Closed-channel block of BK potassium channels by bbTBA requires partial activation

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Blockade of large-conductance Ca\(^{2+}\)-activated K\(^+\) (BK) channels by the bulky quaternary ammonium compound, N-(4-[benzoyl]benzyl)-N,N,N-tributylammonium (bbTBA), exhibits features consistent with blockade of both closed and open states. Here, we examine block of closed BK channels by bbTBA and how it may differ from block of open channels. Although our observations generally confirm earlier results, we describe three observations that are inconsistent with a model in which closed and open channels are equally accessible to blockade by bbTBA. First, block by bbTBA exhibits Ca\(^{2+}\)-dependent features that are inconsistent with strictly state-independent block. Second, the steady-state voltage dependence of bbTBA block at negative potentials shows that any block of completely closed states either does not occur or is completely voltage independent. Third, determination of the fractional unblock by bbTBA at either low or high Ca\(^{2+}\) reveals deviations from a model in which open- and closed-state block is identical. The results support the view that bbTBA blockade of fully closed channels does not occur. We imagine two general types of explanation. First, a stronger voltage dependence of closed-channel block may minimize the contribution of closed-channel block at negative potentials. Second, voltage-dependent conformational changes among closed-channel states may permit block by bbTBA. The analysis supports the latter view, suggesting that bbTBA blockade of fully closed channels does not occur, but the ability of bbTBA to block a closed channel requires movement of one or more voltage sensors. Models in which block is coupled to voltage sensor movement can qualitatively account for (1) the ability of open-channel block to better fit block of conductance–voltage curves at high Ca\(^{2+}\); (2) the voltage dependence of fractional availability; and (3) the fractional unblock at different open probabilities. BK channels appear to undergo voltage-dependent conformational changes among closed states that are permissive for bbTBA block.

INTRODUCTION

Mechanistic studies of ion channel block have provided important insight into many fundamental aspects of ion channel structure and function (Armstrong, 1971; Armstrong and Hille, 1972). This has included examination of the state dependence of drug and blocker access to blocking sites (Liu et al., 1997), the definition of structural elements of channels involved in gated access to channel-blocking sites (del Camino and Yellen, 2001), the relationship of blocker molecules to the ion permeation pathway and blocker interactions with permeant ions (Heginbotham and Kutluay, 2004; Kutluay et al., 2005), and the molecular mechanisms of inactivation (Choi et al., 1991). In Shaker K\(^+\) channels, charged cytosolic channel blockers typically only reach their blocking position when the channel is open. Furthermore, a blocker generally must exit its blocking position before the channel can close (Demo and Yellen, 1991). Thus, the blocking position is considered to be within an aqueous central cavity (Zhou et al., 2001) and, in the closed channel, a constriction at the cytosolic end of the S6 inner helix prevents access to that central cavity (del Camino and Yellen, 2001). This simple block behavior is thought to be shared by a variety of blocking molecules, including both small quaternary ammonium (QA) blockers (Armstrong, 1971) and bulkier peptide blockers derived from the physiologically relevant N-terminal inactivation domains of either α or β subunits (Ruppersberg et al., 1991; Murrell-Lagnado and Aldrich, 1993; Rettig et al., 1994). As an extension of this similarity in blocking action, QA blockers and inactivation domains also exhibit strict competition for pore-blocking sites, with QA blockers producing a slowing of the onset of inactivation consistent with simple competition (Choi et al., 1991). Structural studies coupled with a mutant cycle analysis of Kvβ1.2 peptide interactions with the Kv1.4 channel have provided a compelling view that inactivation domains fit snugly within the central cavity of open Kv channels (Zhou et al., 2001), thereby preventing both ion permeation and the channel-closing conformational change.
For large-conductance Ca$^{2+}$-activated K$^+$ (BK)-type channels, several aspects of block and inactivation have not been easy to explain within the context of a simple open-channel block mechanism: (1) QA blockers typically do not produce the slowing of channel deactivation that is predicted by simple block (Li and Aldrich, 2004); (2) BK channel block by N(4-[benzoyl]benzyl)-N,N,N-tributylammonium (bbTBA) exhibits kinetic features consistent with blockade of both open and closed channels (Wilkens and Aldrich, 2006); (3) QA blockers do not show competitive inhibition of the onset of BK channel inactivation (Solaro et al., 1997; Xia et al., 1999); and (4) BK channel inactivation exhibits features of a two-step inactivation process (Lingle et al., 2001; Benzingier et al., 2006) and, in some cases, recovery from inactivation can occur without obvious channel reopening (Solaro et al., 1997). Some of these properties may be explained by the idea that some blocking species may be able to occupy a position in the BK central cavity, both when the channel is open and when it is closed (Wilkens and Aldrich, 2006). However, block of BK channels by a peptide derived from the ShakerB N terminus exhibits clear evidence of state-dependent block (Li and Aldrich, 2006), indicative that the BK channel does undergo a conformational change during gating that increases access of some peptide blockers to a blocking position. One suggestion regarding the underlying physical basis for the differences between block by the peptides and block by QA compounds is that, unlike for Kv channels, perhaps the entryway into the BK central cavity is not completely occluded in closed channels (Wilkens and Aldrich, 2006). Furthermore, perhaps like the open MthK bacterial channel (Jiang et al., 2002), the overall central cavity of the BK channel may be much broader than that found in Kv channels. The idea of a central cavity of larger dimension is also supported by an analysis of sugar effects on ion conductance in BK channels (Breidhze and Magleby, 2005).

An important implication of the idea that pore-blocking molecules may have access to the BK central cavity both in open and closed conformations is that this suggests that, in BK channels, there may be no cytosolic gate for small molecules. As such, the conformational change that allows gated access of Shaker peptides to the BK channel would not be the movement of a gate directly leading to the increase in ion permeation, but perhaps an additional expansion of an opening to an already accessible central cavity. Definition of the precise temporal sequence through which molecules may gain access to the central cavity is therefore important for elucidating the nature of conformational changes that precede channel opening and which steps are directly coupled to the initiation of ion permeation.

Some of the most intriguing evidence supporting the idea that small molecules may have access to the BK central cavity when the channel is closed concerns the effects of bbTBA, a bulky derivative of tetrabutylammonium (Wilkens and Aldrich, 2006). At 1 or 4 µM Ca$^{2+}$, blockade by bbTBA exhibits properties consistent with both open- and closed-channel block (Wilkens and Aldrich, 2006). Furthermore, at high [bbTBA], current waveforms exhibit kinetic components that can only be explained by block of both closed and open channels. An important question unaddressed by this earlier work is the extent to which block of open and closed states shares similar properties, including affinity and voltage dependence. Given the importance of establishing the validity of this model of block of BK channels, here we have extended this earlier work in several ways. We have undertaken tests to define properties of closed-channel block and to examine block over a range of fractional activation at both 4 and 300 µM Ca$^{2+}$. In addition, we have evaluated the results in terms of physically plausible allosteric models of BK gating (Cui et al., 1997; Horrigan and Aldrich, 2002), which include multiple closed and open states that are traversed during gating. Although the present results confirm some aspects of the earlier observations concerning bbTBA (Wilkens and Aldrich, 2006), the new observations argue that blockade by bbTBA does not occur in a fully state-independent fashion, a possibility raised by Wilkens and Aldrich. Rather, the effects of bbTBA are best explained by the idea that channels in a full resting state, i.e., closed channels with no active voltage sensors, are not sensitive to block by bbTBA. However, blockade of closed channels by bbTBA may occur as soon as at least one voltage sensor becomes active.

**MATERIALS AND METHODS**

**Oocyte handling and expression construct**

The handling of stage IV *Xenopus laevis* oocytes for cRNA injection and expression was as described previously (Xia et al., 1999, 2002). The coding region of mSlO1 (provided by L. Salkoff, Washington University, St. Louis, MO; GenBank accession number NM_010610) was subcloned into an oocyte expression vector pXMX using the 5’ NotI and 3’ SalI restrictions sites in a segment containing multiple cloning sites. The pXMX vector was designed to contain multiple factors that may promote expression or increase RNA stability. At the 5’ end of the multiple cloning site segment, vector pXMX contains a Sp6 promoter, 45 bp (residues -48 to -2 upstream of ATG) of *Xenopus laevis* major beta globin (XLMBG), and 162 bp (residues -109 to -7 upstream of ATG) of *Shaker* channel noncoding sequence. Following the 3’ end of the multiple cloning site vector, the vector contains 142 bp (residues -9 to 130 relative to stop codon TAA) of XLMBG noncoding downstream sequence and a 45-bp poly-A segment, followed by linearization enzyme sites. The pXMX vector has been routinely used by this laboratory for expression of both α and β subunits in oocytes for several years (Xia et al., 2002; Zeng et al., 2003; Zhang et al., 2006).

**Electrophysiology**

Currents were recorded in the inside-out configuration (Hamill et al., 1981) with an Axopatch 200 amplifier (MDS Analytical Technologies). The Clampex program from the pClamp package (MDS Analytical Technologies) was used to drive stimulus protocols.
and digitize currents. Pipettes were typically 1–2 MΩ and were coated with Sylgard 184 (Dow Corning) and fire-polished. Gigaohm seals were formed in frog Ringer ([in mM] 115 NaCl, 2.5 KCl, 1.8 CaCl₂, and 10 HEPES, pH 7.4) and, after excision, moved into flowing test solutions. The standard solution bathing the extracellular membrane face (pipette solution) contained

\[ \text{Composition of solutions used to bathe the cytoplasmic face of patch membranes was as described previously (Wei et al., 1994; Zhang et al., 2001). Solutions were titrated with Ca-MES or methanesulfonic acid solutions to obtain desired Ca}^{2+} \text{concentrations, as defined with a Ca}^{2+}-\text{sensitive electrode calibrated with commercial Ca}^{2+} \text{solutions (WPI). Solutions were exchanged at the pipette tip by a fast perfusion stepper system (SF-77B; Warner Instruments). All experiments were at room temperature (∼22–25°C). Salts for solution preparation were obtained from Sigma-Aldrich. bbTBA was obtained from Spectra Group Limited. Stock solutions of 1 mM bbTBA in different Ca}^{2+} \text{solutions were prepared and stored frozen at −20°C. bbTBA solutions for experiments were prepared by dilution of stock solutions immediately before experiments. Stock solutions were used within 1 month.}

Data analysis

Analysis of current recordings was accomplished either with Clampfit (MDS Analytical Technologies) or with programs written in this laboratory. To define the voltage dependence of blocking equilibria, conductance–voltage (GV) curves were constructed from measurements of steady-state current. For families of GV curves obtained within a patch, conductances were normalized to estimates of maximal conductance obtained in the absence of blocker. For definition of the voltage of half-activation (Vₐₐ), and voltage dependence of current activation (q), GV curves were fit with the following Boltzmann function:

\[
G(V) = \frac{G_{\text{max}}}{1 + \exp \left( \frac{V - V_{\text{h}}}{\kappa} \right)}
\]

At activation potentials of +160 mV and more positive, in our hands there are often slow reductions of BK current in control saline, regardless of the magnitude of the current in a patch. This appears to reflect a slowly developing block, but it is also rapidly reversible because tail currents do not show the same decrement in current with command step duration. An additional complication is that, in the presence of bbTBA (or other blockers), such slow reductions in current are generally not observed. We have not identified any procedure that consistently circumvents this problem. The inclusion of the crown ether Ba³⁺ chelator, (+)-18-crown-6-tetra-carboxylic acid (Neyton, 1996), did not reduce such slow macroscopic current relaxations. To minimize the possibility that estimates of conductance may be distorted by such slow block, we have limited estimates of conductance and block at 100 or 300 µM Ca²⁺ to voltages of +120 mV or more negative and to voltages of +160 mV or lower at 4 µM Ca²⁺.

