Affinity and Location of an Internal K$^+$ Ion Binding Site in *Shaker* K Channels

JILL THOMPSON AND TED BEGENISICH

From the Department of Pharmacology and Physiology, University of Rochester Medical Center, Rochester, New York 14642

ABSTRACT We have examined the interaction between TEA and K$^+$ ions in the pore of *Shaker* potassium channels. We found that the ability of external TEA to antagonize block of *Shaker* channels by internal TEA depended on internal K$^+$ ions. In contrast, this antagonism was independent of external K$^+$ concentrations between 0.2 and 40 mM. The external TEA antagonism of internal TEA block increased linearly with the concentration of internal K$^+$ ions. In addition, block by external TEA was significantly enhanced by increases in the internal K$^+$ concentration. These results suggested that external TEA ions do not directly antagonize internal TEA, but rather promote increased occupancy of an internal K$^+$ site by inhibiting the emptying of that site to the external side of the pore. We found this mechanism to be quantitatively consistent with the results and revealed an intrinsic affinity of the site for K$^+$ ions near 65 mM located ∼7% into the membrane electric field from the internal end of the pore. We also found that the voltage dependence of block by internal TEA was influenced by internal K$^+$ ions. The TEA site (at 0 internal K$^+$) appeared to sense ∼5% of the field from the internal end of the pore (essentially colocalized with the internal K$^+$ site). These results lead to a refined picture of the number and location of ion binding sites at the inner end of the pore in *Shaker* K channels.

KEY WORDS: ion channels • ion permeation • voltage-clamp • tetraethylammonium

INTRODUCTION

The permeation and selectivity properties of K channels are quite complex. These channels can simultaneously be occupied by several ions and they support high flux rates in spite of the presence in the pore of high affinity permeant ion binding sites.

There are several mechanisms that could allow both tight binding and high throughput. As first suggested for Ca channels (Almers and McClesky, 1984; Hess and Tsien, 1984), a tightly bound permeant ion could be destabilized by electrostatic repulsion between it and another permeant ion entering the pore. This mechanism is certainly intuitive but is also reasonable since permeant ions have been localized to no more than ∼7.5 Å apart in the bacterial K channel, KcsA (Doyle et al., 1998). In addition, K$^+$ ions applied to one side of the pore increase the apparent off-rate for blocking ions at the other (Yellen, 1984; Neyton and Miller, 1988b; Hurst et al., 1996; Harris et al., 1998) as if one repels the other. Another way to provide flux in the face of tight binding is the release of a bound ion by a conformational change induced by a newly entering ion. Since K channels (especially maxi-K channels) pass >10^8 ions/s, any such conformational change operating during ion permeation must do so well within 10 ns. Nevertheless,

there are properties of some K channels that may be a result of conformational changes induced by ion binding, especially by binding of blocking ions (Immke et al., 1999; Spassova and Lu, 1999; Wood and Korn, 2000). Finally, Dang and McCleskey (1998) showed that the problem of high affinity binding could be overcome if sites of low affinity flank the high affinity one. In this view, permeation occurs in a series of steps that increase the flux out of the high affinity site. Studies of Maxi-K and *Shaker* K channels have revealed the presence of both high affinity and low affinity K ion binding sites (Neyton and Miller, 1988a,b; Hurst et al., 1995, 1996; Baukrowitz and Yellen, 1996; Harris et al., 1998; Vergara et al., 1999). Kiss et al. (1998) and Stampe et al. (1998) showed that models based on the "stepwise affinity" mechanism are quantitatively consistent with many of the permeation properties of K channels.

These mechanisms are not necessarily mutually exclusive, and sorting out under what conditions and in what channels they operate is a daunting task that will require specific tests of these mechanisms. At the very least, a full accounting of the number, affinity, and location of the permeant ion binding sites must be made. As a first approach to this end, we (Thompson and Begenisich, 2000) recently investigated the antagonism between TEA ions in *Shaker* K channels and showed that electrostatic repulsion is unlikely to occur over the distance between the external and internal TEA binding sites. In the present study, we found that the apparent interaction between external and internal TEA ions required
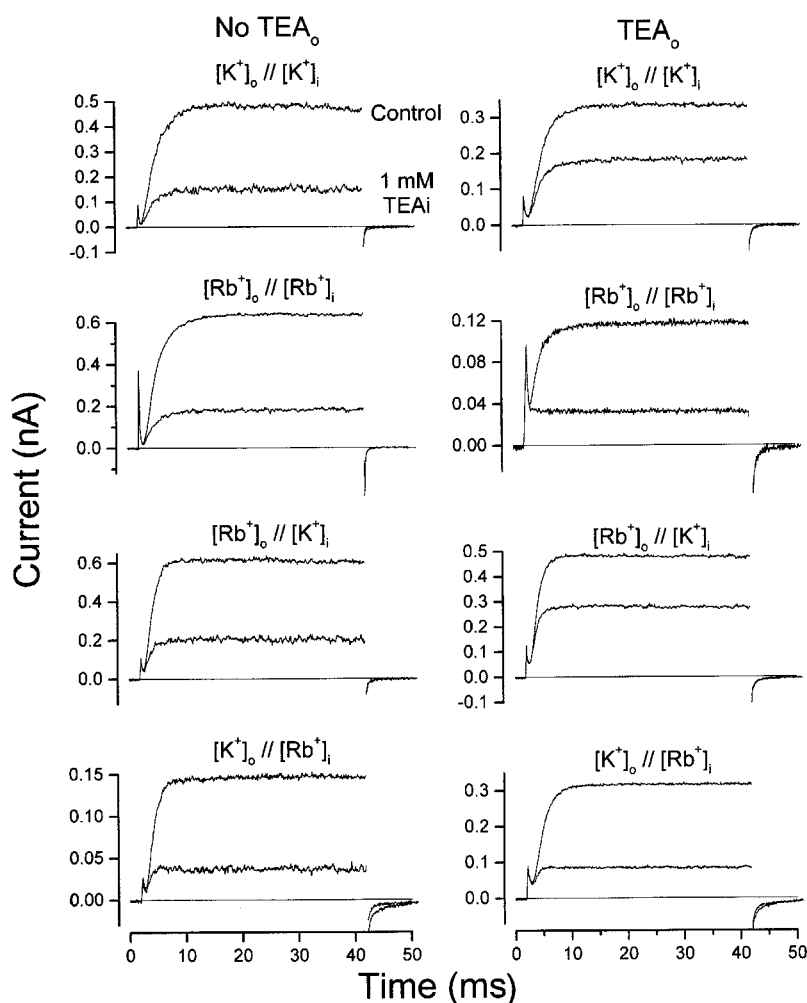
FIGURE 1. Only internal K^+ ions allow external TEA to protect from internal TEA block. Currents recorded at 0 mV in the absence (Control) and in the presence of 1 mM internal TEA (smaller current in each panel, marked 1 mM TEA$_i$ in the first panel) without (left column) and with (right column) 100 mM external TEA. External and internal concentrations of the indicated ions were 5 and 135 mM, respectively.

