Coupled Ion Movement Underlies Rectification in an Inward-Rectifier K^+ Channel

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ABSTRACT We studied block of the internal pore of the ROMK1 inward-rectifier K^+ channel by Mg^{2+} and five quaternary ammoniums (tetramethylammonium, tetraethylammonium, tetrapropylammonium, tetrabutylammonium, and tetrapentylammonium). The apparent affinity of these blockers varied as a function of membrane voltage. As a consequence, the channel conducted K^+ current more efficiently in the inward than the outward direction; i.e., inward rectification. Although the size of some monovalent quaternary ammoniums is rather large, the $z\delta$ values (which measure voltage dependence of their binding to the pore) were near unity in symmetric 100 mM K^+. Furthermore, we observed that not only the apparent affinities of the blockers themselves, but also their dependence on membrane voltage (or $z\delta$), varied as a function of the concentration of extracellular K^+. These results suggest that there is energetic coupling between the binding of blocking and permeating (K^+) ions, and that the voltage dependence of channel blockade results, at least in part, from the movement of K^+ ions in the electrical field. A further quantitative analysis of the results explains why the complex phenomenon of inward rectification depends on both membrane voltage and the equilibrium potential for K^+.

KEY WORDS: inward-rectifier K^+ channel • rectification • ionic blocker • magnesium • tetraethylammonium

INTRODUCTION

Inward-rectifier K^+ channels derive their name from the observation that they pass much larger inward current at negative membrane voltages than they pass outward current at positive voltages, even when the concentration of K^+ on both sides of the membrane is equal (Katz, 1949; Hodgkin and Horowicz, 1959; Horowicz et al., 1968; Noble and Tsien, 1968; Adrian, 1969; Hagiwara and Takahashi, 1974; Hille and Schwarz, 1978; Hille, 1992). This unusual property is commonly referred to as inward rectification. Under physiological conditions, inward-rectifier K^+ channels allow a small outward K^+ flux near the equilibrium potential for K^+, but this flux diminishes as membrane voltage becomes more positive. This feature enables the channel to regulate the resting membrane potential without impeding the generation of action potentials (Katz, 1949; Hille, 1992). By doing so, inward-rectifier K^+ channels accomplish many important biological tasks (Hille, 1992).

Inward rectification undoubtedly results mainly from channel blockade by intracellular cations such as Mg^{2+} and polyamines because many inward-rectifier K^+ channels exhibit nearly symmetric conduction properties in the absence of these blocking ions (Horie et al., 1987; Matsuda et al., 1987; Vandenberg, 1987; Ficker et al., 1994; Lopatin et al., 1994; Lu and MacKinnon, 1994*b*, 1995; Nichols et al., 1994; Stanfield et al., 1994; Taglialatela et al., 1994; Wible et al., 1994; Fakler et al., 1995; Yang et al., 1995; Taglialatela et al., 1995). The apparent affinities of these blocking ions increase as the membrane voltage becomes more positive, similar to blockade of voltage-activated K^+ channels in squid by internal tetraethylammonium (TEA)[1] (Armstrong, 1966, 1969). Consequently, inward-rectifier K^+ channels conduct K^+ current in an inwardly rectifying manner. Although the voltage dependence of channel blockade has been attributed to the binding of blocking ions in the transmembrane electrical field, the interpretation fails to explain some critical features of inward rectification (Katz, 1949; Hagiwara and Takahashi, 1974; Hille and Schwarz, 1978; Lopatin and Nichols, 1996). For example, Katz (1949) observed that the outward current through inward-rectifier K^+ channels was diminished not only when membrane voltage was made more positive, but also when the concentration of extracellular K^+ was decreased (also see Hagiwara and Ta-

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[1]*Abbreviations used in this paper:* I-V, current–voltage; QA, quaternary ammonium; TEA, tetraethylammonium.

kahashi, 1974; Hille and Schwarz, 1978; Hille, 1992). Inward rectification appears to be a function of the difference between the membrane potential and the equilibrium potential of K^+; i.e., $V_m - E_K$ (Katz, 1949; Hodgkin and Horowicz, 1959; Horowicz et al., 1968; Noble and Tsien, 1968; Hagiwara and Takahashi, 1974; Hille and Schwarz, 1978; Hille, 1992). Subsequently, it was discovered that the apparent affinity of intracellular blocking ions that causes rectification also depends on the concentration of extracellular K^+ (Armstrong and Binstock, 1965; Bezanilla and Armstrong, 1972; Hille, 1975; Adelman and French, 1978; Matsuda, 1991; Nichols et al., 1994; Stanfield et al., 1994; Lu and MacKinnon, 1994*a*; Lopatin and Nichols, 1996). This phenomenon is commonly referred to as *trans* knock off of blocking ions by K^+. This K^+ "knock-off" effect has been suggested to underlie the dependence of rectification on extracellular K^+ (Hille and Schwarz, 1978; Hille, 1992; Lopatin and Nichols, 1996).

To better understand why inward rectification is affected by extracellular K^+, we examined how the apparent affinity of blocking ions and its voltage dependence ($z\delta$) are related to extracellular K^+ in the ROMK1 inward-rectifier K^+ channel. We found that both the apparent affinity of blocking ions and the voltage dependence of their affinity varied as a function of the concentration of extracellular K^+. These results indicate that the voltage dependence of channel blockade, which underlies inward rectification, results from the coupled movement of K^+ and the blocking ion in the pore.

METHODS

Molecular Biology and Oocyte Preparation

ROMK1 cDNA cloned into the p-SPORT1 plasmid (GIBCO BRL, Gaithersburg, MD) was kindly provided by Drs. Ho and Hebert (Ho et al., 1993). RNA was synthesized using T7 polymerase (Promega Corp., Madison, WI) from Not1-linearized ROMK1 DNA. Oocytes harvested from *Xenopus laevis* (Xenopus One, Ann Arbor, MI) 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 μM 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., Foster City, CA). The recorded signal was filtered at 1 kHz and sampled at 5 kHz using an analog-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 (I-V) curves were recorded as membrane voltage was linearly ramped (1 mV/20 ms). At the end of each experiment, the membrane patch was exposed to a Mg^{2+}-containing and ATP-free solution (see below) to induce rundown of ROMK1. The I-V curves recorded after complete rundown were used as templates for subsequent off-line background current corrections (Lu and MacKinnon, 1994*b*).