Open-channel block

The basic open-channel blocking mechanism (OB model) is defined in Scheme 1, with the predictions for steady-state Po defined in Eq. 2.

\[
P_{\text{o}}(V, [X]) = \frac{1}{1 + \frac{C}{O} \frac{B}{O} \frac{[X]}{K_{bo}}}
\]

Eq. 2 yields the following well-known expression for the dependence of conductance on voltage and blocker:

\[
G(V, [X]) = \frac{G_{\text{max}}}{1 + \exp \left( \frac{qF(V - V_{\text{h}})}{RT} \right) + \frac{[X]}{K_{x} \exp \left( \frac{-qF}{RT} \right)}}
\]

where \( q \) is charge movement associated with the activation equilibrium, \( V_{h} \) is the voltage of half-activation, and \( K_{x} \) is the 0-voltage dissociation constant of blocker, X, for the open state, with \( z_{b} \) reflecting the intrinsic voltage dependence of the binding equilibrium. Because BK channel activation can be defined by distinct terms reflecting both Ca²⁺ and voltage-dependent allosteric equilibria (Horrigan et al., 1999; Cui and Aldrich, 2000), here we use general equations that, by incorporating both activation and block, allow a more direct assessment of predictions regarding the overall fractional block of conductance as a function of steady-state channel open probability (Po) at different Ca²⁺. We have therefore approximated L in terms of the Horrigan-Cui-Aldrich formulation (Horrigan et al., 1999; Cui and Aldrich, 2000), in which activation of channel conductance by both voltage and Ca²⁺ is given by:

\[
P_{\text{o}}(V, A) = \frac{1}{1 + \left( \frac{1}{L(V)} \left( \frac{1 + \frac{f}{D'} \frac{1 + [Ca^{2+}]/K_{c}^{1}}{1 + [Ca^{2+}]/K_{c}^{2}} \right) \right)}
\]

where \( L(V) = L_{0} \exp \left( \frac{f_{1}A}{RT} \right) \) and \( f_{0} \) exp \left( \frac{f_{1}A}{RT} \right), \) L, defining the C-O equilibrium constant, \( z_{c} \), its voltage dependence, \( f_{0} \) defining the voltage sensor equilibrium constant at 0 mV, with \( z_{f} \) defining the voltage dependence of \( f \), \( D \) is the coupling constant between \( L \) and \( f \), \( K_{c} \) is the binding constant of agonist \([A]\) to the closed conformation, and \( C \) is the coupling constant between the ligand binding equilibrium and \( L \). This formulation ignores the weak coupling (E) that occurs between the Ca²⁺-binding equilibrium and the voltage sensor equilibrium (Horrigan and Aldrich, 2002).

For many experiments, we examine blockade at a single Ca²⁺ concentration. When fitting data obtained in the presence of blocker, it is desirable that parameters describing activation be constrained to those obtained in the absence of blocker. Therefore, Eq. 4 can be simplified to reflect the Po at a single Ca²⁺ concentration:

\[
P_{\text{o}}(V) = \frac{1}{1 + \left( \frac{1}{L(V)} \left( \frac{1 + \frac{f}{D'} \frac{1 + [Ca^{2+}]/K_{c}^{1}}{1 + [Ca^{2+}]/K_{c}^{2}} \right) \right)}
\]

where

\[
L' = L(V) \frac{1 + [Ca^{2+}]/K_{c}^{1}}{1 + [Ca^{2+}]/K_{c}^{2}}.
\]

Here, \( f' \) and \( D' \) differ from \( f \) and \( D \) as defined over all Ca²⁺ and voltage because of the small effect of coupling between the ligand binding and the voltage sensor equilibrium. Eq. 5 provides an empirical description of the activation behavior for Slo1 at a given Ca²⁺. For all cases in which either single or multiple GV curves in the absence of blocker were fit to define activation parameters, \( z_{c} \) and \( z_{f} \) were constrained to 0.5 and 0.58, respectively, whereas
equilibrium and coupling constants were allowed to vary. Once the optimal fit to the activation GV curves was obtained, all activation constants were then fixed during fitting of the GV curves in the presence of blocker.

Using terms for activation, Eq. 5 can then be recast as:

\[
Po(V,[X]) = \frac{1}{1 + \frac{1}{L^*} \left(1 + f \frac{J}{J + D} \right)^4 + \frac{[X]}{K_{bo} \exp \left(-\frac{\gamma X}{RT} \right)}}. \tag{6}
\]

For simplicity in the equations below, we also define the following:

\[
\frac{1}{L} = \frac{1}{L^*} \left(1 + f \frac{J}{J + D} \right)^4.
\]

Eq. 6 can be derived directly from the following open-channel block model (Scheme 1a) for activation and block over a range of voltages at a single agonist concentration.

\[
\begin{array}{cccc}
C_1 & C_2 & C_3 & C_4 \\
\downarrow & \downarrow & \downarrow & \downarrow \\
L & LD & LD^2 & LD^3 \\
\downarrow & \downarrow & \downarrow & \downarrow \\
O & O_2 & O_3 & O_4 \\
\downarrow & \downarrow & \downarrow & \downarrow \\
K_o & K_1 & K_2 & K_3 \\
\downarrow & \downarrow & \downarrow & \downarrow \\
B_1 & B_2 & B_3 & B_4 \\
\end{array}
\]

(Scheme 1a)

Block of both open and closed states

We also evaluate whether a blocker may act by binding to the same site in both open and closed states, as given in Scheme 2 (CB-OB model).

\[
\begin{array}{cccc}
C & C & C_3 & C_4 \to C_5 \\
\downarrow & \downarrow & \downarrow & \downarrow \\
C' & C_2 & C_3 & C_4 \\
\downarrow & \downarrow & \downarrow & \downarrow \\
O & O_2 & O_3 & O_4 \\
\downarrow & \downarrow & \downarrow & \downarrow \\
CB & CB_2 & CB_3 & CB_4 \\
\end{array}
\]

(GENERAL SCHEME 2)

For Scheme 2, the predictions for \(Po\) as a function of voltage and blocker concentration \([X]\) are:

\[
Po(V,[X]) = \frac{1}{1 + \frac{1}{L} \left(1 + f \frac{J}{J + D} \right)^4 + \frac{[X]}{K_{bo} \exp \left(-\frac{\gamma X}{RT} \right)}}. \tag{7}
\]

where \(K_{bc}\) and \(K_{bo}\) reflect the dissociation constants for binding to either closed or open channels, respectively, and \(z_b\) is the valence of the blocking equilibrium, assuming block of both open and closed states has an identical voltage dependence. Defining \(W = K_{bc}/K_{bo}\), an allosteric constant coupling the drug-binding equilibria to \(L'\), Eq. 7 becomes:

\[
Po(V,[X]) = \frac{1}{1 + \frac{1}{L} \left(1 + f \frac{J}{J + D} \right)^4 + \frac{[X]}{W K_{bo} \exp \left(-\frac{\gamma X}{RT} \right)} + \frac{[X]}{K_{bo} \exp \left(-\frac{\gamma X}{RT} \right)}}. \tag{8}
\]

When \(W = 1\), Eq. 8 describes a completely state-independent block scheme. When \(W \gg 1\), i.e., when closed-channel block is minimal, Eq. 8 becomes identical to Eq 6. When \(K_{bc} \ll K_{bo}\) (i.e., \(W \ll 1\)), Eq. 8 describes a strict closed-channel block mechanism.

For situations in which open- and closed-channel block exhibits different voltage dependencies,

\[
W(V) = \frac{K_{bc}}{K_{bo}} \exp \left(-\frac{(z_c - z_o) \gamma V}{RT} \right).
\]

This adds an additional free parameter to Eq. 8, which imparts a voltage-dependent effect on the gating equilibrium:

\[
Po(V,[X]) = \frac{1}{1 + \frac{1}{L} \left(1 + f \frac{J}{J + D} \right)^4 + \frac{[X]}{W K_{bo} \exp \left(-\frac{\gamma X}{RT} \right)} + \frac{[X]}{K_{bo} \exp \left(-\frac{\gamma X}{RT} \right)}}. \tag{9}
\]

Eqs. 8 and 9 can be derived directly from a 20-state blocking scheme (e.g., General Scheme 2) for activation at a single \([Ca^{2+}]\), but including blockade of both open and closed states.

We designate the completely state-independent block scheme in which \(K_{bc} = K_{bo}\) and \(z_b = z_o\) as Scheme 2a (also see Fig. S2). We designate the specific case in which closed-channel block is voltage independent (\(z_c = 0\)) and \(K_{bc}(0) = K_{bo}(0)\) as Scheme 2b, the case in which \(K_{bc}(0) \neq K_{bo}(0)\) and \(z_c = 0\) as Scheme 2b', and the case that \(K_{bc} \neq K_{bo}\) and \(z_b \neq z_o\) as Scheme 2'.

Access to a blocking site depends on movement of voltage sensors

We have also evaluated models in which we postulate that conformational events occur in closed channels in conjunction with voltage sensor movement that then permit access of blockers to blocking sites.

In Scheme 3 (also see Fig. S3), access of blocker to a blocking site does not occur until at least one voltage sensor has been activated.
An alternative version of this scheme would allow blockade of state $O_1$, but not state $C_1$. However, under all conditions evaluated here, occupancy of state $O_1$ is sufficiently negligible that such a model is largely indistinguishable from Scheme 3. For Scheme 3, some of the symmetry of the full allosteric models is lost, yielding a somewhat more complicated steady-state current prediction. For the general case in which $K_{bc} = K_{bo}$ and $z = z_c$, the following general expression can be derived:

$$P_{o}(V,[X]) = \frac{1}{1 + \frac{1}{L^{'}} (1 + J^{'}) D^{'})^4 + C_{term} + O_{term} + Ob_{term} + Ob_{term}$$

where

$$C_{term} = \frac{(1 + J^{'})^4 - 1}{L^{'}(1 + J^{'}) D^{'})^4} \frac{[X]}{K_{o} \exp \frac{-\Delta F}{RT}}$$

and

$$Ob_{term} = \frac{(1 + J^{'}) D^{'})^4 - 1}{(1 + J^{'}) D^{'})^4} \frac{[X]}{K_{o} \exp \frac{-\Delta F}{RT}}$$

We term the specific case that $K_{bc} = K_{bo}$ and $z = z_c$ as Scheme 3a, the case in which $K_{bc} = K_{bo}$ and $z = 0$ as Scheme 3b, the case that $K_{bc} = K_{bo}$ and $z = 0$ as Scheme 3b', and the case in which $K_{bc} = K_{bo}$ and $z = 0$ as Scheme 3c.

Affinity for the blocking site increases with number of active voltage sensors

We also consider a case in which voltage sensor movement is required for block to occur, but that blocking affinity scales with the number of active voltage sensors (Scheme 4 and Fig. S4). For activation at a single $[Ca^{2+}]$, this yields the following expression for steady-state $P_{o}$:

$$P_{o}(V,[X]) = \frac{1}{1 + \frac{1}{L^{'}} (1 + J^{'}) D^{'})^4 + C_{term} + O_{term} + Ob_{term}$$

where

$$C_{term} = \frac{4 (1 + J')^3}{L' (1 + J') D'} \frac{[X]}{K_{o} \exp \frac{-\Delta F}{RT}}$$

and

$$Ob_{term} = \frac{4 (1 + J')^3}{L' (1 + J') D'} \frac{[X]}{K_{o} \exp \frac{-\Delta F}{RT}}$$

The specific case in which $K_{bc} = K_{bo}$ and $z = z_c$ is termed Scheme 4a, the case in which $K_{bc} = K_{bo}$ and $z = 0$ is referred to as Scheme 4b, and the case in which $K_{bc} \neq K_{bo}$ and $z = 0$ is referred to as Scheme 4b'. For simplicity, we omit consideration of Scheme 4 from most sections of this paper.

Equations for block in conjunction with a full allosteric activation model

For some datasets, we also used equations based on a full allosteric activation model for BK gating (Horrigan and Aldrich, 2002), thereby allowing simultaneous fitting of GV curves obtained at multiple $Ca^{2+}$ and multiple bBTBA. For open-channel block (Scheme 1), the blocking equilibrium is defined by:

$$P_{o}(Ca,V,[X]) = \frac{1}{1 + \frac{1}{L} (1 + J + K + [JKE])^3 \frac{[X]}{L} + \frac{1}{K_{o} \exp \frac{-\Delta F}{RT}}}$$

where $J$, $K$, and $L$ represent the voltage sensor, Ca$^{2+}$ binding, and C-O equilibria, and $D$, $C$, and $E$ represent coupling constants (Horrigan and Aldrich, 2002). Similarly, the equilibrium predictions for a model in which both open and closed channels can be blocked (Scheme 2) is the following:

$$P_{o}(Ca,V,[X]) = \frac{1}{1 + \frac{1}{L} \frac{1}{C_{term}} \frac{[X]}{L} + \frac{1}{K_{o} \exp \frac{-\Delta F}{RT}}}$$

$$P_{o}(Ca,V,[X]) = \frac{1}{1 + \frac{1}{L} \frac{1}{O_{term}} \frac{[X]}{L} + \frac{1}{K_{o} \exp \frac{-\Delta F}{RT}}}$$

where $C_{term} = (1+J+K+JE)^4$ and $O_{term} = (1+J+K+KE)^4$.