the presence of K^+ ions on the inner side of the pore. The results support a mechanism for the apparent antagonism between TEA ions that involves a competition between internal K^+ ions and internal TEA for a site in the pore. Our analysis indicates that the affinity of this site for K^+ ions is near 65 mM. We also found that the apparent location of the internal TEA site in the membrane electric field was influenced by internal K^+ ions. The intrinsic location of this site (extrapolated to 0 internal K^+) was near 5% of the way into the field from the internal end of the pore. We found the location of the K^+ binding site to be near 7% into the field from the inside, which is essentially colocalized with the internal TEA site. These results lead to a refined picture of the number and location of ion binding sites at the inner end of the pore in *Shaker* K channels.

MATERIALS AND METHODS

K Channel Constructs

The experiments reported here were done on the inactivation-deletion version of *Shaker* B, ShB Δ6-46 (Hoshi et al., 1990).

Oocyte Isolation and Microinjection

Frogs, *Xenopus laevis*, were maintained as described by Goldin (1992). Isolated ovarian lobes were rinsed with calcium-free OR-2 solution (82.5 mM NaCl, 2.5 mM KCl, 1 mM MgCl and 5 mM HEPES, pH 7.6 with NaOH) and defolliculated by incubation for 60–90 min with 2 mg/ml collagenase Type IA (Sigma-Aldrich). Cleaned oocytes were transferred and maintained for 2 h in ND-96 solution (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl, 1 mM MgCl, 5 mM HEPES, and 2.5 mM sodium pyruvate, pH 7.6 with NaOH) before injection of RNA coding for the channel of interest. Injected oocytes were transferred to multiwell tissue culture plates and incubated at 18°C in ND-96 solution supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin.

Electrophysiological Recordings

Potassium channel currents were assayed electrophysiologically 1–5 d after RNA injection. Electrophysiological recordings were done at room temperature (20–22°C) with excised inside-out or outside-out macropatches using an Axopatch 1-D amplifier (Axon Instruments). Patch pipets had tip diameters of ∼2–4 μm constructed from glass (Corning 7052; Garner Glass Co.). The measured junction potentials for the solutions used were all within 4 mV of one another and so no correction for these was applied. The holding potential was −70 mV in most cases; however, to minimize the amount of slow inactivation in solutions of
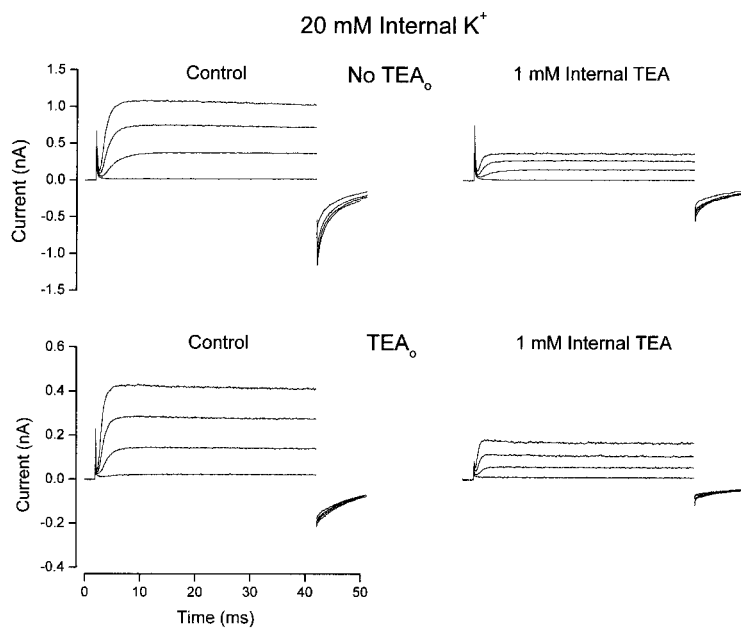
FIGURE 2. Effects of internal TEA in the presence and absence of external TEA with 20 mM intracellular K^+ ions. Currents recorded at -30, -10, $+10$, and $+30$ mV (smallest to largest in each panel) in the absence (left column) and the presence (right column) of 1 mM internal TEA without (top row) and with (bottom row) 100 mM external TEA.

reduced K^+ (Baukrowitz and Yellen, 1995, 1996), we used a holding potential of -90 mV. Data acquisition was performed using a 12-bit analogue/digital converter controlled by a personal computer. Current records were filtered at 5 kHz.

The standard external solution contained the following (in mM): 5 KCl, 135 NMDG-Cl, 2 $CaCl_2$, 2 $MgCl_2$, and 10 mM HEPES, pH 7.2 (with NMDG). TEA was added to this solution by equimolar replacement of NMDG. The standard internal solution consisted of the following (in mM): 110 KCl, 25 KOH, 10 EGTA, and 10 HEPES, pH 7.2 (with HCl). In some experiments, Rb^+ completely replaced the K^+ in these solutions. NMDG replaced K^+ in solutions used for experiments with reduced K^+ content.

Data Analysis

Dose-response relations for external and internal TEA block were determined from at least three independent measurements at four or more concentrations of blockers. As described in RESULTS, several different equations were fit to these and other data. The fits were done using the Levenberg-Marquardt algorithm as implemented in Origin 5.0 (Microcal Software Inc.). Error limits for the fitted parameters are the estimated errors from the fitting routine.

R E S U L T S

External TEA ions inhibit the ability of internal TEA to block the pore in K channels (Newland et al., 1992). This is illustrated by the top row of records in Fig. 1. On the left are currents recorded at 0 mV in the absence and presence (smaller current) of 1 mM internal TEA. In this experiment, this concentration of internal TEA blocked 69% of the control current. In the presence of external TEA (Fig. 1, right), this same concentration of internal TEA blocked only 46% of the current.

We (Thompson and Begenisich, 2000) previously showed that the ability of external TEA to protect from block by internal TEA was lost if K^+ ions in both the internal and external solutions were replaced by Rb^+. This is illustrated in the second row of currents in Fig.

1. Channel current was blocked by 1 mM TEA by about the same amount (72 and 74% in this experiment) independent of external TEA.

Internal K^+ Ions Are Required for TEA Interactions

To determine if K^+ ions are required in the internal or external solution (or both), we tested the ability of external TEA to protect from internal TEA block by replacing K^+ with Rb^+ in each solution. With K^+ ions only in the internal solution (Fig. 1, third row), external TEA was able to protect from block by internal TEA (66 vs. 42% block). In contrast, external TEA was not able to protect from block by internal TEA if K^+ ions were only in the external solution (Fig. 1, bottom row, 74 and 73% block).