Recording Solutions

Pipette solutions contained specified concentrations of K^+ with 0.3 mM $CaCl_2$, 1.0 mM $MgCl_2$, and 10 mM HEPES, pH 7.6. Sodium was used to maintain a constant ionic strength such that the total concentration of KCl and NaCl was 100 mM. In the experiments involving quaternary ammoniums, bath solutions contained the specified concentrations of quaternary ammoniums (QAs) with 90 mM KCl, 5 mM K_2EDTA, and 10 mM HEPES, pH 7.6 ($[K^+]_{total}$ = 100 mM). In the experiments involving intracellular Mg^{2+}, K_2ATP and $MgCl_2$ were included in the bath solution described above (QAs were excluded.). Free $[Mg^{2+}]$ was achieved (maintaining 4 mM MgATP) by adjusting the total ATP and $MgCl_2$ using a stability constant of 1.64×10^4 M^{-1}. The solution used to induce ROMK1 rundown contained (mM): 100 KCl, 3 $MgCl_2$, and 10 HEPES, pH 7.6.

Data Analysis and Equation Derivation

Refer to the state diagram in Fig. 6 *B* and Eq. 5. The dissociation constants ($^{TEA}K_{us}$ and $^{K}K_{ext}$) for the two competitors (TEA_{int} and K^+_{ext}) are defined as

$$^{TEA}K_{us} = \frac{[^{K}Ch]\,[TEA]_{int}}{[^{TEAK}Ch]} \quad (1)$$

and

$$^{K}K_{ext} = \frac{[^{K}Ch]\,[K^+]_{ext}}{[^{KK}Ch]}. \quad (2)$$

The fraction of unblocked channels (θ) is given by

$$\theta = \frac{[^{K}Ch] + [^{KK}Ch]}{[^{K}Ch] + [^{KK}Ch] + [^{TEAK}Ch]}, \quad (3)$$

where $[^{KK}Ch]$, $[^{K}Ch]$, and $[^{TEAK}Ch]$ are concentrations of the channel with one K^+, two K^+, and one K^+ plus one TEA, respectively. Combining Eqs. 1–3, we obtain

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

where $^{TEA}K_{obs} = {}^{TEA}K_{us}([K^+]_{ext}/{}^{K}K_{ext} + 1)$ (see Eq. 5 in DISCUSSION).

RESULTS

Voltage Dependence of ROMK1 Channel Blockade by Intracellular TEA

We first examined how intracellular TEA alters the I-V relation in the ROMK1 channel. Macroscopic I-V curves of the channel were recorded in the inside-out configuration while membrane voltage was linearly

ramped. In the presence of 100 mM K^+ on both sides of the membrane and in the absence of intracellular blocking ions, the ROMK1 channel displayed a linear current-voltage relationship (Fig. 1 *A*). Addition of TEA to the intracellular solution caused a voltage-dependent reduction of outward current; i.e., inward rectification. Fractions of unblocked currents at four representative membrane voltages were plotted against the concentration of TEA in Fig. 1 *B*. The curves superimposed on the data correspond to fits assuming 1:1 stoichiometry between TEA and the channel. The observed dissociation constants ($^{TEA}K_{obs}$), determined as shown in Fig. 1 *B*, were plotted against the corresponding membrane voltages in Fig. 1 *C*. As we analyzed the voltage dependence of channel blockade with the Woodhull equation (Woodhull, 1973), we observed a striking phenomenon: channel blockade by TEA is highly voltage dependent and associated with a surprisingly large value of $(z\delta)_{obs}$, 0.86. [$(z\delta)_{obs}$ is an empirical measure of voltage dependence.] This value is much larger than that observed for blockade of voltage-activated K^+ channels by intracellular TEA ($z\delta$ = 0.15; Hille, 1975; French and Shoukimas, 1981; Yellen et al., 1991; Choi et al., 1993).

Extracellular K^+ Dependence of Channel Blockade by Internal TEA

We next examined how the concentration of extracellular K^+ ($[K^+]_{ext}$) affects channel blockade by internal TEA. We found that the strong voltage dependence of

