ and Ba^{2+} ions (Neyton and Miller, 1988a; also see Harris et al., 1998), although the absolute K_d values for all four ions determined in the present study are generally much larger than those determined in the quoted studies (see also Baukrowitz and Yellen, 1996). (As already discussed, the affinity of a given ion binding site in the pore depends on the presence of other ions in the pore.) Our data show that the external site in the ROMK1 channel strongly selects against smaller Na^+, but only modestly selects among the larger ions. Furthermore, we found that the binding of intracellular K^+ versus Rb^+ to an internal site has dramatically different effects on how the pore interacts with other ions. This finding argues that this internal site may be more selective among the larger ions. The idea that both internal and external sites in a K^+ pore are selective has previously been used by Neyton and Miller (1988a,b) to account for the fact that both intra- and extracellular permeating ions selectively interact with blocking Ba^{2+} in a Ca^{2+}-activated K^+ channel. This view is supported by a recent crystallographic study on KcsA, a bacterial K^+ channel, showing that two permeating ions can simultaneously reside in the narrow region of the pore, and that the locations of the binding sites are the same for various alkali metal ions (Doyle et al., 1998).

A multi-ion theory, often used to explain ion selectivity in K^+ channels (e.g., Neyton and Miller, 1988a,b; Doyle et al., 1998), was originally proposed to explain ion selectivity in the voltage-activated Ca^{2+} channels (Almers and McCleskey, 1984; Hess and Tsien, 1984). The theory hypothesizes that there are two (or more) K^+-selective ion-binding sites in the narrow region of the pore. K^+ ions can bind to either site with high affinity, which accounts for the K^+ selectivity of the pore. To account for the high throughput rate of the channel, the binding of K^+ to the second site is hypothesized to dramatically lower the affinity of both sites for K^+ due to electrostatic repulsion between the bound K^+ ions.

We found here that the external site in the ROMK1 channel has very different affinities for extracellular permeating ions depending on whether the internal site is exposed to K^+ or Rb^+, which provides experimental evidence for the hypothesized permeant ion interactions in the pore. This finding also argues that the interactions between permeating ions in the pore are not merely electrostatic. Conceivably, binding of ions of different sizes at one site (e.g., the internal site in this case) in the pore can induce different degrees of structural "deformation" at a second site (e.g., the external site) elsewhere in the pore by propagating the binding energy along the pore-lining protein elements. Since

the interactions between ions and the narrow part of the pore almost certainly are ion–dipole interactions, they should be highly sensitive to a change in the distance between permeating ions and the dipole-generating atoms, such as carbonyl oxygen, in the channel protein (Heginbotham et al., 1994; Doyle et al., 1998). Thus, even a small change in the distance between a permeating ion and the dipole-generating atoms in the channel protein, induced by the binding of another ion elsewhere, could dramatically alter their interactions.

In summary, the external ion site in the ROMK1 pore binds various alkali metal ions (Na^+, K^+, Rb^+, and Cs^+) with different affinities, which can in turn be altered by the binding of various permeating ions at the internal site through a nonelectrostatic mechanism. Consequently, the saturation level of the external ion site depends on the ion species on both sides of the membrane. Since rectification is determined by the movement of all energetically coupled ions in the transmembrane electrical field along the pore, various degrees of rectification are observed with various combinations of extra- and intracellular ions. Although both the external and internal ion sites in the ROMK1 pore appear to be ion selective, they likely have different ion selectivity: the external site selects strongly against smaller Na^+ but only modestly among the three larger ions, whereas the internal site interacts quite differently with the larger ions K^+ and Rb^+.

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