Similarly, Scheme 3, in which voltage sensor movement is required for block to occur, can be described by:

$$P_{o}(Ca,V,[X]) = \frac{1}{1 + \frac{1}{L} \frac{1}{C_{term}} \frac{[X]}{L} + \frac{1}{K_{o} \exp \frac{-\Delta F}{RT}}}$$

where

$$C_{term} = \frac{1}{L} \frac{1}{C_{term}} \frac{[X]}{L} + \frac{1}{K_{o} \exp \frac{-\Delta F}{RT}}$$

and

$$O_{term} = \frac{1}{L} \frac{1}{O_{term}} \frac{[X]}{L} + \frac{1}{K_{o} \exp \frac{-\Delta F}{RT}}$$

This equation reflects that Ca$^{2+}$-binding steps among states with 0 active voltage sensors are removed from the set of all blocked-closed states and the set of all blocked-open states.

Derivation of a similar equation for the case in which the blocking equilibria scales linearly with the number of active voltage sensors (Scheme 4) is less straightforward because in the full allosteric model, the scaled blocking rates result in allosteric effects on gating equilibrium steps that involve subunit-specific coupling between voltage sensors and Ca$^{2+}$ sensors (Horrigan and Aldrich, 2002).
However, if we assume that this coupling is minor, i.e., that $E = 1$, one can derive the following equation:

$$Po \left( Ca, V_{\infty} \right) = \frac{1}{1 + \frac{1}{L} \left( 1 + f + K + JK \right)^4 + \text{Oblck} + \text{Oblk} \left( j + K + fK \right)^4}.$$  

(15)

where

$$\text{Oblck} = \frac{4 f (1 + f)^4 (1 + K)^4 \left[ X \right]}{L (1 + fD + KC + fDKC)^4} \exp \frac{-zFV}{RT}$$

and

$$\text{Oblk} = \frac{4 JD (1 + JD)^4 (1 + JC)^4 \left[ X \right]}{K_J \exp \frac{-zFV}{RT}}.$$  

When comparing Scheme 4 to other models, all fitting was done with the assumption of $E = 1$.

**Two distinct blocking sites**

We also considered a blocking model involving two distinct binding sites for bbTBA, where one site is accessible in both open and closed channels (states $B_1$ and $B_2$), and one site ($B_c$) is only reached when channels are open. Because this model did not better explain the results than the single-site models, we only briefly mention this model in the Results. The specific two-site model we considered is summarized in Scheme 5:

$$C \overset{L}{=} O \overset{\left[ X \right] / K_{2a}}{=} B_c \overset{\left[ X \right] / K_{2a}}{=} B_2 \overset{(K_{1c} / K_{1o}) \left[ X \right]}{=} B_{2c},$$

(SCHEM 5)

We define two allosteric coupling constants: $W = K_{1c} / K_{1o}$ and $Y = K_{2o} / K_{2a}$. From Scheme 4, the dependence of $Po$ on voltage, $[bbTBA]$, and $Ca^{2+}$ is:

$$Po \left( Ca, V_{\infty} \right) = \frac{1}{1 + \frac{1}{L \text{ Oterm}} + \text{Trm1} + \text{Trm2} + \text{Trm3}},$$  

(16)

where

$$\text{Trm1} = \frac{\left[ X \right]}{W \ast K_o \exp \frac{-zFV}{RT}},$$

$$\text{Trm2} = \frac{\left[ X \right]}{K_o \exp \frac{-zFV}{RT} + \left[ X \right] \exp \frac{-zFV}{RT}},$$

and

$$\text{Trm3} = \frac{Y \left[ X \right] \left[ X \right]}{K_a K_o \exp \frac{-zFV}{RT} \exp \frac{-zFV}{RT}}.$$  

When $W = 1$, binding to site 1 is identical both in open and closed channels. When $Y = 1$, binding to either site does not influence binding to the other site. In practice, the number of free parameters in this function can limit its utility. However, we explored this model with the simplifying assumptions that the sites are independent ($Y = 1$) and that $W = 1$. In addition, we assumed that binding to site 1 is voltage independent.

**Kinetic simulations**

Simulations of the kinetic behavior of different blocking models was done with a modified version of the IchSim program (see Online supplemental material below) for single-channel and macroscopic current simulations developed by J.A. De Santiago-Castillo (Jefferson University, Philadelphia, PA). All simulations used a population of 100 channels with an open-state conductance of 200 pS. Using simulated currents activated with various kinds of standard stimulation protocols, predicted GVs, fractional unblock curves, fractional availability curves, and tail currents were generated for each model. MathCad 8.0 (PTC Corporation) was also used to generate predicted GV curves, fractional unblock curves, and fractional availability curves to confirm results from the kinetic simulations. In all cases, steady-state predictions derived from analysis of simulated currents generated by IchSim agreed with the predictions based on steady-state equations implemented in MathCad.

**Online supplemental material**

Figs. S1–S4 summarize details of blocking Schemes 1–4, respectively. Fig. S5 shows that voltage dependence of closed-channel block, by altering the effective gating charge in the CB–OB transition, predicts unusual current activation and deactivation behavior. Fig. S6 highlights the inability of particular forms of Scheme 2 to produce triphasic current relaxations. Fig. S7 expands on Fig. 12 and examines the predictions of various closed-channel blocking schemes for the behavior of fractional availability in the presence of blocker species. Fig. S8 expands on Fig. 13 and shows that deactivation behavior is not particularly useful for distinguishing among different closed-channel blocking schemes. The modified version of the IchSim program used for kinetic simulations is also included in the supplemental material. The supplemental material is available at http://www.jgp.org/cgi/content/full/jgp.200910251/DC1.

**RESULTS**

In this paper, we show that, although bbTBA does block both open and closed channels, bbTBA does not block in a fully state-independent fashion. The results support the view that, independent of specific blocking model, closed channels with inactive voltage sensors are insensitive to block by bbTBA. Such a result might arise either because (1) a strong closed-state voltage dependence minimizes bbTBA block at negative potentials, or (2) there are voltage-dependent conformational changes among closed states permissive for block. Our analysis tends to exclude the possibility of strong closed-state voltage dependence, while supporting the view that block is coupled to conformational changes among closed states. However, the results provide no direct insight into the physical basis for the inability of bbTBA to block closed channels with inactive voltage sensors. We envision two primary mechanistic explanations for the dependence of closed-channel block
To assess the ability of different channel block models to describe bbTBA behavior, we fit each family of GV curves over all bbTBA concentrations both with the strict open-channel block model (OB model; Scheme 1a) and with a model in which both open and closed channels are blocked identically (CB-OB model; Scheme 2a). The GV curves at each [Ca²⁺] were fit with Eq. 12 (Fig. 1 B) to assess the adequacy of the OB model, and then fit with Eq. 13 (Fig. 1 C) to assess the CB-OB model. Block parameters are given in Fig. 1 and its legend. Neither scheme does a good job of fitting the GV curves over all Ca²⁺. As highlighted by the blue ovals (Fig. 1 B, 1 and 2), at 0 and 4 µM Ca²⁺, the OB model failed to describe the extent of block produced by the higher concentrations of bbTBA, particularly at lower fractional conductances. In contrast, at 100 µM Ca²⁺, the CB-OB model overestimated the extent of block observed with higher bbTBA at lower fractional conductances (Fig. 1 C, 3). Furthermore, the best fit of the CB-OB model does not describe the upward trend in conductance at the most positive voltages with bbTBA (e.g., Fig. 1 C, 2).

We also fit each of the GV curves individually with Eq. 8, with W constrained to either 100,000 (the OB assumption) or 1 (the CB-OB assumption). Fitting of the GV curves at a single Ca²⁺ resulted in better approximations of the data. However, consistent with Fig. 1, at 0 and 4 µM, the CB-OB model did a much better job of reproducing the specific curvature of the data. Similarly, at 100 (or 300 µM) Ca²⁺, the OB model better describes the GV curves. This was also supported by a comparison of the sum of the squares of the fit obtained with each model. Another factor supporting this idea is on closed-state conformation changes: (1) a change in bbTBA access to the central cavity perhaps involving an expansion of an aperture separating the central cavity from the bulk cytosol, and (2) a change in bbTBA binding affinity within the central cavity. Although this issue is unanswered in this paper, these potential explanations of physical mechanism may help guide understanding of the results and analysis.

The apparent state dependence of block by bbTBA differs between low and high Ca²⁺

Slo1 currents were activated in inside-out patches at various voltage steps with 0, 4, or 100 µM cytosolic Ca²⁺. At each [Ca²⁺], currents were generated in the presence and absence of 0, 1, 5, and 20 µM bbTBA in the solution bathing the cytosolic face of the membrane (Fig. 1). At higher [Ca²⁺] in which current activation is faster, the slower block by bbTBA produces a time-dependent reduction in outward current (traces at 100 µM Ca²⁺). Typically, any individual patch was only used to generate GV curves at one [Ca²⁺], but over the full range of [bbTBA]. Because rapid unblock occurred during tail currents, measurements of steady-state current levels at different voltages were used for the generation of GV curves at each [Ca²⁺] (Fig. 1, B and C). Note that identical GV curve values are plotted in B and C, but each panel was fit with a different blocking model. Qualitatively, the features of block, particularly with 20 µM bbTBA, exhibit differences at lower and higher Ca²⁺. In particular, at low open probabilities, bbTBA produces more appreciable block at lower Ca²⁺ concentrations than it does at higher Ca²⁺ concentrations.

To assess the ability of different channel block models to describe bbTBA behavior, we fit each family of GV curves over all bbTBA concentrations both with the strict open-channel block model (OB model; Scheme 1a) and with a model in which both open and closed channels are blocked identically (CB-OB model; Scheme 2a). The GV curves at each [Ca²⁺] were fit with Eq. 12 (Fig. 1 B) to assess the adequacy of the OB model, and then fit with Eq. 13 (Fig. 1 C) to assess the CB-OB model. Block parameters are given in Fig. 1 and its legend. Neither scheme does a good job of fitting the GV curves over all Ca²⁺. As highlighted by the blue ovals (Fig. 1 B, 1 and 2), at 0 and 4 µM Ca²⁺, the OB model failed to describe the extent of block produced by the higher concentrations of bbTBA, particularly at lower fractional conductances. In contrast, at 100 µM Ca²⁺, the CB-OB model overestimated the extent of block observed with higher bbTBA at lower fractional conductances (Fig. 1 C, 3). Furthermore, the best fit of the CB-OB model does not describe the upward trend in conductance at the most positive voltages with bbTBA (e.g., Fig. 1 C, 2).
that the parameters of the best fit for either the OB or CB-OB model differ among low and high \([\text{Ca}^{2+}]\). In particular, the voltage dependence of block switches from block being favored by more positive potentials at higher \([\text{Ca}^{2+}]\) to being weakened at higher voltages with low \([\text{Ca}^{2+}]\). Similarly, the estimated K_b values are not consistent across \([\text{Ca}^{2+}]\) concentrations. The inability of either model to yield a generally consistent set of parameters for the blocking reaction can only indicate that neither model is an appropriate description of block of BK channels by bbTBA. The dependence of block on \([\text{Ca}^{2+}]\) suggests that block by bbTBA may not be entirely state independent.