Thus, K^+ but not Rb^+, ions in the internal solution supported the external TEA-mediated protection from block by internal TEA. The data in Fig. 2 confirm this suggestion showing a greatly reduced protection from internal TEA block by external TEA when the intracellular K^+ concentration is reduced. The top row in Fig. 2 contains currents recorded with 20 mM internal K^+ at several potentials in the absence (Fig. 2, left panel) and presence (Fig. 2, right panel) of 1 mM internal TEA. The block at 0 mV was 62% in this experiment. The bottom row shows currents recorded at the same potentials in the presence of 100 mM external TEA. Unlike the protection afforded by external TEA in 135 mM internal K^+ (Fig. 1, top row), internal TEA block was the same in the presence of external TEA (62% block at 0 mV, independent of the external TEA).

The top panel of Fig. 3 A illustrates the fraction of current blocked by internal TEA in the absence ($\blacksquare$) and presence ($\bigcirc$) of external TEA with 20 mM internal
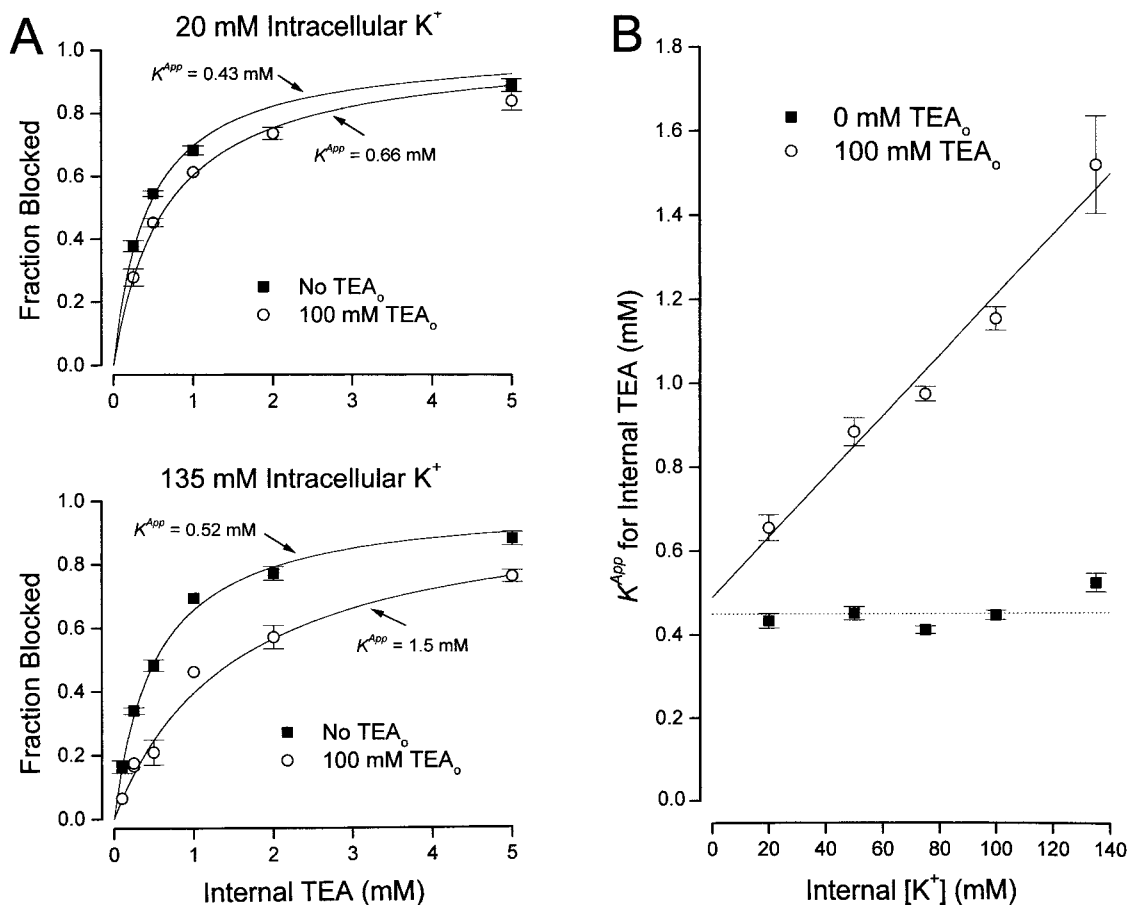
FIGURE 3. Actions of external TEA on block by internal TEA with different concentrations of intracellular K^+. (A, top) Fraction of current blocked (at 0 mV) by the indicated internal TEA concentrations in a 20-mM internal K^+ solution without (■) and with (○) 100 mM external TEA. (A, bottom) Fraction of current blocked (at 0 mV) by the indicated internal TEA concentrations in a 135-mM internal K^+ solution without (■) and with (○) 100 mM external TEA. Includes data from Thompson and Begenisich (2000). (lines) Fits of Eq. 1 to the data with the indicated K^{App} values: 0.43 ± 0.017 mM and 0.66 ± 0.031 mM in the absence and presence of 100 mM TEA_o with 20 mM internal K^+ and 0.52 ± 0.022 mM (0 TEA_o) and 1.5 ± 0.12 mM (100 mM TEA_o) in 135 mM K^+. (B) K^{App} (at 0 mV) for block by internal TEA (obtained as in A) as a function of the concentration of internal K^+ ions without (■) and with (○) 100 mM external TEA. The broken line represents the mean K^{App} value (0.45 mM) obtained in the absence of external TEA. The solid line is the fit of Eq. 3 to the data with 100 mM external TEA with values for K_o and K_K of 0.49 ± 0.06 and 68 ± 14 mM, respectively.

K^+. The fit to the pooled data of a standard binding relation (see Eq. 1) reveals an apparent affinity for internal TEA block of 0.43 ± 0.017 mM. The presence of external TEA produced a modest decrease in the apparent internal TEA affinity represented by the somewhat increased K^{App} value of 0.66 ± 0.031 mM. As shown in the bottom panel of Fig. 3 A, this same concentration of external TEA produced a threefold decrease in the internal TEA affinity if the internal solution contained 135 mM K^+.

We obtained data similar to those illustrated in Fig. 3 A over a range of internal K^+ concentrations, and the results are contained in Fig. 3 B. Shown is the apparent affinity for internal TEA block (K^{App}) at internal K^+ concentrations between 20 and 135 mM, both in the absence (■) and presence (○) of external TEA. Without external TEA, block by internal TEA is essentially independent of the internal K^+ concentration with a mean value of 0.45

mM (Fig. 3 B, dashed line). In contrast, in the presence of external TEA, the apparent affinity for internal TEA block is a linear function of the internal K^+ concentration.