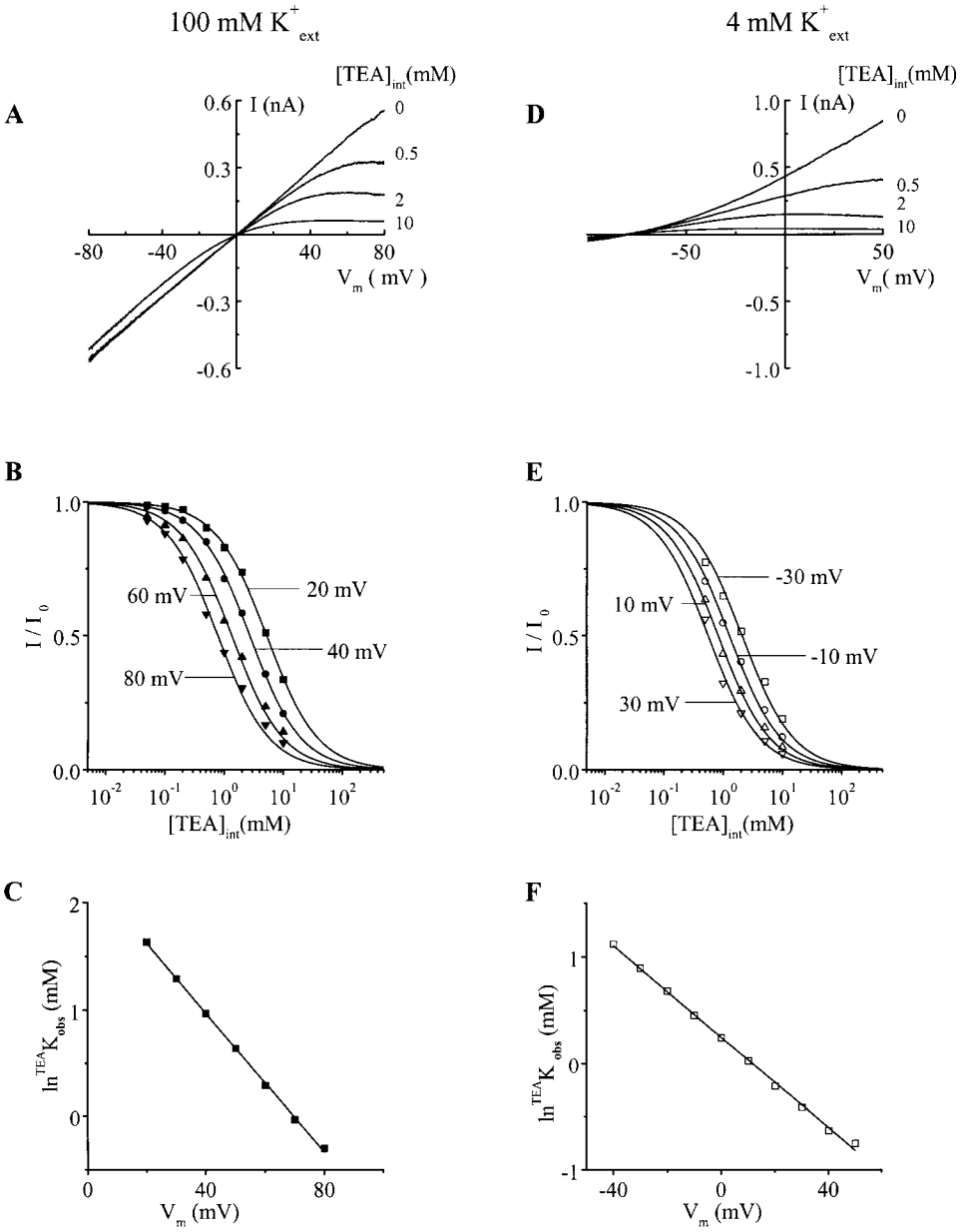


FIGURE 1. Voltage and extracellular K^+ dependence of ROMK1 channel blockade by internal TEA. (*A* and *D*) Macroscopic I-V curves of ROMK1 channels recorded in the inside-out configuration in various $[TEA]_{int}$. $[K^+]_{int}$ was 100 mM and $[K^+]_{ext}$ was as indicated. (*B* and *E*) Fractions of unblocked currents (I/I_0) obtained from *A* and *D* were plotted against $[TEA]_{int}$ for several membrane voltages (V_m). Curves superimposed on the data correspond to least-squares fits using $I/I_0 = {}^{TEA}K_{obs}/({}^{TEA}K_{obs} + [TEA]_{int})$ ($^{TEA}K_{obs}$ is the observed TEA equilibrium dissociation constant). (*C* and *F*) $\ln {}^{TEA}K_{obs}$ obtained from *B* and *E* were plotted against V_m, respectively. The lines superimposed on the data correspond to least-squares fits using the Woodhull equation (Woodhull, 1973), $\ln {}^{TEA}K_{obs} = \ln {}^{TEA}K_{obs}(0\text{ mV}) - {}^{TEA}(z\delta)_{obs}FV_m/RT$.

blockade by TEA was dramatically reduced as we lowered the concentration of extracellular K^+ from 100 to 4 mM (Fig. 1, *A–C* vs. *D–F*). This effect of extracellular K^+ manifested itself as a dramatic reduction both in the TEA-induced curvature of the I-V curves (Fig. 1, *A* vs. *D*) and in the slope of a semilogarithmic plot of ${}^{TEA}K_{obs}$ versus membrane voltage (V_m) (Fig. 1, *C* vs. *F*; note the different voltage scale), as extracellular K^+ concentration was lowered.

We also observed an increase in channel affinity for TEA as a result of reducing $[K^+]_{ext}$ (Fig. 1, *A–F*), similar to what was originally observed by Armstrong and Binstock (1965) for blockade of squid voltage-activated K^+ channels by internal TEA.

To quantify the effects of $[K^+]_{ext}$, we determined ${}^{TEA}K_{obs}$ (0 mV) (the observed TEA equilibrium dissociation constant at 0 mV) and ${}^{TEA}(z\delta)_{obs}$ (which measures the voltage dependence of TEA binding) at various $[K^+]_{ext}$ using the Woodhull equation (Fig. 1, *C* and *F*). As shown in Fig 2 *A*, ${}^{TEA}K_{obs}$ (0 mV) increased linearly with increases in $[K^+]_{ext}$, as if internal TEA and external K^+ competed for the pore. Moreover, ${}^{TEA}(z\delta)_{obs}$ increased with $[K^+]_{ext}$ in a saturating fashion, from 0.39 at nominal zero external K^+ to 0.86 at 100 mM external K^+ (Fig. 2 *B*). The apparent dissociation constant for this effect of external K^+ was 9.6 mM. (We wish to point out that, depending on the model used, ${}^{TEA}(z\delta)_{obs}$ at $[K^+]_{ext}$ between zero and infinity may not have a straightforward physical meaning.)

The results described thus far suggest that the voltage dependence of channel blockade does not simply reflect the fraction of the electrical field traversed by the blocking ion (TEA). Instead, they suggest that the voltage dependence results from a coupled movement of K^+ and TEA ions in the pore.

Channel Blockade by TEA Derivatives

We also examined blockade of the ROMK1 channel by four other symmetric QAs, tetramethylammonium (TMA), tetrapropylammonium (TPrA), tetrabutylammonium (TBA), and tetrapentylammonium (TPeA). Like TEA, all these QAs blocked the channel in a voltage-dependent manner (Fig. 3, *A–D*). In the presence of symmetric high concentration of K^+ (100 mM), zδ values for all but one of the QAs approached unity; the exception was TMA, whose zδ value was 0.53. We did not observe relief of block by any QA even at the most positive voltages (up to +80 mV). Thus, in spite of these large zδ values, none of the QAs appeared to permeate the channel. Near-constant levels of voltage dependence associated with blockade of voltage-activated K^+ channels by the same QAs have previously been observed, although the magnitude of zδ (≈0.15) was much smaller (French and Shoukimas, 1981).

The apparent affinity of each QA and its dependence on membrane voltage were again strongly dependent on $[K^+]_{ext}$: both the ${}^{QA}K_{obs}$ (0 mV) values and the ${}^{QA}(z\delta)_{obs}$ values decreased significantly as $[K^+]_{ext}$ was lowered (Figs. 3 and 4).

Channel Blockade by Intracellular Mg^{2+}

To determine if $[K^+]_{ext}$ dependence of zδ values was unique to monovalent blocking cations, we examined