Physically plausible models for activation and block

The above results suggest that not all open or closed states are equivalent in their sensitivities to bbTBA. Here, we ask whether there is a blocking scheme that may better account for the apparently differential blocking effects of bbTBA at low and high \([\text{Ca}^{2+}]\). We base our examination of the predictions for bbTBA block on physically plausible gating models of BK channel activation (Cui and Aldrich, 2000; Horrigan and Aldrich, 2002). To examine predictions for blockade at a single \([\text{Ca}^{2+}]\), blocking models were constructed based on a basic 10-state activation model (see Scheme 1a and Fig. S1 A), in which activation of BK channels at a given \([\text{Ca}^{2+}]\) involves movement of four voltage sensors defined by equilibrium constant (J') and a closed-open conformational equilibrium (L'(0)) (Horrigan and Aldrich, 2002). Channel activation is coupled to voltage sensor activation via coupling constant, D'. The "prime" designations are used to highlight that these values are effectively altered by the fact that a complete gating model would also include tiers accounting for \([\text{Ca}^{2+}]\)-dependent activation, such that the values J', L', and D' are not identical to the constants used in the full allosteric gating scheme. Predictions of different models of block were examined using two nominal \([\text{Ca}^{2+}]\) concentrations, 4 and 300 \(\mu\text{M}\) (see Materials and methods and Figs. S1–S4 for additional detail of different blocking models). Values chosen for rates of voltage sensor movement and channel gating rates (Table I) were guided by previous estimates from Horrigan and Aldrich (2002) and adjusted to yield an overall V_h that generally approximates experimental observations obtained at 4 and 300 \(\mu\text{M}\) \([\text{Ca}^{2+}]\), with channel activation and deactivation rates generally consistent with observed data.

We evaluated three main categories of blocking mechanism: (1) only open channels are blocked (Scheme 1); (2) both open and closed states are blocked (Scheme 2); and (3) both open and closed channels are blocked, but at least one voltage sensor must be activated in order for block to occur (Scheme 3). We also considered one variation of Scheme 3, in which block depends on voltage sensor movement, but the forward rate of block scales with the number of active voltage sensors (Scheme 4). One earlier observation that justifies consideration of the idea that voltage sensor movement may result in steps permissive for block is that closed-state inactivation of BK channels depends on voltage-dependent steps that occur in the absence of channel opening (Ding and Lingle, 2002). For Schemes 2–4, we considered several specific cases. First, for Schemes 2a, 3a, and 4a, we assumed that the open- and closed-channel blocking equilibria were identical as were their voltage

<table>
<thead>
<tr>
<th>4 (\mu\text{M}) Ca^{2+}</th>
<th>C1–C2</th>
<th>C2–C3</th>
<th>C3–C4</th>
<th>C4–C5</th>
<th>(\gamma)</th>
<th>(\zeta)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\alpha(0)_c)</td>
<td>4,480</td>
<td>3,560</td>
<td>2,240</td>
<td>1,120</td>
<td>0.29</td>
<td>(\epsilon)</td>
</tr>
<tr>
<td>(\beta(0)_c)</td>
<td>30,000</td>
<td>60,000</td>
<td>90,000</td>
<td>120,000</td>
<td>(\alpha(0)_c)</td>
<td>25</td>
</tr>
<tr>
<td>(\delta(0))</td>
<td>0.4</td>
<td>7.544</td>
<td>120</td>
<td>288</td>
<td>z</td>
<td>(\epsilon)</td>
</tr>
<tr>
<td>(\gamma(0))</td>
<td>3,200</td>
<td>2,350.08</td>
<td>1,536</td>
<td>147.456</td>
<td>7.168</td>
<td>(\epsilon)</td>
</tr>
<tr>
<td>(\alpha(0)_o)</td>
<td>24</td>
<td>24</td>
<td>24</td>
<td>24</td>
<td>(\epsilon)</td>
<td></td>
</tr>
<tr>
<td>(\beta(0)_o)</td>
<td>12,000</td>
<td>24,000</td>
<td>36,000</td>
<td>48,000</td>
<td>(\epsilon)</td>
<td></td>
</tr>
<tr>
<td>(\delta(0))</td>
<td>0.4</td>
<td>7.544</td>
<td>120</td>
<td>288</td>
<td>z</td>
<td>(\epsilon)</td>
</tr>
<tr>
<td>(\gamma(0))</td>
<td>2,227</td>
<td>841,511</td>
<td>395,911</td>
<td>10,557</td>
<td>0.2815368</td>
<td>(\epsilon)</td>
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<tr>
<td>(\alpha(0)_o)</td>
<td>4,000</td>
<td>4,000</td>
<td>4,000</td>
<td>4,000</td>
<td>(\epsilon)</td>
<td></td>
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<tr>
<td>(\beta(0)_o)</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>(\epsilon)</td>
<td></td>
</tr>
</tbody>
</table>

Numbers are rates (s\(^{-1}\)) for the indicated transitions (e.g., C1–C2, state C1 to state C2). Calculated allosteric constants based on microscopic rates: L' (4 \(\mu\text{M}\) = 0.000125; D' (4 \(\mu\text{M}\) = 25; J' (4 \(\mu\text{M}\) = 0.057333; L' (300 \(\mu\text{M}\) = 0.0107768; D' (300 \(\mu\text{M}\) = 37.5; J' (300 \(\mu\text{M}\) = 0.1333333; \(\zeta\) = 0.3 \(\epsilon\) \(\gamma\) = 0.58 \(\epsilon\).
dependencies. In Schemes 2b, 3b, and 4b, we assumed that closed-state block was voltage independent, but $K_{bc} = K_{bo}$. Finally, for all schemes, we also examined variations in which both the binding affinities and voltage dependencies of open- and closed-channel block varied independently (termed $2^\prime$, $3^\prime$, and $4^\prime$). Because Scheme 4 was not clearly superior to Scheme 3 in accounting for the key aspects of block by bbTBA, to simplify this presentation we minimize further consideration of Scheme 4. Rates of block for simulations were chosen to approximate the macroscopic behavior of bbTBA (Table II).

Simulations with models in which block is dependent on voltage sensor movement reproduce differential effects of bbTBA on GV curves at low and high Ca$^{2+}$

Examples of simulated currents based on various blocking schemes (Schemes 1a, 2a, and 3a) are shown in Fig. 2 for a blocker concentration that is approximately sixfold the $K_b$ for block of the open channel at 0 mV. For all models, this produces an ~90% block at a potential of +200 mV. Resulting GV curves were determined at different effective blocker concentrations, both for 4 µM Ca$^{2+}$ (Fig. 3, left-hand panels) and for 300 µM Ca$^{2+}$ (Fig. 3, middle panels). In each case, the families of GV curves were fit with Eq. 8, either with $W = 1$ (red lines; full OB-CB model equivalent to the predictions of Scheme 2a) or $W = 100,000$ (blue lines; full OB model equivalent to the predictions of Scheme 1a). For the GV curves explicitly generated by Scheme 2a, Eq. 8 with $W = 1$ yielded an essentially perfect fit, whereas similarly for the GV curves generated by Scheme 1a, Eq. 8 with $W = 100,000$ yielded an essentially perfect fit. However, for GV curves generated by alternative blocking models, it can be seen that the families of GV curves exhibit interesting differences in their ability to be fit under the constraints of Eq. 8. We used two types of comparisons to assess the ability of either the strictly state-independent model or open-channel block model to fit the GV curves. First, we simply determined the total sum of squares error for different fits (not depicted). Second, we determined the absolute error of a given fit for the full set of GV curves at each voltage (Fig. 3, right-hand panels). The former provides a direct measure of whether a given set of GV curves is better fit by one or the other model, whereas the latter provides a visual picture of the range of voltages over which deviations of a particular model are most pronounced.

The primary conclusion here is that, when currents are simulated by a scheme in which blockade depends on voltage sensor movement (Scheme 3a), the CB-OB dependencies. In Schemes 2b, 3b, and 4b, we assumed that closed-state block was voltage independent, but $K_{bc} = K_{bo}$. Finally, for all schemes, we also examined variations in which both the binding affinities and voltage dependencies of open- and closed-channel block varied independently (termed $2^\prime$, $3^\prime$, and $4^\prime$). Because Scheme 4 was not clearly superior to Scheme 3 in accounting for the key aspects of block by bbTBA, to simplify this presentation we minimize further consideration of Scheme 4. Rates of block for simulations were chosen to approximate the macroscopic behavior of bbTBA (Table II).
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this model tend to converge to values similar to the single-site models, with the open-channel block site having very low affinity. We will not consider these latter models in more detail.

Models in which block is coupled to voltage sensor movement better fit GV curves at both low and high Ca\textsuperscript{2+}. In Fig. 1, data for the GV curves at each Ca\textsuperscript{2+} were obtained in separate sets of patches. To better constrain blocking parameters, it would be desirable to fit GV curves obtained at both low and high Ca\textsuperscript{2+} simultaneously. Data were therefore collected from a set of patches in which the effects of 0, 1, 5, and 20 µM bbTBA were tested at both 4 and 300 µM (Fig. 4). With activation parameters defined from fitting GV curves with 0 bbTBA, the full family of GV curves were then fit simultaneously with equations for Scheme 2a (Eq. 13, complete state-independent block), Scheme 1a (Eq. 12, strictly open-channel block), and Scheme 3a (Eq. 14, block dependent on initial voltage sensor movement), in all cases with the assumption that block of open and closed states has an identical affinity (Kbc = Kbo) and voltage dependence (z_o = z_c). It can be clearly seen that both Scheme 2a (Fig. 4 A) and Scheme 1a (Fig. 4 B) fail to describe important features of the GV curves, consistent with the simulations of Fig. 3. In contrast, Scheme 3a, in which block depends on activation of at least one voltage sensor, captures the shape of the GV curves particularly at low Po for both 4 and 300 µM Ca\textsuperscript{2+} (Fig. 4 C).

equation (Eq. 8; W = 1) provides a better fit to the 4-µM GV curves, whereas the OB equation (Eq. 8; W = 100,000) provides the better fit to the 300-µM GV curves. This qualitatively approximates the situation observed for the bbTBA block of BK currents shown in Fig. 1 and suggests that neither a fully state-independent CB-OB model nor a strict OB model adequately describes bbTBA block.

As shown below, the basic OB model (Scheme 1a) clearly results in behaviors that are distinct from our observations, as also noted previously (Wilkens and Aldrich, 2006). For similar reasons, another scheme, not mentioned above, in which only channels with four activated voltage sensors are blocked, can also be excluded. We also found that a trapping model in which closed channels cannot be blocked, but open-blocked channels can close (Lingle, 1983; Neely and Lingle, 1986; Wilkens and Aldrich, 2006), makes predictions inconsistent with observations. We also tested a two-site model (see Materials and methods) in which block is posited to occur at two sites: an open-channel block site, and a peripheral site that is accessible in both open and closed states. This model potentially involves a large number of parameters, although various simplifying assumptions can be made to reduce model complexity. However, two aspects of this model led us to exclude it from consideration: first, the open-channel block portion of the model predicts properties of tail currents inconsistent with observation and, second, the best fits of this model tend to converge to values similar to the single-site models, with the open-channel block site having very low affinity. We will not consider these latter models in more detail.
potential issue when $z_c > z_o$ is that, in a full allosteric scheme in which microscopic reversibility is assumed, a pronounced voltage dependence of closed-channel block requires a very pronounced voltage-dependent effect on the CB–OB transition (see Scheme 6 for one blocking tier in which voltage dependence is split evenly within the CB–OB equilibrium). In Scheme 6, $K_{bc}(V) = (k_r/k_f)\exp(-0.1V/kT)$, $K_{bo}(V) = (k_r/k_f)\exp(-0.8V/kT)$, with $L(V) = \delta(V)/\gamma(V)$. If one requires that microscopic reversibility be maintained, a voltage dependence equivalent to the difference in the voltage dependence of closed- versus open-channel block must be distributed between transitions in the CB $\leftrightarrow$ OB equilibrium. When such voltage dependence is distributed identically to both the channel-opening and -closing transitions, this predicts strong slowing effects on macroscopic current activation and deactivation kinetics (Fig. S5), which are inconsistent with the observed effects of bbTBA on gating kinetics (Figs. 1 and 8 for activation and Fig. 13 for deactivation). In fact, regardless of how voltage dependencies are distributed to the CB–OB transitions in Scheme 6, as long as microscopic

We next relaxed the constraints that $K_{bc} = K_{bo}$ and $z_o = z_c$, and GV curves were fit with Schemes 2 and 3 with $K_{bo}$, $K_{bc}$, $z_o$, and $z_c$, with all allowed to vary independently (Fig. 4, D and E). With all block parameters unconstrained, Schemes 2 and 3 each gave very reasonable fits of the GV curves. The deviations (SSQ/pt) that arose solely from the GVs obtained in bbTBA were 0.00925 (Scheme 3) and 0.010225 (Scheme 2), corresponding to an $\sim 10\%$ increase in quality of fit with Scheme 3 relative to Scheme 2.