Thus, internal K^+ ions had a significant effect on the interaction between internal and external TEA. The records of Fig. 1 suggest that, in contrast, external K^+ ions had little effect on the interaction between TEA ions. This was confirmed by determining the apparent affinity for internal TEA block in the absence and presence of 100 mM external TEA in solutions with 0.2 and 40 mM external K^+ in addition to the standard 5-mM amount. These results are summarized in Table I. The apparent affinity for internal TEA block was little affected by external K^+ ions, with or without external TEA. We also determined the apparent affinity for external TEA block with and without internal TEA and, again, these were little affected by external K^+.

TABLE I
External K⁺ and TEA Block

Ext. K⁺	K^{App}	K_i^{App} (TEA$_o$)	K_o^{App}	K_o^{App} (TEA$_i$)
mM	*mM*	*mM*	*mM*	*mM*
0.2	0.41 ± 0.006	1.1 ± 0.035	16 ± 1.2	33 ± 2.6
5	0.52 ± 0.022	1.5 ± 0.12	15 ± 0.89	39 ± 2.3
40	0.63 ± 0.028	1.5 ± 0.035	17 ± 1.2	34 ± 2.4

Apparent affinity for internal TEA block (K_i^{App}) determined as in Fig. 3 with 135 mM internal K⁺. K_i^{App} (TEA$_o$) is the apparent affinity for internal TEA block with 100 mM external TEA. Apparent affinity for external TEA block, K_o^{App}, determined similarly; K_o^{App} (TEA$_i$) is the apparent affinity for external TEA block in 2 mM internal TEA.

Competition between Internal TEA and Internal K⁺ Ions: A Model

In the presence of external TEA, there was a linear relation between the apparent affinity for internal TEA block and internal K⁺ concentration (Fig. 3 B, ○). This suggests a competition between internal K⁺, and TEA that was apparent only when the channel was blocked by TEA. Thus, we consider the existence of a site in the pore of *Shaker* K channels accessible to both internal TEA and internal K⁺ ions and that the binding of these ions to this site is mutually exclusive. In the absence of external TEA, this site is rarely occupied by K⁺ ions because they readily move through the pore and exit on the external side. Under these conditions, internal TEA is able to block the pore. However, when the external TEA binding site is occupied, the flow of K⁺ ions to the outside is eliminated and there is now a true thermodynamic equilibrium between internal K⁺ ions and the inner part of the pore. Since, in this scheme, occupancy of this site by internal TEA and K⁺ is mutually exclusive, internal TEA will be less effective in the presence of external TEA. Further, occupancy of the internal site by K⁺ ions will be decreased in reduced internal K⁺ and so block by internal TEA under these conditions would not be antagonized by external TEA.

To determine if channels with this type of site can quantitatively reproduce the experimental findings, it is necessary to derive the expectations for how the apparent affinity for internal TEA depends on internal K⁺ and external TEA. Thus, consider that internal TEA ions bind to the channel with an intrinsic affinity of K_i according to the reaction:

$$\text{TEA}_i + \text{channel} \xleftarrow{\quad K_i \quad} \text{TEA}_i\text{-channel}$$

(SCHEME 1)
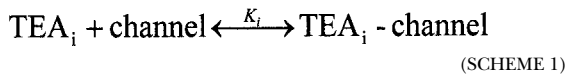
and that external TEA can also bind to the channel with an intrinsic affinity, K_o:

$$\text{TEA}_o + \text{channel} \xleftarrow{\quad K_o \quad} \text{TEA}_o\text{-channel}$$
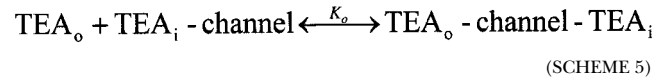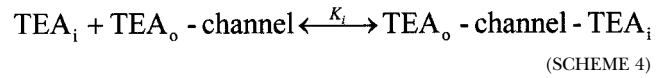
(SCHEME 2)
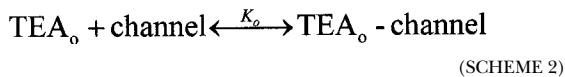
As discussed above, we consider that when external TEA occupies its binding site and shuts off K⁺ efflux, internal K⁺ ions can occupy a site in the pore:

$$\text{K}_i^+ + \text{TEA}_o\text{-channel} \xleftarrow{\quad K_K \quad} \text{TEA}_o\text{-channel-K}^+$$

(SCHEME 3)
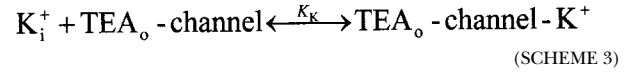
where K_i^+ represents an internal K⁺ ion, and K_K the affinity for binding internal K⁺ to the site. In this simple model, internal TEA ions cannot bind to channels occupied by K⁺ ions nor can internal K⁺ ions bind to channels already occupied by internal TEA. Of course K channels have several K⁺ binding sites, some of very high affinity (Neyton and Miller, 1988a,b; Hurst et al., 1995; Baukrowitz and Yellen, 1996; Starkus et al., 1997; Harris et al., 1998). The site in this model is defined as one that is significantly occupied only when in true equilibrium with the internal K⁺ solution and that binds internal TEA and K⁺ ions in a mutually exclusive manner.

In this model, external and internal TEA are considered to bind independently, and so the only remaining reactions to consider are the following:

$$\text{TEA}_i + \text{TEA}_o\text{-channel} \xleftarrow{\quad K_i \quad} \text{TEA}_o\text{-channel-TEA}_i$$

(SCHEME 4)

$$\text{TEA}_o + \text{TEA}_i\text{-channel} \xleftarrow{\quad K_o \quad} \text{TEA}_o\text{-channel-TEA}_i$$

(SCHEME 5)

With these reactions, the block of channels by internal TEA in the presence of external TEA is given by:

$$\text{Fraction blocked} = \frac{\text{TEA}_i}{\text{TEA}_i + K_i^{App}}, \qquad (1)$$

where K_i^{App} is given by:

$$K_i^{App} = K_i \frac{1 + \dfrac{\text{TEA}_o}{K_o}\left(1 + \dfrac{[\text{K}^+]_i}{K_K}\right)}{1 + \dfrac{\text{TEA}_o}{K_o}}. \qquad (2)$$

Affinity of a Binding Site for Internal K⁺ Ions

Under conditions where the external TEA concentration is much larger than its intrinsic affinity Eq. 2 reduces to the simple form:

$$K_i^{App} = K_i\left(1 + \frac{[\text{K}^+]_i}{K_K}\right). \qquad (3)$$

That is, with high concentrations of external TEA, the apparent affinity for internal TEA block of the channel is expected to be a linear function of internal K⁺ concentration with a zero K⁺ intercept equal to the intrinsic affinity (K_i) and a slope that is the ratio of K_i and K_K. As noted above, the experimental data (Fig. 3 B, ○) have this linear relationship. Eq. 3 provides a reason-

able description of the data (Fig. 3 B, solid line) with values of K_i and K_K of 0.49 ± 0.06 and 68 ± 14 mM, respectively. The intrinsic affinity for internal TEA determined this way (0.49 mM) is consistent with the mean value of the affinity obtained in the absence of external TEA (0.45 mM; Fig. 3 B, dotted line).