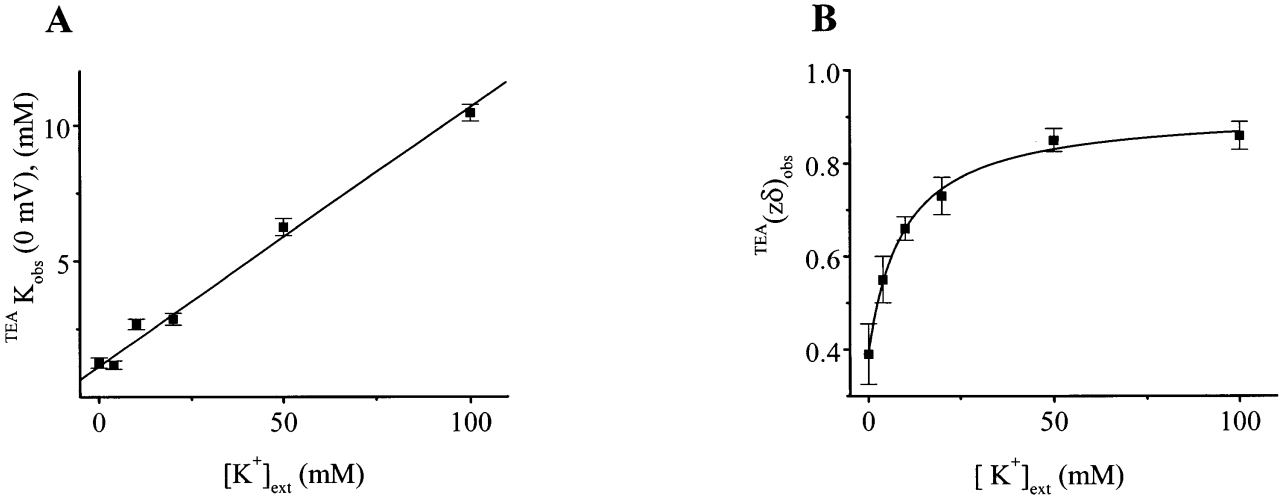


FIGURE 2. Summary of extracellular K^+ dependence of ROMK1 channel blockade by internal TEA. (*A*) ${}^{TEA}K$ (0 mV) was plotted against $[K^+]_{ext}$. The line corresponds to the least-squares fit of a linear equation. (*B*) ${}^{TEA}(z\delta)_{obs}$ (mean ± SEM, $n = 5$) was plotted against $[K^+]_{ext}$. The curve corresponds to the least-squares fit by the equation ${}^{TEA}(z\delta)_{obs} = \{{}^{TEA}(z\delta)_{inf} - {}^{TEA}(z\delta)_0\ [K^+]_{ext}\}/([K^+]_{ext} + {}^{K}K_{app}) + {}^{TEA}(z\delta)_0$, where ${}^{K}K_{app}$ is the apparent (empirical) dissociation constant for the binding of external K^+ to the pore, while ${}^{TEA}(z\delta)_0$ and ${}^{TEA}(z\delta)_{inf}$ are ${}^{TEA}(z\delta)_{obs}$ when concentrations of external K^+ ($[K^+]_{ext}$) are zero and infinity, respectively. The fit gives that ${}^{TEA}(z\delta)_0 = 0.39$, ${}^{TEA}(z\delta)_{inf} = 0.92$, and ${}^{K}K_{app} = 9.6$ mM. The data points corresponding to 0 mM external K^+ were obtained in a nominal K^+ free solution.

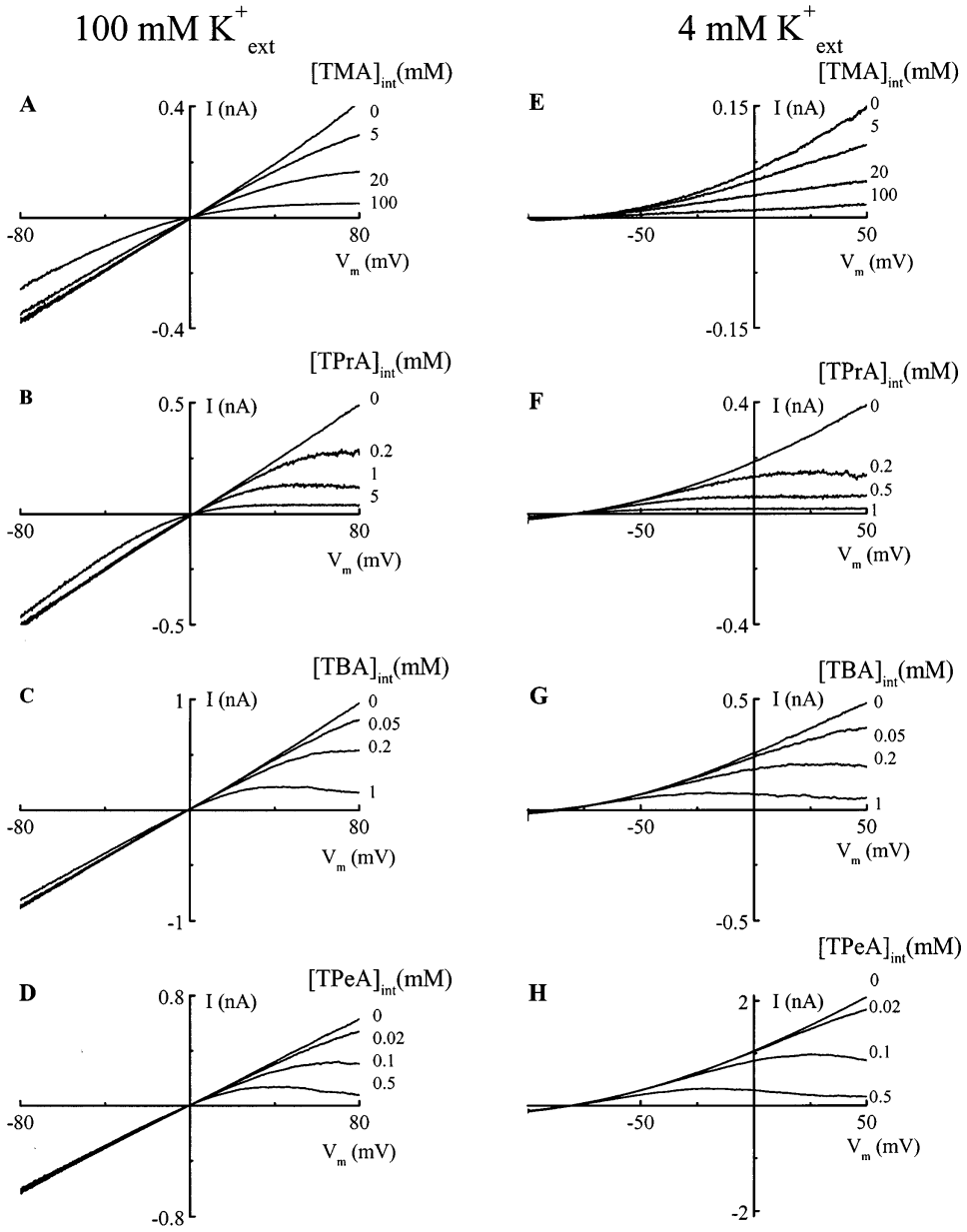


FIGURE 3. Voltage and extracellular K^+ dependence of ROMK1 channel blockade by TEA derivatives. (*A–H*) Macroscopic I-V curves recorded in various concentrations of quaternary ammoniums with either 100 mM external K^+ (*left*) or 4 mM external K^+ (*right*). TMA, TPrA, TBA, and TPeA stand for tetramethylammonium, tetrapropylammonium, tetrabutylammonium and tetrapentylammonium, respectively. $[K^+]_{int}$ was 100 mM and $[K^+]_{ext}$ was as indicated.