Despite the overall adequacy of the fits for both models (Fig. 4, D and E), we were concerned about the physical plausibility of the actual parameter values. In particular, in each case, the best fit required that $z_c$, the voltage dependence of closed-channel block, be rather large, with $0.8 \varepsilon$ for Scheme 2 and $0.44 \varepsilon$ for Scheme 3. Voltage dependence in closed-channel block cannot be excluded out of hand and, in fact, calculations based on physical dimensions of the KcsA open- and closed-channel structures (Roux et al., 2000) suggest that the effective field will extend farther into the central cavity from the selectivity filter in closed channels. However, a potential issue when $z_c > z_o$ is that, in a full allosteric scheme in which microscopic reversibility is assumed, a pronounced voltage dependence of closed-channel block requires a very pronounced voltage-dependent effect on the CB–OB transition (see Scheme 6 for one blocking tier in which voltage dependence is split evenly within the CB–OB transition). In Scheme 6, $K_{bo}(V) = (k_r/k_f)\exp(-0.1V/kT)$, $K_{bc}(V) = (k_r/k_f)\exp(-0.8V/kT)$, with $L(V) = \delta(V)/\gamma(V)$. If one requires that microscopic reversibility be maintained, a voltage dependence equivalent to the difference in the voltage dependence of closed- versus open-channel block must be distributed between transitions in the CB $\leftrightarrow$ OB equilibrium. When such voltage dependence is distributed identically to both the channel-opening and -closing transitions, this predicts strong slowing effects on macroscopic current activation and deactivation kinetics (Fig. S5), which are inconsistent with the observed effects of bbTBA on gating kinetics (Figs. 1 and 8 for activation and Fig. 13 for deactivation). In fact, regardless of how voltage dependencies are distributed to the CB–OB transitions in Scheme 6, as long as microscopic

![Figure 3. Models in which block is coupled to voltage sensor movement can account for differential ability of OB and CB–OB models to fit data at low and high Ca2+.](image)
We next considered the case in which closed-state block is completely voltage independent (Fig. 4, F and G). With this assumption and one less free parameter, the fit of Scheme 3b was quite a bit better than for Scheme 2b, with the deviations \( \text{SSQ/pt} \) arising from the GV curves in bbTBA being 0.0154 (Scheme 3) and 0.0415 (Scheme 2).

Overall, this comparison of the ability of Schemes 2 and 3 to fit the GV curves at low and high Ca\(^{2+}\) indicates that a model in which the resting closed state is unblocked by bbTBA provides a better description of the data than a model in which bbTBA blocks all closed states. Although we have considered the consequences of different assumptions for the voltage dependence of closed-channel block, the analysis does not allow strong conclusions regarding \( z_c \), except that, if microscopic

![Scheme 6 Diagram](image)

**Scheme 6**

\[
\begin{align*}
C & \xrightarrow{k_f \exp^{0.4V/kT}} CB \\
\text{exp}^{0.212V/kT} & \xrightarrow{k_r \exp^{-0.888V/kT}} \text{exp}^{0.138V/kT} \\
O & \xrightarrow{k_f \exp^{0.05V/kT}} OB \\
\text{exp}^{0.262V/kT} & \xrightarrow{k_r \exp^{-0.05V/kT}} \text{exp}^{0.262V/kT}
\end{align*}
\]

reversibility is maintained, the net effect is to alter some aspect of activation or deactivation kinetics in a fashion that contradicts observation.

We next considered the case in which closed-state block is completely voltage independent (Fig. 4, F and G). With this assumption and one less free parameter, the fit of Scheme 3b' was quite a bit better than for Scheme 2b', with the deviations \( \text{SSQ/pt} \) arising from the GV curves in bbTBA being 0.0154 (Scheme 3) and 0.0415 (Scheme 2).

We next considered the case in which closed-state block is completely voltage independent (Fig. 4, F and G). With this assumption and one less free parameter, the fit of Scheme 3b' was quite a bit better than for Scheme 2b', with the deviations \( \text{SSQ/pt} \) arising from the GV curves in bbTBA being 0.0154 (Scheme 3) and 0.0415 (Scheme 2).
Model dependence of fractional unblock at low and high open probabilities

An important result supporting the idea that bbTBA blocks both closed and open channels in a state-independent fashion was the demonstration that the fractional unblock of single BK channels either at low or high Ca\(^{2+}\) was largely independent of channel Po (Wilkens and Aldrich, 2006). Fractional unblock corresponds to the fraction of BK current that remains unblocked at given bbTBA and activation conditions. For state-independent block, the fraction of unblocked channels will be identical under all conditions of channel activation.

Using the GV curves in Fig. 1, we determined fractional unblock at 4 and 300 µM Ca\(^{2+}\) with 5 µM bbTBA (Fig. 5 A). Estimates of fractional unblock for Po values >0.4 obtained with 4 µM Ca\(^{2+}\) overlapped estimates of fractional unblock obtained with 300 µM Ca\(^{2+}\). This is generally consistent with the earlier observations of Wilkens and Aldrich (2006), in which fractional unblock for different [Ca\(^{2+}\)] was only compared at relatively high Po. However, our estimates of fractional unblock obtained at Po < 0.4 with 4 µM Ca\(^{2+}\) exhibited marked deviations from the fractional unblock with 300 µM Ca\(^{2+}\) at each voltage. The differences between our results and those of Wilkens and Aldrich may reflect the more limited range of estimates at lower Po provided in the earlier study.

Based on Fig. 5 A, two aspects of these fractional unblock relationships contradict the expectations of a completely state-independent blocking scheme: first, the difference in fractional unblock observed at low and high Ca\(^{2+}\) over some voltages and, second, the upward curvature in unblock observed at 300 µM Ca\(^{2+}\).

To compare predictions of different models, we determined fractional unblock from the GV curves generated using the GV curves in Fig. 1, the fractional unblock (-G(bbTBA/G(control)) with 5 µM bbTBA was determined over the full range of voltages for both 300 µM Ca\(^{2+}\) (red circles) or 4 µM Ca\(^{2+}\) (blue circles) and compared with GV curves for current activation. Over the range of +20 to +60 mV, the fractional unblock at 4 µM Ca\(^{2+}\) is larger than that for 300 µM Ca\(^{2+}\), whereas fractional unblock with 300 µM Ca\(^{2+}\) approaches 1.0 at the most negative activation voltages.

(E) Block and fractional unblock were determined from currents simulated using blocking constants derived from simultaneous fitting of GV curves to Scheme 2b (Fig. 4 G, with K_{bc} of ~3 K_{bo}). (F) Predictions derived from currents simulated with Scheme 3a are plotted.

(G) Predictions based on Scheme 3b (z_c = 0.0) are plotted. (H) Predicted block and fractional unblock are shown based on currents simulated with blocking constants from simultaneous fitting of GV curves to Scheme 3b (Fig. 4 H, with K_{bo} = 3 K_{bc}).

(E) Differences in fractional unblock of BK channels by bbTBA between 4 and 300 µM Ca\(^{2+}\) are not supportive of fully state-independent block. (A) From measurements of macroscopic conductance (Fig. 1), the fractional unblock (-G(bbTBA/G(control)) with 5 µM bbTBA was determined over the full range of voltages for both 300 µM Ca\(^{2+}\) (red circles) or 4 µM Ca\(^{2+}\) (blue circles) and compared with GV curves for current activation. Over the range of +20 to +60 mV, the fractional unblock at 4 µM Ca\(^{2+}\) is larger than that for 300 µM Ca\(^{2+}\), whereas fractional unblock with 300 µM Ca\(^{2+}\) approaches 1.0 at the most negative activation voltages.

(B) Predictions for simple open-channel block (Scheme 1a) are illustrated. GV curves at 4 (open circle) and 300 (closed circle) µM Ca\(^{2+}\) are plotted along with GV curves in the presence of bbTBA (blue with 4 µM Ca\(^{2+}\) and red with 300 µM Ca\(^{2+}\)). Fractional unblock is also plotted for 4 µM (blue circle) and 300 µM (red circle) at each voltage. (C) Similar plots for currents generated with Scheme 2a show that fractional unblock is identical for both 4 and 300 µM Ca\(^{2+}\) at each voltage. (D) The relationships for Scheme 2b, in which closed-channel block is voltage independent, are shown. The limiting fractional unblock at negative voltages reflects the voltage independence of closed-channel block.
by different blocking schemes (Fig. 5, B–H). With Scheme 1a (Fig. 5 B; simple open-channel block), fractional unblock is identical at 4 and 300 µM Ca²⁺ over Po values for 4 µM above ~0.6. Below 0.6, the fractional unblock curves exhibit marked differences, although both approach 1.0 at low Po values. In contrast, for the fully state-independent Scheme 2a (Fig. 5 C; \( K_{bc} = K_{ob} \) and \( z_c = z_o \)), the fractional unblock curves at 4 and 300 µM completely overlap as expected (Wilkens and Aldrich, 2006). However, when \( z_c = 0 \) as in Scheme 2b (Fig. 5 D), deviations between fractional unblock are observed at lower and higher Ca²⁺, with both reaching an asymptote at negative voltages corresponding to the voltage-independent \( K_{ob} \). The fractional unblock predictions based on fitted values for Scheme 2b’ (Fig. 4 F; \( K_{bo} \neq K_{bc} \) and \( z_c = 0 \)) also showed discrepancies between fractional unblock at lower and higher Ca²⁺ (Fig. 5 E) for models with block coupled to voltage sensor movement (Fig. 5, F–H), the differences between fractional unblock curves at 4 and 300 µM Ca²⁺ tend to be intermediate between those observed for Schemes 1a and 2a. However, in all cases, fractional unblock approaches 1 at the lowest Po values both for low and high Ca²⁺. This reflects the fact that the fully resting closed state is not subject to block by bbTBA in these models. It is also noteworthy that, for all the variants of Scheme 3 shown in Fig. 5 (F–H), the fractional unblock curves for 4 and 300 µM overlap down to a Po at 4 µM of ~0.5.

These considerations indicate that a variety of models that incorporate closed-channel block, but are not strictly state independent, can produce the correspondence of fractional unblock over the ranges previously examined by Wilkens and Aldrich (2006). Given challenges (small currents and large variance) that arise in the estimates of fractional unblock obtained at low Po for lower [Ca²⁺], the datasets used for this type of analysis may have limitations for rigorous model discrimination. However, the clear tendency of fractional unblock at 300 µM to approach 1.0 and the discrepancies between fractional unblock at 4 and 300 µM Ca²⁺ over the range of low Po in 4 µM Ca²⁺ (Fig. 5 A) suggest that the fully state-independent version of Scheme 2 is not consistent with observations.

Another important component of the analysis of Wilkens and Aldrich (2006) was an examination of the Po dependence of fractional unblock. Although their observation that similar fractional unblock with bbTBA is observed at both low and high Po over a range of voltages is consistent with the expectations of state-independent block (Fig. 6 A), we have found that this behavior could also be reproduced by Scheme 3 (Fig. 6, D–F), dependent on particular parameters for block of open and closed states. Scheme 3a (Fig. 6 D) predicts a difference in fractional block at low and high Po that is intermediate between that for open-channel block (Fig. 6 A, 1a) and Scheme 2a. For schemes in which \( K_{bc} = K_{ob} \), but \( z_c = 0 \), there is an additional increase in slope in the fractional block versus Po relationship (Fig. 6, B, Scheme 2b, and E, Scheme 3b). Interestingly, the fractional unblock versus Po relationship based on the fitted values (\( z_c = 0.0 \) and \( K_{bc} \neq K_{ob} \)) from Fig. 4 G for Scheme 3 results in relatively Po-independent estimates of fractional unblock (Fig. 6 F). This indicates that the absence of detectable slope in the fractional unblock
versus Po relationship does not allow a definitive test of whether a fully state-independent block model applies. Furthermore, it can be seen that a model in which block is dependent on voltage sensor activation is able to produce a Po independent of fractional unblock, similar to that observed for fully state-independent block.