This simple model is obviously quantitatively consistent with the data and allows a simple understanding of the mechanism by which external TEA ions inhibit block by internal TEA. In the absence of external TEA, an inner K^+ ion binding site is very rarely occupied even at physiological (135 mM) internal K^+ concentrations. Under these conditions, there is steady-state occupancy of K^+ sites in the pore, but there is no thermodynamic equilibrium with K^+ ions in the internal solution. Thus, under these conditions, there is little interaction between internal K^+ and internal TEA ions.[1] However, when the external TEA binding site is occupied, the flow of K ions to the outside is eliminated and there is now a true thermodynamic equilibrium between internal K^+ ions and the inner part of the pore. If, in the presence of external TEA, there is only a single accessible site, then this site has an affinity for K^+ ions of ∼68 mM.

Additional Tests of the Model

If this picture of the inner part of the pore is correct, the interaction of external TEA with the pore will not be independent of internal K^+ (Scheme 3). The presence of internal K^+ ions, in essence, adds an additional blocked state and will increase the apparent affinity for block by external TEA, K_o^{App}. Solving Schemes 2 and 3 reveals the relationship between K_o^{App} and the internal K^+ concentration:

$$K_i^{App} = \frac{K_o}{1 + \frac{[K^+]_i}{K_K}}, \tag{4}$$

where, as above, $[K^+]_i$ and K_K represent the internal K^+ concentration and the affinity of the K^+ site, respectively. That is, if this view of the TEA and K^+ binding sites is correct, then external TEA should appear to have a higher affinity for its receptor at increased internal K^+ ion concentrations.

Fig. 4 shows that this prediction was realized. The apparent affinity for block by external TEA increased with internal K^+. Eq. 4 provided a reasonable description of these data with an affinity for the K^+ ion binding site of 64 ± 12 mM, which is consistent with the value of 68 mM obtained from the analysis of the data in Fig. 3 B. According to this analysis, the intrinsic affinity of the external TEA site (the zero concentration asymptote) is

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[1]However, there is some interaction since we show (Fig. 6) that the voltage dependence of internal TEA block depends on the internal K^+ concentration.
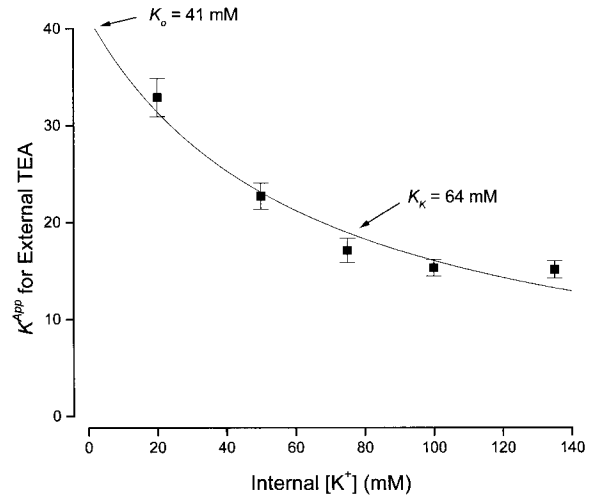

FIGURE 4. Internal K^+ and block by external TEA. K^{App} (at 0 mV) for block by external TEA as a function of the concentration of internal K^+ ions. The solid line is a fit of Eq. 4 to the data with K_o and K_K values of 41 ± 3.9 and 64 ± 12 mM, respectively.

near 41 mM, which is a considerably lower affinity than the apparent value obtained at physiological internal K^+ concentrations. Note that the observed effect of internal K^+ on TEA block is the opposite of that expected for electrostatic repulsion (or any other antagonistic interaction) between these ions. The presence of internal K^+ ions enhances block by external TEA as expected if internal K^+ ions bind to channels blocked by external TEA (Scheme 3).

Thus, this simple model can quantitatively account for the internal K^+ dependence of both the apparent affinity of external TEA (Fig. 4) and the ability of external TEA to antagonize block by internal TEA (Fig. 3). The analysis of these data reveals the existence of a K^+ ion binding site with an affinity near 65 mM. The data in Figs. 3 and 4 were obtained at 0 mV. By extending the analysis to other potentials, the location of the external TEA site and the K^+ ion binding site within the membrane electric field can be determined. Fig. 5 presents the results of extending the type of analysis in Fig. 4 to include a range of membrane potentials.

Location of the Sites within the Electric Field

Shown in the main part of Fig. 5 are the affinities of the external TEA and internal K^+ ion binding sites (K_o and K_K, respectively in Eq. 4) obtained (as in Fig. 4) at the indicated membrane voltages. The external TEA affinity (K_o) exhibits a voltage dependence (■) as if this ion must cross 29% of the membrane voltage to reach its binding site. A similar value of 24% was obtained from a direct determination of the voltage dependence of the apparent affinity for block by external TEA with 135 mM internal K^+ (Fig. 5, inset). These values are
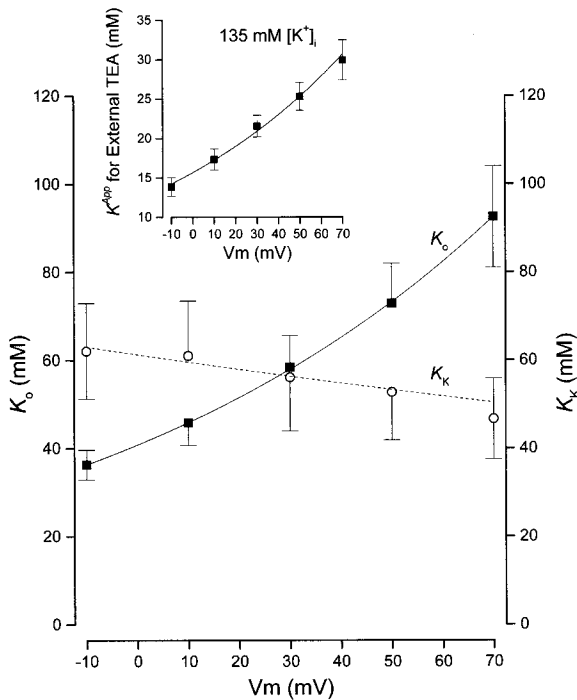
FIGURE 5. Voltage dependence of external TEA site and internal K⁺ site. Intrinsic affinity of external TEA site (K_o, ■, left ordinate) and K⁺ site (K_K, ○, right ordinate) at the indicated membrane voltages. (solid line) Fit to the K_o data of the standard (Woodhull, 1972) equation for block by an external monovalent cation: $K_o = K_o(0)\exp(\delta_o V_m F/RT)$, where $K_o(0)$ is the affinity at 0 mV, and δ_o is the fraction of the membrane voltage through which the external ion has to move to reach the site with values of 41 ± 0.11 mM and 0.29 ± 0.002 mM, respectively. (dashed line) Fit to the K_K data of the equivalent equation for block by an internal monovalent cation. (inset) Voltage dependence of the apparent affinity for external TEA block determined with 135 mM internal K⁺. Solid line is the fit of the Woodhull equation with a zero voltage affinity of 16 ± 0.8 mM and δ value of 0.24 ± 0.032.