channel blockade by intracellular Mg^{2+}, which also produces inward rectification under physiological conditions. As in the case of quaternary ammoniums, Mg^{2+} blocked the channel with a zδ value that approached unity in symmetric 100 mM K^+. Lowering $[K^+]_{ext}$ caused significant reductions in ${}^{Mg}(z\delta)_{obs}$ and ${}^{Mg}K_{obs}$ (0 mV) (Fig. 5). Thus, the influence of $[K^+]_{ext}$ on both the observed blocker affinity and the voltage dependence of block, $(z\delta)_{obs}$, is a general phenomenon. Interestingly, the zδ values for monovalent TEA and divalent Mg^{2+} were nearly identical, not only in the presence of symmetric 100 mM K^+ but also when $[K^+]_{ext}$ was lowered to 4 mM [${}^{TEA}(z\delta)_{obs}$ = 0.86 ± 0.03 and ${}^{Mg}(z\delta)_{obs}$ = 0.85 ± 0.01 in 100 mM external K^+; ${}^{TEA}(z\delta)_{obs}$ = 0.55 ± 0.01 and ${}^{Mg}(z\delta)_{obs}$ = 0.56 ± 0.03 in 4 mM external K^+].

DISCUSSION

We studied voltage-dependent blockade of the ROMK1 channel by QAs and Mg^{2+}, and its dependence on extracellular K^+. We made two principal observations. First, in the presence of symmetric 100 mM K^+, the observed zδ values for several monovalent symmetric quaternary ammoniums and Mg^{2+} were all near unity, yet there was no sign of these blockers being permeant. Second, both the apparent affinities of all the blocking ions and their voltage dependence varied as a function

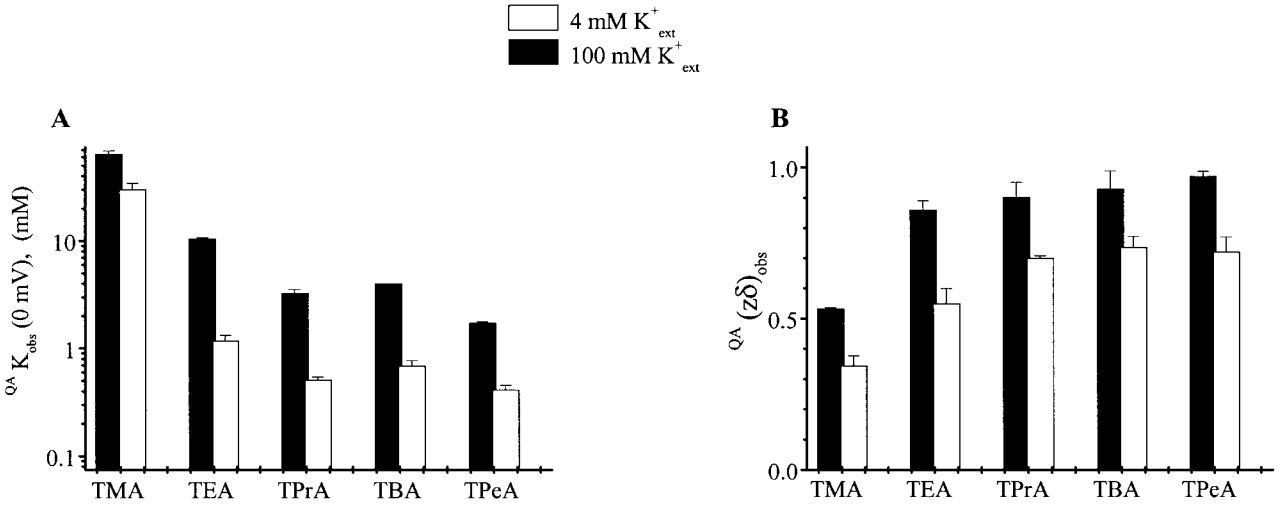


FIGURE 4. Summary of extracellular K^+ dependence of ROMK1 channel blockade by TEA and its derivatives. ${}^{QA}K_{obs}$ (0 mV) and ${}^{QA}(z\delta)_{obs}$ (mean ± SEM, $n = 5$) values obtained as described in Fig. 1.

of $[K^+]_{ext}$. Clearly, the finding of near-unity values for ${}^{QA}(z\delta)_{obs}$ cannot be interpreted in the conventional way, because it would mean that monovalent QAs with diameters of ~6–12 Å (French and Shoukimas, 1981) move across virtually the entire electrical field along a K^+-selective pore whose narrowest region is ~2–3 Å (Hille, 1992; Doyle et al., 1998). The extracellular K^+ dependence of channel blockade, however, strongly suggests an alternative explanation; namely, that the unusually large values of ${}^{QA}(z\delta)_{obs}$ result from the coupled movement of K^+ and QAs in the electrical field.