**bbTBA does not decrease NPo at negative potentials**

The results of Fig. 5 A indicate that bbTBA may not produce the same fractional block at all open probabilities. We reasoned that, if block of BK channels by bbTBA does exhibit state dependence, this issue could best be tested by an examination of BK channel Po at very negative activation potentials. At potentials negative to about −120 mV, voltage sensors are predominantly inactive and BK Po is very low (Horrigan and Aldrich, 2002). In patches with a large number of channels, it is possible to measure NPo for channels with inactive voltage sensors and thus test the effects of bbTBA on NPo. In Fig. 7 A, the predictions for log(NPo) as a function of voltage are displayed for BK conductance at 30 µM Ca²⁺, along with the predictions for changes in log(NPo) with 100 µM bbTBA in accordance with open-channel block (Scheme 1a), state-independent block (Scheme 2a), or block dependent on voltage sensor movement (Scheme 3a). Whereas Scheme 2a predicts a similar fractional reduction in NPo at all voltages, both Scheme 1a and Scheme 3a predict no reduction in NPo at potentials where voltage sensors are inactive. 100 µM bbTBA was chosen for this evaluation because, based on the 0-voltage Kd for block of open and/or closed BK channels by bbTBA (~5 µM) and its voltage dependence (z ~0.1 e), we predict that 100 µM bbTBA should be at least 10 times the predicted Kd (~9.05 µM) at −150 mV. Thus, examination of NPo at negative potentials should provide a direct test for the idea that channels with resting voltage sensors may be insensitive to bbTBA.

In our experiments, patches were held at −150 mV with 30 µM Ca²⁺ to help elevate NPo. Under these conditions, voltage sensors remain inactive (Horrigan and Aldrich, 2002). A brief activation step to +20 mV was used to provide a measure of the total net BK conductance in the patch and to confirm the sensitivity of BK current to bbTBA under conditions where voltage sensors are activated. Example traces in Fig. 7 B show the marked inhibition of current at +20 mV by 100 µM bbTBA, with little obvious effect on channel activity at −150 mV. Expanded time base records of channel activity at −150 mV (Fig. 7 C) reveal no obvious decrease in overall NPo. In fact, total amplitude histograms of current at −150 mV reveal a small increase in NPo in the presence of 100 µM bbTBA (Fig. 7 D). Similar increases in NPo were observed in four other patches tested with 100 µM bbTBA at −150 mV. Although we not know the basis for the increase in NPo, the results show that blockade by bbTBA is markedly reduced at −150 mV relative to that occurring at voltages where voltage sensors are active (e.g., at +20 mV over 90% block in Fig. 7 B). In contrast, the state-independent blocking model predicts a much smaller difference in reduction in NPo between −150 and −20 mV. Specifically, with a voltage dependence of block of 0.1 e and low Po, the fractional reduction in NPo in the presence of bbTBA should be only about twofold less at −150 mV than at +20 mV. Although it might be suggested that the bbTBA-elicited increase in NPo almost
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whereas \( K_{bo} = K_{bc} \). In this case, triphasic relaxations were abolished for Scheme 2b (Fig. 9 C), while persisting for Scheme 3b (Fig. 9 F). Adjustments of parameters of block within the context of Scheme 2b failed to reveal conditions producing a triphasic relaxation. The difference between Schemes 2a, 2b, 3a, and 3b in producing triphasic relaxations is highlighted in Fig. S6. This outcome argues strongly that a blocking scheme in which all closed states can be blocked by bbTBA, but closed-channel block is voltage independent, is inconsistent with experimental observations. We also compared current simulations using parameters of block obtained from the fits in Fig. 4 (F and G), where \( z_c = 0.0 \), whereas \( K_{bo} = K_{bc} \). In this case, triphasic relaxations were abolished for Scheme 2b (Fig. 9 C), while persisting for Scheme 3b (Fig. 9 F). Adjustments of parameters of block within the context of Scheme 2b failed to reveal conditions producing a triphasic relaxation. The difference between Schemes 2a, 2b, 3a, and 3b in producing triphasic relaxations is highlighted in Fig. S6. This outcome argues strongly that a blocking scheme in which all closed states can be blocked by bbTBA, but closed-channel block is voltage independent, is inconsistent with experimental observations. We also compared current simulations using parameters of block obtained from the fits in Fig. 4 (F and G), where \( z_c = 0.0 \), but \( K_{bo} \neq K_{bc} \). Again, the simulations based on Scheme 2 (Fig. 9 D) fail to exhibit triphasic relaxations, whereas those based on Scheme 3 (Fig. 9 G) exhibit triphasic relaxations. Overall, none of the schemes and sets of
Fractional availability of BK channels in the presence of bbTBA

Given that bbTBA blocks both open and at least some closed channels, the peak current during a depolarization in the presence of bbTBA should reflect, in addition to the time course of block of open channels, the resting equilibrium between channels in C and CB states before depolarization. Varying the conditioning potential before depolarization might therefore be expected to provide some information regarding the voltage dependence of the C–CB equilibrium. We therefore examined the dependence of peak BK current in the presence of different [bbTBA] on conditioning potentials between $-300$ and $+100$ mV. We term this the fractional availability of BK channels, reflecting in part the relative occupancy of channels in closed-unblocked states.

Parameters we tested completely mimics all features of the experimental data. In particular, differences exist in regards to the relative change in steady-state current levels at different voltages and also in the relative magnitude of the initial time-dependent block at different [bbTBA]. However, this arises in part because the experimental data were collected with a 5-ms prepulse to $-140$ mV, whereas the simulations used a 20-ms prepulse that was found to better approximate a steady-state condition.

In principle, the distinctions among schemes in terms of the relative amplitudes of the three components in such relaxations might be used to evaluate whether one or the other scheme better approximates the actual behavior of bbTBA. However, practically, we have found it difficult to reliably measure such relaxations at high bbTBA over a range of voltages because robust sets of such relaxations require a large number of channels and/or averaging of a large number of runs. Thus, at this point we simply state that, when $z_o = z_c$, all schemes permit triphasic relaxations, whereas if $z_o = 0.0$, triphasic relaxations only occur in schemes (both Schemes 3 and 4) in which block is coupled to voltage sensor movement.

Figure 9. Triphasic relaxations arise from closed-channel block, but do not clearly distinguish among different models of closed-channel block. (A) These traces replot Fig. 8 A to allow comparison to simulated currents. Red traces correspond to $+240$ mV. (B) Currents were simulated using Scheme 2a with activation rates given in Table I, with the effective [blocker] = $5.71 * K_{db}$, with $z_o = z_c = 0.2$. Top traces correspond to 4 µM Ca$^{2+}$ and the bottom traces to 300 µM. When $z_o = z_c = 0.1$, the triphasic relaxations were not as obvious. (C) Currents were simulated with Scheme 2b with $z_o = 0.0$. No triphasic relaxations are observed. (D) Currents were simulated with Scheme 2b’ but with a ratio of $K_{db}/K_{bc}$ based on fitted values from Fig. 4 F. Again, no triphasic relaxation is observed. (E) Currents were simulated with Scheme 3a with $z_o = z_c = 0.1$. (F) Currents were simulated with Scheme 3b ($z_o = 0.1$ and $z_c = 0.0$). (G) Currents were stimulated with values corresponding to the fitted values of Scheme 3b’ from Fig. 4 G.
The change in peak current amplitude as a function of prepulse potential was also examined for BK currents activated by 100 µM Ca²⁺ (Fig. 10, E and F). With 100 µM [Ca²⁺], the reduction of peak current was shifted to more negative potentials, but at potentials from −140 to −500 mV, there was still little change in peak current amplitude (Fig. 10 G) or fractional availability (Fig. 10 H). The leftward shift in Vₕ with increases in Ca²⁺ is to be expected in conjunction with the leftward shift in the activation curve for the BK channels. The dependence of peak current on prepulse voltage was fit with a Boltzmann function with values given in the legend to Fig. 10. An increase either in [Ca²⁺] or in [bbTBA] shifts the availability curves leftward.

Well-defined half-availability curves were difficult to achieve, even with the averaging of many runs, because of the stochastic variability in the peak current amplitude. To better define such curves, we examined availability with prepulse voltages from −300 to −100 with larger numbers of runs in one set of patches, while in another set we examined voltages from −200 to +100 (+150 mV with 4 µM Ca²⁺) mV. This allowed a larger number of sweeps and the use of 20-mV increments in conditioning potentials. Fractional availability curves covering the range of −300 to +150 mV were generated by normalizing the curves at overlapping voltages (Fig. 11).

At 4 µM Ca²⁺, with either 20 or 75 µM bbTBA, the range of voltages over which changes in fractional availability of BK current occurs exhibits substantial overlap with the activation curve obtained under the same [Ca²⁺] concentrations (Fig. 11 A), with a leftward shift in fractional availability with increases in [bbTBA]. The magnitude of the voltage dependence of the fractional availability (for 20 µM bbTBA, z = 0.76 ± 0.22 e; for 75 µM bbTBA, z = 0.59 ± 0.01 e) is less than that observed for the voltage dependence of activation by Ca²⁺ (q = 1.32 ± 0.07 e) but much greater than that of macroscopic steady-state current block (z = −0.1 e).

Figure 10. Fractional availability of current in the presence of bbTBA exhibits voltage independence at negative potentials. (A) Slo1 currents from excised inside-out patches were activated by the indicated voltage protocol (top; prepulse voltages from −300 to +100 mV with 40-mV increments) with 4 µM of internal Ca²⁺ and 20 µM bbTBA. The peak amplitude of the rapidly decaying current exhibits little change until prepulse voltages of 0 mV or more positive. Red trace corresponds to a −300-mV prepulse and blue to a +60-mV prepulse. (B) In the same patch, currents were activated with 4 µM Ca²⁺ along with 75 µM bbTBA. Red trace, −300-mV prepulse; blue trace, −20-mV prepulse. (C) The peak current amplitudes are plotted as a function of prepulse potential for both 20 µM (open circles) and 75 µM (filled circles) bbTBA. Red lines correspond to fits of a Boltzmann function with 4 µM Ca²⁺ and 20 µM bbTBA. Red lines correspond to fits of a Boltzmann function with Vₕ = 64.9 ± 54.4 mV (z = 0.62 ± 0.33 e) and 22.9 ± 34.9 mV (z = 0.46 ± 0.13 e) for 20 and 75 µM bbTBA, respectively. (D) Normalized fractional availability was determined based on the fit of the Boltzmann function in C, better illustrating the leftward shift in availability with the increase in bbTBA. (E) The same protocol was used to activate currents in 100 µM Ca²⁺ (a different patch) with 20 µM bbTBA. The peak current at −100 mV (blue trace) shows some reduction compared with −300 mV (red trace). (F) The same patch was exposed to 75 µM bbTBA, producing more rapid and complete block. (G) The peak current is plotted as a function of prepulse potential either with 20 µM bbTBA (Vₕ = −39.4 ± 3.3 mV and z = 0.72 ± 0.09 e) or 75 µM bbTBA (Vₕ = −72.7 ± 4.5 mV and z = 0.72 ± 0.09 e). (H) The normalized fractional availability curves for the data in G are replotted.
The shift in availability curves at a fixed [bbTBA] as Ca\(^{2+}\) is altered is highlighted in Fig. 11 (C and D, 20 µM bbTBA and 75 µM bbTBA, respectively). The availability curves shift in a Ca\(^{2+}\)-dependent fashion, indicating that the blocking events that limit the amplitude of the peak transient current must be state dependent.

These results allow two mutually exclusive interpretations. One possibility is that, if there is block of closed channels over the range of \(-120\) to \(-300\) mV, that block must be entirely voltage independent. If closed-channel block had a voltage dependence similar to open-channel block (\(\sim 0.11\) \(e\)), a clear change in fractional availability would be expected over the range of \(-120\) to \(-300\) mV (see below). The second possibility is that there may be no block of BK channels in fully resting states. The latter interpretation is supported by the close overlap of the fractional availability curve with the activation curve and the similarities in voltage dependence, suggesting that changes in fractional availability are strongly coupled to states involved in channel activation, rather than direct block of closed channels.