reasonably consistent with the value of 19% obtained by Heginbotham and MacKinnon (1992).

The interaction of K⁺ ions with the binding site appears to exhibit very little voltage sensitivity (Fig. 5, ○). The dashed line represents a site only 7% into the membrane field from the internal end of the channel. Since we consider that internal TEA and K⁺ ions compete for a single site, internal TEA block should have a similar voltage dependence. However, block by internal TEA, measured in normal internal K⁺ solutions, has a voltage dependence consistent with binding to a site 15–20% into the field (Yellen et al. 1991; Choi et al., 1993). The discrepancy between these results may arise from the difference between the intrinsic voltage dependence and the apparent voltage dependence of a blocking ion in a multi-ion pore (Hille and Schwarz, 1978; Begenisich and Smith, 1984; Pérez-Cornejo and Begenisich, 1994). For example, Spassova and Lu (1999) recently showed that the voltage dependence of internal TEA block of ROMK1 channels was a function
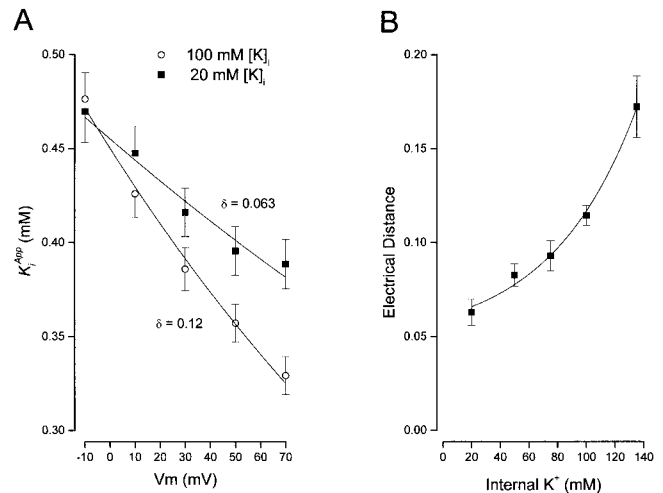

FIGURE 6. Internal K⁺ ions affect the voltage sensitivity of internal TEA block. (A) Voltage dependence of the apparent affinity of block by internal TEA in solutions of 20 (■) and 100 mM (○) internal K⁺. Values obtained from fits of Eq. 1 to data consisting of at least three independent measurements from at least four different TEA concentrations. (lines) Fits to the Woodhull equation (see Fig. 5 legend) with δ as the fraction of the membrane voltage (measured from the inner surface) through which internal TEA moves to reach its blocking site. (B) Apparent electrical distance (δ) determined as in A at the indicated internal K⁺ concentrations.

of the type and concentration of the internal permeant ion. To determine if the voltage dependence of internal TEA block of *Shaker* channels is sensitive to internal K⁺ ions, we determined the apparent affinity for block in solutions of different internal K⁺.

Fig. 6 A shows the voltage dependence of the apparent affinity for block by internal TEA in 100 (○) and 20 mM (■) internal K⁺. The lines are fits of the Woodhull (1972) equation (see Fig. 5 legend) from which the apparent position of the TEA site in the membrane electric field can be obtained. In 100 mM internal K⁺, block by TEA had a voltage dependence as if the ion had to cross ~12% of the electric field. This value was smaller in 20 mM K⁺, as if the site were only ~6% into the field. Fig. 6 B shows that the apparent electrical distance for block by internal TEA depended rather strongly on the internal K⁺ concentration. The electrical distance was ~17% into the field for 135 mM K⁺, which is similar to the values previously reported for normal physiological levels of K⁺ (Yellen et al. 1991; Choi et al., 1993). The apparent electrical distance decreased with decreasing internal K⁺ concentration, perhaps approaching a value near 5% in the absence of internal K⁺. This latter value is quite close to the estimate above (7%) for the location of the K⁺ site in the membrane electric field.

Internal TEA Can Protect from Block by External TEA

Newland et al. (1992) showed that not only could external TEA protect from block by internal TEA, but also

that the opposite is also true: internal TEA can protect K channels from block by external TEA (Table I, last two columns). However, they found that these effects were not quantitatively the same: internal TEA affords less protection from external TEA than does external TEA from internal TEA. This asymmetry in the TEA antagonism data is inconsistent with an electrostatic mechanism for the interaction between the TEA ions.

The data and analysis presented here suggest an alternative mechanism, a competition between internal TEA and internal K^+ ions, which is inherently asymmetric. Therefore, another test of this model is the prediction that reduced internal K^+ should reduce the ability of internal TEA to protect from block by external TEA. We found that in 135 mM internal K^+, 2 mM internal TEA decreased the apparent K_d for block by external TEA by a factor of 2.6 ± 0.22; in 20 mM, the decrease was only 1.5 ± 0.12. That is, reducing the internal K^+ concentration from 135 to 20 mM, reduced the ability of internal TEA to inhibit block by external TEA by a factor of 1.7. Thus, these data qualitatively support the idea that TEA antagonism, even the ability of internal TEA to inhibit block by external TEA, is due to competition between internal TEA and internal K^+ ions. A quantitative test of this idea can be made from the predicted affinity for block by external TEA in the presence of TEA and internal K^+ (solving Schemes 1–5 for the apparent affinity of external TEA in the presence of internal K^+ and internal TEA):

$$K_i^{App} = K_o \frac{1 + TEA_i / K_i}{1 + TEA_i / K_i + [K^+]_i / K_K},$$

where the parameters are as defined above.