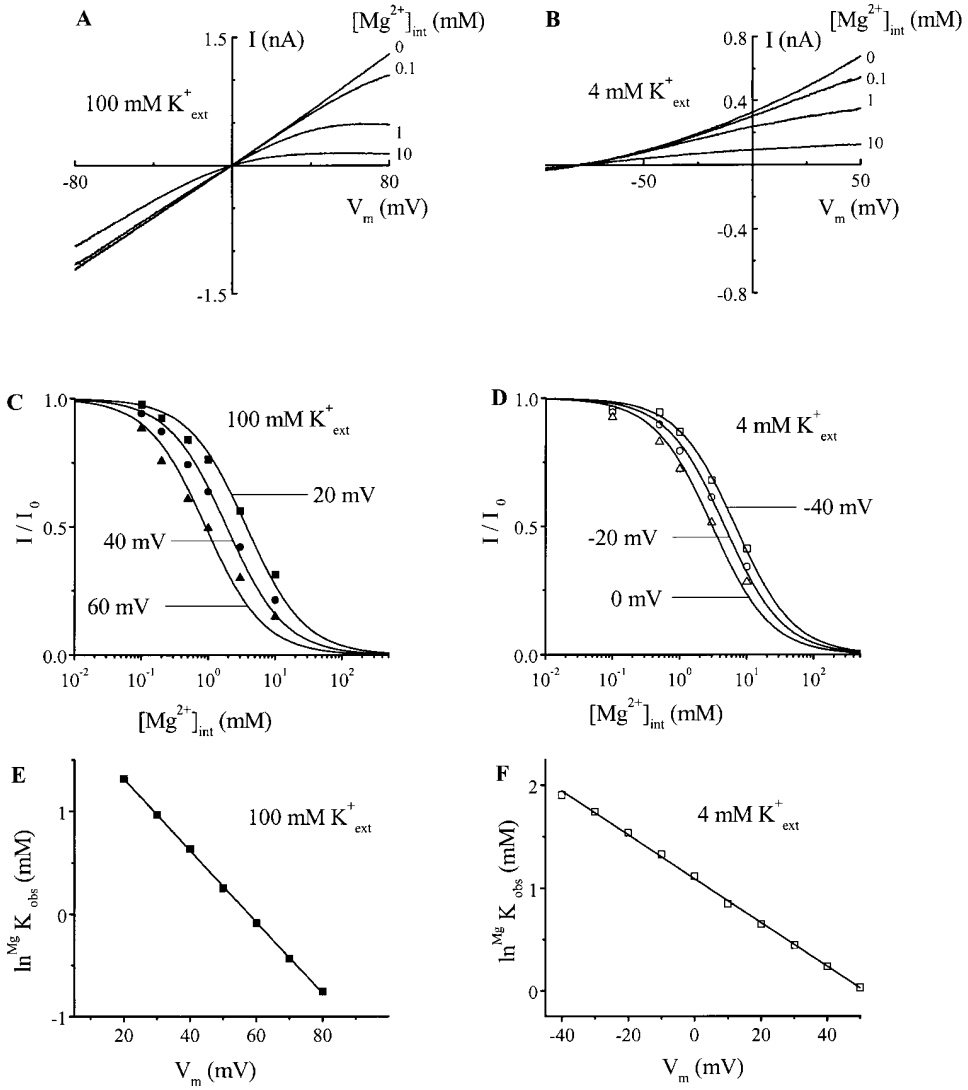
The concept of coupled ion movement was introduced by Hodgkin and Keynes (1955), who suggested that multiple K^+ ions move along a K^+ pore in a coupled manner. The concept was later extended to blocking ions. For example, in some K^+ channels the zδ value of a permeant monovalent blocking ion, Cs^+, was shown to exceed unity (Hille, 1975; Hagiwara et al., 1976; Gay and Stanfield, 1977; Adelman and French, 1978; Yellen, 1984; Cecchi et al., 1987). The excessive zδ value of Cs^+ was attributed to coupled-ion movement. French and Shoukimas (1981) also considered the possibility that the zδ value of TEA (~0.15) for block of a voltage-activated K^+ channel is inflated by an interaction between K^+ and TEA ions in the pore.

To analyze our data, we employed a two-site single-vacancy model (Kohler and Heckmann, 1979; Schumaker and MacKinnon, 1990; Lu and MacKinnon, 1994*a*); extension to more complete models is straightforward but yields cumbersome equations. We assume that an intracellular blocking ion can interact with the channel in any state (i.e., with one or the other, or both sites occupied by K^+). Whether the intracellular blocking ion (e.g., TEA) binds to the inner K^+ binding site, or a distinct site nearby, is irrelevant (Fig. 6 *A* shows that TEA binds to the inner site). In cases where the inner site is occupied by K^+, we treat TEA binding and K^+ outward movement as a "concerted" step (Armstrong and Neyton, 1992), as if TEA electrostatically knocks off the K^+ ion bound to the inner K^+ site. This is a reversal of the classic process by which external K^+ knocks off internal bound TEA, as originally proposed by Armstrong and Binstock (1965). In a fully saturated pore (both K^+ sites occupied), the binding of TEA causes the outer K^+ to be released to the external solution, resulting in transfer of a positive charge from the internal to the external side. More realistically, we can view the binding of internal TEA to the pore as the formation of a transition state in which TEA destabilizes K^+ bound at the inner site and causes it to move. If the transition state is very unstable, we can lump TEA binding and K^+ movement into a single step.

Assuming furthermore that the inner K^+ site is nearly always occupied (i.e., is saturated; see discussion below), the state diagram can be simplified further (Fig. 6 *B*). The model in Fig. 6 *B* clearly illustrates that internal TEA and external K^+ compete for the pore with inner K^+ site occupied, compatible with the experimental observation that ${}^{TEA}K_{obs}$ (0 mV) depends linearly on $[K^+]_{ext}$ (Fig. 2 *A*):

$$ {}^{TEA}K_{obs} = \frac{{}^{TEA}K_{us}}{{}^{K}K_{ext}}[K^+]_{ext} + {}^{TEA}K_{us}, \qquad (5) $$

(see METHODS for derivation of Eq. 5). In the model (Fig. 6 *B*), ${}^{TEA}K_s$ equals the ratio of ${}^{TEA}K_{us}$ to ${}^{K}K_{ext}$. Substituting ${}^{TEA}K_s$ into Eq. 5, we have



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	$^{Mg}K_{obs}$ (mM)	$^{Mg}(z\delta)_{obs}$	n
4 mM K^+	2.5±0.5	0.56±0.03	5
100 mM K^+	9.3±1.5	0.85±0.01	5

FIGURE 5. Voltage and extracellular K^+ dependence of ROMK1 channel blockade by internal Mg^{2+}. (*A* and *B*) Macroscopic I-V curves of ROMK1 channels recorded in various $[Mg^{2+}]_{int}$. $[K^+]_{int}$ was 100 mM and $[K^+]_{ext}$ was as indicated in the figure. (*C* and *D*) Fractions of unblocked currents (I/I_o) obtained from *A* and *B* were plotted against $[Mg^{2+}]_{int}$ for several membrane voltages. Curves superimposed on the data correspond to least-squares fits using $I/I_0 = {}^{Mg}K_{obs}/({}^{Mg}K_{obs} + [Mg^{2+}]_{int})$. (*E* and *F*) $\ln {}^{Mg}K_{obs}$ obtained from *C* and *D* were plotted against membrane voltage (V_m). The lines superimposed on the data correspond to least-squares fits using the Woodhull equation, $\ln {}^{Mg}K_{obs} = \ln {}^{Mg}K_{obs}(0\text{ mV}) - {}^{Mg}(z\delta)_{obs}FV_m/RT$. (*G*) ${}^{Mg}K\ (0\text{ mV})_{obs}$ and ${}^{Mg}(z\delta)_{obs}$ (mean ± SEM, $n = 5$) for both $[K^+]_{ext}$.

$$^{TEA}K_{obs} = {}^{TEA}K_s[K^+]_{ext} + {}^{TEA}K_{us}. \quad (6)$$

Based on Eqs. 5 and 6, we determined $^{TEA}K_s$ (slope), $^{TEA}K_{us}$ (intercept), and $^{K}K_{ext}$ (intercept/slope) at various membrane voltages from the plot in Fig. 6 *C*. We then analyzed their voltage dependence using the Woodhull equation. The analyses yield $^{TEA}K_s\ (0\text{ mV}) = 0.1$, $^{TEA}(z\delta)_s = 1.00$; $^{TEA}K_{us}\ (0\text{ mV}) = 1.0$ mM, $^{TEA}(z\delta)_{us} = 0.42$; $^{K}K_{ext}\ (0\text{ mV}) = 9.3$ mM, $^{K}(z\delta)_{ext} = -0.58$.