Predictions for fractional availability exclude strict state-independent block by bbTBA

For each scheme, currents were simulated to allow determination of the voltage dependence of fractional availability (Fig. 12; also see Fig. S7). Simulated currents (both 4 and 300 µM Ca\(^{2+}\)) were activated by a depolarization to +200 mV after a conditioning voltage spanning the range of \(-400\) to 100 mV (Fig. 12 A). Qualitatively, the completely state-independent Scheme 2a (Fig. 12 C) predicts a behavior that clearly distinguishes it from both the open-channel block of Scheme 1a (Fig. 12 B) as well as other schemes incorporating closed-channel block (Fig. 12, D–F). For Scheme 2a, as the conditioning potential is made more negative, the peak transient current activated at +200 mV increases continuously down through \(-400\) mV. This reflects the fact that the fractional availability in Scheme 2a will be defined solely by the weak voltage dependence of the C–CB equilibrium of the channels with four inactive voltage sensors. In contrast, Schemes 3a and 3b (and 4a and 4b in Fig. S7) both exhibit saturation in fractional availability.

The fractional availability curves were plotted relative to activation GVs at nominal 4 µM Ca\(^{2+}\) (Fig. 12 G). When only open states can be blocked (Scheme 1a), the slope of the half-availability curve closely approximates the slope of the activation GV, whereas for strictly state-independent block (Scheme 2a), the slope of the half-availability curve reflects the voltage dependence of closed-channel block. For schemes in which block is coupled to activation of voltage sensors, the slope of the half-availability curves approaches that of the activation curves, and is clearly greater than that expected for the voltage dependence of either closed- or open-channel block. These model-specific availability curves place some constraints on the types of models that may account for the fractional availability curves obtained for expressed BK channels in the presence of bbTBA (Fig. 11). The fully state-independent Scheme 2a is clearly inconsistent with the observed data.
Different block models also predict useful differences in the Ca\(^{2+}\) dependence of fractional availability curves. Models in which closed-channel block is coupled to voltage sensor movement (Schemes 3 and 4) exhibit Ca\(^{2+}\)-dependent shifts in the fractional availability curves (Fig. S7, F and H) because increased inactivation is favored by transitions involved in activation. In contrast, in any version of Scheme 2 in which block of all closed channels can occur, fractional availability becomes largely uncoupled from Ca\(^{2+}\) (Fig. 7 D) because availability becomes dominated largely by block of closed channels with inactive voltage sensors.

Modification of BK tail current decay by bbTBA depends on internal [Ca\(^{2+}\)].

Wilkens and Aldrich (2006) showed that 5 µM bbTBA produces little alteration in BK deactivation rates. Although this is generally consistent with the idea that blocked but open channels can close without blocker first dissociating from the channel, we find that the absence of tail current prolongation is not only predicted by state-independent block. Here, we examine BK tail currents in the presence of different [bbTBA] with either 4 or 300 µM Ca\(^{2+}\). For activation with 4 µM Ca\(^{2+}\) (Fig. 13 A), even with 20 µM bbTBA (which produces a >50% reduction in tail current amplitude measured at −150 mV), there is little discernible effect on tail current time course (Fig. 13, B and C), consistent with earlier observations (Wilkens and Aldrich, 2006). However, with 300 µM Ca\(^{2+}\) (Fig. 13 D), both 5 and 20 µM bbTBA exhibited clear effects on tail current behavior, producing both a slowing of tail current decay and a slight unblocking hook at the beginning of the tail current (Fig. 13 E). The slowing of the tail current decay was similar both at 5 and 20 µM bbTBA (Fig. 13 F), although 20 µM bbTBA produces almost a 30% greater reduction in peak tail current than 5 µM bbTBA (Fig. 13 E). Although the unblocking relaxation may seem counterintuitive if blocked channels can close, such a relaxation is expected when return of blocked channels to open states occurs more rapidly than closure of open or blocked channels.

We next examined the predictions for tail current behavior for various blocking models (Fig. 14). For Scheme 2a, there is little discernible effect of blocker on tail current deactivation for currents activated with a nominal 4 µM Ca\(^{2+}\) (Fig. 14 B, 1). However, with 300 µM Ca\(^{2+}\) (Fig. 14 B, 2), Scheme 2a exhibits some slight slowing of deactivation along with a clear unblocking hook in the tail current at the highest effective [blocker]. Similar effects are observed for most models (Fig. 14, C–G). For activation at a nominal 4 µM Ca\(^{2+}\), bbTBA magnitude of the voltage dependence is less than that predicted for open-channel block, characteristic of bbTBA of Slo1 current.
The overall conclusion from this analysis is that several blocking models that incorporate block of closed channels do a reasonable job of approximating the observed lack of effect of bbTBA on tail currents with 4 µM Ca\(^{2+}\), while reproducing the slight prolongation and tail current hook observed at 300 µM Ca\(^{2+}\). As a consequence, we do not consider analysis of tail currents to be particularly useful in discriminating among the various models evaluated here.

**DISCUSSION**

The primary conclusion of this work is that the QA blocker, bbTBA, does not block BK channels in a completely state-independent fashion. The results generally support the conclusions of a previous study (Wilkens and Aldrich, 2006) indicating that bbTBA does block both open and closed BK channels. However, the difference with the earlier study is that here the results argue that bbTBA does not block BK channels in closed states with four inactive voltage sensors. We infer that voltage sensor movement is coupled to conformational changes in active-not-open channels that are permissive for block of closed channels by bbTBA. An important issue that we address below concerns the physical/structural basis by which closed channels with inactive voltage sensors are resistant to block by bbTBA.

**bbTBA does not block BK channels by a completely state-independent mechanism**

The simplest form of a fully state-independent blocking model (Scheme 2a) posits that both open and closed channels are blocked with equal affinity and voltage dependence. Our experiments present several observations inconsistent with this model. First, families of GV curves at different [bbTBA] obtained with either 100 or 300 µM of internal Ca\(^{2+}\), with tail current at −150 mV. (B) Tail currents in A were normalized to the peak tail current amplitude showing no difference in time course. (C) Deactivation time constants are plotted as a function of tail current potential for control and 5- and 20-µM bbTBA conditions. (D) Currents were activated by steps to +100 mV with 300 µM of internal Ca\(^{2+}\), with tail current at −150 mV. (E) Tail currents in D were normalized to the peak current amplitude and the baseline for an exponential fitted to the decay phase, showing the slowing of deactivation and a slight hook of unblock in the current before deactivation. (F) The time constant of deactivation is plotted for the same three conditions as shown in C. The time constants in 5 and 20 µM bbTBA were indistinguishable from each other, but clearly were slowed relative to control time constants.

Generally produces little effect on tail current decay, whereas for activation at 300 µM Ca\(^{2+}\), there is typically more slowing of deactivation and a clear unblocking hook at the highest bbTBA concentrations. For the general Scheme 3a (Fig. 14 E), there is substantially more tail current prolongation predicted than for Scheme 2a, and some prolongation is observed, even at 4 µM Ca\(^{2+}\). However, in all cases the magnitude of any slowing of tail current deactivation is rather minor. For Scheme 3b (Fig. 14 F), in which closed-channel block is voltage independent, no slowing of deactivation is observed at 4 µM. For the various models, we have plotted the deactivation time constant for tail current decay as a function of effective [blocker] (see Fig. S8). In most cases, blocker-dependent tail current prolongations observed in 300 µM Ca\(^{2+}\) exhibit saturation at the two highest [blocker], consistent with the similarity of tail current slowing produced by 5 and 20 µM bbTBA in native BK channels (Fig. 13).
We also addressed whether the results might be explained by a form of Scheme 2 in which all open and closed channels can still be blocked by bbTBA, but open- and closed-channel block differ. We considered models in which closed-channel block is voltage independent ($z_c = 0.0$ e; Scheme 2b) or all block parameters varied independently (Scheme 2'). With $z_c = 0.0$ e, the triphasic relaxations in the current time course are not predicted. Furthermore, Scheme 2b is unable to account for the disparities in block by bbTBA between low and high Ca$^{2+}$ (Fig. 3 B), and does not provide adequate fits of GV curves at low and high Ca$^{2+}$ (Fig. 4 F). This inability of Scheme 2b to account for the observations holds even when $K_{bc} \neq K_{bo}$. For Scheme 2', the optimal fit required that $z_c$ is $\sim 0.8$ e, which predicts...
What is the correct blocking model?

Our results argue that closed BK channels with inactive voltage sensors are resistant to block by bbTBA and support the idea that voltage-dependent conformational changes occurring in closed channels are permissive for bbTBA block. These conclusions do not depend on any particular model of channel block. However, a major part of this work involved an attempt to distinguish among different channel block models in which block was coupled to voltage sensor movement. For BK channels, voltage sensor movement both in closed and open channels is very rapid relative to channel opening and closing conformational changes (Horrigan and Aldrich, 1999, 2002). Therefore, after a voltage step, at least some voltage sensors will be rapidly activated at any Ca\textsuperscript{2+} concentration, perhaps producing a conformational change sufficient to permit block of closed channels. We focused our evaluation of models in which block was coupled to movement of voltage sensors on Scheme 3, in which activation of a single voltage sensor was considered sufficient to confer full sensitivity of a closed channel to block. Although we have not presented detailed examples, we also considered a Scheme 4 (Fig. S4), in which block affinity scaled linearly with the number of active voltage sensors. Both models were able to account for many essential features of the data, including the differences in block by bbTBA at low and high Ca\textsuperscript{2+}, the fractional availability curves, and also fractional unblock curves. However, because Scheme 4 was not obviously superior to Scheme 3 and any choice for how block affinity may scale with voltage sensor movement is rather arbitrary, we have omitted detailed consideration of Scheme 4.

We have also given consideration to several other possibilities. These include: (1) that perhaps block of the central cavity only occurs when at least two voltage sensors are active (Table III; Scheme 6); (2) that block of closed channels scales with voltage sensor movement, but not block of open channels (Table III, Scheme 5); and (3) that the closed-channel blocking affinity increases in a superlinear fashion with the number of active voltage sensors up to some limit. The available data do not discriminate adequately among different variations of closed-channel block coupled to active voltage sensors, and we have not extensively evaluated the behavior of these alternative models. We also considered and excluded a two-site blocking model (see Materials and methods), one site corresponding to open-channel block and a second site accessible in both open and closed states.

Although schemes based on the coupling of block to voltage sensor movement (such as Scheme 3) can account for many aspects of block by bbTBA, neither Scheme 3 nor other variations of schemes in which block depends on voltage sensor movement provide an optimal description of block of BK channels by bbTBA.

unusual channel kinetic behavior because of a large change in the effective gating charge in the CB–OB transitions (Fig. S8). Based on the inability of any form of Scheme 2 to describe several important features of block by bbTBA, we propose that the absence of appreciable change in fractional block of BK channels over holding potentials from −100 to −300 mV indicates that the fully closed BK channel is not blocked by bbTBA. This conclusion is also directly supported by the apparent absence of a reduction in NPo by 100 µM bbTBA. This conclusion is also directly supported by the apparent absence of a reduction in NPo by 100 µM bbTBA. However, a complication in the measurement of NPo was that 100 µM bbTBA produced a small net increase in NPo, an effect not predicted by any blocking scheme. This effect complicates interpretation of the absence of any blocking effect because one might argue that a blocking effect is being masked by the simultaneous increase in NPo. However, we calculate that, if the observed increase in NPo is masking a block effect of this magnitude, it would require an over 10-fold increase in NPo, an increase we consider unlikely. At 30 µM Ca\textsuperscript{2+}, the Ca\textsuperscript{2+} activation machinery is already strongly activated (Horrigan and Aldrich, 2002). Furthermore, we know of no other known allosteric regulator pathways of BK channels, e.g., Mg\textsuperscript{2+} (Horrigan and Ma, 2008), that might produce increases in NPo of this magnitude. Finally, if 100 µM bbTBA were causing strong allosteric activation of BK channels at −150 mV, one might also expect this activation to also occur at +20 mV. If that were the case and bbTBA was also blocking BK channels in a completely state-independent fashion, we would have expected to see similar reductions in NPo both at −150 and +20 mV. This is not observed. Thus, the absence of any reduction in NPo at −150 mV with 100 µM bbTBA is best explained by the idea that block by bbTBA at −150 mV is much less than at +20 mV. Although it will be important to identify the basis for the increase in NPo observed with bbTBA, at present the results seem best explained by the idea that bbTBA does not block channels with inactive voltage sensors, but that the quaternary bbTBA also produces some small increase in NPo through some unidentified, perhaps electrostatic effect.