With model parameters obtained from the previous analyses (65, 42, and 0.45 mM for K_K, K_o, and K_i, respectively), the model predicts a change in K_o^{App} of 1.7 for the reduction of internal K^+ from 135 to 20 mM, which is identical to the measured value of 1.7. Thus, it appears that most, if not all, of the apparent antagonism between TEA ions may actually be a competition between internal K^+ ions and internal TEA for a common binding site. As noted above, the role of external TEA is not to directly antagonize internal TEA but, rather, to promote increased occupancy of the internal K^+ site by inhibiting emptying of the site to the external side of the pore.

DISCUSSION

The results of the study presented here reveal several new properties of the pore in *Shaker* K channels. We found that the apparent antagonism between external and internal TEA (Newland et al., 1992; Thompson and Begenisich, 2000) depended on the concentration of K^+ ions in the solution on the inner side of the pore, and disappeared entirely if K^+ ions were replaced by

Rb^+. We also found that internal K^+ ions affected block by external TEA: the apparent affinity increased with increasing K^+ concentration. This latter result is opposite to the expectation if internal K^+ ions (either through electrostatic repulsion or some other mechanism) antagonized binding of external TEA.

Comparison with Previous Results

An interaction between external TEA and internal K^+ ions has been observed in other voltage-gated K channels. In Kv2.1 K channels (Immke et al., 1999; Immke and Korn, 2000), this interaction is both similar to and more complex than the behavior of *Shaker* channels we report here. In wild-type Kv2.1 channels, internal K^+ has a large effect on the efficacy of external TEA with relatively little effect on potency. We have observed no significant changes in efficacy of TEA in *Shaker* channels. However, if two lysines in Kv2.1 (not represented in *Shaker* channels) are replaced with neutral amino acids, internal K^+ ions significantly enhance external TEA block, as we report here for *Shaker* K channels.

A Mechanism for the Interaction between External and Internal TEA

We were able to quantitatively account for all of our results with a rather simple model that suggests a mechanism by which external TEA ions can antagonize block by internal TEA. In this model, internal TEA and K^+ ions compete for a single site located near the inner end of the pore. Occupancy of the pore by external TEA shuts off the flow of K^+ ions to the external side of the pore and so will enhance occupancy of the internal site by K^+ ions. Increased occupancy of the site by K^+ will, necessarily, decrease block by internal TEA. That is, there is no direct interaction between internal and external TEA ions—the apparent antagonism between these blocking ions is entirely mediated through changes in occupancy of a K^+ ion binding site and the competition between K^+ ions and internal TEA for occupancy of this site.

Properties of the Internal Ion Binding Site

The effects of internal K^+ on external TEA block and on the antagonism between external and internal TEA provide two independent means for estimating the intrinsic affinity of the K^+ site. A single K^+ ion binding site with an affinity near 65 mM (at 0 mV) was consistent with both sets of data. Since there is no interaction between TEA ions in Rb^+ solutions (Thompson and Begenisich, 2000; Fig. 1), this site appears to be selective for K^+ ions. The alternative explanation, that the site has a much higher affinity for Rb^+ than for K^+ and so is always filled, is inconsistent with the effectiveness of internal TEA in Rb^+ solutions.

By analyzing the interaction between internal K^+ ions and external TEA at various membrane potentials (Fig. 5, main) we were able to estimate the apparent location within the membrane electric field of both the external TEA binding site and the internal K^+ binding site. From this analysis, we found that the internal K^+ binding site apparently experiences only ~7% of the membrane electric field measured from the inner end of the pore and the external TEA binding site appears to be ~29% into the field from the external surface. This latter value is similar to the values of 24% (Fig. 5, inset) and 19% (Heginbotham and MacKinnon, 1992) determined directly from the voltage dependence of external TEA block, and so provides some confidence in the value obtained for the position of the K^+ binding site.

The observation that the voltage dependence of block by internal TEA was enhanced by increased internal K^+ concentration (Fig. 6) shows that internal K^+ and TEA ions interact in the pore. TEA block in very low internal K^+ places the internal TEA site no deeper than ~5–6% into the membrane field from the inner end of the pore. This is considerably less than the 15–20% value obtained with a normal concentration of internal K^+ (Fig. 6 B; Yellen et al., 1991; Choi et al., 1993). This behavior is expected in a single-file, multi-ion pore, in which the apparent voltage sensitivity of a blocking ion includes the voltage dependence of any movement of permeant ions linked to the blocking event (Hille and Schwarz, 1978). Not only do internal TEA and K^+ ions compete for occupancy of the pore, but the close similarity of the location of the K^+ and TEA sites within the electric field also strongly suggests that these ions compete for the same location.

It is possible that the change in the voltage sensitivity of block by TEA produced by internal K^+ is the result of the ability of TEA to bind to two different locations: a superficial site in low K^+ solutions and a site deeper into the electric field in high K^+ solutions. If so, these two sites must have very similar TEA affinities since, in the absence of external TEA, block by internal TEA (at 0 mV) is relatively insensitive to internal K^+ (Fig. 4). Although certainly possible, the existence of two sites of identical affinity at two separate positions in the pore does not seem particularly likely. Rather, the internal K^+ concentration dependence of the apparent voltage sensitivity of TEA block is a predicted consequence of the competition between TEA and K^+ ions for occupancy of a site near the inner end of a multi-ion pore.

In the simple model presented here, we considered that by shutting off K^+ flux to the external solution, external TEA allowed a true thermodynamic equilibrium between K^+ ions in the internal solution and a single site in the pore. It is possible that the presence of external TEA enhances occupancy of more than a single K^+ site; however, two lines of evidence suggest that, at least

over the range of internal K^+ of 20–135 mM, a significant change in occupancy of only a single site is likely. First, significant occupancy of two sites of different affinities would produce a bimodal shape to the plot in Fig. 4. Although the one-site model (Eq. 4) doesn't fit every data point perfectly, there is also no evidence of a more complex behavior. Second, the very shallow location of the site in the electric field leaves very little room for two independent sites.

Ion Binding Sites in K Channels

Thus, we have presented evidence for the existence of a site in the pore of *Shaker* K channels that has an intrinsic affinity for K^+ ions of ~65 mM. This site is located only a very short distance into the membrane electric field, binds TEA, and is selective for K^+ over Rb^+. Is this a new site or one that has been previously described?