From these results, we draw the following conclusions. First, TEA binding to a K^+-saturated pore results in the transfer of one positive charge across the entire electrical field [$^{TEA}(z\delta)_s = 1$]. Second, the external K^+ binding site is located about halfway through the electrical field [$^{K}(z\delta)_{ext} = -0.58$]. Third, the upper limit for the fraction of the electric field traversed by TEA is ~50% [$^{TEA}(z\delta)_{us} = 0.42$]. The portions of $^{TEA}(z\delta)_{us}$ related to the movement of K^+ versus the movement of TEA in the electrical field are not known. Theoretically, TEA may not even bind within the narrow region of the pore to sense a voltage drop across the membrane. In this case, the voltage dependence of block by

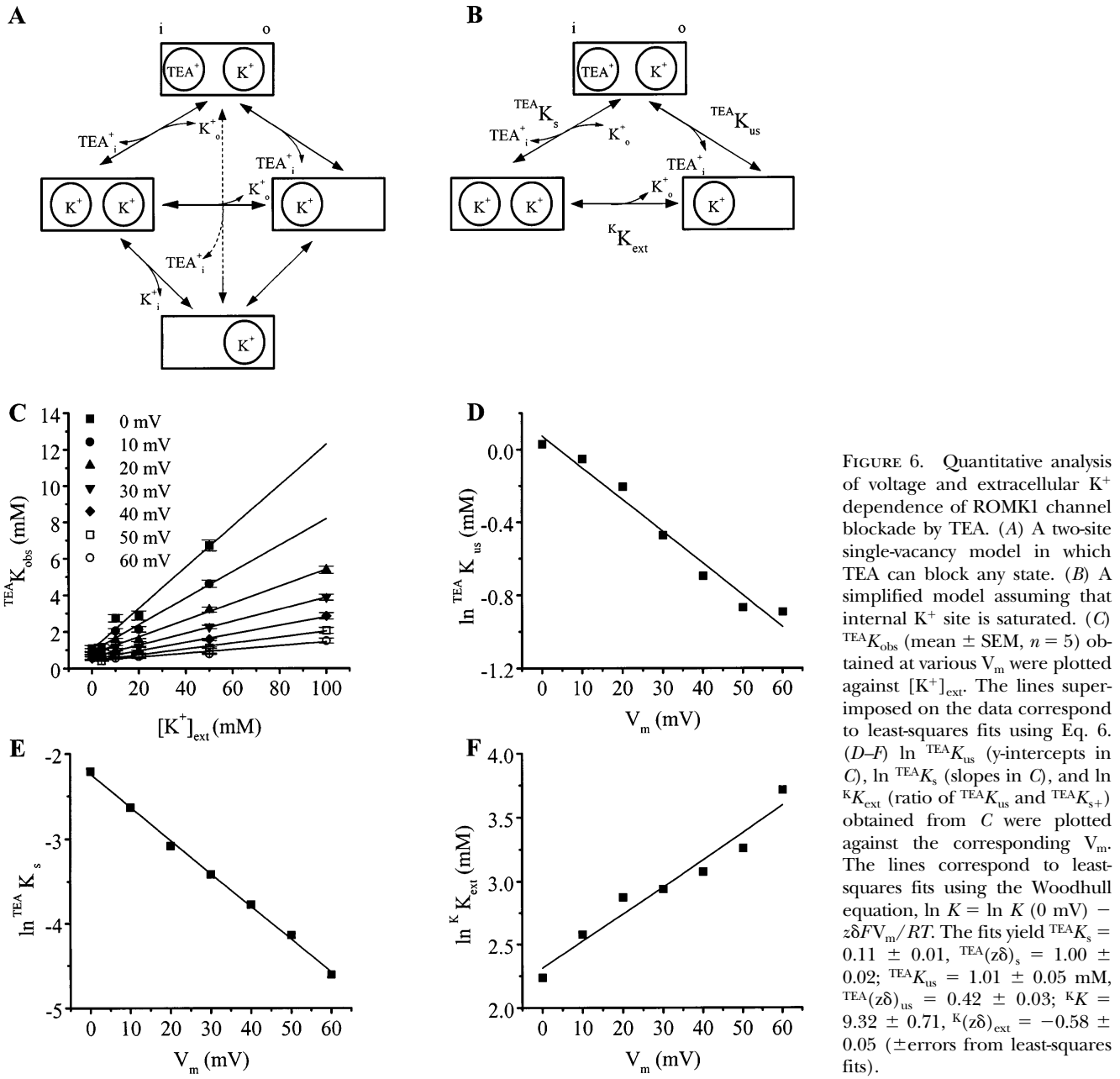

FIGURE 6. Quantitative analysis of voltage and extracellular K^+ dependence of ROMK1 channel blockade by TEA. (*A*) A two-site single-vacancy model in which TEA can block any state. (*B*) A simplified model assuming that internal K^+ site is saturated. (*C*) $^{TEA}K_{obs}$ (mean ± SEM, $n = 5$) obtained at various V_m were plotted against $[K^+]_{ext}$. The lines superimposed on the data correspond to least-squares fits using Eq. 6. (*D–F*) ln $^{TEA}K_{us}$ (y-intercepts in *C*), ln $^{TEA}K_s$ (slopes in *C*), and ln $^{K}K_{ext}$ (ratio of $^{TEA}K_{us}$ and $^{TEA}K_{s+}$) obtained from *C* were plotted against the corresponding V_m. The lines correspond to least-squares fits using the Woodhull equation, $\ln K = \ln K(0\text{ mV}) - z\delta FV_m/RT$. The fits yield $^{TEA}K_s$ = 0.11 ± 0.01, $^{TEA}(z\delta)_s$ = 1.00 ± 0.02; $^{TEA}K_{us}$ = 1.01 ± 0.05 mM, $^{TEA}(z\delta)_{us}$ = 0.42 ± 0.03; ^{K}K = 9.32 ± 0.71, $^{K}(z\delta)_{ext}$ = −0.58 ± 0.05 (±errors from least-squares fits).