Where our results lead to different conclusions than the earlier observations of Wilkens and Aldrich (2006), it is primarily because the conditions over which block by bbTBA was examined in the present study were extended, either by examination of low and high Ca\textsuperscript{2+} or by the use of more negative voltages. Both studies support the view that bbTBA does block closed BK channels, but the present results find that channels with inactive voltage sensors appear insensitive to bbTBA block. Thus, in comparing different blocking models, it is critical that conditions must be identified over which measurable model-dependent differences are expected.
At present, we simply interpret the analysis as supportive of the idea that block of closed BK channels depends on activation of one or more voltage sensors. Models that include this idea allow fitting of bbTBA block of GV curves at both low and high Ca²⁺, and can account for the Po independence of fractional unblock by bbTBA, the fractional availability of channels in bbTBA as a function of voltage, the kinetic aspects of block by bbTBA, features of tail current alteration by bbTBA, and the absence of blocking effects of bbTBA on NPo at negative potentials.

Conceptualizing the physical basis for dependence of closed-channel block on voltage sensor movement

Accepting the view that closed-channel block only occurs subsequent to movement of at least one voltage sensor, the key question that arises is, What is the physical change in the channel that allows blockade by bbTBA once a voltage sensor moves? Our results provide no specific answer to this question. As noted at the beginning of the Results, we envision two primary types of explanation, both of which assume that the bbTBA-binding site is within the BK central cavity: first, a change
in bbTBA accessibility to the central cavity and, second, a change in bbTBA binding affinity within the central cavity. In one case, movement of the voltage sensor is imagined to link to other elements of the pore domain to allow access of bbTBA to a central cavity binding site. In the other, the voltage sensor movement results in a change within the central cavity that increases the bbTBA binding affinity.

The idea that access of bbTBA to the central pathway changes with voltage sensor movement supposes that movement of the voltage sensor, in association with movement of the S4-S5 linker, also results in displacement of the S6 inner helix from its resting position, thereby creating a pathway of access to the central cavity. Thus, in the fully resting condition, the apertures to the central cavity would be sufficiently small to prevent bbTBA access, although perhaps allowing entry of smaller molecules, including permeant ions. Subsequently, the rapid movement of BK voltage sensors, which markedly precedes BK channel activation (Horrigan and Aldrich, 1999, 2002), might lead to an increase in diameter of the aperture, effectively allowing bbTBA to enter the still-closed channel. The actual opening of the BK channel permeation pathway would then involve movement of a gate located elsewhere, presumably at the selectivity filter as suggested by Winkens and Aldrich (2006).

The idea that a portion of the inner helix of K+ channels can move in a not-yet-open channel has been supported by experiments on the Shaker K+ channel by del Camino et al. (2005), who exploited a construct in which the voltage dependence of voltage sensor movement is markedly shifted leftward of the channel-opening equilibrium. This allowed identification of inner helix residues that became accessible in closed channels with activated voltage sensors (activated-not-open channels), but which were not accessible in closed or open states, supporting the idea that inner helix movement in K+ channels may occur in conjunction with voltage sensor movement in the absence of channel openings. Here, for BK channels, in accordance with this explanation of bbTBA block, any rearrangement of the inner helices that may occur upon voltage sensor activation must be sufficient to allow access to the channel central cavity, even while channels remain closed. Given the molecular dimensions of bbTBA, accessibility to the central cavity in a closed channel would imply that a fairly large access path to the central cavity is created upon movement of perhaps a single voltage sensor. Any blocking molecules smaller than bbTBA would be expected to have even more ready access to the central cavity in activated-but-closed channels. In fact, the lack of effect of QA blockers on BK tail currents supports the idea that block of closed states of BK channels by a variety of QA blockers does occur (Li and Aldrich, 2004), although it leaves open the question of whether such compounds also block closed channels with inactive voltage sensors.

Properties of BK channel inactivation also support the idea that voltage-dependent conformational changes precede BK channel opening. At low open probabilities, inactivation of BK channels can occur in the absence of channel openings, consistent with the idea that there are voltage-dependent conformational changes among closed BK channel states that are permissive for the inactivation domains of BK β2 subunits to reach a position of block (Ding and Lingle, 2002). Collectively, these results support the idea that voltage-dependent changes in aperture dimension may occur in BK channels in the absence of overt channel opening.

The idea that there are changes in dimension at the cytosolic end of the BK inner helix during gating is also supported by work with the ShakerB N-terminal peptide (Li and Aldrich, 2006). In contrast to bbTBA and tetrabutylammonium, the ShakerB peptide produces a state-dependent block of BK channels that is also associated with a hindering of channel closing during deactivation (Li and Aldrich, 2006). Li and Aldrich proposed that the differences in behavior of BP and bbTBA arise from the idea that the aperture to the central cavity in closed channels is simply too small to allow passage of the larger BP molecule, while permitting entry of bbTBA.

An alternative explanation for the absence of bbTBA block in closed states with inactive voltage sensors is that perhaps the affinity for bbTBA differs between closed channels with resting or active voltage sensors. To our knowledge, whether binding affinity within the central cavity may change between open and closed states has not been addressed for any K+ channel. Among different K+ channels, block by internal TEA exhibits a wide range of open-state affinities, despite the assumed similarity of architecture at the entrance of K+ channel selectivity filters. For example, estimates of 0-voltage TEA binding affinity include ~75 mM for Kcsa (Kutluay et al., 2005), 0.56 mM for Shaker (Thompson and Begenisich, 2000), and 25 mM for BK channels (Yellen, 1984). Given differences in the blocking affinities of cytosolic TEA among different open K+ channels, it might not be surprising that in association with channel gating, there are also changes in the central cavity or vicinity of the selectivity filter that might influence TEA affinity. However, for a bulky blocker like bbTBA, the determinants of binding are likely to be different than for TEA. In Shaker, residues in the P loop near the selectivity filter have been shown to influence affinity for block by smaller QA blockers, whereas the affinity of block by QA blockers with long hydrophobic side chains is affected by mutations at hydrophobic residues in S6 (Choi et al., 1993). If bbTBA binding affinity differs among different closed states, it would suggest that voltage sensor movement is causing a change in the strength of hydrophobic interactions between bbTBA and central cavity residues.
Overall, the results provide no definitive basis for distinguishing between the access or affinity explanations. However, because of the precedent that conformational changes may occur at the cytosolic end of K+ channels that are coupled to voltage sensor movement, we prefer the view that bbTBA gains access to the central cavity through expansion of an aperture that is sufficient to allow bbTBA passage as soon as at least one voltage sensor is active. We consider it less likely a change in strength of hydrophobic interactions within the central cavity might occur as a consequence of voltage sensor movement.

The above discussion leaves open the possibility that, even with four resting voltage sensors, an aperture of finite open dimension exists that allows molecules smaller than bbTBA to enter the central cavity. The gating behavior of BK channels and the allosteric model of BK activation that best accounts for this behavior implicitly support this possibility, at least for open channels with four resting voltage sensors. Specifically, at very negative potentials, where voltage sensors are almost entirely inactive, BK channels have a finite Po that exhibits a voltage dependence characteristic of the C–O equilibrium defined by the equilibrium constant, L (Horrigan and Aldrich, 2002). The fact that the residual Po is independent of the voltage dependence of the voltage sensor equilibrium requires that the openings at negative potentials occur in channels in which voltage sensors are usually inactive. This necessarily suggests that permeant ions enter the central cavity and permeate through channels with four inactive voltage sensors. However, this separation of Po and voltage sensor activation does not directly identify the position of the gate(s) opened during the C to O transition. Openings that occur in the absence of voltage sensor activation might involve either of two mechanisms. First, perhaps in BK channels rearrangements of the S6 helices permitting ion access to the central cavity may occur independently of movement of the S4-S5 linker typically thought to be involved in pore opening for Kv channels (Lu et al., 2002; Long et al., 2005). Second, it is possible that an aperture sufficiently large to allow hydrated K+ to enter the central cavity is always present in all BK channel conformations, and that the observed openings simply reflect a gating process at the selectivity filter. The latter interpretation seems most consistent with our current understanding of steps in BK gating.

Considerations pertinent to the voltage dependence of closed-channel block

A final issue concerns the properties of closed-channel block and, in particular, the voltage dependence of closed-channel block, \( z_c \). Given that bbTBA appears to block both open and closed channels, it would be desirable to know how the binding affinity and voltage dependence compares for open and closed states. However, no aspect of our data allows any model-independent estimate of the properties of closed-channel block, and our estimates of \( K_{bc} \) and \( z_c \) are based entirely on the use of simultaneous fitting of GV curves at both low and high Ca\(^{2+}\) to the various models. Although the simultaneous fitting is an advantage in parameter estimation, the GV curves cannot be considered to strongly define parameter values for closed-channel block because closed-channel block primarily contributes only at the foot of the GV curves. Yet, it is satisfying that the conclusion that closed-channel block depends on voltage sensor movement, based on our fitting of GV curves, was strongly and directly supported by measurements of NPo at negative potentials. This suggests that at least some aspects of closed-channel block can be reliably assessed by model-dependent fitting of the GV curves.

To ascertain properties of closed-channel block, we examined the ability of different blocking schemes (Schemes 2–4) to fit GV curves, under different assumptions regarding the relationship of \( K_{bc} \) and \( K_{bo} \), as well as \( z_c \) and \( z_o \). Under the most relaxed condition, when \( K_{bc} \neq K_{bo} \) and both \( z_c \) and \( z_o \) were allowed to vary, the best fit to GV curves yielded values for \( z_c \) with large voltage dependence. However, a difficulty with large values of \( z_c \) is that, to maintain microscopic reversibility in tiered reaction schemes, strong allosteric effects on transitions between CB and OB states are required, and such effects were not observed. In contrast, although fits of GV curves with \( z_c = z_o \) or \( z_c = 0.0 \) were not quite as good, the weaker closed-state voltage dependence resulted in simulated currents that better approximate the observed BK current behavior. Yet, overall, for Scheme 3, the fits obtained either when \( z_c \) was allowed to vary or when \( z_c = 0 \) were only slightly different, suggesting that one cannot draw strong conclusions based on different estimates of \( z_c \). Furthermore, it should be noted that, regardless of the assumptions for \( z_c \) under different variations of Scheme 3 (Table III), \( K_{bc} \) and \( K_{bc} \), the 0-voltage open- and closed-channel affinities for bbTBA are fairly similar, suggesting that the determinants for bbTBA binding are similar in open and closed channels.

Theoretical considerations have suggested that closed-channel block might be expected to exhibit stronger voltage dependence than open-channel block. Calculations based both on the crystal structure of the presumed closed KcsA channel and also on an open-state model indicate that substantial differences exist in the expected transmembrane potential drop along the channel axis (Roux et al., 2000). Specifically, at the level of the central cavity, a larger fraction of the total field (relative to the inside) is felt in closed channels (\( \Delta \phi \) of \( \sim 0.46 \)) versus open channels (\( \Delta \phi \) of \( \sim 0.3 \)), suggesting that a larger voltage dependence of block in closed channels is expected. This idea would seem to conflict with our observation that the assumption of \( z_c > z_o \) can
result in predictions for kinetic aspects of currents that are not plausible. However, it should be kept in mind that the models we have evaluated make the assumption of microscopic reversibility in the coupling of blocking steps with gating steps. Thus, under this assumption, our analysis would argue that \( z_c \) is unlikely to be much larger than \( z_o \). However, it may be the case that this assumption may not be necessary or entirely appropriate. For example, the effect of a voltage gradient acting on a charged moiety in the central cavity might provide energy that effectively introduces asymmetry into a block mechanism. Perhaps models in which \( z_c > z_o \) and which allow deviations from microscopic reversibility might better account not only for the steady-state aspects of our results, but also for the various kinetic features of block. Despite the uncertainty on this point, these considerations do not negate our conclusion regarding the inability of bbTBA to block channels with four inactive voltage sensors.

Summary
The results and analysis support the view that bbTBA does not block BK channels in a completely state-independent fashion, but that blockade of closed channels is dependent on movement of at least one or more voltage sensors. In contrast to Shaker \( K^+ \) channels, in BK channels movements of the inner helix associated with voltage sensor movement allow blockers to gain access to the central cavity in the closed channel. The analysis does not define the physical mechanism that accounts for the inability of bbTBA to block closed channels with inactive voltage sensors, but we discuss two possibilities: that access to the central cavity depends on voltage sensor movement, or that bbTBA affinity in the central cavity is altered by voltage sensor movement.

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