Several ion binding sites have been identified in K channels including *Shaker*. Neyton and Miller (1988a,b) described three K^+ and at least a single Ba^{2+} binding site. One of these is accessible to external K^+ ions and "locks" Ba^{2+} into the pore. This site has a very high affinity for K^+ ions (a binding constant as low as 2.7 µM; Vergara et al., 1999) and for Rb^+ (Neyton and Miller, 1988a). A similar site (with an affinity closer to the low millimolar range) has been described in *Shaker* K channels, which also appears to be involved in C-type inactivation (Hurst et al., 1995; Baukrowitz and Yellen, 1996; Starkus et al., 1997; Harris et al., 1998). This site is preferentially occupied by external, not internal, K^+ and is more selective for Rb^+ than K^+. The second site, the "external enhancement site" (Neyton and Miller, 1988b), is preferentially occupied by external ions (with affinities in the hundreds of millimolar range) and appears to have about the same affinity for K^+ and Rb^+, although Rb^+ is not as effective as K^+ in enhancing the Ba^{2+} off-rate. The "internal lock-in site" is accessible to internal ions, is quite nonselective, and appears to be located 36% into the membrane electric field from the internal side (Neyton and Miller, 1988b).

The solution of the KcsA crystal structure identified the location of three ion binding sites (at least for the electron dense Rb^+ or Cs^+ ions): two at either end of the narrow selectivity region, and the third in a cavity about midway through the pore. Ba^{2+} ions occupy a position in the KcsA structure between the cavity ion and the innermost end of the selectivity filter (Jiang and MacKinnon, 2000), which suggests that the cavity site might be the internal lock-in K^+ site. Since the channel affinity for internal TEA can be modified by mutations of amino acids near the inner end of the selectivity filter (Yellen et al., 1991; Doyle et al., 1998), the colocalized internal K^+/TEA site might be considered to be the internal lock-in/cavity site. However, unlike the internal K^+/TEA site, the internal lock-in site has little ion selec-
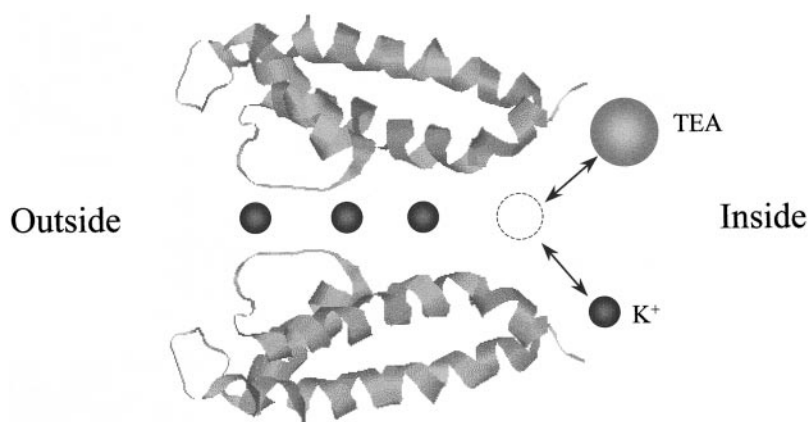
tivity and is located much deeper in the membrane electric field than the internal K^+/TEA site.

Thus, we propose to have identified a fourth K^+ site in the pore of *Shaker* channels as illustrated in the cartoon in Fig. 7. Shown is a representation of the pore in the KcsA K channel incorporating the "bent S6" structure of del Camino et al. (2000). The three small, dark spheres in the pore represent the three K^+ sites in the KcsA crystal (Doyle et al., 1998). Our results suggest the existence of a fourth K^+ ion binding site (dashed circle) at the innermost end of the pore. We place this site quite near the inner end of the pore consistent with the weak voltage dependence of K^+ binding to this site and the weak intrinsic voltage dependence of TEA block. This site has a very low affinity for Rb^+ ions but can, as indicated, bind either K^+ or TEA, but not both.

The presence of four K^+ ion binding sites in the pore of *Shaker* channels is consistent with flux-ratio data showing that these channels can simultaneously be occupied by at least four K^+ ions (Stampe and Begenisich, 1996) at physiological levels of K^+. Unfortunately, the flux-ratio data provide no information on the affinity or fractional occupancy of any particular site. The conductance of *Shaker* channels increases as the symmetrical K^+ concentration is raised into the several hundred millimolar range (Heginbotham and MacKinnon, 1993) as if filling a site with a 300-mM (activity) affinity. This means that at least one site in the *Shaker* channel pore is only partially occupied at normal physiological levels of K^+. As noted above, the innermost K^+ site we propose will be only slightly occupied at physiological levels of internal K^+ in the absence of external TEA. Thus, the existence of a fourth K^+ site that is only occasionally occupied at physiological internal K^+ concentrations can account for both the flux-ratio and conductance saturation data.

The fourth site we propose might not have been apparent in the KcsA structure (Doyle et al., 1998) for several reasons. First, K^+ ions are not sufficiently electron dense to have been detected and the sufficiently dense Rb^+ ions do not significantly occupy the site. Even if the presence of K^+ ions could be detected in the diffraction pattern, the steady-state occupancy of this site is so low that it could escape detection by crystallographic methods. Second, this site may be absent in the truncated KcsA protein used in the structure determination. Finally, this site may be absent in KcsA channels because this protein lacks structures known to contribute to the pore in *Shaker* channels (Isacoff et al., 1991; Slesinger et al., 1993).

Thus, our determination of a shallow location in the membrane field for the internal TEA site is not directly contradicted by existing data. However, it is more difficult to reconcile this superficial location with the ability of amino acid mutations near *Shaker* position 441 to influence block by internal TEA. The equivalent amino acids in the KcsA channel are deep in the pore, just at the inner end of the narrow selectivity filter. How could mutations this deep in the pore affect superficial TEA binding? The movements of ions in a multi-ion, single-file pore are coupled. Thus, mutations near position 441 could alter the affinity of K^+ ion binding at this deep location and, indirectly, also affect block by internal TEA.

Summary

This study has provided a refined view of K^+ and TEA binding near the inner end of the pore in *Shaker* K channels. We showed that there is little intrinsic voltage dependence of internal TEA block, and so the TEA site is located just barely within the membrane electric field near the inner end of the pore. The apparent voltage dependence in normal K^+ concentrations is the result of a coupling between TEA and the movement of K^+ ions in the multi-ion, single-file pore. We identified a site for K^+ ions that has a low intrinsic affinity and, therefore, is infrequently occupied under normal conditions. This site is located at the same electrical position as the internal TEA site and these ions compete for occupancy. When external TEA blocks K^+ efflux, the site becomes significantly occupied and reduces in-

ternal TEA block. This site is K$^+$- (and TEA) selective: the affinity for Rb$^+$ ions is extremely low. If this site is the "cavity" site visualized in the structure of the KcsA channel, then only 5–7% of the membrane electric field appears at this location. However, as discussed above, this K$^+$/TEA site appears to have properties distinct from those of the cavity site, and so likely represents a fourth K$^+$ ion binding site located between the cavity position and the inner end of the pore.

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