TEA might completely result from the movement of K^+ ions in the electrical field.

Locating the QA binding site outside the membrane electrical field would nicely explain why QAs of very different size blocked the ROMK1 channel with very similar voltage dependence. Moreover, if the QAs and Mg^{2+} bind to the same site outside the membrane electrical field, it would be easy to understand why the voltage dependence of channel blockade by these ions of different size and different (actual) valence is nearly identical. This scenario is compatible with the result of a recent crystallographic study on a bacterial K^+ channel (Doyle et al., 1998), which shows that the pore has a large cavity, internal to the narrow ion selectivity filter. Results from studies in voltage-activated K^+ channels are highly compatible with quaternary ammoniums binding in the cavity (Armstrong, 1971; Holmgren et al., 1997; Liu et al., 1997). It is interesting to note that a cavity-lining residue, Phe 103, in the bacterial channel corresponds to residue Asn 171 in the ROMK1 channel (Doyle et al., 1998). Asn 171 in the ROMK1 channel is known to interact with Mg^{2+} (Lu and MacKinnon,

1994*b*, 1995). Therefore, it is very likely that both TEA and Mg^{2+} bind in the large internal cavity.

In our analysis, we assume that the internal K^+ site is largely saturated. Is this valid? Single-channel I-V curves for the ROMK1 channel were shown to be linear over a wide range of $[K^+]$ (10 mM–1 M; $[K^+]_{int} = [K^+]_{ext}$) (Lu and MacKinnon, 1994*a*), arguing that the internal and external K^+ sites likely have similar affinities (i.e., ${}^{K}K_{ext} = {}^{K}K_{int} \approx 9.3$ mM). In the present study, the internal site should then be >90% saturated when $[K^+]_{int}$ is 100 mM. For the more complete model in Fig. 6 *A*, the slope of a plot of ${}^{TEA}K_{obs}$ versus $[K^+]_{ext}$ is described by the equation:

$$\text{slope} = \frac{{}^{TEA}K_{us}}{{}^{K}K_{ext}}\left(1 + \frac{{}^{K}K_{int}}{[K^+]_{int}}\right). \quad (7)$$

Under the conditions that ${}^{K}K_{int} = {}^{K}K_{ext}$ and $[K^+]_{int} =$ 100 mM, ${}^{K}K_{ext}$ (= ${}^{K}K_{int}$) should then be slightly increased from 9.3 to 10.1 mM.

K^+ dissociation constant (${}^{K}K$) values between 20 μM and 2 mM have been reported for Ca^{2+}- and voltage-activated K^+ channels (Neyton and Miller, 1988; Baukrowitz and Yellen, 1996; for Rb^+ see Armstrong et al., 1982). The discrepancy between ${}^{K}K$ values estimated in these previous studies and those presented here may in part be due to the fact that we examined the K^+ site in the presence of other K^+ ions in the pore. Previous studies of $[K^+]$ dependence of single-channel conductance in the ROMK1 channel suggested that ${}^{K}K$ was likely ~17 mM, when the data were analyzed using the same two-site single-vacancy model (Lu and MacKinnon, 1994*a*). Thus, a ${}^{K}K$ value of 10 mM estimated here can account for the single-channel conductance.

To derive an expression (Eq. 4) that describes the dependence of ${}^{TEA}K_{obs}$ on both membrane potential and K^+ equilibrium potential, we combined the Nernst equation, the Woodhull equation, and Eq. 6:

$${}^{TEA}K_{obs} = {}^{TEA}K_s(0\text{ mV})\,[K^+]_{int}\,e^{-\frac{F}{RT}\{{}^{TEA}(z\delta)_s V_m - {}^{K}zE_K\}} + {}^{TEA}K_{us}(0\text{ mV})\,e^{-\frac{F}{RT}{}^{TEA}(z\delta)_{us}V_m}, \quad (8)$$

where E_K and ${}^{K}z$ stand for equilibrium potential and valence of K^+, respectively. Other symbols either have been previously defined or have their usual meaning.

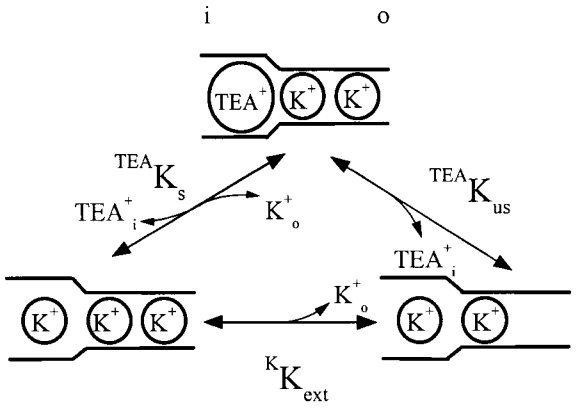

FIGURE 7. A model for TEA binding to a pore with three K^+ sites. The model, modified from the model in Fig. 6 *B*, has two K^+ sites in the narrow region of the pore and a third site in a wider internal region where either K^+ or TEA can bind.

Generally, the first term in Eq. 8 describes TEA binding to a pore saturated with K^+, whereas the second term describes TEA binding to an unsaturated pore. (Modifications and expansions of each term may be needed if there are more than two K^+ sites.) It is interesting to note that since ${}^{TEA}(z\delta)_s$ and ${}^{K}z$ are unity, the first term in Eq. 8 is equivalent to a classic empirical description for inward rectification: at constant $[K^+]_{int}$, the binding affinity of blocking ions, and thus the degree of rectification, changes as a function of electrochemical potential for K^+ ($V_m - E_K$) (Katz, 1949; Hodgkin and Horowicz, 1959; Hagiwara and Takahashi, 1974; Hille and Schwarz, 1978; Hille, 1992).

We started our data analysis using a two-site model as shown in Fig. 6. However, in reality the ROMK1 pore may contain more than two K^+ ions. For example, the recent crystallographic study shows that the outer narrow part of a bacterial K^+ pore contains two permeating ions and the inner cavity contains an additional one (Doyle et al., 1998). If the ROMK1 pore similarly contains three ions, the state diagram in Fig. 6 *B* should be modified to what is shown in Fig 7. The addition of an extra ion in the state diagram does not change the fundamental meaning of ${}^{TEA}K_s$, ${}^{TEA}K_{us}$, or ${}^{K}K_{ext}$. Eq. 8 can still be used to describe the relation between ${}^{TEA}K_{obs}$ and $[K^+]_{ext}$, as well as ${}^{TEA}K_{obs}$'s dependence on membrane voltage.

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